Committee for Risk Assessment

RAC

Annex 1
Background document
to the Opinion proposing harmonised classification and labelling at EU level of

fluopicolide (ISO); 2,6-dichloro-N-[3-chloro-5-(trifluoromethyl)-2-pyridylmethyl]benzamide

EC Number: -
CAS Number: 239110-15-7

CLH-O-0000006820-76-01/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

11 June 2020

CLH report

Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

International Chemical Identification:

Fluopicolide (ISO); 2,6-dichloro-N-[3-chloro-5-(trifluoromethyl)-2-pyridylmethyl]benzamide

EC Number: **not allocated**

CAS Number: **239110-15-7**

Index Number: **not allocated**

Contact details for dossier submitter:

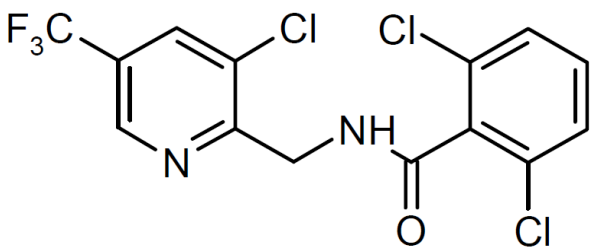
Version number: 02 **Date: June 3, 2019**

CONTENTS

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1-1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	IUPAC name: 2,6-dichloro-N-{[3-chloro-5-(trifluoromethyl)-pyridin-2-yl]methyl}benzamide CAS name: Benzamide, 2,6-dichloro-*N*-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-
Other names (usual name, trade name, abbreviation)	Fluopicolide
ISO common name (if available and appropriate)	Fluopicolide
EC number (if available and appropriate)	Not allocated
EC name (if available and appropriate)	Not allocated
CAS number (if available)	239110-15-7
Other identity code (if available)	CIPAC: 787
Molecular formula	$C_{14}H_8Cl_3F_3N_2O$
Structural formula	
SMILES notation (if available)	FC(F)(F)c1cnc(CNC(=O)c2c(Cl)cccc2Cl)c(Cl)c1
Molecular weight or molecular weight range	383.59 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	Not applicable
Description of the manufacturing process and identity of the source (for UVCB substances only)	Not applicable, fluopicolide is not an UVCB.
Degree of purity (%) (if relevant for the entry in Annex VI)	min. 97.0% w/w

1.2 Composition of the substance

Table 1-2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)
Fluopicolide	Min. 97.0% w/w	No entry in Annex VI	

Table 1-3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
Toluene CAS: benzene, methyl [108-88-3]	Max. 0.3% w/w	Flam. Liq. 2 H225 Skin Irrit. 2 H315 Asp. Tox. 1 H304 STOT SE 3 H336 STOT RE 2 H373 Repr. 2 H361d		No

Table 1-4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
Not relevant					

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria[#]

Table 2-1:

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATEs	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Dossier submitters proposal	n.a.	Fluopicolide (ISO); 2-6-dichloro-N-[3-chloro-5-(trifluoromethyl)-2-pyridylmethyl]benzamide	n.a.	239110-15-7	none	none	none	none	none	none	n.a.
Resulting Annex VI entry if agreed by RAC and COM	n.a.	Fluopicolide (ISO); 2-6-dichloro-N-[3-chloro-5-(trifluoromethyl)-2-pyridylmethyl]benzamide	n.a.	239110-15-7	none	none	none	none	none	none	n.a.

[#] only health hazards are assessed
n.a.: not applicable

Table 2-2: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	Hazard class not assessed in this dossier.	No
Flammable gases (including chemically unstable gases)	Hazard class not assessed in this dossier.	No
Oxidising gases	Hazard class not assessed in this dossier.	No
Gases under pressure	Hazard class not assessed in this dossier.	No
Flammable liquids	Hazard class not assessed in this dossier.	No
Flammable solids	Hazard class not assessed in this dossier.	No
Self-reactive substances	Hazard class not assessed in this dossier.	No
Pyrophoric liquids	Hazard class not assessed in this dossier.	No
Pyrophoric solids	Hazard class not assessed in this dossier.	No
Self-heating substances	Hazard class not assessed in this dossier.	No
Substances which in contact with water emit flammable gases	Hazard class not assessed in this dossier.	No
Oxidising liquids	Hazard class not assessed in this dossier.	No
Oxidising solids	Hazard class not assessed in this dossier.	No
Organic peroxides	Hazard class not assessed in this dossier.	No
Corrosive to metals	Hazard class not assessed in this dossier.	No
Acute toxicity via oral route	Data conclusive but not sufficient for classification	Yes
Acute toxicity via dermal route	Data conclusive but not sufficient for classification	Yes
Acute toxicity via inhalation route	Data conclusive but not sufficient for classification	Yes
Skin corrosion/irritation	Data conclusive but not sufficient for classification	Yes
Serious eye damage/eye irritation	Data conclusive but not sufficient for classification	Yes
Respiratory sensitisation	Data lacking, but hazard is unlikely based on skin sensitisation and acute inhalation data	Yes
Skin sensitisation	Data conclusive but not sufficient for classification	Yes
Germ cell mutagenicity	Data conclusive but not sufficient for classification	Yes
Carcinogenicity	Data conclusive but not sufficient for classification	Yes
Reproductive toxicity	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-single exposure	Data conclusive but not sufficient for classification	Yes
Specific target organ toxicity-repeated exposure	Data conclusive but not sufficient for classification	Yes
Aspiration hazard	Data lacking, but hazard is unlikely based on physical chemical properties	Yes
Hazardous to the aquatic environment	Hazard class not assessed in this dossier.	No
Hazardous to the ozone layer	Hazard class not assessed in this dossier.	No

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Fluopicolide is an active substance in the scope of the Regulation (EC) No 1107/2009 (repealing Council Directive 91/414/EEC). There is no harmonised classification and labelling in Annex VI of regulation 1272/2008 (CLP) and there have been no previous classification and labelling discussions of this active substance. The active substance is therefore subject to the harmonized classification and labelling process in accordance with Article 36(2) of CLP and no further justification is required. However, **only health hazards** are assessed in this dossier. The member state Austria proposed to ECHA on Novmeber 28, 2017, to limit the CLH report to human health hazards only, owing to a lack of resources. Furthermore, clarity is required on human health classification (particularly with regard to reproductive toxicity) prior to the EU approval renewal of the active substance under regulation (EC) 1107/2009, because a reproductive classification would directly impact on the data required for this renewal. This proposal was initially accepted by ECHA on December 22, 2017.

The peer review of the pesticide risk assessment of fluopicolide (EFSA Scientific Report (2009) 299, 1-158) concluded that fluopicolide was of low acute toxicity (via the oral, dermal and inhalation routes), was not a skin irritatant and that only slight eye irritation (insufficient for classification purposes) was observed. The review concluded that fluopicolide was unlikely to be genotoxic and that no classification for carcinogenicity, fertility or development was warranted. There were no effects on specific target organs at doses relevant for classification into STOT SE or STOT RE. All of these health hazards have been addressed in this CLH report.

Overall, following the peer review of fluopicolide, no classification was proposed with regard to the physical and chemical data or the toxicological data; however, based on the fate and behaviour data R53 was proposed for fluopicolide and based on the ecotoxicological data N, R50, R53, S60 and S61 were proposed (see https://www.efsa.europa.eu/en/efsajournal/pub/rn-299 for further details).

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

There is no requirement for justification that action is needed at Community level; fluopicolide is an active substance in the scope of Regulation (EC) No 1107/2009 (repealing Council Directive 91/414/EEC).

5 IDENTIFIED USES

Fluopicolide containing plant protection products are developed for foliar and seed treatment application to control diseases caused by pathogen fungi from the Oomycete (Phycomycete) class including downy mildews and late blight in crops like vines and potatoes.

6 DATA SOURCES

For human health, the data used in this CLH report consist of studies which have been submitted for Annex I inclusion under Council Directive 91/414/EEC and studies which will be additionally submitted for EU approval renewal under Regulation (EC) No 1107/2009 in November 2020.

7 PHYSIOCHEMICAL PROPERTIES

Physical hazards are not assessed in this dossier. Only health hazards are assessed.

8 EVALUATION OF PHYSICAL HAZARDS

Physical hazards are not assessed in this dossier. Only health hazards are assessed.

8.1 Explosives

Please refer to Section 8.

8.1.1 Short summary and overall relevance of the information provided on explosive properties

Please refer to Section 8.

8.1.2 Comparison with the CLP criteria

Please refer to Section 8.

8.1.3 Conclusion on classification and labelling for explosive properties

Please refer to Section 8.

8.2 Flammable gases (including chemically unstable gases)

Please refer to Section 8.

8.2.1 Short summary and overall relevance of the provided information on flammable gases (including chemically unstable gases)

Please refer to Section 8.

8.2.2 Comparison with the CLP criteria

Please refer to Section 8.

8.2.3 Conclusion on classification and labelling for flammable gases

Please refer to Section 8.

8.3 Oxidising gases

Please refer to Section 8.

8.3.1 Short summary and overall relevance of the provided information on oxidising gases

Please refer to Section 8.

8.3.2 Comparison with the CLP criteria

Please refer to Section 8.

8.3.3 Conclusion on classification and labelling for oxidising gases

Please refer to Section 8.

8.4 Gases under pressure

Please refer to Section 8.

8.4.1 Short summary and overall relevance of the provided information on gases under pressure

Please refer to Section 8.

8.4.2 Comparison with the CLP criteria

Please refer to Section 8.

8.4.3 Conclusion on classification and labelling for gases under pressure

Please refer to Section 8.

8.5 Flammable liquids

Please refer to Section 8.

8.5.1 Short summary and overall relevance of the provided information on flammable liquids

Please refer to Section 8.

8.5.2 Comparison with the CLP criteria

Please refer to Section 8.

8.5.3 Conclusion on classification and labelling for flammable liquids

Please refer to Section 8.

8.6 Flammable solids

Please refer to Section 8.

8.6.1 Short summary and overall relevance of the provided information on flammable solids

Please refer to Section 8.

8.6.2 Comparison with the CLP criteria

Please refer to Section 8.

8.6.3 Conclusion on classification and labelling for flammable solids

Please refer to Section 8.

8.7 Self-reactive substances

Please refer to Section 8.

8.7.1 Short summary and overall relevance of the provided information on self-reactive substances

Please refer to Section 8.

8.7.2 Comparison with the CLP criteria

Please refer to Section 8.

8.7.3 Conclusion on classification and labelling for self-reactive substances

Please refer to Section 8.

8.8 Pyrophoric liquids

Please refer to Section 8.

8.8.1 Short summary and overall relevance of the provided information on pyrophoric liquids

Please refer to Section 8.

8.8.2 Comparison with the CLP criteria

Please refer to Section 8.

8.8.3 Conclusion on classification and labelling for pyrophoric liquids

Please refer to Section 8.

8.9 Pyrophoric solids

Please refer to Section 8.

8.9.1 Short summary and overall relevance of the provided information on pyrophoric solids

Please refer to Section 8.

8.9.2 Comparison with the CLP criteria

Please refer to Section 8.

8.9.3 Conclusion on classification and labelling for pyrophoric solids

Please refer to Section 8.

8.10 Self-heating substances

Please refer to Section 8.

8.10.1 Short summary and overall relevance of the provided information on self-heating substances

Please refer to Section 8.

8.10.2 Comparison with the CLP criteria

Please refer to Section 8.

8.10.3 Conclusion on classification and labelling for self-heating substances

Please refer to Section 8.

8.11 Substances which in contact with water emit flammable gases

Please refer to Section 8.

8.11.1 Short summary and overall relevance of the provided information on substances which in contact with water emit flammable gases

Please refer to Section 8.

8.11.2 Comparison with the CLP criteria

Please refer to Section 8.

8.11.3 Conclusion on classification and labelling for substances which in contact with water emit flammable gases

Please refer to Section 8.

8.12 Oxidising liquids

Please refer to Section 8.

8.12.1 Short summary and overall relevance of the provided information on oxidising liquids

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8.12.2 Comparison with the CLP criteria

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8.12.3 Conclusion on classification and labelling for oxidising liquids

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8.13 Oxidising solids

Please refer to Section 8.

8.13.1 Short summary and overall relevance of the provided information on oxidising solids

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8.13.3 Conclusion on classification and labelling for oxidising solids

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8.14 Organic peroxides

Please refer to Section 8.

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Please refer to Section 8.

8.14.2 Comparison with the CLP criteria

Please refer to Section 8.

8.14.3 Conclusion on classification and labelling for organic peroxides

Please refer to Section 8.

8.15 Corrosive to metals

Please refer to Section 8.

8.15.1 Short summary and overall relevance of the provided information on the hazard class corrosive to metals

Please refer to Section 8.

8.15.2 Comparison with the CLP criteria

Please refer to Section 8.

8.15.3 Conclusion on classification and labelling for corrosive to metals

Please refer to Section 8.

RAC General Comment

Fluopicolide is a fungicide used in agriculture to control oomycetes, e.g. *Phyphthora infestans*, causing potato blight.

RAC was unable to evaluate the physical and environmental hazard classes. The Committee was informed that the DS had not provided data, citing resource limitations as the reason. RAC notes that in light of it being mandatory to consider all hazard classes for the harmonised classification of pesticides and biocides (CLP regulation Art. 36(2), Art 37(1) and Annex VI Parts 1 and 2) the remaining hazard classes should therefore become the subject of a future Annex XV dossier without delay.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Toxicokinetics are not assessed in this dossier, only health hazards are assessed; however, as it is relevant to the human health evaluation, a short summary of the available toxicokinetic data is provided below.

Table 9.1: Summary table of toxicokinetic studies

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206: Single high & low dose rat A.D.E. study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The major elimination route was via faeces (82 to 88% of dose), while urine contained 5 to 13% dose. Almost complete excretion occurred within 48 hours for the low dose group and 24 hours for the high dose group. At 168 hours post dose tissues were low (0.75 to 1.25% of the dose). Liver and kidneys contained highest residues, and also skin & fur for females of the high dose group.	ADE report. 2 separate metabolism reports (see below). [Phenyl-U-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw.	Anonymous; 2001; M-204781-01-1
[Phenyl-U-^{14}C]-AE C638206: Rat metabolism following administration of a single oral low dose - (including Amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-fluopicolide was very extensively metabolised in low dose rats (10 mg/kg bw/day) with up to 55 metabolites in urine (9 to 13% dose in urine) and 52 in faecal extracts (81 to 82% dose eliminated in faeces). Biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were oxidised to both sulphones and sulphoxides. The formation of an acetylated version of AE C653711 (M-01, BAM), indicated that fluopicolide could be cleaved which is consistent with results from the other radiolabelled metabolism study with [Pyridyl-2,6 - ^{14}C]-fluopicolide.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 4 male & 4 female rats at 10 mg/kg bw (see above).	Anonymous; 2004; M-227026-02-1

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206: Rat metabolism following administration of a single oral high dose - (including Amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]- fluopicolide was also extensively metabolised in high dose animals (100 mg/kg bw/day) with 46 metabolites detected in urine (4 to 6% dose in urine) and 14 in faecal extracts (86 to 87% dose eliminated in faeces). The same routes of metabolism as seen in the low dose group were observed in high dose animals.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 4 male & 4 female rats at 100 mg/kg bw (see above).	Anonymous; 2004; M-227025-02-1
[Pyridyl-2,6 - ^{14}C]-AE C638206 - Single oral low dose rat A.D.E. study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The major elimination route was via faeces (69 to 72% of dose), while urine contained 21 to 27% dose. Almost complete excretion occurred within 48 hours. At 168 hours post dose tissues contained between 0.7 to 0.5% of the dose. Liver, kidneys and blood consistently contained highest residues.	Separate ADE and Metabolism reports (see below). [Pyridyl-2,6-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw.	Anonymous; 2001; M-202609-02-1
[Pyridyl-2,6-14C]-AE C638206: Rat metabolism following administration of a single oral low dose US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Pyridyl-2,6-^{14}C]- fluopicolide was also very extensively metabolised in the rat with up to 28 metabolites in urine (17 to 21% dose in urine) and 31 in faecal extracts (63% dose eliminated in faeces). The same biotransformations as seen in rats dosed with [phenyl-U-^{14}C]-fluopicolide were observed in rats dosed with [pyridyl-2,6-^{14}C]-fluopicolide. The formation of AE C657188 (M-02, PCA), indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule.	[Pyridyl-2,6-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw (see above).	Anonymous; 2004; M-227023-01-1

Method	Results	Remarks	Reference
[Phenyl-U-14C]-AE C638206: Repeat oral low dose A.D.M.E. study in the rat - (including amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	No evidence of accumulation was observed. Following 14 daily oral administrations of [phenyl-U-^{14}C]-fluopicolide the major route of elimination was via faeces (73 to 79% dose). Repeated dosing enhanced elimination via urine compared with the single oral dose (15 to 22% dose). Tissue levels were consistently low (mean 0.38%). Liver, kidneys (organs of excretion and metabolism) and blood contained the highest concentrations of radioactivity in both sexes. [Phenyl-U-^{14}C]- fluopicolide was also extensively metabolised in repeat dose animals with 46 metabolites detected in urine and 14 in faecal extracts. A large number of metabolites were observed in the excreta (up to 57 in the urine and 45 in the faeces). The observed routes of metabolism included glutathione conjugation and its subsequent biotransformation products, hydroxylation, conjugation with glucuronic acid, conjugation with sulphate and oxidative N-dealkylation.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 5 male & 5 female rats at 10 mg/kg bw	Anonymous; 2004; M-227027-02-1
[Phenyl-U-^{14}C]-AE C638206: Rat bile excretion study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	Biliary elimination was a major route in low dose animals (10 mg/kg bw/day). 77% of the low dose for males and 83% for the females (mean 80%) was detected in the bile of cannulated rats dosed with [phenyl-U-^{14}C]-fluopicolide. At the high dose level, the values were 34% for the males and 41% for the females (mean 37%), demonstrating absorption had been saturated by this dose level.	[Phenyl-U-^{14}C]-Fluopicolide: 4 male & 4 female bile-duct cannulated rats at 10 mg/kg bw; 4 male & 4 female bile-duct cannulated rats at 100 mg/kg bw.	Anonymous; 2002; M-212243-01-1
[Pyridyl-2,6-^{14}C]-AE C638206: Single oral low dose rat bile excretion study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	When dosed with [pyridyl-U-^{14}C]-fluopicolide 59% of the low dose for males and 64% for the females (mean 62%) was detected in the bile of cannulated rats. The difference between the two radiolabels likely indicates a portion of the fluopicolide dose is metabolised to form single ring metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA), which behave differently in the rat.	[Pyridyl-U-^{14}C]-Fluopicolide: 4 male & 4 female bile-duct cannulated rats at 10 mg/kg bw;	Anonymous; 2003; M-230976-01-1

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206 and [pyridyl-2,6-^{14}C]-AE C638206: Rat blood and plasma kinetics study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The general pharmacokinetic profiles were similar between radiolabels and sexes. Fluopicolide was absorbed relatively rapidly with maximal concentrations achieved between 7 and 10 hours post dose at 10 mg/kg bw.	[Phenyl-U-^{14}C]-Fluopicolide: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw. [Pyridyl-U-^{14}C]-Fluopicolide: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw.	Anonymous; 2002; M-221902-01-1
[Phenyl-U-^{14}C]-AE C638206 rat tissue kinetic study	Fluopicolide was rapidly and widely distributed into the tissues. No significant sex difference was found. In rats dosed with [phenyl-U-^{14}C]-fluopicolide, highest tissue concentrations were in the intestine and contents with next highest concentrations observed in liver, kidneys and adrenals, which decreased with time. The compound was extensively metabolised with 13 metabolites detected in liver by 8 hours post dose, of which AE C653711 (M-01, BAM), AE 0717559, AE C643890 (M-06) and AE 0717560 were identified.	[Phenyl-U-^{14}C]-Fluopicolide: 16 male & 16 female rats at 10 mg/kg bw; 16 male & 16 female rats at 100 mg/kg bw. Sacrificed at 8, 24 or 30, 36 or 48, 72 (males) & 120 hours (females).	Anonymous; 2003; M-221892-01-1
[2,6-Pyridyl-^{14}C]-AE C638206: Rat tissue kinetic study	In rats dosed with [pyridyl-U-^{14}C]-fluopicolide, the compound was similarly distributed into tissues, followed by a significant and rapid decrease in tissue concentrations. Again, no significant sex difference was found. Highest radioactivity concentrations were in the intestine and contents presumably as a result of biliary excretion of radioactivity. The next highest concentrations were in the liver, kidneys, adrenals and cardiac blood which declined with time post dose.	[Pyridyl-U-^{14}C]-Fluopicolide: 16 male & 16 female rats at 10 mg/kg bw. Sacrificed at 6 or 7, 24, 36, 48 (males) & 120 hours (females).	Anonymous, 2003; M-221885-01-1

Method	Results	Remarks	Reference
(^{14}C)-AE C638206: Preliminary toxicokinetic studies in the rat Non guideline preliminary study GLP	The findings aer consistent with later studies. Fluopicolide was relatively rapid adsorped with blood C_{max} between 8 to 12 hours. At low dose residues were below 0.10 µg/g in all tissues except the liver, kidney and blood 168 h after dosing. The major metabolic reactions identified were aromatic hydroxylation of the phenyl ring, glucuronidation of the phase I hydroxyl products and sequential metabolism through the mercapturic acid pathway.	Combined ADME & kinetics preliminary study (25 & 500 mg/kg bw) [Phenyl-U-^{14}C]-Fluopicolide: ADME: 2 male & 2 female rats at 25 mg/kg bw; 2 male & 2 female rats at 500 mg/kg bw. Blood kinetic: 2 male & 2 female rats at 25 mg/kg bw; 2 male & 2 female rats at 500 mg/kg bw. [Pyridyl-2,6-^{14}C]-Fluopicolide: ADME: 2 male & 2 female rats at 25 mg/kg bw. Blood kinetic: 2 male & 2 female rats at 25 mg/kg bw.	Anonymous; 2000; M-197858-01-1
Interspecies comparison of in vitro metabolism of [phenyl-UL-^{l4}C] fluopicolide using mouse, rat, dog and human liver microsomes Non guideline preliminary study GLP	[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from mice, rat, dog and humans, with a total of 8 metabolites detected. No human-specific metabolites were observed.	[Phenyl-U-^{14}C]-Fluopicolide: *In vitro* (1 and 10 µM) with liver microsomes from mice, rat, dog and humans.	Anonymous; 2019; M-653630-02-1

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

In vivo studies

Toxicokinetic studies on the absorption, distribution, metabolism and excretion of fluopicolide, were conducted in the rat. Studies were performed using two different radiolabels; [phenyl-U-^{14}C]-fluopicolide or [pyridyl-2,6-^{14}C]-fluopicolide.

The major route of elimination of fluopicolide was via the faeces for both the 10 and 100 mg/kg bw oral dose and for both pyridyl (69 to 72% of the administered dose) and phenyl (82 to 88%) ring radiolabels. No significant sex difference was observed. There was a tendency towards a higher urinary excretion level with the pyridyl radiolabel (19% in males and 22% in females for the 10 mg/kg bw dose) compared to the phenyl radiolabel (10% in males and 13% in females for the 10 mg/kg bw dose). This suggests that a proportion of the metabolites that were formed differed between the two radiolabels and were presumably linked to the formation of AE C657188 (M-02, PCA) from the pyridyl ring moiety and AE C653711 (M-01, BAM) from the phenyl ring.

Following repeated (14x) daily oral administrations of [phenyl-U-^{14}C]-fluopicolide the total recovery of radioactivity was approx. 96% of the administered dose; with the faeces, again, being found to be the major route of elimination representing 79% for the males and 72% for the females. The urine was found to represent 15% of the administered dose for the males and 21% for the females. It appeared that repeated dosing enhanced elimination via urine compared with the single oral dose.

Tissue radioactivity levels were consistently low and ranged between 0.46 to 1.25% of the administered dose for the single dose studies and a mean of 0.38% for the repeat dose study.

Investigations in bile-cannulated rats over 48 hours showed a large proportion of the radioactivity found in the faeces had been absorbed and then eliminated via the bile. The extent of oral absorption based on the biliary excretion study only for the 10 mg/kg bw oral dose, was 80% of the administered dose for the phenyl radiolabel and 62% for the pyridyl radiolabel. However, blood and plasma pharmacokinetic data show the systemic exposure was similar between both the radiolabels and the sexes. The bioavailability of fluopicolide, taking into account the material undergoing entero-hepatic recirculation, was calculated to be 75 to 88% of the administered dose.

Fluopicolide was well distributed into organs and tissues (blood T_{max} 5.5 to 7.5 hours and plasma T_{max} 6.5 to 8 hours for 10 mg/kg bw) followed by a moderately rapid elimination such that the majority was eliminated by 48 hours post dose followed by a slower terminal elimination phase with a mean half-life of approx. 99 hours for blood. A lower mean half-life of 16 hours was observed for plasma due to the difference in limits of quantification.

The highest tissue residues were found in the liver and kidney and (to a lesser extent) in the spleen and blood.

In tissue kinetic studies the highest tissue concentrations were observed in the intestine and contents, reflecting a combination of unabsorbed material and biliary excretion. The next highest concentrations were consistently observed in the liver, kidneys and adrenals albeit that the concentrations were decreasing with time post dosing. AE C653711 (M-01, BAM), AE 0717559, AE C643890 (M-06) and AE 0717560 were identified in the liver 8 hour post dosing with [phenyl-U-^{14}C]-fluopicolide.

Fluopicolide was extensively metabolised in the rat. The formation of the metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA) was confirmed during the course of the biotransformation investigations and indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule. Generally, the biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate and glutathione. The glutathione conjugates were seen to be further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were seen to be further metabolised by acetylation to form the mercapturic acids or to be dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were seen to be oxidised to both sulphones and sulphoxides.

In vitro study

The comparative *in-vitro* metabolism of fluopicolide was studied with liver microsomes from CD-1 mouse, Wistar rat, Beagle dog and human. Incubations were performed with [phenyl-U-^{14}C]-fluopicolide at two concentrations (1 and 10 µM) at 1, 60 and 120 minutes.

[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from all four species. Conversion of fluopicolide was 98% in dog, 82% in mouse, 68% in human and 54% in rat microsomes after 120 minutes of incubation. A total of 8 metabolites were detected, named Metabolite 1 to 8 based on their HPLC retention time. Metabolites accounting for ≥ 5% were considered as main metabolites. Overall, five main metabolites were detected: Metabolite 1 (mouse, rat and human) and Metabolite 2 (mouse and rat), Metabolite 3 and Metabolite 5 (mouse, dog and human) and Metabolite 6 which was detected as a main metabolite in the four species. Metabolite 2 was detected in the mouse and rat microsome incubations only. No human-specific fluopicolide metabolites were detected.

10 EVALUATION OF HEALTH HAZARDS

10.1 Acute toxicity - oral route

The acute oral toxicity of fluopicolide has been investigated in rats.

Table 10-1: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/ group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute oral toxicity OECD 423(1996) GLP	Rat, Hsd: Sprague-Dawley (CD) male & female 5/sex/ dose level	Fluopicolide (purity 97.7%)	Single oral gavage at doses of 5000 mg/kg bw, in 1% w/v aqueous methylcellulose	> 5000 mg/kg bw	Anonymous.; 2000; M-197224-01-1

Table 10-2: Summary table of human data on acute oral toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-3: Summary table of other studies relevant for acute oral toxicity

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No other studies				

10.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

In an acute toxicity study by the acute toxic class method, 5 male and 5 female fasted Sprague-Dawley rats were each administered by gavage a single oral dose of 5000 mg/kg bw.

No mortality was observed. Clinical signs of reaction to treatment were confined to piloerection and hunched posture, seen in all female rats and in three male rats with abnormal gait notable in three females. Recovery of rats, as judged by external appearance and behavior, was complete by Day 3. All animals were considered to have achieved satisfactory body weight gains throughout the study.

The acute lethal oral dose in rats of fluopicolide (AE C638206) was greater than 5000 mg/kg bw.

10.1.2 Comparison with the CLP criteria

The guidance on the application of the CLP criteria (Regulation (EC) No 1272/2008) gives a cut-off LD_{50} value of 2000 mg/kg bw for the classification of acute toxicity via the oral route. Under the conditions of this study the LD_{50} value of fluopicolide for oral toxicity was found to be > 5000 mg/kg bw. Therefore **no classification** for acute oral toxicity is proposed.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

The data on the acute toxicity potential of fluopicolide (AE C638206) are conclusive. Based on the oral LD_{50} of > 5000 mg/kg bw after acute oral administration to rats, an acute toxicity classification is **not warranted** according to Regulation (EC) No 1272/2008 (CLP).

10.2 Acute toxicity - dermal route

The acute dermal toxicity of fluopicolide has been investigated in rats.

Table 10-4: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute dermal toxicity OECD 402 (1987) GLP	Rat, Hsd: Sprague-Dawley (CD) male & female 5/sex/ dose level	Fluopicolide (purity 97.7%)	Single dermal dose of 5000 mg/kg bw, in 1% w/v aqueous methylcellulose	> 5000 mg/kg bw	Anonymous; 2000; M-197225-01-1

Table 10-5: Summary table of human data on acute dermal toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-6: Summary table of other studies relevant for acute dermal toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.2.1 Short summary and overall relevance of the provided information on acute dermal toxicity

A group of ten rats (five males and five females) received a single topical application of the test substance, administered as supplied at a dose level of 5000 mg/kg bw. The application site was occluded for 24 hours. All animals were observed daily for 14 days and body weights were recorded at weekly intervals post dosing.

No mortality was observed. There were no clinical signs of reaction to treatment observed in any animal throughout the study. There was no evidence of a dermal response to treatment observed in any animal throughout the study. A slightly reduced body weight was evident in 2/5 females on Day 8. All other animals were considered to have achieved satisfactory bodyweight gains throughout the study.

No macroscopic abnormalities were observed for animals killed at study termination on Day 15. The acute lethal dermal dose (LD_{50}) to rats of fluopicolide (AE C638206) was greater than 5000 mg/kg bw and thus greater than the trigger value of 2000 mg/kg bw.

10.2.2 Comparison with the CLP criteria

The application on the guidance of the CLP criteria (Regulation (EC) No 1272/2008) gives a cut off LD50 value of 2000 mg/kg bw for acute dermal toxicity classification. Under the conditions of this study fluopicolide (AE C638206) had an LD_{50} of > 2000 mg/kg bw and as such **no classification** for acute dermal toxicity is proposed.

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

The data on the acute dermal toxicity potential of fluopicolide (AE C638206) are conclusive. Based on the LD_{50} value of > 5000 mg/kg bw after acute dermal administration to rats, according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008), an acute dermal toxicity classification of fluopicolide (AE C638206) is **not warranted**.

10.3 Acute toxicity - inhalation route

The acute toxicity of fluopicolide via the inhalation route has been investigated in rats.

Table 10-7: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, form and particle size (MMAD)	Dose levels, duration of exposure	Value LC_{50}	Reference
Acute inhalation toxicity OECD 403 (1981) GLP	Rat, Sprague-Dawley Crl:CD®BR male & female 5/sex/dose level	Fluopicolide (purity 98.3%) Dust MMAD ± GSD = 3.37 ± 2.09 µm	Mean concentration of 5.16 mg/L (9.09 mg/L nominal) was administered by 4 hour nose-only exposure	>5.16 mg/L (4 hours)	Anonymous; 2000; M-197229-01-1

Table 10-8: Summary table of human data on acute inhalation toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-9: Summary table of other studies relevant for acute inhalation toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.3.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

The acute inhalation toxicity of fluopicolide (AE C638206) was investigated by exposing a group of five male and five female Sprague-Dawley (CD) rats to a dust atmosphere of the limit concentration of test substance of 5.16 mg/L. The test group was subjected to a single four-hour, continuous, snout only exposure. Signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were sacrificed at the end of the observation period and were subjected to detailed necropsy.

No mortality was recorded. Common observations noted both during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen. Animals recovered quickly to appear normal on the first day after exposure. Normal bodyweight gain was noted during the study.

No macroscopic abnormalities were noted for 9/10 animals. One male showed dark foci on its lungs. The LC_{50} was therefore > 5.16 mg/L.

10.3.2 Comparison with the CLP criteria

The guidance of the application of the CLP criteria (Regulation (EC) No 1272/2008) gives a 4-hour LC_{50} cut-off value of 5 mg/L to trigger classification for acute inhalation toxicity. Under the conditions of this study the 4-hour LC_{50} of fluopicolide was > 5.16 mg/L. **No classification** for acute inhalation toxicity is proposed.

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

The data on the acute inhalation toxicity potential of fluopicolide (AE C638206) are conclusive. Based on the LC_{50} value of > 5.16 mg/L after acute inhalative administration to rats according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008)) an acute inhalation toxicity classification of fluopicolide (AE C638206) is **not warranted**.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS summarised three acute toxicity studies in the CLH report.

Acute oral toxicity

In an OECD TG 423 compliant oral acute toxicity study (acute toxic class method) conducted in 2000, 5 male and 5 female fasted Sprague-Dawley rats were each administered by gavage a single oral dose of 5000 mg/kg bw. There were no mortality. Clinical signs as piloerection and hunched posture were seen in all female rats and in three male rats, and abnormal gait was noted in three females. Recovery of rats, as judged by external appearance and behavior, was complete by day 3. No significant effect was reported on body weight gains throughout the study. The acute lethal oral dose of fluopicolide in rats was greater than 5000 mg/kg bw. The DS proposed not to classify for acute oral toxicity.

Acute dermal toxicity

An OECD TG 402 acute dermal limit toxicity study was conducted in Hsd Sprague-Dawley rats in 2000 with 97.7% pure fluopicolide. A single topical application of 5000 mg/kg bw of the test substance was applied under occlusion for 24 hours.

No animals died. No clinical signs or dermal response in any animal was observed throughout the study. Two females had a slightly reduced body weight on day 8. There were no macroscopic abnormalities at study termination on day 15. The fluopicolide acute lethal dermal dose (LD_{50}) to rats was greater than 5000 mg/kg bw and thus greater than the trigger value of 2000 mg/kg bw, and therefore the DS proposed not to classify fluopicolide for acute dermal toxicity.

Acute inhalation toxicity

A limit test in accordance with OECD TG 403 exposing five male and five female Sprague-Dawley rats nose only to 5.16 mg/L for four hours did not lead to any mortality.

Clinical signs during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen, but all animals recovered within a day after exposure. At autopsy, one male showed dark foci on its lungs. The LC_{50} was thus > 5.16 mg/L.

No classification for acute inhalation toxicity was proposed by the DS.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Limit acute toxicity tests were conducted with fluopicolide following oral, dermal and inhalation route. For the oral route, a dose of 5000 mg/kg bw was used, and no mortalities were reported. Thus, the criteria for classification (2000 mg/kg bw) for acute oral toxicity was no met, and no classification is warranted.

For the dermal route, no mortalities were reported at the limit dose of 2000 mg/kg bw, which also is the classification limit dose for classification for acute dermal toxicity. No classification for the dermal route is therefore warranted.

With respect to the inhalation route, a concentration of 5.16 mg/L for 4 hours was used. No mortalities at the doses tested were reported. As the highest limit for classification is 5 mg/L for 4h for dusts and mists, no classification for acute toxicity by inhalation is warranted.

RAC agrees with the DS that **no classification for acute toxicity through any route is warranted for fluopicolide.**

10.4 Skin corrosion/irritation

The potential of fluopicolide to induce acute skin corrosion or irritation has been investigated in rabbits.

Table 10-10: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance**	**Dose levels, duration of exposure**	**Results** **- Observations and time point of onset** **- Mean scores/ animal** **- Reversibility**	**Reference**
Acute skin irritation study OECD 404 (1992) GLP	Rabbit, New Zealand White albino Females 3/group	Fluopicolide (purity 97.7%)	0.5 g (powder moistened with water prior applying to skin) 4 hours, semi-occlusive	None of the three rabbits showed any substance-related lesions at the examination time-points 1, 24, 48 and 72 hours after patch removal. The mean irritation score over 24 – 72 h was 0.0 for erythema and oedema respectively.	Anonymous; 2000; M-197226-01-1

Table 10-11: Summary table of human data on skin corrosion/irritation

Type of data/report	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No human data				

Table 10-12: Summary table of other studies relevant for skin corrosion/irritation

Type of study/data	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No other data				

10.4.1 Short summary and overall relevance of the provided information on skin corrosion/irritation

The potential of fluopicolide (AE C638206) to cause inflammatory or corrosive changes upon first contact with skin was assessed by semi-occluded application of 0.5 g of the test material to the closely-clipped dorsa of three New Zealand White rabbits for four hours. Dermal reactions were assessed 1, 24, 48 and 72 hours after removal of the dressings.

The single semi-occlusive application of fluopicolide (AE C638206) to intact rabbit skin for four hours elicited no dermal irritation in any animal during the study. The mean irritation score over 24 – 72 h was 0.0 for erythema and oedemafor all animals.

10.4.2 Comparison with the CLP criteria

The guidance on the application of the CLP criteria (Regulation (EC) No 1272/2008) requires that mean irritation score are > 2.3 for erythema/eschar in at least 2 out of 3 animals, before classification as a skin irritant is triggered. The scores for erythema/eschar for fluopicolide in this study were 0 for all animals. Fluopicolide was found to be not irritating to the skin of the rabbit under the conditions of this study, therefore **no classification** for skin corrosion/irritation is proposed.

10.4.3 Conclusion on classification and labelling for skin corrosion/irritation

Since this valid skin corrosion/irritation study with fluopicolide (AE C638206) in rabbits did not show signs of skin irritation, the results are conclusive, but do **not warrant** classification as skin irritant according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008)).

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS summarised an OECD TG 404 compliant skin irritation study by semi-occluded application of 0.5 g of moistened fluopicolide for four hours using 3 New Zealand White rabbits (Anonymous, 2000). Dermal reactions were assessed 1, 24, 48 and 72 hours after removal of the dressings. No dermal irritation in any animal was elicited (mean irritation score 0.0 for erythema and oedema for all animals over 24 – 72h).

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

As the scores for erythema/eschar for fluopicolide in this guideline compliant dermal irritation study in rabbits were 0 for all animals, the criteria > 2.3 for erythema/eschar in at least 2 out of 3 animals is not met. RAC thus concurs with the DS that no classification for skin corrosion/irritation is warranted.

10.5 Serious eye damage/eye irritation

The potential of fluopicolide to induce serious eye damage / eye irritation has been investigated in rabbits.

Table 10-13: Summary table of animal studies on serious eye damage/eye irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results - Observations and time point of onset - Mean scores/animal - Reversibility	Reference
Acute eye irritation study OECD 405 (1987) GLP	Rabbit, New Zealand White albino Females 3/group	Fluopicolide (purity: 97.7%)	100 mL (93 mg)	Eyes were examined and irritation was assessed at 1, 24, 48 and 72 hours after administration; mean scores were calculated from 24-72 h values The mean eye irritation score (24/48/72 hours) for conjunctival redness was 0.33 in 2 animals and 0 in 1 animal and 0 in all 3 animals for all other endpoints scored. Reactions had resolved in all instances two days after instillation. Thus, fluopicolide was transiently slightly irritant to the rabbit eye.	Anonymous; 2000; M-197227-01-1

Table 10-14: Summary table of human data on serious eye damage/eye irritation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-15: Summary table of other studies relevant for serious eye damage/eye irritation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.5.1 Short summary and overall relevance of the provided information on serious eye damage/eye irritation

The eye irritating potential of fluopicolide was investigated in rabbits. A single dose of 100 mL of fluopicolide powder was administered to one eye each of three Himalayan rabbits. Eye irritation was assessed at 1, 24, 48 and 72 hours.

A single instillation of fluopicolide into the unrinsed eye of the rabbit elicited slight conjunctival irritation in all animals (in one animal minimal (grade 1) conjunctival redness only at the 1 hour observation). The ocular reactions resolved in all instances within two days after instillation. There were no observations of corneal opacity, iritis or chemosis. Thus, fluopicolide was transiently slightly irritant to the rabbit eye.

10.5.2 Comparison with the CLP criteria

According to the grading criteria as described in Regulation (EC) No 1272/2008, substances that produce in at least 2 of 3 tested animals, a positive response of: (a) corneal opacity ≥ 1 and/or (b) iritis ≥ 1, and/or (c) conjunctival redness ≥ 2 and/or (d) conjunctival oedema (chemosis) ≥ 2 calculated as the mean scores following grading at 24, 48 and 72 hours after installation of the test material, and which fully reverses within an observation period of 21 days shall be classified as category 2 eye irritant.

These criteria were not met at any observation point for any animal in the study. Mean scores for 24, 48 and 72 hours post instillation were 0.0 for all parameters, with the exception of conjunctival redness, which had a score of 0.33 in two out of three animals. The ocular reactions resolved in all instances within two days after instillation.Therefore **no classification** for serious eye damage/eye irritation is proposed.

10.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

The results of the eye irritation study with fluopicolide are conclusive, but they do **not warrant** an eye irritation classification according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008).

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The eye irritating potential of fluopicolide was investigated in 2000 in an OECD TG 405 compliant study in New Zealand White rabbits, using instillation of a single dose of 100 mg of fluopicolide powder to one eye each of three rabbits. Conjunctival redness was seen in two animals with a mean score of 0.33 at the 24, 48 and 72h after exposure readings. The effect was reversed 2 days after exposure. Scores were 0 for chemosis, iritis and corneal opacity at all observation points. Thus, fluopicolide was slightly irritant to the rabbit eye but the effects were transient.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The criteria for classification for eye irritation were not met at any observation point for any animal in the study. All parameters (corneal opacity, iritis and/or conjunctival oedema (chemosis) had mean scores for 24, 48 and 72 hours post instillation of 0.0, whilst the mean score for conjunctival redness was 0.33 in two out of three animals, thus none of the criteria for classification are fulfilled. The slight ocular reactions resolved within two days after instillation. The DS proposed no classification for serious eye damage/eye irritation. RAC concurs with the DS that **no classification of fluopicolide for eye irritation/eye damage is warranted.**

10.6 Respiratory sensitisation

No data on respiratory sensitisation available. Fluopicolide was of low toxicity in an acute inhalation study and was negative in a skin sensitisation study (see below); therefore, it is unlikely that it would induce respiratory sensitisation.

10.6.1 Short summary and overall relevance of the provided information on respiratory sensitisation

Medical surveillance data on manufacturing personnel was obtained during the pilot-scale production of fluopicolide (2004-2005 in Lyon, France, and Dormagen, Germany). No incidences of adverse reactions were reported during the pilot-scale manufacture/formulation of fluopicolide (fluopicolide DAR 2005). No formally recognised and validated animal tests currently exist for respiratory sensitisation. However data from some animal studies may be indicative of the potential of a substance to cause respiratory sensitisation in humans and may provide supportive evidence in case human evidence is available. This information may also be combined with information on structural alerts for respiratory sensitisation and information on the skin sensitising properties of a substance and should be used in a weight of evidence assessment.

In the animal studies conducted with fluopicolide, no evidence of respiratory tract irritation (local cytotoxic effects) was obvious; also the acute rat inhalation data did not provide evidence for functional impairment of the respiratory system. Moreover, fluopicolide has no skin sensitizing potential. According to the CLP criteria, a substance which is negative in sensitization assays, most probably also lacks the potential for respiratory allergy so that based on this no evidence of a respiratory sensitization potential of fluopicolide exists. Since in addition the skin and eye irritation studies in rabbits, and the rat acute and repeated dose dermal toxicity studies did not indicate an irritating potential on skin and mucous membranes, a respiratory irritation potential of fluopicolide is not likely.

10.6.2 Comparison with the CLP criteria

According to the CLP criteria for respiratory sensitization, evidence that a substance can lead to specific hypersensitivity will normally be based on human experience. In this context, hypersensitivity is normally seen as asthma, but other hypersensitivity reactions such as rhinitis/conjunctivitis and alveolitis are also considered. The condition will have the clinical character of an allergic reaction.

In the animal studies with fluopicolide, no evidence of respiratory tract irritation (local cytotoxic effects) was obvious, also the acute rat inhalation data do not provide evidence for functional impairment of the respiratory system. According to the CLP criteria, substances which are negative in sensitization studies, most likely have no potential for respiratory allergy. Therefore, based on this no evidence of a respiratory sensitization potential of fluopicolide exists. Also the skin and eye irritation studies in rabbits, and the rat acute and repeated dose dermal toxicity studies did not indicate a severe irritating or corrosive potential on skin and mucous membranes, so that a respiratory irritation potential of fluopicolide (AE C638206) is not likely.

10.6.3 Conclusion on classification and labelling for respiratory sensitisation

Since in the animal studies with fluopicolide (AE C638206) no evidence of respiratory sensitisation was obvious (no evidence of local irritation, respiratory tract impairment or skin sensitisation) and since limited human data (up to 2005) shows no evidence of adverse respiratory effects, no classification for respiratory sensitisation is proposed.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

No data investigating specifically respiratory sensitisation potential of fluopicolide are available from humans nor animals. The DS evaluated the end-point of respiratory sensitisation in a weight of evidence approach including information from medical surveillance of workers in two fluopicolide production plants in which no adverse reactions were reported, and information from available animal studies that showed low systemic toxicity or local reactivity with skin and mucous membranes. Finally, the negative skin sensitisation study (Guinea pig maximization test) described below was also included in the evaluation. The DS concluded overall that fluopicolide is unlikely to induce of respiratory sensitisation.

Assessment and comparison with the classification criteria

According the criteria for classification for respiratory sensitisation the evidence for this endpoint will normally be based on human experience. The criteria further state that (a) the size of the population exposed, and (b) the extent of exposure is taken into account. There is no positive evidence of a respiratory sensitisation potential of fluopicolide in humans. However, RAC's assessment is that due to the lack of details on e.g. exposure levels provided in the information from production sites, the negative data are insufficient to be used to conclude that the substance is not a respiratory sensitiser.

None of the alerts included in the IR&CSA guidance are present in fluopicolide.

The DS included information from acute toxicity and irritation tests to support the evaluation that fluopicolide. RAC agrees with the DS that there is a low potential for respiratory sensitisation, although the nature of tests to be included is not clear from the criteria nor the guidance on classification.

RAC notes that reference is made in the guidance to using skin sensitising properties of the substance, e.g. from non-standard versions of the LLNA in the assessment. For fluopicolide, no LLNA is available, but a negative GPMT was included by the DS in the weight of evidence evaluation leading to the conclusion that the data available were sufficient to conclude that fluopicolide is not a respiratory sensitizer.

RAC agrees with the DS that fluopicolide should not be classified for this hazard class. However, RAC was not prepared to base their conclusion solely on negative data from studies that are not designed for the assessment of respiratory sensitisation.

In conclusion, RAC considers that **fluopicolide does not warrant classification for respiratory sensitisation due to insufficient data.**

10.7 Skin sensitisation

The skin sensitising potential of fluopicolide has been investigated in a guinea-pig maximisation test (GMPT).

Table 10-16: Summary table of animal studies on skin sensitisation

Method, guide-line, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results	Reference
Guinea-pig maximisation test (GPMT according to Magnusson and Kligman) OECD 406 (1992) GLP	Guinea pigs, Dunkin-Hartley guinea-pigs Females 20 animals for the test item group and 10 control animals	Fluopicolide (purity: 97.7%)	Intradermal induction 10% w/v fluopicolide in sterile water or in a 50:50 mixture of Freund's complete adjuvant in sterile water Topical induction 6 days later 100% w/v fluopicolide in sterile water, occlusive dressing for 48 hours. Challenge Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank.	**Negative** Slight erythema was observed in 2/20 test animals (10%) at the 24 and 48 hour reading compared to slight to well-defined erythema for 2/10 control animals (20%) at the 48 hour reading only. As the reactions observed were of similar severity and the incidence was greater in control animals (20% of controls compared with 10% of tested animals) and no reactions were observed for any of the remaining animals the reactions in the test animals are not considered relevant.	Anonymous; 2000; M-197228-01-1

Table 10-17: Summary table of human data on skin sensitisation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-18: Summary table of other studies relevant for skin sensitisation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

The potential of fluopicolide to cause delayed contact hypersensitivity in guinea pigs was investigated in a GPMT according to Magnusson-Kligman. Based on a preliminary study, the closely-clipped backs of twenty female Dunkin-Hartley guinea-pigs were subject to intradermal injections of Freund's Complete Adjuvant, 10% w/v fluopicolide (AE C638206) in sterile water and 10% w/v fluopicolide (AE C638206) in a 50:50 mixture of Freund's complete adjuvant in sterile water on Day 1. Six days later, the same area of skin was treated by topical application of 100% w/v fluopicolide in sterile water and the test site was covered by an occlusive dressing for 48 hours. The same induction procedures were carried out on 10 control animals, except that the test material was replaced by vehicle in all doses. Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank. The occlusive dressings were removed on the following day and the condition of the test sites was assessed approx. 24 and 48 hours later.

There were no deaths or signs of ill health or toxicity. Body weight changes were similar between control and treated animals. Necrosis was observed at sites receiving Freund's Complete Adjuvant in all test and control animals following intradermal injections. Slight irritation was seen in 6/20 animals on the site treated with 10% w/v fluopicolide in sterile water. No irritation was observed in controls. After topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was seen in one control guinea-pig.

The challenge application produced no dermal reactions indicative of skin sensitization in any of the animals. Slight erythema was observed in two test animals at the 24 and 48 hour reading compared with slight to well-defined erythema for two control animals at the 48 hour reading only. The reactions observed were noted to be of similar incidence and severity and as no reactions were observed for any of the remaining test or control animals, the overall response was considered negative.

Fluopicolide was not a skin sensitizer in this GPMT.

10.7.2 Comparison with the CLP criteria

In a GPMT with fluopicolide a similar incidence and severity of reactions were observed (10% of animals of the test group and 20% of the control responded); Therefore, the overall response is considered negative. Moreover, the incidence of 10% in the test group is below the guidance value of responses in ≥ 30% of animals in an adjuvant test that would lead to classification. Therefore **no classification** for skin sensitisation is proposed.

10.7.3 Conclusion on classification and labelling for skin sensitisation

Since for fluopicolide no evidence of a sensitizing potential from a Maximization study in guinea-pigs exists, the data are conclusive that they do **not warrant** a skin sensitization classification of fluopicolide according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008).

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The skin sensitising potential of fluopicolide has been investigated in an OECD TG 406 compliant guinea-pig maximisation test (GMPT) using 10% w/v fluopicolide for intradermal induction with or without Freund's complete adjuvant, whilst the topical induction was performed 6 days later with 100% w/v fluopicolide under occlusive dressing for 48 hours. Challenge two weeks after topical induction under occlusion for 24 hours used 100% fluopicolide and 50% fluopicolide. In all cases, sterile water was used as vehicle.

Intradermal induction sites receiving Freund's Complete Adjuvant in all test and control animals showed necrosis. Slight irritation was seen in 6/20 animals on the site treated with the intradermal injections of 10% w/v fluopicolide in sterile water. No irritation was observed in controls. After topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was seen in one control guinea pig.

Challenge resulted in two out of 20 test animals showing slight erythema at the 24 and 48 hour readings. In the control animals, 2 out of 10 animals reacted with slight to well-defined erythema at the 48-hour reading only.

Due to the comparable reaction in the controls at a higher incidence than in the test group, and the lack of reactions in the other test animals, the DS considered the overall response to be negative. No classification for skin sensitisation is proposed.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

The incidence of slight erythema in the tested animals in 2 out of 20 animals at 24 and 48 hours after challenge is below the value relevant for classification. RAC notes that the reactions in the 2/10 controls at 48 hours after induction may be interpreted as a reduced reliability of the study. In conclusion, RAC agrees with the DS that **fluopicolide should not be classified for skin sensitisation based on the available evidence**.

10.8 Germ cell mutagenicity

The genotoxic potential of fluopicolide has been investigated in nine *in vitro* studies, covering the end-points bacterial- and mammalian-cell mutation and clastogenicity, and in five *in vivo* assays (an unscheduled DNA synthesis assay in rat liver, three mouse micronucleus tests and one Comet assay in mice).

Table 10-19: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide (purity 97.8%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9) Experiment II/III (TA98 only): 50; 160; 500; 1600; 2000; 3000; 4000 and 5000 µg/plate (+S9) <u>Pre-incubation</u> Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9)	**Positive** at precipi-tating dose levels.	Anonymous; 2004; M-197259-02-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide Purity not reported DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202931-01-1

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide (purity 95.6%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202927-01-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide (purity 95.9%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202939-01-1

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide (purity 99.3%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202935-01-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide (purity 98.2%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 TA 100 and TA 102 The following concentrations were tested: Plate incorporation Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (±S9) Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated (see experiment Ia). Experiment Ia (TA102 only): 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (+S9) Pre-incubation Experiment II: 10; 33; 100; 333; 1000; 2500 and 5000 µg/plate (±S9)	**Negative**	Anonymous; 2017; M-595228-01-1

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
In vitro chromosome aberration assay in Chinese hamster lung V79 cells OECD 473 (1997) GLP Deviations: Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide (purity 97.8%) DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: <u>Experiment I:</u> 25; 50; 75 and 100 µg/mL (±S9) <u>Experiment II:</u> 1.6; 3.2 and 6.3 µg/mL (-S9)	**Positive** at cytotoxic concentrations	Anonymous; 2004; M-197260-02-1
In vitro chromosome aberration assay in human lymphocytes OECD 473 (1997) GLP Deviations: Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide (purity 95.9%) DMSO	Test system: Human lymphocytes The following concentrations were tested: <u>Experiment I:</u> 19.53; 78.13 and 156.25 µg/mL (-S9) 78.13; 312.5 and 625 µg/mL (+S9) <u>Experiment II:</u> 1.22; 9.77 and 19.53 µg/mL (-S9) 39.06; 156.25 and 312.5 µg/mL (+S9)	**Negative**	Anonymous; 2001; M-201582-01-1

Method, guideline, deviations if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
In vitro HPRT mutation assay in Chinese hamster lung V79 cells OECD 476 (1997) GLP	Fluopicolide (purity 97.8%) DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: <u>Experiment I:</u> 1.2; 3.8; 12.1; 38.2; 120.8; 382; 1208 and 3820 µg/mL (±S9) <u>Experiment II:</u> 0.4; 0.8; 1.6; 3.2; 6.3; 12.5; 25; 50; 75; 100 and 120 µg/mL (±S9) <u>Experiment III:</u> 0.313; 0.625; 1.25; 2.5; 5; 10; 20; 30; 40; 50 and 60 µg/mL (±S9)	**Negative**	Anonymous; 2005; M-210831-02-1

Table 10-20: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations/ Results	Reference
Mouse micronucleus test OECD 474 (1997) GLP Deviations: Target organ exposure not measured. Only 2000 instead of 4000 erythrocytes were analysed for MN. Only 200 instead of 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide (purity 97.8%), in 1% (w/v) methylcellulose	Test system: Mouse (HsdWin:NMRI) The following concentrations were tested: 200, 600 and 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2005; M-197261-02-1

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations/ Results	Reference
Mouse micronucleus test OECD 474 (1997) GLP Deviations: Target organ exposure not measured. Only 2000, not 4000 erythrocytes, were analysed for MN. Only 200, not 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide (purity 96.1%), in 1% (w/v) methylcellulose	Test system: Mouse (Crl:CD1) The following concentration was tested: 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2003; M-219364-01-1
Mouse micronucleus test (i.p.) OECD 474 (1997) GLP Deviations: Altered NCE/PCE ratio. Only 2000, not 4000 erythrocytes, were analysed for MN.	Fluopicolide (purity 99.4%), in 0.5% cremophor	Test system: Mouse (Crl:CD1) The following concentrations were tested: 150, 300 and 600 mg/kg bw (i.p.)	**Negative**	Anonymous; 2003; M-223119-01-1
Rat UDS assay OECD 486 (1997) GLP	Fluopicolide (purity 97.7%), in 1% (w/v) methylcellulose	Test system: Rat (Hsd/Ola SD) The following concentrations were tested: 600 and 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2000; M-197230-02-1

Method, guideline, deviations if any	Test substance	Relevant information about the study (as applicable)	Observations/ Results	Reference
Comet assay in mice OECD 489 (2016) GLP	Fluopicolide (purity 98.2%), in 1% (w/v) methylcellulose	Test system: Mouse (Hsd:ICR (CD-1)), male, 6/dose, liver and kidney cells examined The following concentrations were tested: 500, 1000 and 2000 mg/kg bw (oral, gavage, dose volume 10 mL/kg) Positive control: methyl methanesulfonate (MMS) 40 mg/kg bw Two doses were administered approx. 21 h apart (positive control dose was administered once on day 2) Animals were euthanized 3-4 h following last treatment 150 cells/animal examined	**Negative** No dose increase observed in % tail DNA Positive control gave the expected results The % tail DNA in the negative control was within the laboratory historical control data	Anonymous; 2018; M-635020-01-1

Table 10-21: Summary table of human data relevant for germ cell mutagenicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

Several genotoxicity studies were performed for fluopicolide: six reverse gene mutation tests in *Salmonella typhimurium* and *Escherichia coli* strains of bacteria, one of them recently (2017), one chromosomal aberration assay in Chinese hamster V79 cells *in vitro*, one chromosomal aberration assay in human lymphocytes *in vitro*, one HPRT mutation assay in Chinese hamster V79 cells, two *in vivo* micronucleus assays in mouse bone marrow cells with oral administration, one *in vivo* micronucleus assay in mouse bone marrow cells with intraperitoneal administration, one *in vivo* UDS assay in rat hepatocytes by oral route and one *in vivo* Comet assay in male mice with oral gavage administration. Several deviations were reported from the current OECD test guidelines for a number of these studies, specifically, two *in vitro* chromosome abberation tests (OECD 473) and three *in vivo* micronucleus tests (OECD 474); however, the dossier submitter considers that these deviations do not affect the validity or results of the studies.

One of the earlier five bacterial reverse mutation assay showed a very slight increase in the number of revertant colonies in only one strain (TA 98) and only with metabolic activation at the highest concentration of 5000 µg/plate where precipitation was observed. Therefore, this result was considered of doubtful biological significance and four additional assays were conducted. No evidence of mutagenic activity of fluopicolide was observed in the four additional bacterial reverse mutation assays performed with five *Salmonella typhymurium* strains and one *Escherichia coli* strain. In addition a recently conducted bacterial reverse mutation assay (2017) was also negative and also confirmed the overall negative outcome in this study type. Furthermore, a Comet assay was also recently (2018) performed to confirm the negative profile for the endpoint gene mutation *in vivo*. In this *in vivo* Comet assay, no statistically significant or dose related increases in % tail DNA were observed in liver or kidney cells of treated male mice (6/dose) up to doses of 2000 mg/kg bw/day. The positive

control gave the expected response and the increase in % tail DNA observed in the vehicle control group was within the range of the laboratory historical control data, thus confirming the validity of the study (a detailed summary of this comet assay is provided in Annex I of this CLH report).

The chromosomal aberration assay performed in Chinese hamster V79 showed a positive response. However, the increase of aberrant cells occured at cytotoxic concentrations where mitotic indices were clearly below the limit of 50% indicating the doubtful biological signifiance of these data. This chromosome aberration assay was therefore repeated in human lymphocytes and gave a clear negative response. Moreover, two *in vivo* micronucleus assays were performed in mice by the oral route up to the limit dose of 2000 mg/kg bw. Both of these assays were negative. However, one was of questionable biological significance due to the slight increase of micronucleated polychromatic erythrocytes in bone marrow of some animals given 2000 mg/kg bw as well as in one control animal. As the ratio of polychromatic to normochromatic erythrocytes was not significantly affected and no clinical signs were observed in both assays, a third assay was performed in mice by the intraperitoneal route to increase the likelihood of bone marrow exposure. This assay gave a clear negative result for clastogencity *in vivo* at dose levels showing clear cytotoxicity of the bone marrow.

The HPRT mutation assay in Chinese hamster V79 cells was negative. Moreover, the *in vivo* rat hepatocyte UDS assay clearly showed that fluopicolide does not induce damage to DNA.

10.8.2 Comparison with the CLP criteria

In summary, 2 out of 15 tests (one *in vitro* bacterial reverse mutation assay and one *in vitro* chromosome aberration assay) gave weak positive responses of doubtful biological significance. The *in vivo* mutagenicity data from Comet assay and micronucleus tests are reliable and it is clear that no mutagenic effects were seen in whole animals. Clearly, chromosomal damage or point mutations do not occur *in vivo*. Overall, therefore, it is concluded that fluopicolide is not genotoxic *in vivo*. Furthermore, in the carcinogenicity studies (see section 10.9) fluopicolide caused an increase in hepatocellular adenomas in male and female mice by a mechanism considered not relevant to humans, and increased neoplasms were observed only at or above the maximum tolerated dose (MTD).

No information is available on the genotoxicity of fluopicolide in humans. Therefore, it clearly does not meet the criteria for classification in category 1A. Since fluopicolide was negative in *in vivo* tests in mammals and there is no information on its mutagenicity in germ cells, classification in category 1B is not appropriate.

The lack of histopathological findings in the male and reproductive organs would suggest (in the absence of relevant toxicokinetic data) that the potential for fluopicolide or its metabolites to reach and interact with the genetic material of the germ cells is low. Classification for germ cell mutagenicity category 2 may be considered on the basis of positive somatic cell mutagenicity tests *in vivo*, in mammals; or other positive *in vivo* somatic cell genotoxicity tests that are supported by positive results from *in vitro* mutagenicity assays; or positive *in vitro* mammalian mutagenicity assays for substances that also show chemical structure activity relationship to known germ cell mutagens. Since none of these conditions was met, classification in category 2 is not appropriate.

No classification for germ cell mutagenicity is proposed.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Since the results of the conducted guideline genotoxicity studies with fluopicolide did not reveal a genotoxic potential, the criteria for genotoxicity classification are not met. Thus, the data for fluopicolide are conclusive, but they do **not warrant** genotoxicity classification according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008).

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The genotoxic potential of fluopicolide has been investigated in nine *in vitro* studies, covering the end-points bacterial- and mammalian-cell mutation and clastogenicity, and in five *in vivo* assays (an unscheduled DNA synthesis assay in rat liver, three mouse micronucleus tests and one Comet assay in mice).

Several genotoxicity studies were performed for fluopicolide: six reverse gene mutation tests in *Salmonella typhimurium* and *Escherichia coli* strains of bacteria, one of them recently (2017), one chromosomal aberration assay in Chinese hamster V79 cells *in vitro*, one chromosomal aberration assay in human lymphocytes *in vitro* and one HPRT mutation assay in Chinese hamster V79 cells. In addition, two *in vivo* micronucleus assays were performed in mouse bone marrow cells with oral administration, one *in vivo* micronucleus assay in mouse bone marrow cells with intraperitoneal administration, one *in vivo* oral UDS assay in rats and one *in vivo* Comet assay in male mice with oral gavage administration.

2 out of 15 tests, one *in vitro* bacterial reverse mutation assay and one *in vitro* chromosome aberration assay, gave weak positive responses of doubtful biological significance. The *in vivo* mutagenicity data from the Comet assay and micronucleus tests are reliable and it is clear that no mutagenic effects were seen in animals *in vivo*. Clearly, chromosomal damage or point mutations do not occur *in vivo*. Overall, the DS concluded that fluopicolide is not genotoxic *in vivo*.

***Table**: Summary table of mutagenicity/genotoxicity tests in vitro*

Method	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide (purity 97.8%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 50, 160, 500, 1600 and 5000 µg/plate (±S9) Experiment II/III (TA98 only): 50, 160, 500, 1600, 2000; 3000, 4000 and 5000 µg/plate (+S9) <u>Pre-incubation</u> Experiment I: 50, 160, 500, 1600; and 5000 µg/plate (±S9)	Positive at precipitating dose levels.	Stammberger, 2004; M-197259-02-1

Method	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide Purity not reported DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6, 8, 40, 200, 1000 and 5000 µg/plate (±S9) Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (+S9)	Negative	Ballantyne, 2001; M-202931-01-1
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide (purity 95.6%) DMSO	Test system: Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and Escherichia coli WP2uvrA The following concentrations were tested: Plate incorporation Experiment I: 1.6, 8, 40, 200, 1000 and 5000 µg/plate (±S9) Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (+S9)	Negative	Ballantyne, 2001; M-202927-01-1
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide (purity 95.9%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6, 8, 40, 200, 1000 and 5000 µg/plate (±S9) Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (+S9)	Negative	Ballantyne, 2001; M-202939-01-1

Method	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide (purity 99.3%) DMSO	Test system: Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and Escherichia coli WP2uvrA The following concentrations were tested: Plate incorporation Experiment I: 1.6, 8, 40, 200, 1000 and 5000 µg/plate (±S9) Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25, 62.5, 125, 250, 500 and 1000 µg/plate (+S9)	Negative	Ballantyne, 2001; M-202935-01-1
Bacterial point mutation assay (Ames test) OECD TG 471 (1997) GLP	Fluopicolide (purity 98.2%) DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 TA 100 and TA 102 The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate (±S9) Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated (see experiment Ia below). Experiment Ia (TA102 only): 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate (+S9) <u>Pre-incubation</u> Experiment II: 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate (±S9)	Negative	Chang, 2017; M-595228-01-1
In vitro chromosome aberration assay in Chinese hamster lung V79 cells OECD TG 473 (1997) GLP	Fluopicolide (purity 97.8%) DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 25, 50, 75 and 100 µg/mL (±S9) Experiment II: 1.6, 3.2 and 6.3 µg/mL (-S9) Cytotoxicity was not measured using the parameters of relative population doubling or relative increase in cell count. Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Positive at cytotoxic concentrations	Stammberger and Graeser, 2004; M-197260-02-1
In vitro chromosome aberration	Fluopicolide (purity 95.9%)	Test system: Human lymphocytes The following concentrations were tested:	Negative	Allais, 2001; M-201582-01-1

Method	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations/ Results	Reference
assay in human lymphocytes OECD TG 473 (1997) GLP	DMSO	<u>Experiment I:</u> 19.53, 78.13 and 156.25 µg/mL (-S9) 78.13, 312.5 and 625 µg/mL (+S9) <u>Experiment II:</u> 1.22, 9.77 and 19.53 µg/mL (-S9) 39.06, 156.25 and 312.5 µg/mL (+S9) Cytotoxicity was not measured using the parameters of relative population doubling or relative increase in cell count. Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.		
In vitro HPRT mutation assay in Chinese hamster lung V79 cells *OECD TG 476 (1997)* *GLP*	Fluopicolide (purity 97.8%) DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 1.2, 3.8, 12.1, 38.2, 120.8, 382, 1208 and 3820 µg/mL (±S9) Experiment II: 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, 25, 50, 75, 100 and 120 µg/mL (±S9) Experiment III: 0.313, 0.625, 1.25, 2.5, 5, 10, 20, 30, 40, 50 and 60 µg/mL (±S9)	Negative	Graeser andStammberger, 2005; M-210831-02-1

***Table**: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo*

Method	Test substance	Relevant information about the study (as applicable)	Observations/ Results	Reference
Mouse micronucleus test OECD TG 474 (1997) GLP	Fluopicolide (purity 97.8%),in 1% (w/v) methylcellulose	Test system: Mouse (HsdWin:NMRI) The following concentrations were tested: 200, 600 and 2000 mg/kg bw (oral) Target organ exposure not measured. Only 2000 instead of 4000 erythrocytes were analysed for MN. Only 200 instead of 500 cells were analysed to obtain the PCE/NCE ratio.	Negative	Anonymous, 2005; M-197261-02-1
Mouse micronucleus test OECD TG 474 (1997) GLP	Fluopicolide (purity 96.1%), in 1% (w/v) methylcellulose	Test system: Mouse (Crl:CD1) The following concentration was tested: 2000 mg/kg bw (oral). Target organ exposure not measured. Only 2000, not 4000 erythrocytes, were analysed for MN. Only 200, not 500 cells were analysed to obtain the PCE/NCE ratio.	Negative	Anonymous, 2003; M-219364-01-1

Method	Test substance	Relevant information about the study (as applicable)	Observations/ Results	Reference
Mouse micronucleus test (i.p.) OECD TG 474 (1997) GLP	Fluopicolide (purity 99.4%), in 0.5% cremophor	Test system: Mouse (Crl:CD1) The following concentrations were tested: 150, 300 and 600 mg/kg bw (i.p.) Altered NCE/PCE ratio. Only 2000, not 4000 erythrocytes, were analysed for MN.	Negative	Anonymous, 2003; M-223119-01-1
Rat UDS assay OECD TG 486 (1997) GLP	Fluopicolide (purity 97.7%), in 1% (w/v) methylcellulose	Test system: Rat (Hsd/Ola SD) The following concentrations were tested: 600 and 2000 mg/kg bw (oral)	Negative	Anonymous, 2000; M-197230-02-1
Comet assay in mice OECD TG 489 (2016) GLP	Fluopicolide (purity 98.2%), in 1% (w/v) methylcellulose	Test system: Mouse (Hsd:ICR (CD-1)) The following concentrations were tested: 500, 1000 and 2000 mg/kg bw (oral, gavage)	Negative	Anonymous, 2018; M-635020-01-1

Based on the available studies, no classification for germ cell mutagenicity was proposed by the DS.

Comments received during public consultation

One MSCA agreed with the DS that classification for germ cell mutagenicity is not justified.

Assessment and comparison with the classification criteria

One of the earlier five bacterial reverse mutation assay showed a very slight increase in the number of revertant colonies in only one strain (TA 98) and only with metabolic activation at the highest concentration of 5000 µg/plate where precipitation was observed. Therefore, this result was considered of doubtful biological significance and four additional assays were conducted. No evidence of mutagenic activity of fluopicolide was observed in the four additional bacterial reverse mutation assays performed with five *Salmonella typhimurium* strains and one *Escherichia Coli* strain. In addition, a recently conducted bacterial reverse mutation assay (2017) was negative. Furthermore, a Comet assay was also recently (2018) performed to confirm the negative profile for the endpoint gene mutation *in vivo*.

The chromosomal aberration assay performed in Chinese hamster V79 showed a positive response. However, the increase of aberrant cells occurred at cytotoxic concentrations where mitotic indices were clearly below the limit of 50% indicating the doubtful biological significance of these data. This chromosome aberration assay was therefore repeated in human lymphocytes and gave a clear negative response. Moreover, two *in vivo* micronucleus assays were performed in mice by the oral route up to the limit dose of 2000 mg/kg bw. Both of these assays were negative. However, one was of questionable biological significance due to the slight increase of micronucleated polychromatic erythrocytes in bone marrow of some animals given 2000 mg/kg bw as well as in one control animal. As the ratio of PCE/NCE erythrocytes was not significantly affected and no

clinical signs were observed in both assays, a third assay was performed in mice by the intraperitoneal route especially to check for bone marrow exposure. This assay gave a clear negative result for clastogencity *in vivo* at dose levels showing clear cytotoxicity of the bone marrow. The PCE/NCE ratio is strongly and significantly altered in this *in vivo* micronucleus test and therefore bone marrow exposure is considered to be demonstrated.

The HPRT mutation assay in Chinese hamster V79 cells was negative. Moreover, the *in vivo* rat hepatocyte UDS assay clearly showed that fluopicolide does not induce damage to DNA.

Based on the available data, RAC agrees with the DS that **classification of fluopicolide for mutagenicity is not warranted.**

10.9 Carcinogenicity

The chronic toxicity and carcinogenic potential of fluopicolide has been investigated in one two-year long-term toxicity/carcinogenicity study in rats and an 18-month carcinogenicity study in mice.

Table 10-22: Summary table of animal studies on carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Combined carcinogenicity and toxicity study by dietary administration to CD rats for 104 weeks. Crl:CD® (SD)IGS BR rat (60/dose/sex carcinogenic phase, 20/dose/sex chronic tox. phase, 10/dose/sex treated for 52-weeks, followed by a 13-week period without treatment) OECD 453 (04/1981) GLP Coagulating gland, Harderian gland, vagina and bone marrow were not sampled, fixed or examined histopathologically	Fluopicolide (purity 95.9%) 0, 50, 200, 750 or 2,500 ppm (equivalent to 0, 2.1, 8.4, 31.5, 109.4 / 0, 2.8, 10.8, 41.0, 142.2 mg/kg bw/day in M/F)	<u>50 ppm</u> No effects observed <u>≥ 200 ppm</u> No adverse effects <u>≥ 750 ppm</u> ↓ bodyweight gain week 1 (M/F) ↑ total protein concentration and ↓ A/G ratio in blood (M/F) ↑ cholesterol in blood (M) ↑ K^+ and/or Ca^{2+} in blood (M/F) ↑ liver and kidney weights (M/F) ↓ incidence of mammary masses (F) ↑ increased incidence and/or severity of centrilobular hepatocyte hypertrophy (M/F), incidence and/or severity of cystic degeneration and foci of alteration (M) and ↑ increased incidence of eosinophilic foci of alteration (F) in the liver ↑ incidence of cortical tubular basophilia and hyperplasia of the papillary epithelium (M/F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids week 104 (M) <u>2,500 ppm</u> ↓ bodyweight gain (M/F) ↓ RBC parameters (M/F) ↑ albumin in blood (M) Most of these changes reversible after a 13-week off-dose period. No evidence of a carcinogenic potential.	Anonymous; 2003; M-225616-01-2 Anonymous; 2005; M-263575-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Carcinogenicity study in mice via diet C57BL/6 mice (50/dose/sex) OECD 451 (04/1981) GLP Coagulating glands were not sampled, fixed or examined histopathologically	Fluopicolide (purity 95.9%) 0, 50, 400, or 3,200 ppm for 78 weeks (equivalent to 0, 7.9, 64.5 , 551.0 / 0, 11.5, 91.9, 772.3 mg/kg bw/day in M/F)	50 ppm (7.9/11.5 mg/kg bw/day) No effects observed 400 ppm (64.5/91.9 mg/kg bw/day) ↑ abs. & rel. liver weight week (M/F) ↑ incidence of hepatocellular hypertrophy (M/F) 3,200 ppm (551/772.3 mg/kg bw/day) ↓ body weight (M/F) ↓ feed intake (M/F) ↑ incidence of altered liver foci week 52 (F) and 78 (M/F) ↑ incidence of liver adenomas week 52 (F) and 78 (M/F)	Anonymous; 2003; M-225595-01-1 Anonymous; 2005; M-263591-01-1

M = male F = female

Table 10-23: Summary table of human data on carcinogenicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-24: Summary table of other studies relevant for carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
28-day explanatory dietary toxicity study in C57Bl/6 female mice to investigate liver cell proliferation and cytochrome P450 induction	Fluopicolide (purity 99.3%) 15 mice for at least 28 days at concentrations of 0 (control) and 3,200 ppm (equivalent to 575 mg/kg bw/day) Satellite subgroups of 20 mice for interim sacrifice after 7 days of treatment	↑ hepatocellular proliferation in C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. ↑ liver weight ↑ increased incidence of hepatocellular hypertrophy ↓ diffuse, mainly centrilobular hepatocellular vacuolation ↑ total cytochrome P-450 and BROD and PROD activities As confirmed by a separate positive control study (Langrand-Lerche, C.; 2004; M-232813-01-1), fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.	Anonymous; 2004; M-229594-01-1

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td>In vitro tests with fluopicolide in cultured male and female C57BL/6 Mouse hepatocytes, CarKO/PxrKO Mouse hepatocytes and human hepatocytes</td><td>Fluopicolide (purity 98.2%)

0, 0.03, 0.1, 0.3, 1, 2, 3 and 10 µM</td><td>Overview of key results relevant for the liver tumor MOA:
<table>
<tr><th>Key results</th><th>Wildtype mouse hepatocytes</th><th>CarKO/PxrKO mouse hepatocytes</th><th>Human hepatocytes</th></tr>
<tr><td>CAR activation</td><td>+</td><td>-</td><td>-</td></tr>
<tr><td>PXR activation</td><td>+</td><td>-</td><td>(+)</td></tr>
<tr><td>Liver cell proliferation</td><td>+</td><td>-</td><td>-</td></tr>
</table>
The in vitro studies confirmed a rodent specific CAR/PXR MOA since they demonstrated that significant CAR and PXR activation and hepatocyte proliferation was induced in WT hepatocytes but not in CarKO/PxrKO or human hepatocytes.</td><td>Anonymous; 2017; M-600904-01-1

Anonymous; 2017; M-603455-01-1

Anonymous; 2017; M-600908-01-1

Anonymous; 2017; M-604080-01-1

Anonymous; 2017; M-600911-01-1

Anonymous; 2017; M-604094-01-1</td></tr>
</table>

10.9.1 Chronic/carcinogenicity study in rats

Fluopicolide was administered to Sprague Dawley rats in the diet at concentrations of 0, 50, 200, 750 or 2,500 ppm for 2 years. After 1-year treatment period, 20 animals/sex/group were killed for assessment of chronic toxicity. In addition, the recovery of any effects seen during the 52-week toxicity phase was assessed in a subsequent 13-week recovery period. After the 2-year treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (60 animals/sex/group). When compared with the controls there was a marked effect on body weight gain in the first week of treatment in animals receiving 2,500 ppm and in females receiving 200 or 750 ppm. Subsequent weight gain by animals receiving 2,500 ppm tended to be lower than that of the controls, though the difference was less than was seen during the first week of treatment. For males and females given 2,500 ppm, the overall weight gain was 5 and 25% lower than controls, respectively, at the end of Week 52 in the Toxicity phase, and 11 and 17% lower than controls at the end of the Carcinogenicity phase. The subsequent body weight gain of females receiving 200 or 750 ppm was similar to that of the controls. The target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm. At the same dose levels, there were histopathological findings in liver comprising an increased incidence of centrilobular hepatocytic hypertrophy and an increased incidence and/or severity of cystic degeneration and foci of alteration in males and an increased incidence of eosinophilic foci of alteration in females after 104 weeks. Secondary to the increased metabolic activity of the liver was an increased incidence of cystic follicular cell hyperplasia in the thyroids of males. In the kidneys at 2,500 ppm there were degenerative and proliferative changes, comprising cortical tubular basophilia at an increased severity in both sexes, the males in particular, had increased incidences of hyaline droplets in the cortical tubules, cortical tubular dilatation and tubular casts. Hyperplasia of the papillary epithelium at 2,500 and 750 ppm was present at an increased incidence and severity in females and this was associated with mineralisation of the pelvic epithelium. No treatment-related adverse changes were observed at 200 ppm; at this dose a slight increase in hepatocellular hypertrophy in males was the only non-neoplastic finding (an adaptive response secondary to

liver enzyme induction), whilst the only neoplastic finding was a decrease in mammary and/or adrenal masses in females (which is not toxicologically relevant). There were no findings at 50 ppm. The overall incidence of tumour-bearing animals, the time of occurrence and the pattern of neoplastic findings did not indicate a carcinogenic effect of fluopicolide.

Therefore, the NOAEL for toxicity was 200 ppm in both males and females, (equivalent to 8.4 and 10.8 mg/kg bw/day, in males and females, respectively). Furthermore, there was no evidence of carcinogenicity with fluopicolide up to and including the dose level of 2,500 ppm (equivalent to 109.4 and 142.2 mg/kg bw/day, in males and females, respectively).

10.9.2 Chronic/carcinogenicity study in mice

In a mouse oncogencity study, fluopicolide was administered to C57BL/6 mice in the diet at concentrations of 0 (control), 50, 400 and 3,200 ppm for 78 weeks. After 52-week treatment period, 10 animals/sex/group were killed for assessment of chronic toxicity. After 78-week treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (50 animals/sex/group).

Fluopicolide administered daily for 78 weeks produced severe reduction of the body weight gain (-45% in males and -35% in females) at 3,200 ppm indicating that the Maximal Tolerated Dose (MTD) was reached. The target organ identified was the liver. Higher liver weights, enlarged liver, increased number of masses and nodules in the liver were observed at 400 and 3,200 ppm at 52 and 78 weeks. These changes were associated with hepatocellular hypertrophy at 52 and 78 weeks, and higher incidence of altered cell foci at 3,200 ppm at 78 weeks.

Generally, the number of animals with neoplasms, the number of animals with more than one primary neoplasm and the number of animals with benign and malignant tumors were comparatively similar in all groups. A significant increased incidence of hepatocellular adenoma was observed at 3,200 ppm at 78 weeks in both males (22% vs. 10% in control)) and females (32% vs. 2% in control) and at 52 weeks in females (30% vs. 0% in control). In addition, the time of onset of the hepatocellular neoplasm was shorter in the treated females when compared with controls. However, no increased incidence of hepatocellular carcinoma were observed in any of the groups after the 78-week treatment period. An overview about relevant liver findings is given in the following tables.

Liver findings in the mouse oncogenicity study at 52 weeks

Parameter	Control data		Low dose 50 ppm		Mid dose 400 ppm		High dose 3,200 ppm	
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**
Final bodyweight (g) and (% control)	37.66	36.23	37.90 (+1%)	34.14 (-6%)	42.56 (+13%)	34.03 (-6%)	**31.25* (-17%)**	**26.58** (-27%)**
Liver: organ weight, relative (g/100g) and absolute (g)	1.59 4.21	1.51 4.21	1.71 4.53	1.44 4.20	**2.06**** **4.84****	1.57 4.61	**2.15**** **6.85****	**2.26**** **8.39****
Non-neoplastic changes:								
Hepatocellular hypertrophy	0/10	0/10	0/10	0/10	**5/10**	**6/10**	**10/10**	**9/10**
Altered cell foci	0/10	0/10	0/10	0/10	0/10	0/10	0/10	**2/10**
Coagulative hepatocyte necrosis	1/10	1/10	0/10	0/10	1/10	0/10	0/10	0/10
Microfoci of necrosis	4/10	3/10	1/10	2/10	2/10	1/10	1/10	1/10
Chronic inflammation	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Neoplastic changes:								
Hepatocellular adenoma	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	1/10 (10)	0/10 (0)	**3/10 (30)**
Hepatocellular carcinoma	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)

* p<0.05, ** p<0.01
m: males; f: females
Findings considered related to treatment with fluopicolide are written in **bold**.

Liver findings in the mouse oncogenicity study at 78 weeks

Parameter	Control data		Low dose 50 ppm		Mid dose 400 ppm		High dose 3,200 ppm	
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**
Final bodyweight (g) and (% control)	38.58	32.61	40.62 (+5%)	33.77 (+4%)	39.09 (+1%)	33.73 (+3%)	**31.15** (-19%)**	**27.17** (-17%)**
Liver: organ weight, relative (g/100g) and absolute (g)	1.62 4.26	1.66 5.18	1.85 4.65	1.64 4.94	**1.91**** **4.90****	**2.20**** **6.62**	**2.37**** **7.62****	**2.59**** **9.37****
Non-neoplastic changes:								
Hepatocellular hypertrophy	0/50	0/49	0/50	0/50	**20/50**	**41/50**	**49/50**	**41/50**
Altered cell foci	1/50	1/49	8/50	3/50	5/50	4/50	**18/50**	**25/50**
Coagulative hepatocyte necrosis	6/50	2/49	3/50	5/50	1/50	4/50	2/50	3/50
Microfoci of necrosis	2/50	2/49	5/50	5/50	8/50	1/50	4/50	2/50
Chronic inflammation	0/50	0/49	1/50	0/50	0/50	0/50	0/50	0/50
Neoplastic changes:								
Hepatocellular adenoma (%)	5/50 (10)	1/50 (2)	0/50 (0)	2/50 (4)	5/50 (10)	0/50 (0)	**11/50* (22)**	**16/50** (32)**
Hepatocellular carcinoma (%)	3/50 (6)	0/50 (0)	1/50 (2)	0/50 (0)	0/50 (0)	2/50 (4)	2/50 (4)	0/50 (0)

* p<0.05, ** p<0.01
m: males; f: females
Findings considered related to treatment with fluopicolide are written in **bold**.

Therefore, an increased number of benign liver tumours occured only at the highest dose reaching the MTD (severe body weight gain reduction in high dose animals) suggesting a threshold mechanism (see also Annex II). In addition, no tumours were observed with increased incidences in other mouse tissues and the liver adenoma did not progress into malignant neoplasia during the lifespan of these animals. Altogether, these findings clearly indicate that the slightly increased incidence of hepatocellular adenoma in mice is a weak carcinogenic response. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital (see below). This MoA is considered of no relevance in humans.

Therefore, the NOAELs of the study are 50 ppm for toxicity (equivalent to 7.9 and 11.5 mg/kg bw/day, in males and females, respectively) and 400 ppm for carcinogenicity (equivalent to 64.5 and 91.9 mg/kg bw/day, in males and females, respectively).

10.9.3 Other studies relevant for carcinogenicity

There are four additional mechanistic studies addressing the observed mouse liver oncogenicity. The first one is a 28-day *in vivo* study in C57BL/6 mice focused on hepatic cellular proliferation as well as morphological changes of the liver and hepatic cytochrome P-450 isoenzymes activity. Furthermore three *in vitro* studies in different hepatocyte cultures (mouse wild type, mouse CarKO/PxrKO and human) were performed.

10.9.3.1 28-day mechanistic study in mice

In an *in vivo* study in mice, fluopicolide (AE C638206) was administered continuously via the diet to a group of 15 female C57BL/6 mice for at least 28 days at concentrations of 0 (control) and the dose at which liver tumors occurred in the carcinogencity study, i.e. 3,200 ppm (equivalent to 575 mg/kg bw/day), with satellite subgroups of 20 female mice per group for interim sacrifice after 7 days of treatment. Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. At both interim and final sacrifice times, liver was weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes of the liver. In addition, at interim sacrifice, hepatic cytochrome P-450 isoenzymes were assessed.

At 3,200 ppm, there were no mortalities or clinical signs during the course of the study. There was a body weight loss (-2.1 g) between Days 1-7. The mean body weight gain was thereafter transiently higher and then again lower than controls between Days 15-28 resulting in a mean body weight reduction throughout treatment (-6 to -9%). Mean food consumption was also lower than controls between Days 1-7 (-25%).

At interim sacrifice, mean terminal body weight was statistically significantly lower (-7%) when compared to controls. Mean absolute and relative liver weights were increased by 27 to 37% compared to controls, 9/20 livers appeared to be dark and 1/20 livers was enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals and a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/20 treated animals when compared to controls. An increased number of mitotic cells and some foci of single cell necrosis/apoptosis were seen in 5/20 treated animals. The mean BrdU labeling index was approx. 6.5-fold higher in treated animals, when compared to controls, indicative of a marked hepatocellular proliferation in the liver.

At final sacrifice, mean terminal body weight was not affected. Mean absolute and relative liver weights were increased by 48 to 56% compared to controls, 11/15 livers appeared to be dark and 3/15 livers were enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals together with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/15 treated animals, when compared to controls. Minimal single cell necrosis/apoptosis were seen in only 1/15 treated animals and an increased number of mitotic cells in 2/15 treated animals. There was no increased hepatocellular proliferation based on the results of the BrdU assay.

Fluopicolide also induced a marked increase in total cytochrome P-450 content (+97%) as well as in BROD (+1785%) and PROD (+1143%) activities. EROD activity was only slightly induced and lauric acid hydroxylation decreased compared to control mean as shown in the following table.

Total cytochrome P-450 content and enzymatic activities at interim sacrifice

Parameter	**Fluopicolide at 3,200 ppm** % change compared to control mean
P450	+ 97 %
BROD	+ 1785 %
EROD	+ 79 %
PROD	+ 1143 %
Lauric acid	- 67 %

In conclusion, fluopicolide at 3,200 ppm in the diet induced a transient and marked hepatocellular proliferation in C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, fluopicolide is a marked total cytochrome P-450 and BROD and PROD activities inducer. As confirmed by a separate positive control study (Anonymous.; 2004; M-232813-01-1), fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.

10.9.3.2 Mechanistic studies in hepatocytes

This MoA is further supported by *in vitro* studies in hepatocytes from wildtype mice and CAR/PXR-knockout mice, since under the assumption of a CAR/PXR-mediated liver tumor MoA, a proliferative effect in mice which are lacking CAR/PXR receptors should not occur.

The goal of these *in vitro* studies was to investigate the potential of fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female C57BL/6 mouse hepatocyte cultures in comparison to isolated male and female constitutive androstane receptor knockout/pregnane x receptor knockout (CarKO/PxrKO) mouse hepatocyte cultures and to cryopreserved male and female human hepatocyte cultures from three independent donors (Anonymous.; 2017; M-603455-01-1 / Anonymous.; 2017; M-604080-01-1 / Anonymous.; 2017; M-604094-01-1).

In these studies, cytotoxicity was evaluated by adenosine 5'-triphosphate (ATP) depletion and Phenobarbital (PB) was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of Cyp2b and Cyp3a- activities and increased cell proliferation). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

Mechanistic study in wildtype C57BL/6 male and female mouse hepatocytes

Fluopicolide administration to C57BL/6 male and female mouse hepatocytes in culture induced replicative DNA synthesis in a dose-dependent manner with maximal induction at 0.3 µM (1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes). PB induced replicative DNA synthesis to a maximum of 1.8-fold and 2.2-fold in the male and female hepatocytes, respectively; the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

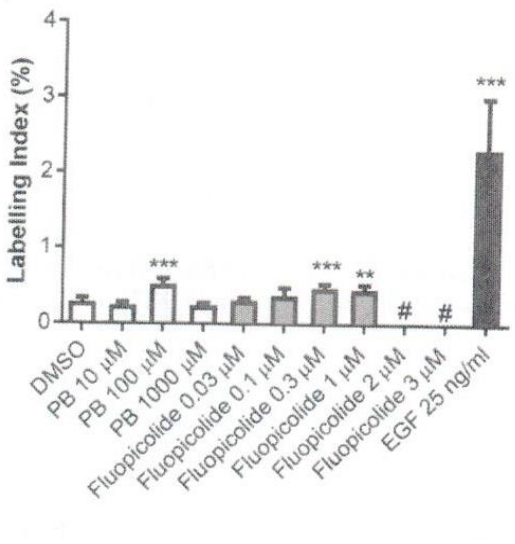


Figure 10- 1: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in male C57BL/6 mouse primary hepatocytes

Effect of Fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in female C57BL/6 mouse primary hepatocytes

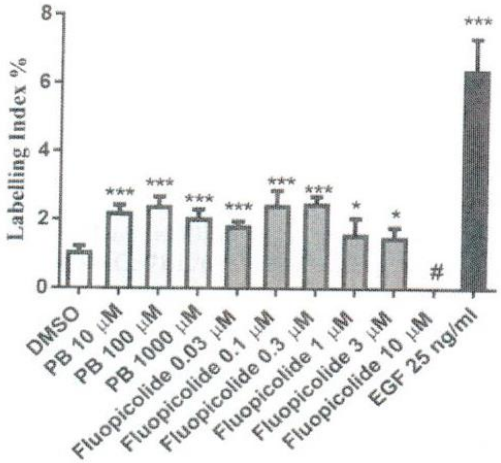


Figure 10- 2: Figure 10- 1: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in female C57BL/6 mouse primary hepatocytes

Hepatic pentoxyresorufin-O-dependentylation (PROD), benzyloxyresorufin O-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of Cyp2b and Cyp3a induction. In male C57BL/6 mouse hepatocytes, fluopicolide caused a dose dependant increase in PROD and BROD (up to 3-and 2.6-fold of control respectively). BQ was also slightly increased in these cells following administration of Fluopicolide at 1 and 2 µM (1.4- and 1.5-fold respectively). In female C57BL/6 mouse hepatocytes, fluopicolide induced a dose dependent increase in PROD (up to 1.7-fold), but not BROD or BQ activities.

PB (1 mM) caused significant increases in PROD (6.5-fold), BROD (4.9-fold) and BQ (7.7-fold) activities in the male mouse hepatocytes. PB (1 mM) also caused significant increases in PROD, BROD and BQ activities in the female mouse hepatocytes, increasing activities by 2.6-, 1.6- and 4.4-fold, respectively. Therefore, treatment with the positive control items PB and EGF gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide induced both hepatocellular S-phase replicative DNA synthesis and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures. These data suggest that fluopicolide activated the nuclear hormone receptor constitutive androstane receptor (CAR) in male and female C57BL/6 mouse hepatocytes.

Mechanistic study in male and female CarKO/PxrKO Mouse hepatocytes

Treatment with fluopicolide or PB did not induce replicative DNA synthesis in male or female CarKO/PxrKO hepatocytes at any concentration but the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

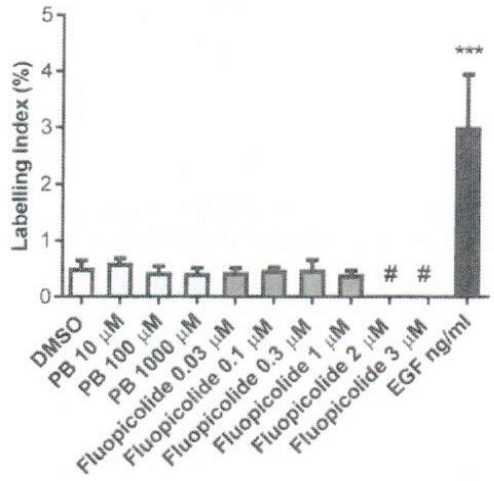


Figure 10- 3: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in male CarKO/PxrKO mouse primary hepatocytes

Effect of Fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in female *Car*KO/*Pxr*KO mouse primary hepatocytes

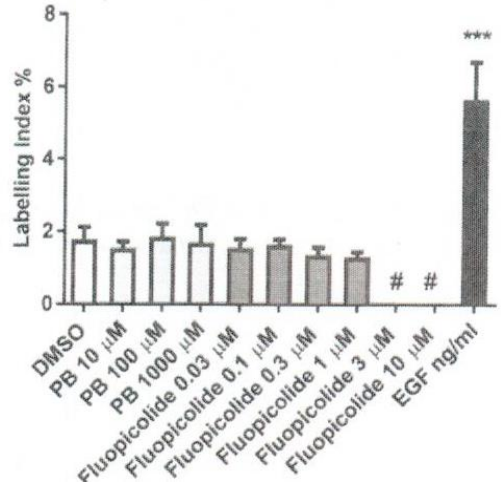


Figure 10- 4: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in female CarKO/PxrKO mouse primary hepatocytes

Similarily, fluopicolide did not cause any increases in PROD, BROD or BQ in male or female CarKO/PxrKO mouse hepatocytes. PB administration (1000 µM only) to male mouse hepatocytes slightly induced PROD, BROD and BQ to 3.5-, 1.5- and 1.7-fold respectively. 1000 µM PB also caused induction in female mouse hepatocytes in PROD and BROD 1.6- and 1.8-fold respectively, with no induction observed in BQ.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, Cyp2b or Cyp3a enzyme activity in male or female CarKO/PxrKO mouse primary hepatocyte cultures. These data suggest that fluopicolide requires the presence of the nuclear hormone receptors CAR and/or PXR to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

Mechanistic study in human hepatocytes from three Individual Donors

Neither administration with fluopicolide nor PB induced replicative DNA synthesis in cultured male or female human hepatocytes. However, the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

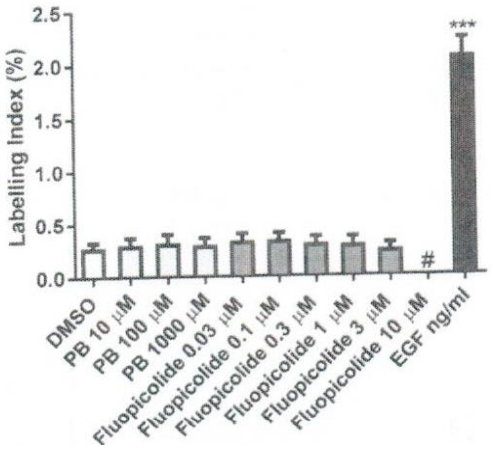


Figure 10- 5: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-Phase) in male human hepatocytes, donor 8210

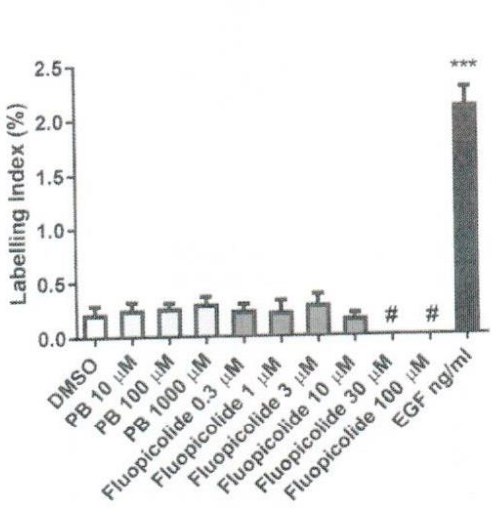


Figure 10- 6: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-Phase) in female human hepatocytes, donor 8239

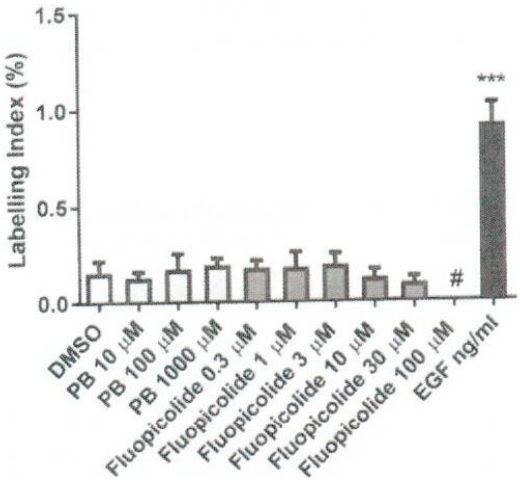


Figure 10- 7: Effect of fluopicolide, PB or EGF on replicative DNA synthesis (S-Phase) in female human hepatocytes, donor 1765

In male human hepatocytes, fluopicolide caused slight increases in BROD and BQ activities to a maximum of 1.5 - and 2.6-fold respectively. All levels of PB resulted in statistically significant increases in BROD and BQ activities in the male human hepatocytes, with maximum increases observed at 1 mM (2.0- and 5.3-fold respectively). PROD activity could not be analysed as levels were below the level of quantification, therefore, no results are presented for this assay in the male Donor 8210.

Treatment with fluopicolide resulted in dose-dependent increases in BQ activity in female hepatocytes from both donors to a maximum of 1.7- and. 2.8-fold induction compared to control. Fluopicolide caused no relevant increase in PROD or BROD activity in female hepatocytes, however, slight, but significant, decreases in BROD activity were observed at the top concentrations. In female human hepatocytes, PB consistently induced BROD and BQ activites in both donors, however, only donor 1765 responded in a dose-dependent manner after treatment with PB.

In summary, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and females)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

These data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels) which in humans is normally involved in xenobiotic metabolism with no effect on DNA-synthesis in male or female human hepatocytes.

Most importantly, in addition to the CarKO/PxrKO Mouse hepatocyte study, this study in human hepatocytes strongly supports human non-relevance of this CAR/PXR-mediated liver tumor MoA.

The following table summarizes the overall results regarding CAR/PCR activation and liver cell proliferation in wildtype mouse, CarKO/PxrKO Mouse and human hepatocytes:

Overview of key effects relevant for the liver tumor MOA

Key events	Wildtype mouse hepatocytes	CarKO/PxrKO mouse hepatocytes	Human hepatocytes
CAR activation	+	-	-
PXR activation	+	-	(+)
Liver cell proliferation	+	-	-

This overview makes clear that the key event of CAR/PXR activation which lead to liver cell proliferation and eventually to liver tumors in rodents, is not relevant to humans in which the important key events, CAR induction and liver cell proliferation do not occur after exposure to fluopicolide.

10.9.3.3 Proposed mode of action (MOA) overview

An evaluation of the mode of action (MOA) of the liver tumor induction according to the IPCS/human Framework method is provided in Annex II to this document. The main conclusions are summarized in the following.The weight of evidence indicates a hypothesized MOA via the constitutive androstane receptor and/or pregnane X receptor (CAR/PXR) activation leading to the observed liver tumours in mice. The key events involved are summarized in the following table.

Listing of key events and associative events for a CAR-mediated liver tumour MOA

Events	Description
Key events (KE)	
KE 1	Activation of CAR/PXR nuclear receptor
KE 2	Altered gene expression secondary to CAR/PXR activation
KE 3	Increased hepatocellular proliferation
KE 4	Increased clonal expansion, leading to altered foci
KE 5	Increased incidence of hepatocellular tumours
Associative events (AE)	
AE 1	Increased CYP2B, CYP3A enzyme activity and/or protein
AE 2	Hepatocellular hypertrophy
AE 3	Increased liver weight

(From Elcombe et al, Crit Rev Toxicol. 2014;44(1): 64-82)

MOA studies demonstrated these key events for fluopicolide. Fluopicolide induced BROD and PROD activities in the conducted mechanistic 28-day in vivo mouse study (Langrand-Lerche, C.; 2004; M-229594-01-1) which indicates that CAR/PXR receptors were activated by fluopicolide with subsequently increased protein expression/enzyme activity of CYP2B and CYP3A (KE 1, 2 and AE 1). Moreover, a marked transient hepatocellular proliferation (KE 3) were demonstrated at 7-day interim sacrifice. At terminal sacrifice significantly increased absolute and relative weights and a diffuse, perilobular to panlobular hepatocellular hypertrophy was seen in all treated animals (AE 2). Moreover, the PCNA assessment on liver tissue from animals at 3200 ppm in the subchronic mouse study (Wason, S. M.; 2006; M-205579-02-1) showed that fluopicolide did not produce hepatocellular proliferation on Day 90. This is completely consistent with the lack of cell proliferation observed on Day 28 with the BrdU assessment. These findings emphasize that a transient liver cell proliferation followed by a return to control levels is a prerequisite for the development of hepatocellular adenoma following a long term exposure period to fluopicolide.

Hepatocellular hypertrophy (AE 2), increased liver weights (AE 3), elevated incidences of altered foci (KE 4) and eventually liver adenomas (KE 5) were seen in the standard subchronic and chronic mouse studies with fluopicolide (Anonymous.; 2006; M-205579-02-1, Anonymous.; 2000; M-197623-01-1, Anonymous.; 2003; M-225595-01-1).Therefore, all key and associative events for a CAR-mediated liver tumour MOA were observed after fluopicolide treatment *in vivo*.

These rodent-specific findings are very similar to that demonstrated for phenobarbital which causes liver tumours in rodents. In a 28-day study in the same strain of mice (Anonymous.; 2004; M-232813-01-1) phenobarbital at 80 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which remained statistically significant but slight in males and returned to control levels in females after 28 days of treatment. In addition, phenobarbital was found to be a strong inducer of hepatocellular hypertrophy and of total cytochrome P-450 and BROD and PROD activities. Clofibric acid at 300 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, clofibric acid was found to be a strong inducer of hepatocellular hypertrophy and of lauric acid hydroxylation activities. That this transient effect on hepatocellular cell proliferation is the key event for non-genotoxic induction of hepatocellular carcinogenesis by xenobiotic compounds is supported by widely published work (e.g. Schulte-Hermann, R. (1974)[1]; Schulte-Hermann, R. (1979)[2]; Hildebrand, B. et al. (1991)[3]). However, despite the long history of phenobarbital use in human medicine no evidence of an increased carcinogenic risk for humans was seen. Therefore, also for fluopicolide (AE C638206) which caused similar tumors via CAR and PXR human relevance can be excluded.

In addition to the *in vivo* MOA study also *in vitro* hepatocyte studies were conducted with exposure of wild-type (WT) mice (Chatham, L.; 2017; M-603455-01-1), CAR/PXR-knockout (CarKO/PxrKO) mice (Chatham, L.; 2017; M-604080-01-1) and human (Chatham, L.; 2017; M-604094-01-1) hepatocyte cultures to fluopicolide. These studies confirmed the CAR/PXR MOA since they demonstrated that hepatocyte proliferation was induced in WT mice hepatocytes but not in CarKO/PxrKO mice hepatocytes. Human hepatocytes did not show a proliferation or significant CAR(PXR activation which clearly confirms that human hepatocytes are not sensitive to this liver tumour MOA and thus that this MOA is not relevant to humans.

[1] Schulte-Hermann R. Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit Rev Toxicol. 1974 Sep;3(1):97-158.

[2] Schulte-Hermann R. Adaptive liver growth induced by xenobiotic compounds: its nature and mechanism. Arch Toxicol Suppl. 1979;(2):113-24. Review.

[3] Hildebrand B, Grasso P, Ashby J, Chamberlain M, Jung R, van Kolfschoten A, Loeser E, Smith E, Bontinck WJ. Validity of considering that early changes may act as indicators for non-genotoxic carcinogenesis. Mutat Res. 1991 Jun;248(2):217-20. Review

Other MOAs can be excluded based on the available results. Thus, a genotoxic MOA can be excluded based on the results of the genotoxicity studies which did not indicate a genotoxic potential. Furthermore, the results of the 28-day mechanistic study in mice (Anonymous.; 2004; M-232813-01-1) show that Arylhydrocarbon receptor- or PPARα-mediated effects and thus such MOAs can be excluded since no relevant effects on EROD or Lauric acid, respectively were noted.

Also oxidative stress and severe liver cytotoxicity as a mode of action for the liver tumours can be excluded, since in the repated dose toxicity studies with fluopicolide even at the highest doses, no signs of severe cytotoxicity in the liver, like inflammatory signs, broad hepatic necrosis, hepatocellular death, fibrosis, cirrhosis or severely increased transaminase activities were observed.

Thus, it can be summarized that the investigations demonstrated a non-genotoxic rodent-specific CAR/PXR mediated MOA for the liver tumours after fluopicolide treatment which has a clear threshold for the underlying key events and the final liver tumour induction (see also Annex II: Evaluation of the proposed MOA of fluopicolide according to the IPCS/human Framework method). This MOA is the same that has been demonstrated for phenobarbital and many other non-genotoxic compounds which caused liver tumours in rodents. This MOA has no relevance to humans which was shown in the mechanistic *in vitro* hepatocyte studies and supported by the fact that despite the long history of phenobarbital use in human medicine no evidence of an increased carcinogenic risk for humans was seen.

10.9.4 Comparison with the CLP criteria

According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017), category 1A (known to have carcinogenic potential for humans) classification is largely based on human evidence, or Category 1B (presumed to have carcinogenic potential for humans) classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations. Such evidence may be derived from human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals.

The placing of a substance in Category 2 is done on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies.

No human studies or epidemiological publications regarding effects on humans are known, which could trigger classification of fluopicolide.

With regard to animals studies, it can be summarized that the dietary administration of fluopicolide produced higher incidence of hepatocellular adenoma only at very high doses reaching MTD in male and female mice following chronic treatment (Anonymous.; 2003; M-225595-01-1). Given that these hepatocellular adenoma were not observed at lower dose levels in mice, not observed in rats following a 2-year treatment period and taken into account the lack of genotoxicity potential of fluopicolide, the higher incidence of hepatocellular adenoma was thus considered to be subsequent to a threshold mechanism with a Phenobarbital-like mechanism of action (hepatocellular hypertrophy and transient cell proliferation) which is a well-known mechanism of action specific to the mouse and of no relevance to humans. This is also confirmed by *in vivo* and *in vitro* mode of action studies. Other MOAs can be excluded based on the available study and literature results.

Based on the MOA of a CAR/PXR-mediated effect together with the high dose reaching the MTD at which liver adenomas were seen, the liver adenomas in mice are not regarded as relevant to humans.

10.9.5 Conclusion on classification and labelling for carcinogenicity

No carcinogenic potential in humans is known from epidemiological literature due to an absence of any publications in this regard. Also no carcinogenic potential of fluopicolide was evident from the rat carcinogenicity study.

The liver adenomas at the highest dose in the mouse oncogencity study were caused by a rodent-specific liver enzyme induction via a CAR/PXR receptor-mediated MOA, like that of phenobarbital which is known to be non-relevant to humans.

Therefore, the data are conclusive but do **not warrant** a carcinogenicity classification according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS concluded that data for carcinogenicity are conclusive and that fluopicolide does not warrant a carcinogenicity classification.

A summary of submitted studies with carcinogenicity endpoints are presented below:

Method	Test substance, dose levels duration of exposure	Results	Reference
Combined carcinogenicity and toxicity study by dietary administration to CD rats for 104 weeks. Crl:CD® (SD)IGS BR rat (60/dose/sex carcinogenic phase, 20/dose/sex chronic tox. phase, 10/dose/sex treated for 52-weeks, followed by a 13-week period without treatment) OECD TG 453 (04/1981) GLP Coagulating gland, Harderian gland, vagina and bone marrow were not sampled, fixed or examined histopathologically	Fluopicolide (purity 95.9%) 0, 50, 200, 750 or 2500 ppm (equivalent to 0, 2.1, 8.4, 31.5, 109.4 / 0, 2.8, 10.8, 41.0, 142.2 mg/kg bw/day in M/F)	<u>50 ppm</u> No effects observed <u>≥ 200 ppm</u> No adverse effects <u>≥ 750 ppm</u> ↓ bodyweight gain week 1 (M/F) ↑ total protein concentration and ↓ A/G ratio in blood (M/F) ↑ cholesterol in blood (M) ↑ K^+ and/or Ca^{2+} in blood (M/F) ↑ liver and kidney weights (M/F) ↓ incidence of mammary masses (F) ↑ increased incidence and/or severity of centrilobular hepatocyte hypertrophy (M/F), incidence and/or severity of cystic degeneration and foci of alteration (M) and ↑ increased incidence of eosinophilic foci of alteration (F) in the liver ↑ incidence of cortical tubular basophilia and hyperplasia of the papillary epithelium (M/F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids week 104 (M) <u>2500 ppm</u> ↓ bodyweight gain (M/F) ↓ RBC parameters (M/F) ↑ albumin in blood (M) Most of these changes reversible after a 13-week off-dose period. No evidence of a carcinogenic potential.	Anonymous, 2003; M-225616-01-2 Anonymous, 2005; M-263575-01-1
Carcinogenicity study in mice via diet C57BL/6 mice (50/dose/sex) OECD TG 451 (04/1981) GLP Coagulating glands were not sampled, fixed or examined histopathologically	Fluopicolide (purity 95.9%) 0, 50, 400, or 3200 ppm for 78 weeks (equivalent to 0, 7.9, 64.5 , 551.0 / 0, 11.5, 91.9, 772.3 mg/kg bw/day in M/F)	<u>50 ppm (7.9/11.5 mg/kg bw/day)</u> <u>No effects observed</u> <u>400 ppm (64.5/91.9 mg/kg bw/day)</u> <u>↑ abs. & rel. liver weight week (M/F)</u> <u>↑ incidence of hepatocellular hypertrophy (M/F)</u> <u>3200 ppm (551/772.3 mg/kg bw/day)</u> <u>↓ body weight (M/F)</u> <u>↓ feed intake (M/F)</u> <u>↑ incidence of altered liver foci week 52 (F) and 78 (M/F)</u> <u>↑ incidence of liver adenomas week 52 (F) and 78 (M/F)</u>	Anonymous,2003; M-225595-01-1 Anonymous, 2005; M-263591-01-1

In the combined carcinogenicity and toxicity study in rats, fluopicolide was administered at concentrations of 0, 50, 200, 750 or 2500 ppm for 2 years. After 1-year treatment period, 20 animals/sex/group were sacrificed for assessment of chronic toxicity. In addition, the recovery of any effects seen during the 52-week toxicity phase was assessed in a subsequent 13-week recovery period. After the 2-year treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (60 animals/sex/group). When compared with the controls there was an effect on body weight gain. The target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm. At the same dose levels, there were histopathological findings in liver comprising an increased incidence of centrilobular hepatocyte hypertrophy, an increased incidence and/or severity of cystic degeneration and foci of alteration in males, and an increased incidence of eosinophilic foci of alteration in females after 104 weeks. Secondary to the increased metabolic activity of the liver was an increased incidence of cystic follicular cell hyperplasia in the thyroids of males. In the kidneys, at 2500 ppm, there were degenerative and proliferative changes, comprising cortical tubular basophilia, at an increased severity relative to controls in both sexes. The males, in particular, had increased incidences of hyaline droplets in the cortical tubules, cortical tubular dilatation and tubular casts. Hyperplasia of the papillary epithelium at 2500 and 750 ppm was present at an increased incidence and severity in females and this was associated with mineralisation of the pelvic epithelium. No treatment-related adverse changes were observed at dose levels of ≤ 200 ppm. The overall incidence of tumour-bearing animals, the time of occurrence and the pattern of neoplastic findings did not indicate a carcinogenic effect of fluopicolide.

In a mouse oncogenicity study, fluopicolide was administered to mice in the diet at concentrations of 50, 400 and 3200 ppm for 78 weeks. After 52-weeks, 10 animals/sex/group were killed for assessment of chronic toxicity. After the 78-week treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (50 animals/sex/group).

Fluopicolide administered daily for 78 weeks produced severe reduction of the body weight gain (-45% in males and -35% in females) at 3200 ppm indicating that the maximum tolerated dose (MTD) was reached. The target organ identified was the liver. Higher liver weights, enlarged liver, increased number of masses and nodules in the liver were observed at 400 and 3200 ppm. These changes were associated with hepatocellular hypertrophy, and higher incidence of altered cell foci at 3200 ppm.

Significantly increased incidence of hepatocellular adenoma was observed at 3200 ppm at 78 weeks in both males (22% in treated animals vs. 10% in controls) and females (32% in treated animals vs. 2% in control) and at 52 weeks in females (30% vs. 0% in controls). In addition, the time of onset of the hepatocellular neoplasm was shorter in the treated females when compared with controls.

The incidence of these lesions was close to the range of the laboratory HCD (males up to 14% and females up to 22%). Moreover, no increased incidence of hepatocellular carcinoma were observed in any of the groups after the 78-week treatment period. An overview of relevant liver findings after 78-weeks is given in the following table (based on the Table in Section 10.9.2 of the CLH report).

Parameter	**Control data**		**Low dose 50 ppm**		**Mid dose 400 ppm**		**High dose 3200 ppm**	
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**
Final bodyweight (g) and (% control)	38.58	32.61	40.62 (+5%)	33.77 (+4%)	39.09 (+1%)	33.73 (+3%)	**31.15** ** **(-19%)**	**27.17** ** **(-17%)**
Liver: organ weight, relative (g/100g) and absolute (g)	1.62 4.26	1.66 5.18	1.85 4.65	1.64 4.94	**1.91** ** **4.90** **	**2.20** ** **6.62**	**2.37** ** **7.62** **	**2.59** ** **9.37** **

Parameter	Control data		Low dose 50 ppm		Mid dose 400 ppm		High dose 3200 ppm	
	m	f	m	f	m	f	m	f
Non-neoplastic changes:								
Hepatocellular hypertrophy	0/50	0/49	0/50	0/50	**20/50**	**41/50**	**49/50**	**41/50**
Altered cell foci	1/50	1/49	8/50	3/50	5/50	4/50	**18/50**	**25/50**
Coagulative hepatocyte necrosis	6/50	2/49	3/50	5/50	1/50	4/50	2/50	3/50
Microfoci of necrosis	2/50	2/49	5/50	5/50	8/50	1/50	4/50	2/50
Chronic inflammation	0/50	0/49	1/50	0/50	0/50	0/50	0/50	0/50
Neoplastic changes:								
Hepatocellular adenoma (%)	5/50 (10)	1/50 (2)	0/50 (0)	2/50 (4)	5/50 (10)	0/50 (0)	**11/50* (22)**	**16/50** (32)**
Hepatocellular carcinoma (%)	3/50 (6)	0/50 (0)	1/50 (2)	0/50 (0)	0/50 (0)	2/50 (4)	2/50 (4)	0/50 (0)

* p<0.05, ** p<0.01
m: males; f: females
Findings considered related to treatment with fluopicolide are written in **bold**.

The increased number of benign liver tumours occurred only at the highest dose reaching the MTD (severe body weight gain reduction in high dose animals) suggesting a threshold mechanism. In addition, no tumours were observed with increased incidences in other mouse tissues and the liver adenoma did not progress into malignant neoplasia during the lifespan of these animals. Altogether, these findings clearly indicate that the slightly increased incidence of hepatocellular adenoma in mice is a weak carcinogenic response. The mode of action (MoA) for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital. This MoA is considered of no relevance in humans and therefore the DS concluded that fluopicolide does not warrant classification for carcinogenicity.

Comments received during public consultation

One MSCA supported the proposal for no carcinogenicity based on the argument that the hepatic neoplasia was limited to both sexes in one species, the lack of progression to malignancy and the plausible but incomplete mechanistic information that adenoma likely resulted from a rodent specific mechanism.

Assessment and comparison with the classification criteria

The main findings related to assessment of carcinogenicity of fluopicolide were limited to hepatocellular adenoma which were only observed in mice at the highest dose tested in both males and females. A summary of relevant findings from the 78-week mouse study is presented below:

Parameter	Control data		Low dose 50 ppm		Mid dose 400 ppm		High dose 3200 ppm	
	m	f	m	f	m	f	m	f
Neoplastic changes:								
Hepatocellular adenoma (%)	5/50 (10)	1/50 (2)	0/50 (0)	2/50 (4)	5/50 (10)	0/50 (0)	**11/50* (22)**	**16/50** (32)**
Hepatocellular carcinoma (%)	3/50 (6)	0/50 (0)	1/50 (2)	0/50 (0)	0/50 (0)	2/50 (4)	2/50 (4)	0/50 (0)

* p<0.05, ** p<0.01; m: males; f: females
Findings considered related to treatment with fluopicolide are written in **bold** text.

The DS concluded that a possible CAR/PXR mechanism could be responsible for the observed effects in the liver of the mouse. There are four additional mechanistic studies addressing the observed mouse liver oncogenicity. The first is a 28-day *in vivo* study in C57BL/6 mice focused

on hepatic cellular proliferation as well as morphological changes of the liver and hepatic cytochrome P-450 isoenzyme activity. In addition, three *in vitro* studies in different hepatocyte cultures (mouse wild type, mouse CAR/PXR KO and human) were performed.

In the 28-day *in vivo* study in mice, fluopicolide was administered continuously via the diet to a group of 15 females for at least 28 days at concentrations of 0 and the dose at which liver tumours occurred in the carcinogenicity study, i.e. 3200 ppm (equivalent to 575 mg/kg bw/day), with satellite subgroups of 20 female mice per group for interim sacrifice after 7 days of treatment. Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. At both the interim and final sacrifice times, liver was weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes of the liver. In addition, at interim sacrifice, hepatic cytochrome P450 isoenzymes were assessed.

At 3200 ppm, there were no mortalities or clinical signs during the course of the study.

At interim sacrifice, mean absolute and relative liver weights were increased by 27 to 37% compared to controls, 9/20 livers appeared to be dark and 1/20 livers was enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals and a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/20 treated animals when compared to controls. An increased number of mitotic cells and some foci of single cell necrosis/apoptosis were seen in 5/20 treated animals. The mean BrdU labelling index was approx. 6.5-fold higher in treated animals, when compared to controls, indicative of hepatocellular proliferation in the liver.

At final sacrifice, mean absolute and relative liver weights were increased by 48 to 56% compared to controls, 11/15 livers appeared to be dark and 3/15 livers were enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals together with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/15 treated animals, when compared to controls. Minimal single cell necrosis/apoptosis were seen in only 1/15 treated animals and an increased number of mitotic cells in 2/15 treated animals. There was no increased hepatocellular proliferation based on the results of the BrdU assay.

Fluopicolide also induced an increase in total cytochrome P450 content (+97%) as well as in benzyloxyresorufin O-dealkylation (BROD, +1785%) and pentoxyresorufin O-dealkylation (PROD, +1143%) activities. Ethoxyresorufin O-deethylation (EROD) activity was only slightly induced and lauric acid hydroxylation decreased compared to control mean as shown in the following table.

Parameter	**Fluopicolide at 3200 ppm** % change compared to control mean
P450	+ 97 %
BROD	+ 1785 %
EROD	+ 79 %
PROD	+ 1143 %
Lauric acid	- 67 %

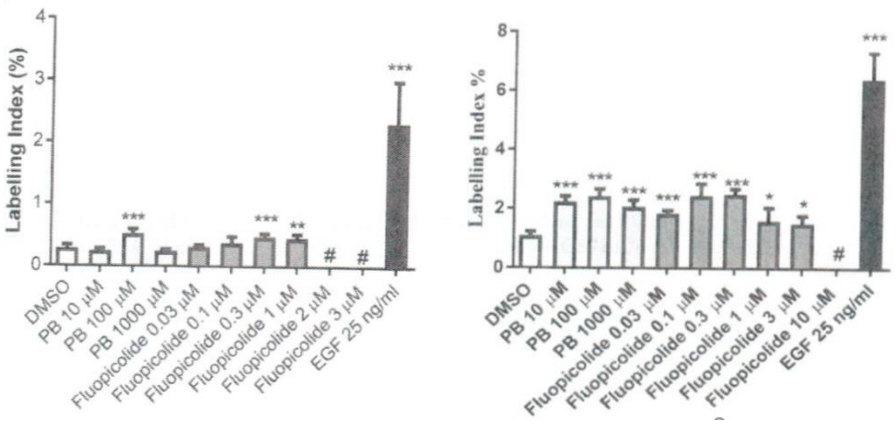
In conclusion, fluopicolide at 3200 ppm in the diet induced a transient and marked hepatocellular proliferation in C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. Fluopicolide is an inducer of BROD and PROD activities.

In the mechanistic studies hepatocytes from wildtype mice and CAR/PXR knockout (KO) mice were used *in vitro*. In these studies, cytotoxicity was evaluated by adenosine 5'-triphosphate depletion and phenobarbital (PB) was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of CYP2B and CYP3A

activities and increased cell proliferation). In addition, epidermal growth factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

In wildtype mouse hepatocytes fluopicolide was administrated to C57BL/6 male and female mouse hepatocytes in culture induced replicative DNA synthesis in a dose-dependent manner with maximal induction at 0.3 µM (1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes). Phenobarbital induced replicative DNA synthesis to a maximum of 1.8-fold and 2.2-fold in the male and female hepatocytes, respectively; the proliferative capability of these cells in culture was confirmed using EGF.

Effects of fluopicolide, PB or EGF on DNA synthesis (S-phase) in male / female mouse hepatocytes:



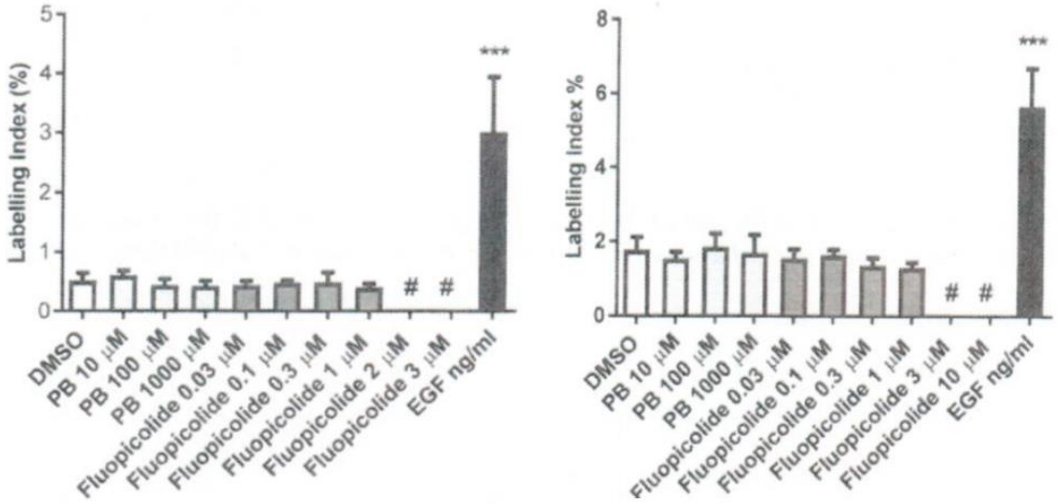
PROD, BROD and 7-benzyloxyquinoline O-debenzylase (BQ) rates are indicative of CYP2B and CYP3A induction. In male mouse hepatocytes, fluopicolide caused a dose dependent increase in PROD and BROD (up to 3-and 2.6-fold of control respectively). BQ was also slightly increased in these cells following administration of fluopicolide at 1 and 2 µM (1.4- and 1.5-fold respectively). In female mouse hepatocytes, fluopicolide induced a dose dependent increase in PROD (up to 1.7-fold), but not BROD or BQ activities.

PB (1 mM) caused significant increases in PROD, BROD and BQ activities, which were 6.5-, 4.9- and 7.7-fold, respectively, in male mouse hepatocytes and 2.6-, 1.6- and 4.4-fold, respectively, in female mouse hepatocytes.

In conclusion, fluopicolide induced both hepatocellular S-phase replicative DNA synthesis and CYP2B enzyme activity in both male and female mouse primary hepatocyte cultures.

Treatment of **CAR/PXR KO mouse hepatocytes** with fluopicolide or PB did not induce replicative DNA synthesis in males or females at any concentration but the proliferative capability of these cells in culture was confirmed using EGF.

Effects of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) (male / female):

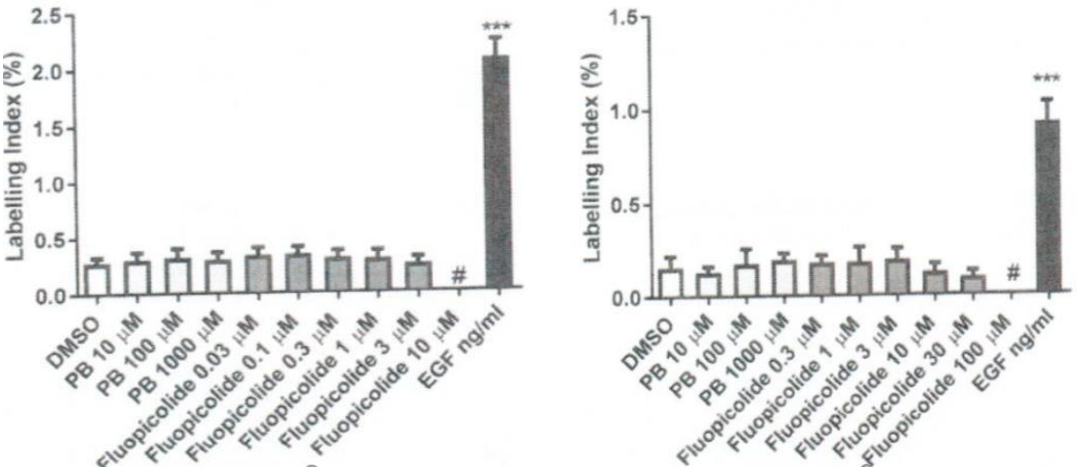


Similarly, fluopicolide did not cause any increases in PROD, BROD or BQ in male or female CAR/PXR KO mouse hepatocytes. PB administration (1000 µM) to male mouse hepatocytes slightly induced PROD, BROD and BQ to 3.5-, 1.5- and 1.7-fold respectively. 1000 µM PB also caused induction in female mouse hepatocytes in PROD and BROD 1.6- and 1.8-fold respectively, with no induction observed in BQ.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, CYP2B or CYP3A enzyme activity in male or female CAR/PXR KO mouse primary hepatocyte cultures. These data suggest that fluopicolide requires the presence of the nuclear hormone receptors CAR and/or PXR to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

In human hepatocytes from three individual male/female donors, fluopicolide or PB administration did not induce replicative DNA synthesis. The proliferative capability of these cells was confirmed using EGF.

Effects of fluopicolide, PB or EGF on replicative DNA synthesis (S-phase) in male/females human hepatocytes:



In male human hepatocytes, fluopicolide caused slight increases in BROD and BQ activities to a maximum of 1.5 - and 2.6-fold respectively. All levels of PB resulted in statistically significant increases in BROD and BQ activities in the male human hepatocytes, with maximum increases observed at 1 mM (2.0- and 5.3-fold respectively). PROD activity could not be analysed as levels were below the level of quantification, therefore, no results are presented for this assay in the male.

In females, treatment with fluopicolide resulted in dose-dependent increases in BQ activity in hepatocytes from both donors to a maximum of 1.7- and. 2.8-fold induction compared to controls. Fluopicolide caused no relevant increase in PROD or BROD activity in female hepatocytes, however, slight, but significant, decreases in BROD activity were observed at the top concentrations. In female human hepatocytes, PB consistently induced BROD and BQ activities in both donors, however, only one donor responded in a dose-dependent manner after treatment with PB.

In summary, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and females)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

These data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels).

In addition to the CAR/PXR KO mouse hepatocyte study, this study in human hepatocytes supports human non-relevance of this CAR/PXR-mediated liver tumour MoA.

The following table summarises the overall results regarding CAR/PXR activation and liver cell proliferation in the wildtype mouse, CAR/PXR KO Mouse and in human hepatocytes:

Key events	Wildtype mouse hepatocytes	CAR/PXR KO mouse hepatocytes	Human hepatocytes
CAR activation	+	-	-
PXR activation	+	-	Weak activator
Liver cell proliferation	+	-	-

Fluopicolide treatment resulted in the activation of CAR and weak activation of the PXR in the liver. This resulted in the altered expression of CAR-responsive genes that promoted a pro-proliferative and anti-apoptotic environment in the liver and an early, transient, increase in hepatocellular proliferation. Increased hepatocellular foci because of clonal expansion of spontaneously mutated cells in the mouse resulted in slight increases in liver adenomas incidence compared to concurrent controls. This MoA was supported by a series of associative events including: increased expression of genes encoding CYPs, particularly CYP2B and (to a lesser extent) CYP3A isoforms, increased proliferation and hepatocellular hypertrophy and increased liver weight. The MoA hypothesis as postulated by the DS is presented below, with the causal key events and associative events identified:

Proposed MoA for liver adenomas in mice:

Events	Description
Key events (KE)	
KE 1	Activation of CAR/PXR nuclear receptor
KE 2	Altered gene expression secondary to CAR/PXR activation
KE 3	Increased hepatocellular proliferation
KE 4	Increased clonal expansion, leading to altered foci
KE 5	Increased incidence of hepatocellular tumours
Associative events (AE)	
AE 1	Increased CYP2B, CYP3A enzyme activity and/or protein
AE 2	Hepatocellular hypertrophy
AE 3	Increased liver weight

RAC agrees with the dossier submitter that the available data provide enough evidence to support the postulated MoA (CAR activation) to be the underlying MoA of liver adenomas observed in

mice. Similar to phenobarbital (a known CAR inducer), fluopicolide did not induce DNA replication (prerequisite for tumour formation) in human hepatocytes nor in CAR/PXR KO mouse hepatocytes following induction of human CAR, in contrast to rats. Due to this qualitative difference, the liver adenomas because of CAR-activation by fluopicolide were considered to be of little relevance to humans.

AhR enzyme induction can be excluded as a potential mode of action. Fluopicolide did not produce a large increase in CYP1A EROD activity in the 28-day mechanistic study; only a slight increase of 79% was noted (compared with 1785% and 1143% increases in BROD and PROD, respectively). This increase followed a similar pattern to that observed with PB in a separate control study, in which PB induced an 83% increase in EROD activity (compared with a 6326% and 1920% increase in BROD and PROD respectively).

RAC notes that no information regarding the PPaR receptor and the gene expression of CYP4A activities was measured in the MoA studies.

The occurrence of hepatic neoplasia is limited to both sexes of one species. Furthermore, RAC considered the lack of progression to malignancy and plausible mechanistic information that the adenomas likely resulted from a rodent specific mechanism (CAR/PXR). Therefore RAC agrees with the DS that **classification for carcinogenicity is not warranted**.

10.10 Reproductive toxicity

10.10.1Adverse effects on sexual function and fertility

Table 10-25: Summary table of animal studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Preliminary study on reproductive per-formance in by dietary administration to CD rats 8 male and 8 female rats/group OECD 451 (1981) GLP	Fluopicolide (purity 95.9%) Oral administration via diet at 0, 50, 200, 750 or 2,500 ppm (equivalent to 0, 5.2/6.4, 25.5/32.9 and 103.4/127.3 mg/kg bw/day for M/F premating) Treatment started 15 days prior to pairing and continued uninterrupted until termination after weaning of the resulting litters.	50 and 200 ppm No effects ≥ 750 ppm ↓ bodyweight gain and food consumption in parental females 2,500 ppm ↓ bodyweight gain parental males and offspring Reproductive parameters were considered to be unaffected by treatment with fluopicolide up to and including the highest tested dose of 2,500 ppm.	Anonymous.; 2002; M-215068-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Two generation reproduction study in rats. Crl: CD® (SD) IGS BR rats 28 male and 28 female rats/group OECD 416, Draft (1999) GLP	Fluopicolide (purity 95.9%) Oral administration via diet at concentrations of 0, 100, 500 or 2,000 ppm throughout the two generations Equivalent to: F0: 0, 5.2, 25.5 & 103.4 mg/kg bw/day in males and 0, 6.4, 32.9 & 127.3 mg/kg bw/day in females during premating F1: 0, 5.7, 28.3 & 117.1 mg/kg bw/day in males and 0, 6.8, 34.6 & 141.6 mg/kg bw/day in females during premating	<u>100 and 500 ppm</u> No effects <u>2,000 ppm</u> *Parental:* ↓ bodyweight gain and food consumption (M/F) ↑ liver and kidney weights (M/F) ↓ spleen weights (F) *Offspring:* ↓ bodyweight gain Reproductive parameters and sexual maturation were considered to be unaffected by treatment with fluopicolide up to and including the highest tested dose of 2000 ppm.	Anonymous; 2003; M-232532-01-1
Additional microscopic examination to the 2-generation reproduct-ion study in rats	Fluopicolide (purity 95.9%) Oral administration via diet at concentrations of 0, 100, 500 or 2,000 ppm throughout the two generations.	<u>100 and 500 ppm</u> No adverse effects <u>2,000 ppm</u> *Parental:* ↑ incidence of centrilobular hepatocyte hypertrophy ↑ incidence of degenerative and regenerative changes in kidneys (M/F)	Anonymous; 2004; M-247289-01-1
Supplementary information: External expert statement regarding fluopicolides reproductive and developmental toxicity potentials	-	This document contains a summary and review of the two-generation and the developmental toxicity studies with rats and rabbits by an external expert for reproductive toxicity concluding that on the basis of the available studies, fluopicolide should not be classified as a reproductive toxicant.	Anonymous; 2018; M-638869-01-1

M = male F = female

Table 10-26: Summary table of human data on adverse effects on sexual function and fertility

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

The data of a preliminary dose range finding and main 2-generation study in rats are available.

In a **preliminary study** treatment levels for a two-generation study in **rats** were examined. Fluopicolide was administered orally, via the diet, at concentration levels of 0, 50, 200, 750 or 2,500 ppm to groups of 8 males and 8 females, 15 days prior to pairing until termination after weaning of the resulting litters. Selected offspring (constituting an F1 generation) continued to receive the diets from about the time of weaning until termination following attainment of sexual maturation.

This preliminary study showed that dietary concentrations of 2,500 ppm of fluopicolide induced general toxicity observed as bodyweight gain reductions in F0 males during the premating period, and in F0 females during the gestation and lactation period. A transient effect in F0 females was also observed during the gestation period at 750 ppm. F1 offspring body weight gains were also reduced at 2,500 ppm. Reproductive parameters were considered to be unaffected by treatment with fluopicolide up to and including the highest tested dose of 2,500 ppm. Therefore, a dose level of maximum up to 2,500 ppm was regarded as suitable as the high concentration in a main study of reproductive performance.

In the main **2-generation study in rats** the influence of fluopicolide on the fertility and reproductive performance of two successive generations was assessed in male and female rats of the Crl: CD® (SD) IGS BR strain. Fluopicolide was administered continuously in the diet at concentrations of 0, 100, 500 or 2,000 ppm to groups of rats throughout the two generations. The F0 generation, which comprised 28 males and 28 females in each group, received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation.

Parental toxicity

Body weight gain and food consumption were low for adult animals treated at 2,000 ppm throughout the study, with the exception of the low body weight gain which was not apparent in the females following parturition.

At 2,000 ppm, kidney and liver weights were high for parental males and females in both generations, when compared with the controls and a retrospective histopathological examination showed treatment-related findings in both organs (centrilobular hepatocyte hypertrophy and degenerative and regenerative changes in kidneys) at this dose level. Group mean body weight-relative liver weights were also slightly higher for females treated at 500 ppm, when compared with the controls and centrilobular hepatocyte hypertrophy was also present in in males at 500 ppm from both generations. Since these findings are common in the livers of rodents which have been administered xenobiotics, they are considered to be an adaptive change and not a toxic effect of treatment at this dose level.

Reproductive parameters

Oestrous cycles, mating performance, fertility and fecundity were similar in all groups. Gestation length, parturition process and sperm parameters were unaffected by treatment. The return of females to oestrous cycling following lactation was not influenced by treatment in either generation.

Offspring toxicity

Litter parameters at birth of the F1 and F2 progeny and their survival to weaning showed no detrimental effects of treatment. Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from day 14 through to weaning, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect. Sexual maturation, as assessed by the age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation, was also not affected by treatment with fluopicolide at doses up to and including 2,000 ppm.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL). The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. Since reproductive parameters were considered to be unaffected the NOEL for reproductive parameters is considered to be the highest tested dose of 2000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing. Reproductive parameters and sexual maturation were considered to be unaffected.

10.10.3 Comparison with the CLP criteria

According to the CLP criteria, reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring. The definitions presented in the CLP criteria are adapted from those agreed as working definitions in IPCS/EHC Document N°225, Principles for Evaluating Health Risks to Reproduction Associated with Exposure to Chemicals. In this classification system, reproductive toxicity is subdivided under two main headings:

- Adverse effects on sexual function and fertility;

- Adverse effects on development of the offspring.

According to the CLP criteria, a ‘Known or presumed human reproductive toxicant’ is a substance known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. Such a substance is classified as Category 1A (evidence for classification is primarily from human data) or Category 1B (evidence for classification is primarily from animal data).

According to the CLP criteria, a ‘Suspected human reproductive toxicant’ or reprotoxicant Category 2 is a substance for which is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

As described before, the rat dietary multigeneration reproductive toxicity study, including the dose range finding study with oral administration of fluopicolide (AE C638206) via diet at concentrations of up to 2,000 and 2,500 ppm did not reveal any effects on reproductive parameters, like fertility, oestrous cycling, spermatogenic function and capacity, mating, gestation or partiurition. Moreover, litter parameters at birth of the F1 and F2 progeny (litter size, sex ratios, neonatal toxicity), their survival to weaning and their sexual maturation (balano-preputial separation, vaginal opening) showed no adverse effects of treatment. The only offspring effect was a reduced bodyweight gain in both male and female offspring at 2,000 and 2,500 ppm from day 14 through to weaning which is considered due to a palatability effect and/or systemic toxicity due to direct consumption of the test diet.

With reference to the CLP criteria (Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures) there are no results from this study of reproduction and fertility in the rat that determine a need for classification i.e. there are no adverse effects on sexual function and fertility and no adverse effect on prenatal or postnatal development of the offspring, in the presence of parental toxicity. Lower body weight from postnatal day 14, in the F1 offspring at 2000 ppm, was not a developmental effect but a palatability effect and/or systemic toxicity resulting from direct consumption of the diet. Fluopicolide is not a reproductive toxicant.

10.10.4 Adverse effects on development

Table 10-27: Summary table of animal studies on adverse effects on development

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels, duration of exposure**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Rat oral gavage range finding develop-mental toxicity study Sprague Dawley rats 4 inseminated female rats/group OECD 414 (1981) GLP	Fluopicolide (purity 97.6%) 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy. No statistical analysis was performed in this range-finding study	<u>Maternal toxicity:</u> <u>There were no deaths at any dose</u> ***1000 mg/kg bw/day*** Clinical signs: pultaceous faeces in 2 females (Days 10-13 of gestation) ↓ food consumption on Days 7-10 (-16% compared with Days 4-7) & lower than at 500 mg/kg bw/day throughout. ↓ bodyweight gain on Days 0-21 (-30% compared with 500 mg/kg bw/day dose-group) ***500 mg/kg bw/day*** ↓ food consumption on Days 7-10 (-7% compared with Days 4-7) <u>Developmental toxicity</u> ***1000 mg/kg bw/day*** ↑ Incidence of post-implantation loss 31.7% (total litter loss in 1 female and 75% resorptions in another) ↓ fetal weight (-24% compared with main study control) ↓ crown-rump length (-10% compared with main study control) ***500 mg/kg bw/day*** ↓ fetal weight (-16% compared with main study control) ↓ crown-rump length (-6% compared with main study control) Based on the results of this study, the high dose in the main study should be between 500 and 1,000 mg/kg bw/day.	Anonymous; 2000; M-198488-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels, duration of exposure**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Rat oral gavage developmental toxicity study Sprague Dawley rats 23 dams/group OECD 414 (1981) GLP dose levels were not set in two- to four-fold intervals thyroid-related parameters were not assessed & anogenital distance of all live rodent foetuses was not measured as required in the newest guideline version	Fluopicolide (purity 97.6%, 97.8%) 0, 5, 60 or 700 mg/kg bw from Day 7-20 of pregnancy	Maternal toxicity: There were no deaths or clinical signs of toxicity ***700 mg/kg bw/day*** ↓ body weight gain (-12%* on Days 1-21; maximum -24%* on Days 7-10) ↓ food consumption (-3% on Days 1-21) ***60 mg/kg bw/day*** No effects ***5 mg/kg bw/d*** No effects Developmental toxicity: ***700 mg/kg bw/day*** ↓ mean foetal weight (-8%*) ↓ crown-rump length (-4%*) ↓ ossification (foetus/litter incidence %): caudal vertebrae (82.4/100*), sternebrae (71.8/100*) & forepaw (71.8/100*), hindpaw metatarsal (7/28.6[1]) & hindpaw toe (5.6/23.8[1]) ↑ incidence of minor defects (foetus/litter %): thoratic vertebrae centra (7/28.6*), thoratic vertebral arches (2.8/14.3[1]), sternebrae (2.1/14.3[1]) , various minor rib defects (4.2/14.3[1], 3.5/9.5[1] and 3.5/14.3[1]) ***60 mg/kg bw/day*** ↓ ossification (foetus/litter incidence %): caudal vertebrae (24.2/72.7*), sternebrae (20.9/63.6*) & forepaw (19/63.6*) ***5 mg/kg bw/day*** ↓ ossification (foetus/litter incidence %): caudal vertebrae (34/81*), sternebrae (20.7/47.6*) & forepaw (27.3/66.7*) There was no evidence of treatment-related teratogenic effects at any dose level. NOEL: 60 mg/kg bw/day for maternal toxicity and for developmental toxicity	Anonymous; 2004; M-202155-02-1

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels, duration of exposure**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Rabbit oral gavage range finding developmental toxicity study. Himalayan rabbits 4 dams/group OECD 414 (1981) GLP	Fluopicolide (purity 97.6%-97.8%) 25, 50, 100, 250, 500 or 1,000 mg/kg bw from Day 6-28 of pregnancy	<u>Maternal toxicity:</u> ***1000 mg/kg bw/day*** ↑ mortality/moribundity: 4/4 dead (Days 13-15) Clinical signs in all animals: impairment of mobility/consciousness, respiratory sounds, coat bristling, ↓ defication, hyper/hypoactivity, discoloured urine and tray, ↓ hay consumption Necropsy findings: beige discoloured heart, liver & kidneys & petechial bleeding in stomach (all animals) ↓ body weight: (10% lower than 25 mg/kg bw/day dose-group at 100% mortality on Day 13) ↓ food consumption throughout study (-97% on Days 10-13 compared with pre-treatment values) ***500 mg/kg bw/day*** ↑ mortality/moribundity: 4/4 dead (Days 15-16) Clinical signs in all animals: impairment of mobility/consciousness, respiratory sounds, coat bristling, ↓ defication, hyper/hypoactivity, discoloured urine and tray, ↓ hay consumption Necropsy findings: beige discoloured heart, liver & kidneys & petechial bleeding in stomach (all animals) Necropsy findings: beige discoloured heart, liver & kidneys & petechial bleeding in stomach (all animals) ↓ body weight: (9% lower than 25 mg/kg bw/day dose-group at 100% mortality on Day 16) ↓ food consumption throughout study (-98% on Days 13-16 compared with pre-treatment values) ***250 mg/kg bw/day*** ↑ mortality/moribundity: 4/4 dead (Days 17-23) Clinical signs in all animals: impairment of mobility/consciousness, respiratory sounds, coat bristling, ↓ defication, hyper/hypoactivity, discoloured urine and tray, ↓ hay consumption Necropsy findings: beige discoloured heart, liver & kidneys & petechial bleeding in stomach (all animals) ↓ body weight: (17% lower than 25 mg/kg bw/day dose-group at 100% mortality on Day 19) ↓ food consumption throughout study (-92% on Days 16-19 compared with pre-treatment values) ***100 mg/kg bw/day*** ↑ mortality/moribundity: 4/4 dead (Days 16-22) Clinical signs in all animals: impairment of mobility/consciousness, respiratory sounds, coat bristling, ↓ defication, hyper/hypoactivity, discoloured urine and tray & ↓ hay consumption Necropsy findings: beige discoloured heart, liver & kidneys & petechial bleeding in stomach (all animals)	Anonymous; 2000; M-211192-01-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels, duration of exposure	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
		1 dam aborted (Day 22) ↓ body weight: (16% lower than 25 mg/kg bw/day dose-group at 100% mortality on Day 19) ↓ food consumption throughout study (-98% on Days 16-19 compared with pre-treatment values) ***50 mg/kg bw/day*** There were no deaths Clinical signs (1 animal): ↓ defication & discoloured tray 1 dam aborted (Day 29) ↓ body-weight gain: (57% lower than 25 mg/kg bw/day dose-group throughout study) ↓ total food consumption (-20% on Days 0-29 compared with 25 mg/kg bw/day dose-group) ***25 mg/kg/bw/day*** No findings <u>Developmental toxicity:</u> Gravid uterus and fetal weights were normal and embryofoetal development was unaffected at 25 and 50 mg/kg bw/day. Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.	

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels, duration of exposure**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Prenatal oral gavage developmental toxicity study in rabbits. Himalayan rabbits 23 dams/dose) OECD 414 (1981) GLP the high dose resulted in a mortality rate >10%. Therefore, the number of females with implantation sites that were available at necropsy was <16 animals	Fluopicolide (purity 97.8%) 0, 5, 20 or 60 mg/kg bw on Days 6-28 of gestation	<u>Maternal toxicity:</u> ***60 mg/kg bw/day*** ↑ mortality: 18/23 dams dead (3 found dead on Days 24, 25 & 29 and 15 killed after premature delivery) Clinical signs: ↓ defecation, hypoactivity, bristling coat, pultaceous faeces & discoloured urine & ↓ hay consumption ↓ body weight gain (-86% Days 6-29 & -57% Days 0-29) ↓gravid uterine weight (-29%) ↓feed consumption (-43%* Days 23-26 & -54%* Days 26-29) Necropsy findings: tautly filled stomach (6/23), red fluid in urinary bladder (2/23), red fluid in uterus (2/23) & yellow discoloured liver (1/23) ↑ incidence of abortions (12/23 dams aborted litter) ↑ incidence of premature deliveries in 3/23 dams (0/7, 1/5 and 7/10 foetuses born dead in these litters) ***20 mg/kg bw/day*** 1 dam killed after aborting on Day 28 ↓ defecation (dam killed on Day 29) ↓ food consumption (-19% on Day 26 in dam killed on Day 29) ***5 mg/kg bw/day*** No findings <u>Developmental toxicity:</u> ***60 mg/kg bw/day*** ↓ mean foetal weight (-14%) ↓ crown-rump length (-6%) ***20 mg/kg bw/day*** No findings ***5 mg/kg bw/day*** No findings No teratogenic effects were observed in the fetuses at any dose level. NOAEL: 20 mg/kg bw/day for maternal toxicity and developmental toxicity	Anonymous; 2004; M-202513-02-1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels, duration of exposure	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
Supplementary information: External expert statement regarding fluopicolides reproductive and developmental toxicity potentials	-	This document contains a summary and review of the two-generation and the developmental toxicity studies with rats and rabbits by an external expert for reproductive toxicity concluding that on the basis of the available studies, fluopicolide should not be classified as a reproductive toxicant.	Anonymous; 2018; M-638869-01-1

% difference refers to concurrent control unless otherwise stated
* Statistically different from control
[1] not statistically significant but outside historical data (based on 21 studies in Sprague Dawley rats)

Table 10-28: Summary table of human data on adverse effects on development

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-29: Summary table of other studies relevant for developmental toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

10.10.5.1 Rat developmental toxicity studies

A rat range finding study was conducted to select suitable doses of fluopicolide for a subsequent developmental toxicity (teratogenicity) study in Sprague Dawley rats. Groups of 4 mated female Sprague Dawley rats received technical fluopicolide in aqueous methylcellulose (1% w/v) by oral gavage once daily at the dose levels of 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy and were sacrificed on Day 21 of pregnancy.

The key maternal findings in this study included a reduced gain in body weight throughout the period of treatment at 1,000 mg/kg bw/day (-34% compared to 500 mg/kg bw/day and -27% when corrected for gravid uterine weight). Food consumption showed a marked initial (Days 7-10) decrease at this dose level and a slight reduction at 500 mg/kg bw/day. No compound-related effects were observed at necropsy of the animals. Post-implantation loss was elevated at 1,000 mg/kg bw/day and included one total resorption. Mean fetal weight and crown-rump length were reduced at 1000 and 500 mg/kg bw/day. Therefore, the highest dose selected for the definitive study of developmental toxicity was 700 mg/kg bw/day.

In the main developmental toxicity study in rats, groups of 23 mated female Sprague Dawley rats received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 60 or 700 mg/kg bw/day from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection). They were sacrificed on Day 21 of pregnancy.

Body weights and weight gains were decreased in the animals from the high dose group, especially at the beginning of the treatment period during gestational Days 7-10 (-24% when compared with the control value), a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. These animals showed also a slight initial decrease in food consumption after beginning of treatment. Overall body weight gain (Days 1-21) was 9% lower than the concurrent control value and 12% lower when corrected for gravid uterine weight.

Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group. However, litter size, number of live and dead foetuses as well as sex ratios were unaffected by the administration of the test substance. Incidences of early and late conceptuses undergoing resorption were also not affected by the administration of the test compound up to and including the highest tested dose level of 700 mg/kg bw/day.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor skeletal defects at the thoracic vertebrae, sternebrae and ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses. In addition a delayed ossification was detected at 700 mg/kg bw/day which indicated together with the decreased foetal weight and length a generally retarded foetal development at this maternally toxic dose level.

In conclusion, oral administration of fluopicolide to the pregnant rat at the dose of 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gains and slightly decreased food consumption. Mean foetal body weights and crown-rump lengths were also slightly decreased at700 mg/kg bw/day. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification were observed more frequently at this dose level and are considered secondary to the above described maternal toxicity. Fluopicolide was not teratogenic in this developmental toxicity study in rats.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 60 mg/kg bw/day or below. Therefore, with regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

10.10.5.2 Rabbit developmental toxicity studies

A rabbit range finding study with dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw/day was conducted in rabbits in order to select a suitable high dose level of fluopicolide for a subsequent developmental toxicity study in Himalayan rabbits. All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study. At the dose of 50 mg/kg bw/day one animal aborted on Day 29 and decreased bodyweight gain and food consumption was recorded. No significant effects were observed at 25 mg/kg bw/day. Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.

In the main rabbit developmental toxicity study, groups of 23 mated female Himalayan rabbits received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 20 or 60 mg/kg bw/day from Day 6-28 of gestation and were sacrificed on Day 29 of gestation.

The study report author stated that three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation; however, further examination of the raw data has revealed that 12 dams aborted whole litters (with no live pups), whilst 2 dams delivered prematurely with partial live litters (dam 178 delivered 1 dead and 4 live pups and dam 180 delivered 7 dead and 3 live pups). In addition a further dam prematurely delivered 7/7 live pups. These dams showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous feces, and discoloured urine. One animal from the intermediate dose group (20 mg/kg bw/day) was killed after aborting on Day 28 of

gestation. This animal showed decreased defecation and reduced hay consumption. The dossier submitter considers this to be a spuorious finding and not related to treatment with fluopicolide, owing to its isolated occurrence in this dose group, supported by the the dose-range finding study, in which no abortions were observed at a similar dose (25 mg/kg bw/day) and only one dam aborted at a much higher dose (50 mg/kg bw/day). Furthermore, according to published historical data, up to 20% abortions have been reported for this strain of rabbit (Viertel & Trieb 2002[4]). Therefore, this isolated single abortion at this dose is considered incidental and not treatment-related.

Body weight gains and food consumption were markedly decreased in the animals from the high dose group (-57% for Days 0-29 and -54% for Days 26-29 for body-weight gain and food consumption respectively when compared with the control group). Gravid uterus weights were 29% lower than controls in the animals from the high-dose group; nonetheless, when adjusted for gravid uterine weight, the dams in the high-dose group showed mean body-weight losses (for Days 0-29) of 148 g, compared with a mean loss of 15 g in the control group.

At necropsy, tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discoloration of the liver were observed in single animals from the high dose group. No compound-related effects were observed in the low and intermediate dose group.

Twelve dams aborted their litters whilst two dams prematurely delivered litters that contained 1/5 and 7/10 dead foetuses respectively; a further dam prematurely delivered a litter conaining only live foetuses (7/7 live foetuses). It is more likely that the observed abortions are secondary to the maternal toxicity observed at this dose, and not a consequence of malformations in the aborted foetuses; no malformations were detected in these foetuses or in foetuses at the lower doses. Furthermore, variations which could potentially progress into malformations (with an increase in dose) were not detected at 50 or 20 mg/kg bw/day. Mean fetal body weights, crown-rump lengths and placental weights were decreased in the animals from the high dose group. Of the remaining surviving litters, litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound (litters from dams found dead or killed after abortion/premature delivery were excluded from subsequent calculations). Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test substance. Morphological examination of the fetuses did not reveal any compound-related effects up to and including the highest tested dose level of 60 mg/kg bw/day.

There are several publications describing the effects of feed restriction during gestation on developmental parameters in rabbits (Matsuzawa *et al.* 1981[5]; Petrere *et al.* 1993[6]; Cappon *et al.* 2005[7]; Menchetti *et al.* 2015[8]). In most of them feed was restricted during the phase of organogenesis between approx. GD 6-19 resulting in abortions, decreased foetal and placental weight, reduced foetal ossification and also higher rate of pre-, peri- and postnatal death. A feed restriction by 87.7-90% resulted in all three publications with feed restriction during organogenesis in an increased rate of abortions whereas pregnancy was not affected at the next higher tested feed level (feed restriction to 23.3%, 40% or 50% of control, respectively).

Reduced fetal weight and delayed ossification was already detected by Cappon *et al.*[7] (2005) starting at a feed restriction of 50% of control. In addition, Menchetti *et al.*[8] (2015) showed that the late gestational phase from

4 Viertel B, Trieb G. The Himalayan rabbit (Oryctolagus cunuiculus L.): Spontaneous incidences of endpoints from prenatal developmental toxicity studies, Laboratory animals. 2003; 27, 19-36.

5 Matsuzawa T, Nakata M, Goto I, Tsushima M. Dietary deprivation induces fetal loss and abortion in rabbits. Toxicology. 1981;22(3):255-9.

6 Petrere JA, Rohn WR, Grantham LE 2nd, Anderson JA. Food restriction during organogenesis in rabbits: effects on reproduction and the offspring. Fundam Appl Toxicol. 1993 Nov;21(4):517-22.

7 Cappon GD, Fleeman TL, Chapin RE, Hurtt ME. Effects of feed restriction during organogenesis on embryo-fetal development in rabbit. Birth Defects Res B Dev Reprod Toxicol. 2005 Oct;74(5):424-30.

8 Menchetti L, Brecchia G, Canali C, Cardinali R, Polisca A, Zerani M, Boiti C. Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming. Res Vet Sci. 2015 Feb;98:7-12

GD 19-28 is even more sensitive regarding secondary effects of decreased feed consumption. A feed restriction of 30% during this gestational period already lead to a minimal increase of abortions and slightly decreased number of live born pups whereas this was not observed if feed was restricted to the same proportion from GD 0-9 or GD 9-18.

Therefore, the increased incidence of abortions (12/23 dams) and premature deliveries with some dead foetuses (2/23 dams), along with the reduced fetal weights and crown-rump lengths in the highest dose group in the present study are considered as secondary consequences of severe maternal toxicity as evidenced by mortality (3/23 dams found dead) and decreases in body weight gain due to markedly reduced food consumption. As shown in the table below the food consumption was drastically reduced to 9% of control in the high dose dams that died or had abortions from gestation day 19 till death or sacrifice whereas the high dose dams that survived till termination showed a food consumption reduction to 60% of control which is in good agreement with the published results in rabbits.

Mean maternal feed consumption in g/animal/day during gestation (% of control)

Parameter	Dose Group (mg/kg bw/day)				
	0	5	20	60	
				Died or aborted during gestation	Survived until termination
Number of dams	21	20	21	18	5
Day 6-19	92.4	87.2 *(94 %)*	87.5 *(95 %)*	64.0 *(69 %)*	79.9 *(86 %)*
Day 19 - sacrifice[a]	98.5	96.7 *(98 %)*	70.0 *(71 %)*	9.1 *(9 %)*	58.9 *(60 %)*

[a] death in case of 3 animals in the high dose group

In conclusion, oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused severe maternal toxicity as evidenced by mortality, clinical signs, decreases in body weight gain and drastically reduced food consumption. Only secondary to this severe maternal toxicity were an increased incidence of abortion (12/23 dams), reduced foetal body weights and reduced crown lump lengths observed. There was no evidence of treatment-related teratogenic effects at any dose level.

At doses up to and including 20 mg/kg bw/day, fluopicolide did not cause any maternal toxicity or embryotoxicity. Therefore, the No Observed Adverse Effect Level (NOAEL) is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity in the present study.

10.10.6 Comparison with the CLP criteria

There is no data on humans to inform on the developmental toxicity of fluopicolide, and thus classification in category 1A is not appropriate.

Classification in category 1B for developmental toxicity is not appropriate as there is no clear evidence of an adverse effect on development in experimental animals.

Substances are classified in category 2 when there is some evidence from humans or experimental animals of an adverse effect on development, and where the evidence is not sufficiently convincing to place the substance in category 1. Furthermore, the effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

In the 2-generation study, there were no effects on embryo or fetal lethality or on pup survival during lactation and weaning. It is concluded that slightly reduced bodyweight development of pups during lactation was likely a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a specific toxic effect of fluopicolide on postnatal development.

In the rat developmental toxicity study, dam body weights and bodyweight gains were decreased in the high dose group during the treatment phase, especially at the beginning of the treatment period during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. Fluopicolide was found not to be teratogenic in the rat; the incidence of major malformations was low and clearly incidental to the administration of fluopicolide. Foetuses from the high dose group showed treatment-related increased incidences of minor skeletal defects at the thoracic vertebrae, sternebrae and ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses. For this reason, it is considered that the anomalies should be considered non-adverse such that classification of fluopicolide as a developmental toxicant is not warranted with reference to the CLP guidance statement that 'classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in fetal/pup body weight or retardation of ossification when seen in association with maternal toxicity'. In addition a delayed ossification was detected at 700 mg/kg bw/day which indicated together with the decreased foetal weight and length a generally retarded foetal development at this maternally toxic dose level.

In the rabbit developmental toxicity study, 15 animals of the 60 mg/kg bw/day group were killed after abortion or premature delivery on Days 22-29 of gestation. Twelve dams aborted their litters, whilst three dams delivered prematurely (two of these dams produced litters with 1/5 and 7/10 dead foetuses respectively and the remaining dam delivered a live litter). Mean fetal body weights, crown-rump lengths and placental weights were decreased in the animals from this dose group. However, all these findings are considered secondary to severe maternal toxicity as evidenced by mortality (3 animals) and decreases in body weight gain due to drastically reduced feed consumption at the hightest tested dose. There was no evidence of treatment-related teratogenic effects at any dose level.

It can be summarized that in both species developmental effects only occurred at high doses which caused maternal toxicity and even mortality in rabbits. Therefore, the developmental findings are considered to be secondary, non-specific consequences of maternal toxicity in the conducted developmental toxicity studies in rats and rabbits.

In conclusion, the data regarding adverse effects on development are conclusive but do **not warrant** a developmental toxicity classification.

10.10.7 Adverse effects on or via lactation

Table 10-30: Summary table of animal studies on effects on or via lactation

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
No special studies on lactation available			
Preliminary study on reproductive performance by dietary administration to CD rats 8 male and 8 female rats/group OECD 451 (1981) GLP	Fluopicolide (purity 95.9%) Oral administration via diet at 0, 50, 200, 750 or 2,500 ppm (equivalent to 0, 5.2/6.4, 25.5/32.9 and 103.4/127.3 mg/kg bw/day for M/F premating) Treatment started 15 days prior to pairing and continued uninterrupted until termination after weaning of the resulting litters.	Dietary concentrations of 2500 ppm induced toxicity observed as body weight gain reductions in F0 males during the premating period, and in F0 females during the gestation and lactation period. Thus, F1 offspring body weight reductions from approx. day 14 of age at the same dose level suggest a systemic toxicity due to direct consumption of the test diet and /or a palatability effect and no direct lacational effect.	Anonymous; 2002; M-215068-01-1
Two generation reproduction study in rats. Crl: CD® (SD) IGS BR rats 28 male and 28 female rats/group OECD 416, Draft (1999) GLP	Fluopicolide (purity 95.9%) Oral administration via diet at concentrations of 0, 100, 500 or 2,000 ppm throughout the two generations	Litter parameters at birth (F1 and F2) and survival to weaning not affected and sexual maturation was not affected. Body weight at birth were also comparable with control. Reduced body weight apparent from approx. day 14 of age on in both generations is considered to be due to a palatability effect and/or systemic toxicity due to direct consumption of the test substance and not a direct lactational effect.	Anonymous; 2003; M-232532-01-1
Supplementary information: External expert statement regarding fluopicolides reproductive and developmental toxicity potentials	-	This document contains a summary and review of the two-generation and the developmental toxicity studies with rats and rabbits by an external expert for reproductive toxicity concluding that on the basis of the available studies, fluopicolide should not be classified as a reproductive toxicant.	Anonymous; 2018; M-638869-01-1

Table 10-31: Summary table of human data on effects on or via lactation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-32: Summary table of other studies relevant for effects on or via lactation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
ADME study following repeated oral administration to the lactating cow	Fluopicolide (purity 99.3%) (^{14}C)-Fluopicolide	The absorption, distribution, metabolism and excretion of (^{14}C)-fluopicolide were investigated after twice daily oral administrations for 7 consecutive days in two lactating cows. Excretion balance and tissue distribution investigations were performed at two dose levels, nominally at 1 ppm (equivalent to 0.024 mg/kg bw/day) and 10 ppm (0.28 mg/kg bw/day) in the diet.	In the milk, the level of radioactive residues reached a steady state with a maximum value of 18.8 ng equivalents/g (19 ppb) 5 days after the first dose at the high dose. Therefore, the residues in milk were below the concentration requiring extensive analysis (<50 ppb). The major compound identified was unchanged fluopicolide, which accounted for 29% of the TRR in the milk.	Anonymous; 2008; M-218626-02-1
Distribution and Metabolism of [^{14}C]-fluopicolide in the lactating cow	Fluopicolide (purity 99.3%) (^{14}C)-Fluopicolide	Two lactating cows were orally dosed by gelatine capsules twice daily with [^{14}C]- fluopicolide for 7 consecutive days, by gelatin capsule at a daily dose level of 1 ppm (0.03 mg/kg bw/day) and 10 ppm (0.35 mg/kg bw/day). Milk was collected from each animal twice daily, immediately prior to the morning and afternoon dosing.	The concentrations of total radioactivity in milk were low for both dose levels, with a maximum concentration of 10 ng equivalents/g reached at 32 h post dose at the 10 ppm dose level. For the 1 ppm dose level, levels did not rise above 1 ng equivalents/g.	Anonymous; 2009; M-233391-02-1
Residues and major metabolites in milk and edible cattle tissues following 28 days dosing of fluopicolide to lactating cows	Fluopicolide (purity 96.1%)	3 lactating cows/group were dosed with gelatine capsules at 0, 0.5, 1.5 and 5 mg/kg bw/day fluopicolide daily for 28-days. Cows were milked twice daily and residues of fluopiclode and ist major metabolites were quantified.	Apart from two values above 0.01 mg/kg, all milk samples analysed gave results below the LOQ of 0.01 mg/kg for fluopicolide and its main metabolites. The two values above the LOQ were from Day 4 and Day 28 in one animal from the top dose group.	Anonymous; 2004; M-219457-01-1

10.10.8 Short summary and overall relevance of the provided information on effects on or via lactation

The potential of fluopicolide to elicit adverse effects on or via lactation has been investigated in a preliminary dose range finding and in a two-generation reproduction toxicity study in rats (see Section 10.10.1). No data from humans are available.

Reduced body weight apparent in offspring from approx. day 14 through to weaning, coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect. This is supported by the fact that reduced food consumption and body weight development was observed in all repeated dose rat studies during the initial treatment phase and also in the parental animals of the present reproductive studies. Therefore, in the preliminary and main rat dietary multigeneration reproductive toxicity study, no effects on the lactation or any negative subsequent effects on the offspring which could be mediated via lactation are considered.

In conclusion, it can be summarized that based on these results there is **no clear evidence** of an adverse effect due to transfer in the milk, or an adverse effect on the quality of the milk.

In addition, three studies are available conducted in lactating cows designed to investigate the concentration of fluopicolide and its major metabolites in milk (see Table 10-32) showing a low to very low transfer into milk (less than 0.2% of radioactivity was excreted in the milk) with no evidence of any accumulation.

10.10.9 Comparison with the CLP criteria

Under CLP, substances that are absorbed by women and have been shown to interfere with lactation shall be classified and labelled to indicate this property hazardous to breastfed babies. Effects in the mother can adversely impact the breast milk (either in terms of the quantity produced or the quality produced). However, if a substance causes overt toxicity in the mother, this may indirectly impair milk production or impair maternal care as a non-specific secondary effect and should not lead to classification.

a) Human evidence indicating a hazard to babies during the lactation period

No data from humans are available.

b) Results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk

Reduced body weight apparent in offspring from approx. day 14 through to weaning, coinciding with the time when the offspring started to eat the diet suggest a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect. This is supported by the fact that reduced food consumption and body weight development was observed in all repeated dose rat studies during the initial treatment phase and also in the parental animals of the present reproductive studies. In conclusion, in the preliminary and main rat dietary multigeneration reproductive toxicity study there is no clear evidence of an adverse effect due to transfer in the milk, or an adverse effect on the quality of the milk.

c) Absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk

The available toxicokinetic data suggest that fluopicolide is extensively metabolised to more polar, water soluble molecules followed by a moderately rapid elimination primarily via bile. On this basis, it is unlikely that fluopicolide or its metabolites would be transferred in significant amounts into the milk. This is supported by studies conducted in lactating cows, which show low to very low transfer of fluopicolide to the milk with no evidence of any accumulation.

Based on the above assessment and comparison with the classification criteria, fluopicolide **does not meet** the criteria for classification for effects on or via lactation.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

On the basis of the results of the two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, there is **no justification for classification** of fluopicolide as a reproductive toxicant based on the CLP criteria (Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures). This is supported by an external expert review of the experimental study results in relation to the criteria for classification (Anonymous; 2018; M-638869-01-1).

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS summarised three acute toxicity studies in the CLH report.

Acute oral toxicity

In an OECD TG 423 compliant oral acute toxicity study (acute toxic class method) conducted in 2000, 5 male and 5 female fasted Sprague-Dawley rats were each administered by gavage a single oral dose of 5000 mg/kg bw. There were no mortality. Clinical signs as piloerection and hunched posture were seen in all female rats and in three male rats, and abnormal gait was noted in three females. Recovery of rats, as judged by external appearance and behavior, was complete by day 3. No significant effect was reported on body weight gains throughout the study. The acute lethal oral dose of fluopicolide in rats was greater than 5000 mg/kg bw. The DS proposed not to classify for acute oral toxicity.

Acute dermal toxicity

An OECD TG 402 acute dermal limit toxicity study was conducted in Hsd Sprague-Dawley rats in 2000 with 97.7% pure fluopicolide. A single topical application of 5000 mg/kg bw of the test substance was applied under occlusion for 24 hours.

No animals died. No clinical signs or dermal response in any animal was observed throughout the study. Two females had a slightly reduced body weight on day 8. There were no macroscopic abnormalities at study termination on day 15. The fluopicolide acute lethal dermal dose (LD_{50}) to rats was greater than 5000 mg/kg bw and thus greater than the trigger value of 2000 mg/kg bw, and therefore the DS proposed not to classify fluopicolide for acute dermal toxicity.

Acute inhalation toxicity

A limit test in accordance with OECD TG 403 exposing five male and five female Sprague-Dawley rats nose only to 5.16 mg/L for four hours did not lead to any mortality.

Clinical signs during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen, but all animals recovered within a day after exposure. At autopsy, one male showed dark foci on its lungs. The LC_{50} was thus > 5.16 mg/L.

No classification for acute inhalation toxicity was proposed by the DS.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

Limit acute toxicity tests were conducted with fluopicolide following oral, dermal and inhalation route. For the oral route, a dose of 5000 mg/kg bw was used, and no mortalities were reported. Thus, the criteria for classification (2000 mg/kg bw) for acute oral toxicity was no met, and no classification is warranted.

For the dermal route, no mortalities were reported at the limit dose of 2000 mg/kg bw, which also is the classification limit dose for classification for acute dermal toxicity. No classification for the dermal route is therefore warranted.

With respect to the inhalation route, a concentration of 5.16 mg/L for 4 hours was used. No mortalities at the doses tested were reported. As the highest limit for classification is 5 mg/L for 4h for dusts and mists, no classification for acute toxicity by inhalation is warranted.

RAC agrees with the DS that **no classification for acute toxicity through any route is warranted for fluopicolide.**

10.11 Specific target organ toxicity-single exposure

The acute studies that are relevant for the assessment of the specific target organ toxicity of fluopicolide after single exposure are reported in Sections 10.1 to 10.3. An acute neurotoxicity study is also available and is summarised below.

Table 10-33: Summary table of animal studies relevant for STOT SE

Study, species, test substance, purity	Doses	Main effects	Reference
Acute oral, dermal and inhalation toxicity studies in rats	Fluopicolide as tested in the acute oral, dermal and inhalation toxicity studies	No specific target organ toxicity, also no narcotic effects, which would fall under any STOT SE criteria were noted in the acute toxicity studies.	See Sections 10.1 to 10.3
Acute neurotoxicity Oral (gavage) US OPPTS 870.6200 (1988) GLP Rat, CD Males & Females 10/sex/group Fluopicolide (purity 95.9%) Vehicle: aqueous 1% methylcellulose	0, 10, 100 and 2,000 mg/kg bw Single dose	There were no deaths or specific neurotoxicity findings. <u>2,000 mg/kg bw</u> Transiently decreased body temperature in both sexes <u>100 mg/kg bw</u> No treatment-related effects <u>10 mg/kg bw</u> No treatment-related effects	Anonymous; 2002; M-208046-01-1

Table 10-34: Summary table of human data on STOT SE

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-35: Summary table of other studies relevant for STOT SE

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No other data				

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

Studies which can be used to evaluate potential STOT SE effects were already summarized in Sections 10.1 to 10.3. These studies on acute oral, dermal and inhalation toxicity demonstrated a low acute toxic potential of fluopicolide (AE C638206) with oral LD_{50} values of > 5,000 mg/kg bw after oral and dermal administration and an inhalation LC_{50} value of > 5.16 mg/L. These values are above the classification criteria for STOT-SE classification. In addition, an acute neurotoxicity study is available in which no specific acute neurotoxicity or any narcotic effect was observed.

There was no indication of any sex-specific susceptibility in any of the acute studies. Since no specific, non-lethal target organ toxicity, or other significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed, arising from a single exposure were seen in the studies on acute toxicity, according to the ECHA Guidance a classification in STOT SE Category 1 and also in 2 is not applicable. The STOT SE criteria are not fulfilled based on the results of the acute toxicity studies with fluopicolide, as shown in the following table:

Summary of acute toxicity study results

Study	Toxicological effects at LOAEL
Acute oral rat	> 5,000 mg/kg bw: unspecific clinical signs
Acute dermal rat	> 5,000 mg/kg bw: No clinical signs, no mortalities
Acute inhalation rat	> 5.16 mg/L/4h (highest tested dose): unspecific clinical signs, no mortalities
Acute oral neuro-toxicity rat	No effects related to specific neurotoxicity up to 2,000 mg/kg bw, NOEL: 100 mg/kg bw for general toxicity

Moreover, there were no acute effects observed in repeated dose toxicity studies with fluopicolide.

Furthermore, the ECHA Guidance specifies criteria that trigger a classification for STOT SE Category 3. These criteria are generally independent from the aforementioned guidance values and include transient target organ effects, focusing on overt narcotic effects and respiratory tract irritation (respiratory tract irritation covers two different effects: ‘sensory irritation’ and ‘local cytotoxic effects’). Specifically, the following examples for findings from single and repeated inhalation toxicity studies are mentioned as possible triggers for a STOT SE Category 3 classification: clinical signs of toxicity (dyspnoea, rhinitis etc.) and histopathology (e.g. hyperemia, edema, minimal inflammation, thickened mucous layer) which are reversible.

According to the CLP criteria, also physicochemical properties, such as pH, physical form, solubility, vapour pressure, particle size, which can be important parameters in evaluating toxicity studies and in determining the most appropriate classification especially with respect to inhalation where physical form and particle size can have a significant impact on toxicity. For fluopicolide none of these parameters indicate a potential to fall under STOT SE Category 3.

10.11.2 Comparison with the CLP criteria

According to the CLP criteria, a classification for STOT SE needs to be considered if the substance causes non-lethal target organ toxicity after a single exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitisation, genotoxicity, carcinogenicity and reproductive toxicity should be taken into consideration).

Based on the results after acute exposure to fluopicolide (AE C638206) in toxicological studies with single and repeated dosing, no significant toxic effects on specific target organs were observed at non-lethal dose levels at or below reference values assigned in the guidance on the application of the CLP criteria. Thus, classification of fluopicolide for STOT SE Category 1 or 2 is not warranted.

There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to fluopicolide. Therefore, classification of fluopicolide for STOT SE Category 3 is therefore also **not warranted**.

10.11.3 Conclusion on classification and labelling for STOT SE

A comparison of the toxicological effects in acute oral, dermal and inhalation toxicity studies, furthermore of the lack of effects in other studies, like in an acute neurotoxicity rat study, with the aforementioned classification criteria reveals that the results are conclusive and that a STOT SE Category 1, 2 and 3 classification of fluopicolide (AE C638206) according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017) is **not warranted**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

The acute studies that are relevant for the assessment of the specific target organ toxicity of fluopicolide after single exposure are assessed in the acute toxicity section. An acute neurotoxicity study is also available and is summarised below.

***Table**: Summary table of animal studies relevant for STOT SE*

Study	Doses	Main effects	Reference
Acute oral, dermal and inhalation toxicity studies in rats	Fluopicolide as tested in the acute oral, dermal and inhalation toxicity studies	No specific target organ toxicity, also no narcotic effects, which would fall under any STOT SE criteria were noted in the acute toxicity studies.	Sec. Acute toxicity
Acute neurotoxicity Oral (gavage) US OPPTS 870.6200 (1988) GLP Rat, CD Males & Females 10/sex/group Fluopicolide (purity 95.9%) Vehicle: aqueous 1% methylcellulose	0, 10, 100 and 2000 mg/kg bw Single dose	There were no deaths or specific neurotoxicity findings. <u>2000 mg/kg bw</u> Transiently decreased body temperature in both sexes <u>100 mg/kg bw</u> No treatment-related effects <u>10 mg/kg bw</u> No treatment-related effects	Anonymous, 2002; M-208046-01-1

The studies on acute oral, dermal and inhalation toxicity demonstrated a low acute toxic potential of fluopicolide with oral and dermal LD_{50} values of > 5000 mg/kg bw and an inhalation LC_{50} value of > 5.16 mg/L. These values are above the classification criteria for STOT-SE classification. In addition, an acute neurotoxicity study is available in which no specific acute neurotoxicity or any narcotic effect was observed.

There is also no indication of transient effects such as respiratory tract irritation and narcotic effects after single exposure to fluopicolide. Therefore, the DS proposed no classification of fluopicolide for STOT SE.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

There was no indication of any sex-specific susceptibility in any of the acute studies. No specific, non- lethal target organ toxicity, or other significant health effects that can impair function, either reversible or irreversible, immediate and/or delayed, arising from a single exposure were seen in the acute toxicity studies. In the acute neurotoxicity study, no specific neurotoxicity effects including narcotic effects were observed. The STOT SE criteria are not fulfilled based on the results of the acute toxicity studies with fluopicolide, as shown in the following table:

***Table**: Summary table of acute toxicity study results*

Study	**Toxicological effects at LOAEL**
Acute oral rat	> 5000 mg/kg bw: unspecific clinical signs
Acute dermal rat	> 5000 mg/kg bw: No clinical signs, no mortalities
Acute inhalation rat	> 5.16 mg/L/4h (highest tested dose): unspecific clinical signs, no mortalities
Acute oral neuro-toxicity rat	No effects related to specific neurotoxicity up to 2000 mg/kg bw, NOEL: 100 mg/kg bw for general toxicity

On the basis of the available studies, RAC agrees with the DS that **no classification for STOT SE is justified.**

10.12 Specific target organ toxicity-repeated exposure

The following tables provide a detailed overview of potentially classification-relevant toxicological findings of fluopicolide (AE C638206) with the respective applicable CLP criteria (following the Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, July 2017) for specific target organ toxicity after repeated exposure (STOT RE). The specific target-organ toxicity of fluopicolide upon repeated exposure has been investigated in nine short-term oral studies in rats, mice and dogs ranging from 28-days to one-year in dogs. Furthermore one 4-week dermal study in rats was performed (see Table 10-36). Additional information is provided by the chronic / carcinogenicity studies in rats and mice, the findings in the parental animals and offspring in the 2-generation study, the developmental toxicity studies and by the subchronic neurotoxicity study in rats (see Table 10-38).

Table 10-36: Summary table of animal studies on STOT RE

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
Oral route					
Rat 28-day dietary toxicity study OECD 407 (1995) GLP Fluopicolide (purity 99.0%)	Sprague Dawley CRL:(IGS)CDBR rats 5/dose/sex	0, 20, 200, 2000 or 20,000 ppm Equivalent to: 1.78, 17.7, 179 and 1,770 mg/kg bw/day (combined sexes) 28 days	Cat 1 ≤ 30 Cat 2 ≤ 300	<u>There were no deaths or clinical signs of toxicity</u> <u>20 ppm (1.78 mg/kg bw/day)</u> no effects observed <u>≥ 200 ppm (17.7 mg/kg bw/day)</u> **↑ incidence of centrilobular hepatocytic hypertrophy in 2/5 M (1 minimal & 1 slight) & 3/5 F (minimal)** <u>≥ 2,000 ppm (179 mg/kg bw/day)</u> **↓ bodyweight F (-11.3%) on Day 29** **↓ Body-weight gain[#] in F (-30%) on Days 0-29** **↑ water consumption in M (+18.4%) in week 3** **↑ cholesterol in M (+50%**) & F (+29%**)** **Pale kidneys in 3/5 M** **↑ severity of phloxine tartrazine-positive granulation (hyaline droplets) in kidneys (M)** **↑ incidence of centrilobular hepatocytic hypertrophy in 5/5 M (1 minimal & 4 slight) & 2/5 F (minimal)** <u>20,000 ppm (1,770 mg/kg bw/day)</u> ↓ bodyweight M ↑ water consumption ↓ feed intake ↓ ALT ↑ relative liver weights ↑ absolute liver weights Enlarged livers	Anonymous; 2000; M-199377-01-1

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Mouse 28-day dietary toxicity study OECD 407 (1995) GLP Fluopicolide (purity 99.0%)	CD-1 mice 5/dose/sex	0, 6, 64, 640 or 6,400 ppm Equivalent to: 1.07, 11.6, 115 and 1,111 mg/kg bw/day (combined sexes) 28 days	Cat 1 ≤ 30 Cat 2 ≤ 300	There were no deaths or clinical signs of toxiicty 6 ppm (1.07 mg/kg bw/day) No effects observed 64 ppm (11.6 mg/kg bw/day) No effects observed ≥ 640 ppm (115 mg/kg bw/day) **↑ ALT in M (+81%**) & F (+49%**)** **↑ rel. liver weight in F (+19%**)** **↑ incidence & severity of hypertrophy of centrilobular hepatocytes in 5/5 M (1 minimal, 3 slight & 1 moderate) & 4/5 F (1 minimal & 3 slight)** 6,400 ppm (1,111 mg/kg bw/day) ↑ ALT in M (+148%**) & F +54%**) ↑ AP in M (+134%) ↑ rel. liver weight in M (+42%**) & F (+58%**) ↑ abs. liver weight in M (+33%**) & F (+50%**) ↑ incidence & severity of hypertrophy of centrilobular hepatocytes in 5/5 M (4 moderate & 1 slight) & 5/5 F (moderate)	Anonymous; 2000; M-197343-01-1

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Mouse 28-day dietary mechanistic toxicity study No applicable guideline GLP Fluopicolide (purity 99.3%)	C57BL/6 mice 15/females/dose	0 or 3,200 ppm Equivalent to: 575 mg/kg bw/day 28 days	Cat 1 ≤ 30 Cat 2 ≤ 300	3,200 ppm (575 mg/kg bw/day) ↓ body weight ↑ abs. & rel. liver weight ↑ activity of drug metabolizing enzymes in the liver ↑ incidence of perilobular to panlobular hepatocellular hypertrophy ↑ no. of mitotic cells in liver	Anonymous; 2004; M-229594-01-1
Dog 28-day oral gavage toxicity study OECD 409 (1998) GLP Fluopicolide (purity 96.9%)	Beagle dogs 2/sex/group	0, 10, 100 and 1,000 mg/kg bw/day 28 days	Cat 1 ≤ 30 Cat 2 ≤ 300	10 mg/kg bw/day No effects observed 100 mg/kg bw/day No effects observed 1,000 mg/kg bw/day ↑ cholesterol in blood in 1 M (+98% on day 29 compared with day 1) ↑ abs. (+34%) & rel. (+44%) liver weight (M)	Anonymous; 2000; M-197350-01-1
90-day dietary toxicity study in rats with 4-week recovery period OECD 407 (1995) GLP Fluopicolide (purity 96.9 and 97.5%)	Sprague Dawley rats 10/dose/sex	0, 100, 1,400 or 20,000 ppm equivalent to: 0, 7.9, 114 or 1,671 mg/kg bw/day (combined sexes) 13 weeks (+ 4 weeks for recovery group animals, high-dose and control)	Cat 1 ≤ 10 Cat 2 ≤ 100	100 ppm (7.9 mg/kg bw/day) No effects observed ≥ 1,400 ppm (114 mg/kg bw/day) ↑ cholesterol in blood (M) ↑ epithelial cells in urinary sediment (M) ↑ urine volume & ↓ specific gravity (F) ↑ rel. liver weight (M) ↓ abs. and rel. spleen weight (F) ↑ rel. kidney weight (M) ↑ incidence hypertrophy of centrilobular hepatocytes (M) ↑ severity & incidence of trabecular hyperostosis of the bone joint (F) ↑ severity accumulation of hyaline droplets in the proximal kidney tubule (M)	Anonymous; 2000; M-197622-01-1

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
				↑ single cell death in the proximal kidney tubule epithelium (M) ↑ foci of basophilic (regenerating) tubules and granular casts (M) <u>20,000 ppm (1,671 mg/kg bw/day)</u> ↑ hair loss & body soiling (M/F) ↓ bodyweight (M/F) ↓ feed intake (M/F) ↑ water intake (F) ↓ red blood cell parameters (M/F) ↑ APTT (M) ↑ cholesterol, protein & GGT in blood (M/F) ↑ abs. & rel.liver weight (F) ↓ abs. & rel. spleen weight (M) ↑ severity and incidence of hypertrophy of the zona glomerulosa in the adrenals (M/F) ↑ severity & incidence of trabecular hyperostosis of the bone joint (M) ↓ cellularity of the bone marrow (M/F) ↑ incidence hypertrophy of centrilobular hepatocytes (F) <u>Recovery group 1,671 mg/kg bw/day</u> <u>Clinical signs: Hair loss in M & F and urogenital soiling in F</u> ↑ body-weight gain in M (+111%) & F (+300%) ↓ food consumption in M (-4%) Slightly ↑ urine volume in F Full or partial recovery of all other previous findings in the high-dose group	

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
90-day dietary toxicity study in mice OECD 408 (1998) GLP Fluopicolide (purity 97.3%)	Crl: CD-1 (ICR) BR mice 10/sex/ dose	0, 32, 320, 3,200 and 6,400 ppm Equivalent to: 0, 5.5, 53, 545 and 1,092 mg/kg bw/day (both sex combined) 13 weeks	Cat 1 ≤ 10 Cat 2 ≤ 100	32 ppm (5.5 mg/kg bw/day) No effects observed ≥ 320 ppm (53 mg/kg bw/day) ↑ **incidence hypertrophy of centrilobular hepatocytes in 9/10 M (6 minimal & 3 slight) & 2/10 F (minimal)** ≥ 3,200 ppm (545 mg/kg bw/day) ↓ body weight gain (F) ↑ALT (M/F) and AST (M) ↑ abs. & rel. liver weight (M/F) ↑ incidence of hepatocytic necrosis (F) 6,400 ppm (1,092 mg/kg bw/day) ↓ body weight gain (M) ↑AP (M) ↑ cholesterol and creatinine in blood (F) ↑ incidence of hepatocytic necrosis (M)	Anonymous; 2000; M-197623-01-1

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
90-day dietary toxicity study in mice OECD 408 (1998) GLP Fluopicolide (purity 95.9%)	C57BL/6JICO mice 10/sex/ dose	0, 50, 200, 800 and 3,200 ppm Equivalent to: 10.4/12.6, 37.8/52.8, 161/207, 770/965 mg/kg bw/day (M/F) 90 days	Cat 1 ≤ 10 Cat 2 ≤ 100	50 ppm (10.4/12.6 mg/kg bw/day) No effects observed ≥ 200 ppm (37.8/52.8 mg/kg bw/day) ↓ **cholesterol in blood in M (-26%**) & F (-21%**)** ≥ 800 ppm (161/207 mg/kg bw/day) ↓ albumin in blood (M/F) ↑ incidence centrilobular hepatocellular hypertrophy (M/F) ↑ rel. liver weight (M/F) ↑ abs. liver weight (F) 3,200 ppm (770/965 mg/kg bw/day) ↑ abs. liver weight (M) ↓ body weight gain (M/F) ↑ AP (M)	Anonymous; 2006; M-205579-02-1
Dog 90-day oral gavage toxicity study OECD 409 (1998) GLP Fluopicolide (purity 97.7%)	Beagle dogs 4/sex/group	0, 5, 70 or 1,000 mg/kg bw/day 13 weeks	Cat 1 ≤ 10 Cat 2 ≤ 100	5 mg/kg bw/day No effects observed 70 mg/kg bw/day No effects observed 1,000 mg/kg bw/day ↓ body weight gain (M/F) ↑ abs. & rel. liver weight (M/F)	Anonymous; 2000; M-199397-01-1
52-week toxicity study by oral route (gavage) in dogs OECD 452 (1981) GLP Fluopicolide (purity 95.9%)	Beagle dogs 5/sex/group	0, 70, 300 or 1,000 mg/kg/day 52 weeks	Cat 1 ≤ 2.5 Cat 2 ≤ 25	70 mg/kg bw/day No effects observed ≥ 300 mg/kg bw/day ↑ incidence of liver enlargement (M/F) 1,000 mg/kg bw/day ↓ bodyweight gain (M) ↑ cholesterol in blood (F)	Anonymous; 2002; M-216694-01-1

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
Dermal route					
Subacute dermal toxicity study in rats OECD 410 (1981) GLP Fluopicolide (purity 97.7%)	Wistar rats 10/dose/sex	0, 100, 250, 500, and 1,000 mg/kg bw/day semi-occlusive covering five days/ week for four weeks at	Cat 1 ≤ 30 Cat 2 ≤ 300	100, 250, 500 and 1,000 mg/kg bw/day No effects observed	Anonymous; 2003; M-220782-01-1

↑ / ↓ = increased/decreased compared with control.
M = male, F = female
* p < 0.05 ; ** p < 0.01 ; *** p < 0.001 statistically different to controls
# = no statistical analyses performed

Table 10-37: Summary table of human data on STOT RE

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No human data				

Table 10-38: Summary table of other studies relevant for STOT RE

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
Chronic toxicity and carcinogenicity study in rats OECD 453 (1981) GLP Fluopicolide (purity 95.9%)	Crl: CD® (SD) IGS BR rats 60/sex/dose	0, 50, 200, 750 and 2,500 ppm Equivalent to: 2.1/2.8, 8.4/10.8, 31.5/41.0, 109.4/142.2 mg/kg bw/day (M/F) 104 weeks (52 week interim sacrifice)	Cat 1 ≤ 1.25 Cat 2 ≤ 12.5	50 ppm (2.1/2.8 mg/kg bw/day) No effects observed ≥ 200 ppm (8.4/10.8 mg/kg bw/day) **↓ bodyweight gain week 1 in F (-36%)** **↑ protein in blood in M in weeks 13 (+3%*) & 26 (+3%)** **↑ incidence centrilobular hepatocytic hypertrophy week 104 (9/60** M, slight)**	Anonymous; 2003; M-225616-01-1

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
				<u>≥ 750 ppm (31.5/41.0 mg/kg bw/day)</u> ↓ bodyweight gain week 1 (M) ↑ incidence yellow perigenital staining (F) ↑ cholesterol in blood (M) ↑ K^+ and/or Ca^{2+} in blood week 52 & 104 (M/F) ↑ abs. & rel. kidney weights week 52 & 104 (M) ↑ rel. liver weights week 52 (M) ↑ incidence centrilobular hepatocytic hypertrophy week 52 (M) ↑ incidence and/or severity of foci of alteration in liver week 104 (M/F) ↑ incidence and/or severity of cortical tubular basophilia in kidneys week 52 and 104 (M) ↑ incidence and/or severity of hyperplasia of the papillary epithelium in kidney week 104 (F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids week 104 (M) <u>2,500 ppm (109.4/142.2 mg/kg bw/day)</u> ↑ Brown staining on the dorsal body surface (F) ↓ bodyweight gain (M/F) ↓ RBC parameters (M/F) ↑ protein in blood up to week 52 (M/F) ↑ albumin in blood week 13 (M) ↑ creatinine in blood (M) ↑ rel. kidney weights week 52 (F) ↑ rel. liver weights week 52 (F) ↑ abs. & rel. liver weights week 104 (M)	

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
				↑ thyroid weights week 104 (M) ↑ incidence and/or severity of cystic degeneration in liver (M) ↑ incidence and/or severity of degenerative changes in kidneys week 104 (M) ↑ incidence and/or severity of cortical tubular basophilia in kidneys (F) ↑ mineralisation of the papillary/pelvic epithelium (F) ↑ increased incidence and/or severity of acinar atrophic change in pancreas week 104 (M/F) ↑ incidenceof acinar atrophy with reduced colloid in prostate week 104 (M)	
Chronic toxicity and carcinogenicity study in mice OECD 451 (1981) GLP Fluopicolide (purity 95.9%)	C57BL/6 mice 60/sex/dose	0, 50, 400 and 3,200 ppm Equivalent to: 7.9/11.5, 64.5/91.9, 551/772.3 mg/kg bw/day (M/F) 78 weeks (52 week interim sacrifice)	Cat 1 ≤ 1.25 Cat 2 ≤ 12.5	<u>50 ppm (7.9/11.5 mg/kg bw/day)</u> No effects observed <u>400 ppm (64.5/91.9 mg/kg bw/day)</u> ↑ abs. & rel. liver weight week 52 and 78) (M/F) ↑ incidence of hepatocellular hypertrophy week 52 and 78 (M/F) <u>3,200 ppm (551/772.3 mg/kg bw/day)</u> ↓ body weight (M/F) ↓ feed intake (M/F) ↑ incidence of altered liver foci week 52 (F) and 78 (M/F) ↑ incidence of liver adenomas week 52 (F) and 78 (M/F)	Anonymous; 2003; M-225595-01-1;

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Two-generation dietary study in rats[a] OECD 416 (1999) GLP Fluopicolide (purity 95.9%)	Crl: CD® (SD) IGS BR rats	0, 100, 500 and 2,000 ppm Equivalent to: <u>F0 pre-maiting (Week 1-10)</u> 7.4/8.1, 36.4/41.0, 147.3/159.7 mg/kg bw/day (M/F) <u>F0 gestation (GD 0-20)</u> 7.4, 38.1, 150.8 mg/kg bw/day <u>F0 lactation (LD 0-14)</u> 13.5, 70.5, 281.4 mg/kg bw/day <u>F1 pre-maiting (Week 1-10)</u> 8.8/9.4, 43.7/46.9, 179.9/193.9 mg/kg bw/day (M/F) <u>F1 gestation (GD 0-20)</u> 7.7, 39.2, 156.2 mg/kg bw/day <u>F1 lactation (LD 0-14)</u> 15.8, 74.8, 320.4 mg/kg bw/day	Cat 1 ≤ 10 Cat 2 ≤ 100	<u>100 ppm (7.4-15.8 mg/kg bw/day)</u> No effects on reproductive organs, liver & kidney observed <u>≥ 500 ppm (36.4-74.8 mg/kg bw/day)</u> ↑ **incidence of centrilobular hepatocyte hypertrophy (slight) in 9/28** F0 M & 8/24**F1 M** <u>2,000 ppm (147.3-320.4 mg/kg bw/day)</u> ↑ incidence of cortical tubular basophilia in F0 (M) and F1 (M/F) ↑increased incidence of cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scaring in the kidneys in both generations (M) ↑ incidence of cortical tubular dilatation and corticomedullary mineralization in kidneys of both generations (F)	Anonymous; 2004; M-247289-01-1

Method, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**CLP guideline value for classification (mg/kg bw/day)**	**Results (% difference refers to difference from concurrent control unless otherwise stated)**	**Reference**
Developmental toxicity study in rats OECD 414 (1981) GLP Fluopicolide (purity 97.6-97.8%)	Hsd: Sprague Dawley SD 23 mated females/group	0, 5, 60 or 700 mg/kg bw/day GD 7-20	Cat 1 ≤ 60 Cat 2 ≤ 600	5 mg/kg bw/day No effects observed 60 mg/kg bw/day No effects observed 700 mg/kg bw/day ↓ bodyweight gain ↓ feed consumption ↓ foetal weights and crown-rump length ↑ incidence of minor skeletal defects and delayed ossification	Anonymous; M-202513-02-1
Developmental toxicity study in rabbits OECD 414 (1981) GLP Fluopicolide (purity 97.8%)	Chbb:HM(SPF) Himalayan rabbit	0, 5, 20 or 60 mg/kg bw/day GD 6-28	Cat 1 ≤ 36.5 Cat 2 ≤ 365	5 mg/kg bw/day No effects observed 20 mg/kg bw/day No effects observed 60 mg/kg bw/day **↑ mortality: 78% mortality (3/23 found dead on Days 24, 25 & 29 and 15/23 killed after abortion/premature delivery)** **↑ incidence of premature deliveries/abortions (12/23 dams aborted, 2/23 dams prematurely delivered a partially dead litter & 1 dam prematurely delivered a live litter)** **↑ clinical signs: ↓ defecation, ↓ hay consumption, hypoactivity, coat bristling, pultaceous faeces & discoloured urine (all animals)** **↓ bodyweight gain (-57% Days 0-29)** **↓ feed consumption (-43%* Days 23-26 & -54%* Days 26-29)** **↓ uterus weight (-19%)**	Anonymous; 2004; M-202513-02-1

Method, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	CLP guideline value for classification (mg/kg bw/day)	Results (% difference refers to difference from concurrent control unless otherwise stated)	Reference
				↓ foetal weights (-14%) and crown-rump length (-6%)	
Subchonic dietary neurotoxicity study in rats USEPA : OPPTS 870.6200 (1998) GLP Fluopicolide (purity 97.8%)	CD rats 10/sex/dose	0, 200, 1,400 or 10,000 ppm Equivalent to: 15.0/18.0, 107/125, 781/866 mg/kg bw/day (M/F) 13 weeks	Cat 1 ≤ 10 Cat 2 ≤ 100	200 ppm (15.0/18.0 mg/kg bw/day) No effects observed ≥ 1,400 ppm (107/125 mg/kg bw/day)[b] **↓ body weight gains in F (-13%*)** **↑ incidence of centrilobular hepatocyte hypertrophy in 9/10 M*** (slight)** **↑ incidence and/or severity of hyaline droplets in the cortical tubules in the kidneys in 10/10 M* (8 slight & 2 moderate)** 10,000 ppm (781/866 mg/kg bw/day) ↓ food consumption (M/F) ↑ incidence of centrilobular hepatocyte hypertrophy (F) ↑incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation (M)	Anonymous; 2002; M-208051-01-1

[a] focused on specific organ effects, [b] adaptive and/or rat specific effects not relevant for classification
* p < 0.05 ; ** p < 0.01; *** p < 0.001 statistically different to controls

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Repeated dose toxicity studies on fluopicolide (AE C638206) have been performed in rats, mice and dogs. The liver was identified as target organ in all species following repeated oral exposure to fluopicolide. In rats the kidney was additionally affected, especially in male animals. Effects on other organs were only observed at high dose levels not relevant for STOT RE classification (e.g. effects on blood related parameters, bones and adrenals in the subchronic rat study). The dog was less sensitive than the rodents and generally showed no effects at dose levels relevant for STOT RE classification. No adverse effects were observed after subacute exposure by the dermal route in rats up to 1,000 mg/kg bw/day.

Liver

The predominant effects on the liver at dose levels relevant for STOT RE classification observed in rats and mice are considered adaptive responses indicating induction of drug metabolizing enzymes in the liver consequent to the extensive hepatic metabolism of fluopicolide. These include increases in relative liver weights and increased incidences of minimal to slight centrilobular hepatocyte hypertrophy. In addition some slight and inconsistent changes in clinical chemical parameters were reported indicating an influence on liver function in a dose range relevant for STOT RE category 2. However, since these changes were slight and inconsistent (increased or decreased cholesterol level in blood, increased plasma transaminase activities (male mice in subacute study only) and increased protein concentration in blood (male rats in the chronic study week 13 and 26 only) they are not considered triggering a STOT RE classification.

Kidney

Kidney effects in a STOT RE classification relevant dose range were only observed in male rats and characterised by a minimally increased severity of hyaline droplets in the 28-day study and a slightly increased absolute kidney weight (+9%) in the chronic toxicity and carcinogencity study. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin. Since the α2μ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2μ-globulin accumulation are unlikely to occur in humans[9]. Therefore, the available toxicity studies do not show significant or severe kidney effects that are relevant for humans at dose levels requiring classification as STOT RE.

10.12.2 Comparison with the CLP criteria

Classification for STOT-RE is warranted when repeated exposure to a substance results in ‘significant’ or ‘severe’ toxicity, generally at doses that are around or below the reference values assigned in the guidance on the application of the CLP criteria. For a 90-day oral study in the rat, the guidance cut-off value for category 2 is ≤ 100 mg/kg bw/day; this value is adjusted to ≤ 300 mg/kg bw/day for a 28-day study and ≤ 12.5 mg/kg bw/day for a one-year study. For category 1, the guidance cut-off value for an oral 90-day study in rats is ≤ 10 mg/kg bw/day. In the context of classification, ‘significant’ is taken to mean morphological changes that are toxicologically significant, or effects that clearly indicate functional disturbance. ‘Severe’ refers to more profound effects of an adverse nature or effects which significantly impact on health.

No effects were observed when fluopicolide was administered dermally to rats for 28 days at doses up to 1,000 mg/kg bw/day; therefore classification for STOT RE via the dermal route is not warranted.

9 Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of alpha 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ Health Perspect. 1993 Mar;99:313-49.

In the available oral subacute and subchronic studies in rats, mice and dogs and in the subacute neurotoxicity study in rats no consistent changes in clinical biochemistry, haematology or urinalysis parameters that indicate severe organ dysfunction were seen in a dose range relevant for STOT RE classification. Severe organ damage apparent in microscopic examination following autopsy was only observed at very high doses. In addition, in the chronic toxicity and carcinogenicity studies in rats and mice and in the reprotoxicity rat studies no relevant effects occurred within the respective ranges of the CLP guideline values for classification for STOT RE. Histopathological changes observed in liver described as minimal to slight centrilobular hepatocyte hypertrophy are indicative of adaptive reversible changes and not considered adverse. Kidney effects observed in male rats in a STOT RE classification relevant dose range were graded as minimal to slight and assumed to be caused by an non-human relevant mode of action.

In the rabbit developmental toxicity study with doses of 0, 5, 20 or 60 mg/kg bw/day from Day 6-28 of gestation, at the hightest tested dose of 60 mg/kg bw/day severe maternal toxicity as evidenced by mortality, marked decreases in body weight gain and food consumption was observed resulting in high incidences of premature delivery. According to the CLP guideline values for classification for STOT RE these could be considered as results triggering STOT RE classification since the increased mortality occurred at a dose below 365 mg/kg bw/day which is the trigger value for STOT RE category 2 adapted to a exposure duration of 23 days. However, mortality was the main sign and no other consistent or siginificant organ damage was seen so that such a classification appears to be not appropriate. The kind of effect (mortality) is also not relevant for STOT SE classification, because STOT SE refers to non lethal effects. An acute toxicity classification is also not justified since only mortalities during the first 72 hours after first treatment in a repeated dose study should be considered for the assessment of acute toxicity (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017). Since the rabbit is known to be overly sensitive to some chemicals it is generally questionable if its mortality can be used for classification purposes. In conclusion, this study type and its results are not regarded as appropriate for an acute, STOT SE or possible STOT RE classification.

10.12.3 Conclusion on classification and labelling for STOT RE

In rodents and dogs, effects which could be relevant for STOT RE classification did neither occur in the short-term toxicity studies nor in the chronic toxicity and oncogenicity studies or in the reproduction and developmental toxicity studies, nor in a subchronic neurotoxicity study in rats with fluopicolide (AE C638206).

Overall, therefore, the data are conclusive, but do **not warrant** a STOT RE classification according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017).

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

Nine subchronic repeated dose study in rats, mice and dogs conducted with fluopicolide are summarised in the table below. Information from other studies using repeated exposure for specific investigations (neurotoxicity, developmental toxicity, effects on fertility or chronic/carcinogenic effects) in rats or rabbits described under the respective heading of carcinogenicity and toxicity to reproduction) are also considered in the evaluation of specific target toxicity by repeated exposure.

Table: *Summary table of animal studies on STOT RE*

Method, publication year, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**Results**
Rat 28-day dietary toxicity study, OECD TG 407 (1995) GLP Fluopicolide (purity 99.0%) Year: 2000	Sprague Dawley CRL:(IGS)CDBR rats 5/dose/sex	0, 20, 200, 2000 or 20000 ppm Equivalent to: 1.78, 17.7, 179 and 1770 mg/kg bw/day (combined sexes) 28 days	<u>20 ppm (1.78 mg/kg bw/day)</u> no effects observed <u>≥ 200 ppm (17.7 mg/kg bw/day)</u> ↑ incidence of centrilobular hepatocyte hypertrophy 2/5 M (1 minimal & 1 slight) & 3/5 F (minimal) <u>≥ 2000 ppm (179 mg/kg bw/day)</u> ↓ bodyweight F (-11.3%) on day 29 ↓ body weight gain# in F (-30%) on days 0-29 ↑ water consumption in M (+18.4%) in week 3 ↑ cholesterol in M (+50%**) & F (+29%**) Pale kidneys in 3/5 M ↑ severity of phloxine tartrazine-positive granulation (hyaline droplets) in kidneys (M) ↑ incidence of centrilobular hepatocyte hypertrophy in 5/5 M (1 minimal & 4 slight) & 2/5 F (minimal) <u>20000 ppm (1770 mg/kg bw/day)</u> ↓ bodyweight (M) ↑ water consumption (F) ↓ feed intake (M/F) ↓ ALT (M) ↑ relative liver weights (M/F) ↑ absolute liver weights (M) enlarged livers (M)
Mouse 28-day dietary toxicity study, OECD TG 407 (1995) GLP Fluopicolide (purity 99.0%) Year: 2000	CD-1 mice 5/dose/sex	0, 6, 64, 640 or 6400 ppm Equivalent to: 1.07, 11.6, 115 and 1111 mg/kg bw/day (combined sexes) 28 days	<u>6 ppm (1.07 mg/kg bw/day)</u> No effects observed <u>64 ppm (11.6 mg/kg bw/day)</u> No effects observed <u>≥ 640 ppm (115 mg/kg bw/day)</u> ↑ ALT in M (+81%**) & F (+49%**) ↑ rel. liver weight in F (+19%**) ↑ incidence & severity of hypertrophy of centrilobular hepatocytes in 5/5 M (1 minimal, 3 slight & 1 moderate) & 4/5 F (1 minimal & 3 slight) <u>6400 ppm (1111 mg/kg bw/day)</u> ↑ AP (M) ↑ abs. & rel. liver weight (M/F) ↑ ALT in M (+148%**) & F +54%**) ↑ AP in M (+134%) ↑ rel. liver weight in M (+42%**) & F (+58%**) ↑ abs. liver weight in M (+33%**) & F (+50%**)

Method, publication year, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	Results
			↑ incidence & severity of hypertrophy of centrilobular hepatocytes in 5/5 M (4 moderate & 1 slight) & 5/5 F (moderate)
Mouse 28-day dietary mechanistic toxicity study, No applicable guideline GLP Fluopicolide (purity 99.3%) Year 2004	C57BL/6 mice 15/females/dose	0 or 3200 ppm Equivalent to: 575 mg/kg bw/day 28 days	<u>3200 ppm (575 mg/kg bw/day)</u> ↓ body weight ↑ abs. & rel. liver weight ↑ activity of drug metabolizing enzymes in the liver ↑ incidence of perilobular to panlobular hepatocellular hypertrophy ↑ no. of mitotic cells in liver
Dog 28-day oral gavage toxicity study, OECD TG 409 (1998) GLP Fluopicolide (purity 96.9%) Year 2000	Beagle dogs 2/sex/group	0, 10, 100 and 1000 mg/kg bw/day 28 days	<u>10 mg/kg bw/day</u> No effects observed <u>100 mg/kg bw/day</u> No effects observed <u>1000 mg/kg bw/day</u> ↑ cholesterol in blood (M) ↑ abs. & rel. liver weight (M)
Subacute (28-days) dermal toxicity study in rats, OECD TG 410 (1981) GLP Fluopicolide (purity 97.7%) Year: 2003	Wistar rats 10/dose/sex	0, 100, 250, 500, and 1000 mg/kg bw/day semi-occlusive covering five days/ week	<u>100, 250, 500 and 1000 mg/kg bw/day</u> No effects observed
90-day dietary toxicity study in rats with 4-week recovery period, OECD TG 407 (1995) GLP Fluopicolide (purity 96.9 and 97.5%) Year: 2000	Sprague Dawley rats 10/dose/sex	0, 100, 1400 or 20000 ppm equivalent to: 0, 7.9, 114 or 1671 mg/kg bw/day (combined sexes) 13 weeks (+ 4 weeks for recovery group animals)	<u>100 ppm (7.9 mg/kg bw/day)</u> No effects observed <u>≥ 1400 ppm (114 mg/kg bw/day)</u> ↑ cholesterol in blood (M) ↑ epithelial cells in urinary sediment (M) ↑ urine volume & ↓ specific gravity (F) ↑ rel. liver weight (M) ↓ abs. and rel. spleen weight (F) ↑ rel. kidney weight (M) ↑ incidence hypertrophy of centrilobular hepatocytes (M) ↑ severity accumulation of hyaline droplets in the proximal kidney tubule (M) ↑ single cell death in the proximal kidney tubule epithelium (M) ↑ foci of basophilic (regenerating) tubules and granular casts (M) ↑ severity & incidence of trabecular hyperostosis of the bone joint (F) <u>20000 ppm (1671 mg/kg bw/day)</u> ↑ hair loss & body soiling (M/F)

Method, publication year, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	Results
			↓ bodyweight (M/F) ↓ feed intake (M/F) ↑ water intake (F) ↓ red blood cell parameters (M/F) ↑ APTT (M) ↑ cholesterol, protein & GGT in blood (M/F) ↑ abs. & rel. liver weight (F) ↓ abs. & rel. spleen weight (M) ↑ severity and incidence of hypertrophy of the zona glomerulosa in the adrenals (M/F) ↑ severity & incidence of trabecular hyperostosis of the bone joint (M) ↓ cellularity of the bone marrow (M/F) ↑ incidence hypertrophy of centrilobular hepatocytes (F)
90-day dietary toxicity study in mice, OECD TG 408 (1998) GLP Fluopicolide (purity 97.3%) Year: 2000	Crl: CD-1 (ICR) BR mice 10/sex/ dose	0, 32, 320, 3,200 and 6400 ppm Equivalent to: 0, 5.5, 53, 545 and 1092 mg/kg bw/day (both sex combined)	<u>32 ppm (5.5 mg/kg bw/day)</u> No effects observed <u>≥ 320 ppm (53 mg/kg bw/day)</u> ↑ incidence hypertrophy of centrilobular hepatocytes in 9/10 M (6 minimal & 3 slight) & 2/10 F (minimal) <u>≥ 3200 ppm (545 mg/kg bw/day)</u> ↓ body weight gain (F) ↑ ALT (M/F) and AST (M) ↑ abs. & rel. liver weight (M/F) ↑ incidence of hepatocyte necrosis (F) <u>6400 ppm (1092 mg/kg bw/day)</u> ↓ body weight gain (M) ↑ AP (M) ↑ cholesterol and creatinine in blood (F) ↑ incidence of hepatocyte necrosis (M)
90-day dietary toxicity study in mice, OECD TG 408 (1998) GLP Fluopicolide (purity 95.9%) Year: 2006	C57BL/6JICO mice 10/sex/ dose	0, 50, 200, 800 and 3200 ppm Equivalent to: 10.4/12.6, 37.8/52.8, 161/207, 770/965 mg/kg bw/day (M/F)	<u>50 ppm (10.4/12.6 mg/kg bw/day)</u> No effects observed <u>≥ 200 ppm (37.8/52.8 mg/kg bw/day)</u> ↓ cholesterol in blood in M (-26%**) & F (-21%**) <u>≥ 800 ppm (161/207 mg/kg bw/day)</u> ↓ albumin in blood (M/F) ↑ incidence centrilobular hepatocellular hypertrophy (M/F) ↑ rel. liver weight (M/F) ↑ abs. liver weight (F) <u>3200 ppm (770/965 mg/kg bw/day)</u> ↑ abs. liver weight (M) ↓ body weight gain (M/F) ↑ AP (M)

Method, publication year, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**Results**
Subchronic dietary neurotoxicity study in rats USEPA : OPPTS 870.6200 (1998) GLP Fluopicolide (purity 97.8% Year: 2002	CD rats 10/sex/dose	0, 200, 1400 or 10000 ppm Equivalent to: 15.0/18.0, 107/125, 781/866 mg/kg bw/day (M/F) 13 weeks	<u>200 ppm (15.0/18.0 mg/kg bw/day)</u> No effects observed <u>≥ 1400 ppm (107/125 mg/kg bw/day)</u> ↓ body weight gains (M/F) ↑ incidence of centrilobular hepatocyte hypertrophy (M) ↑ incidence and/or severity of hyaline droplets in the cortical tubules in the kidneys (M) <u>10000 ppm (781/866 mg/kg bw/day)</u> ↓ food consumption (M/F) ↑ incidence of centrilobular hepatocyte hypertrophy (F) ↑ incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation (M)
Dog 90-day oral gavage toxicity study, OECD TG 409 (1998) GLP Fluopicolide (purity 97.7%) Year: 2000	Beagle dogs 4/sex/group	0, 5, 70 or 1000 mg/kg bw/day	<u>5 mg/kg bw/day</u> No effects observed <u>70 mg/kg bw/day</u> No effects observed <u>1000 mg/kg bw/day</u> ↓ body weight gain (M/F) ↑ abs. & rel. liver weight (M/F)
52-week toxicity study by oral route (gavage) in dogs, OECD TG 452 (1981) GLP Fluopicolide (purity 95.9%) Year: 2002	Beagle dogs 5/sex/group	0, 70, 300 or 1000 mg/kg/day	<u>70 mg/kg bw/day</u> No effects observed <u>≥ 300 mg/kg bw/day</u> ↑ incidence of liver enlargement (M/F) <u>1000 mg/kg bw/day</u> ↓ bodyweight gain (M) ↑ cholesterol in blood (F)
Chronic toxicity and carcinogenicity study in mice, OECD TG 451 (1981) GLP Fluopicolide (purity 95.9%) Year: 2003	C57BL/6 mice 60/sex/dose	0, 50, 400 and 3200 ppm Equivalent to: 7.9/11.5, 64.5/91.9, 551/772.3 mg/kg bw/day (M/F) 78 weeks (52 week interim sacrifice)	<u>50 ppm (7.9/11.5 mg/kg bw/day)</u> No effects observed <u>400 ppm (64.5/91.9 mg/kg bw/day)</u> ↑ abs. & rel. liver weight week 52 and 78) (M/F) ↑ incidence of hepatocellular hypertrophy week 52 and 78 (M/F) <u>3200 ppm (551/772.3 mg/kg bw/day)</u> ↓ body weight (M/F) ↓ feed intake (M/F) ↑ incidence of altered liver foci week 52 (F) and 78 (M/F) ↑ incidence of liver adenomas week 52 (F) and 78 (M/F)

Method, publication year, guideline, test substance purity	**Species, strain, sex, no/group**	**Dose levels, duration of exposure**	**Results**
Two-generation dietary study in rats[a], OECD TG 416 (1999) GLP Fluopicolide (purity 95.9%) Year: 2004	Crl: CD® (SD) IGS BR rats	0, 100, 500 and 2000 ppm Equivalent to: <u>F0 pre-mating (Week 1-10)</u> 7.4/8.1, 36.4/41.0, 147.3/159.7 mg/kg bw/day (M/F) <u>F0 gestation (GD 0-20)</u> 7.4, 38.1, 150.8 mg/kg bw/day <u>F0 lactation (LD 0-14)</u> 13.5, 70.5, 281.4 mg/kg bw/day <u>F1 pre-mating (Week 1-10)</u> 8.8/9.4, 43.7/46.9, 179.9/193.9 mg/kg bw/day (M/F) <u>F1 gestation (GD 0-20)</u> 7.7, 39.2, 156.2 mg/kg bw/day <u>F1 lactation (LD 0-14)</u> 15.8, 74.8, 320.4 mg/kg bw/day	<u>100 ppm (7.4-15.8 mg/kg bw/day)</u> No effects on reproductive organs, liver & kidney observed <u>≥ 500 ppm (36.4-74.8 mg/kg bw/day)</u> ↑ incidence of centrilobular hepatocyte hypertrophy in both generations (M/F) <u>2000 ppm (147.3-320.4 mg/kg bw/day)</u> ↑ incidence of cortical tubular basophilia in F0 (M) and F1 (M/F) ↑ increased incidence of cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scaring in the kidneys in both generations (M) ↑ incidence of cortical tubular dilatation and corticomedullary mineralization in kidneys of both generations (F)
Developmental toxicity study in rats, OECD TG 414 (1981) GLP Fluopicolide (purity 97.6-97.8%) Year: 2003	Hsd: Sprague Dawley 23 mated females/group	0, 5, 60 or 700 mg/kg bw/day GD 7-20	<u>5 mg/kg bw/day</u> No effects observed <u>60 mg/kg bw/day</u> No effects observed <u>700 mg/kg bw/day</u> ↓ bodyweight gain ↓ feed consumption ↓ foetal weights and crown-rump length ↑ incidence of minor skeletal defects and delayed ossification
Developmental toxicity study in rabbits OECD TG 414 (1981) GLP Fluopicolide (purity 97.8%)	Chbb:HM(SPF) Himalayan rabbit	0, 5, 20 or 60 mg/kg bw/day GD 6-28	<u>5 mg/kg bw/day</u> No effects observed <u>20 mg/kg bw/day</u> No effects observed <u>60 mg/kg bw/day</u> ↑ mortality ↑ incidence of premature deliveries ↑ clinical signs ↓ bodyweight gain ↓ feed consumption

Method, publication year, guideline, test substance purity	Species, strain, sex, no/group	Dose levels, duration of exposure	Results
Year: 2004			↓ uterus weight ↓ foetal weights and crown-rump length
Chronic toxicity and carcinogenicity study in rats OECD TG 453 (1981) GLP Fluopicolide (purity 95.9%) Year: 2003	Crl: CD® (SD) IGS BR rats 60/sex/dose	0, 50, 200, 750 and 2500 ppm Equivalent to: 2.1/2.8, 8.4/10.8, 31.5/41.0, 109.4/142.2 mg/kg bw/day (M/F) 104 weeks (52 week interim sacrifice)	<u>50 ppm (2.1/2.8 mg/kg bw/day)</u> No effects observed <u>≥ 200 ppm (8.4/10.8 mg/kg bw/day)</u> ↑ incidence centrilobular hepatocytic hypertrophy week 104 (M) <u>≥ 750 ppm (31.5/41.0 mg/kg bw/day)</u> ↑ abs. & rel. kidney weights week 52 & 104 (M) ↑ rel. liver weights week 52 (M) ↑ incidence centrilobular hepatocyte hypertrophy week 52 (M) ↑ incidence and/or severity of foci of alteration in liver week 104 (M/F) ↑ incidence and/or severity of cortical tubular basophilia in kidneys week 52 and 104 (M) ↑ incidence and/or severity of hyperplasia of the papillary epithelium in kidney week 104 (F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids week 104 (M) <u>2500 ppm (109.4/142.2 mg/kg bw/day)</u> ↑ Brown staining on the dorsal body surface (F) ↑ protein in blood up to week 52 (M/F) ↑ albumin in blood week 13 (M) ↑ creatinine in blood (M) ↑ rel. kidney weights week 52 (F) ↑ rel. liver weights week 52 (F) ↑ abs. & rel. liver weights week 104 (M) ↑ thyroid weights week 104 (M) ↑ incidence and/or severity of cystic degeneration in liver (M) ↑ incidence and/or severity of degenerative changes in kidneys week 104 (M) ↑ incidence and/or severity of cortical tubular basophilia in kidneys (F) ↑ mineralisation of the papillary/pelvic epithelium (F) ↑ increased incidence and/or severity of acinar atrophic change in pancreas week 104 (M/F) ↑ incidence of acinar atrophy with reduced colloid in prostate week 104 (M)

↑ / ↓ = increased/decreased compared with control.
M = male, F = female
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ statistically different to controls; # = no statistical analyses performed

In the rabbit developmental toxicity study, severe maternal toxicity as evidenced by mortality, marked decreases in body weight gain and food consumption were observed at the highest tested dose of 60 mg/kg bw/day. The DS considered the sensitivity of rabbits to certain chemicals and concluded that the effects in the pregnant rabbits are not relevant for classification for STOT RE.

The DS further acknowledged that severe effects (blood related parameters, bones and adrenals) observed in some repeated dose studies at high doses were above the guidance values for classification as STOT RE.

The liver was the target organ in the repeated dose toxicity studies in rats and mice. Liver effects included increases in relative weights and increased incidences of centrilobular hepatocyte hypertrophy. Mild effects were reported in rats from around 20 mg/kg bw/day and more severe effects in mice around 100 mg/kg bw/day in 28 day-studies. The effects were also seen with longer exposure period. In addition, clinical chemical parameters (e.g. changes in blood cholesterol levels, increased plasma transaminase levels and increased protein concentration in blood) were also affected in some, but not all studies. The DS considered these liver effects to be adaptive reversible changes resulting from induction of chemical metabolising enzymes in the liver.

In males rats, the kidney was also targeted by fluopicolide with findings in several studies on kidney weights and deposition of hyaline droplets (179 mg/kg bw/day in a 28 day study, and at around 110 mg/kg bw/d in a 90 day neurotoxicity study). The droplets are a consequence of accumulation of the rat specific protein α2μ-globulin, a mechanism of low human relevance.

Effects on other organs in mice and rats occur only at doses higher than the guidance values for classification (effects on blood related parameters, bones and adrenals).

The dog was less sensitive than the rodents and generally showed no effects at dose levels relevant up to 1000 mg/kg bw/day in a 90-day study and 300 mg/kg bw/day in a one-year study.

No adverse effects were observed in a rat dermal 28-day study up to doses of 1000 mg/kg bw/day.

Comments received during public consultation

In a comment from a company, historical control data (HCD) were provided, which related to the findings of interstitial cell hyperplasia in the testes and acinar cell atrophy with associated reduced colloid in the prostate observed in mid- and high-dose males in the 2-year combined rat chronic/carcinogenicity study were provided.

Another comment from an MSCA concerned the mortality in the rabbit developmental study. The DS responded that the mortality were related to rabbit specific gastro-intestinal tract sensitivity due to a high degree of bacteria-mediated digestion in the caecum, which differs from that of e.g. rats and humans and that the mortalities in the rabbits were therefore not relevant for classification.

Assessment and comparison with the classification criteria

The criteria for classification as STOT RE refer to significant and/or severe morphological chance or clear indication of functional disturbance in studies following repeated exposure. Guidance values are provided to use in a weight of evidence evaluation of the hazard class. For STOT RE category 1, the guidance value for an oral 90-day study in rats is ≤ 10 mg/kg bw/day, whilst the guidance value for STOT RE category 2 is ≤ 100 mg/kg bw/day.

Extrapolation in accordance with Haber's rule can be used to include studies of shorter or longer durations. Thus, for classification as STOT RE 1 a 28-day study the guidance value

would be around 30 mg/kg bw/day - multiplying by a factor 3, whilst an exposure duration of 21 days would warrant classification as STOT RE 1 when occurring at ≤ 40 mg/kg bw/d. In a one-year study, classifiable effects would lead to classification in category 1 when occurring at ≤ 1.25 mg/kg bw/day. For classification in category 2, the corresponding guidance values are ≤ 300 mg/kg bw/ day for a 28 day study, ≤ 600 mg/kg bw/day for a 14-day study and ≤ 12.5 mg/kg bw/day for effects seen in a one-year study.

Mortality:

RAC notes that mortality, when occurring after repeated exposure, is a relevant end-point for consideration under the heading of STOT RE, as described under point 3.9.2.7.3 of Annex I to the CLP Regulation.

In the rabbit developmental toxicity study (described further under "toxicity to reproduction" below), the highest dose tested was 60 mg/kg bw/day. This led to the death of 3 out of 23 dams whilst fifteen dams were killed in relation to early deliveries following exposure duration of between 16 and 22 days (GD 6-28 of gestation, mortalities occurring from day 22 of gestation). One dam of the mid-dose group was killed after early delivery. No deaths occurred in the low dose group.

In the high dose group, mean food consumption and mean body weight were decreased by up to 40% and 8.3%, respectively, compared to controls at the end of the study, but were unaffected in the low and mid-dose groups. The clinical signs seen at the highest dose level included hypoactivity, decreased hay consumption, decreased defecation, decreased body weight and/or discoloured urine for 0-4 days before death or filled stomach at autopsy in 9 out of 18 animals. In the remaining 9 animals that were killed after premature delivery, there were no clinical signs but body weight gain was reduced and filled stomach was reported.

Rabbits are known to be sensitive to antimicrobial xenobiotics interfering with the predominantly bacteria-mediated digestion. Fluopicolide is an anti-fungal agent. Therefore, RAC considers that impairment of normal gastro-intestinal tract function lead to poor condition and deaths of nine or more dams in this developmental toxicity study. In addition, pregnant rabbits are known to be sensitive to reduced food intake and decreases in body weight gain, which could be the cause of some cases of premature deliveries. In Annex I, paragraph 3.7.2.4.4 of the CLP Regulation, it is stated that "in rabbits, the body weight gain may not be useful indicators of maternal toxicity because of normal fluctuations in body weight during pregnancy".

RAC notes that no excessive mortality was reported in repeated dose studies, neurotoxicity studies or reproductive toxicity studies in rats, mice or dogs and that the rabbit is the sole species presenting a high sensitivity to oral intake of fluopicolide which supports the hypothesis that the mortality seen can be considered to be specific to the rabbit.

In conclusion, RAC considers that no classification for as STOT RE for mortality is warranted for fluopicolide.

Findings in the liver (increased weights, centrilobular hepatocyte hypertrophy, changes in blood chemistry) in studies in rats and mice of various exposure duration indicated a clear adverse effect on the liver. The severity of the effects were variable across the studies and species, but were reported to occur at relatively lower exposure levels in studies of shorter duration. The DS considered that the liver effects were the result of adaptive change to the chemical and thus not relevant for classification.

RAC notes that fluopicolide treatment resulted in the activation of CAR and weak activation of PXR in the liver, which is described below in the section on carcinogenicity. This MoA was supported by a series of associative events including the following: increased expression of genes encoding cytochrome P450s (CYPs), particularly CYP2B and (to a lesser extent) CYP3A isoforms, increased proliferation and hepatocellular hypertrophy and increased liver weight.

The kidney effects observed were increased severity of hyaline droplets and increased absolute kidney weight. The effects were only observed in male rats. Hyaline droplets have been recognized as an indicator of changes associated with the accumulation of α2μ-globulin. However, no immune-histochemical investigations are described in the CLH report and most likely these have not been performed. Hence human relevance cannot be excluded. The effects in the relevant dose range for STOT RE classification were only observed in male rats and were characterised by increased severity of hyaline droplets in the 28-day and 90-day studies and a slightly increased absolute kidney weight. The incidences of the hyaline droplets were seen at doses above the guidance value for STOT RE Cat. 2 classification in the 13 week neurotoxicity study (107/125 mg/kg bw/day) and in the 90 day rat study (114 mg/kg bw/day). Because the effect was recorded at doses above the guidance value for STOT RE 2, RAC concludes that classification is not required. However, RAC cannot conclude that the effect is not relevant to humans, as the appropriate immune-histochemical investigations, to conclude that the effects are mediated through the rat specific protein α2μ-globulin,have not been performed.
Effects on other target organs, e.g. blood related parameters, bones and adrenals, were seen in repeated dose toxicity studies at doses above the guidance values for classification.

Overall, RAC considers that the available data on repeated dose toxicity by the oral route of fluopicolide is conclusive. No classification of the substance for STOT RE via the oral route, is warranted.

No effects were observed when fluopicolide was administered dermally to rats for 28 days at doses up to 1000 mg/kg bw/day; RAC agrees with the DS that classification for STOT RE via the dermal route is not warranted.

Overall, RAC considers that, based on the available data on repeated dose toxicity, **fluopicolide should not be classified for STOT RE**.

10.13 Aspiration hazard

Table 10-39: Summary table of evidence for aspiration hazard

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No specific studies on aspiration hazard are available, no evidence of this hazard for fluopicolide				

10.13.1 Short summary and overall relevance of the provided information on aspiration hazard

No specific studies on aspiration hazard are available. However, on the basis of existing animal studies and expert judgment that takes into account surface tension, water solubility, boiling point, volatility and chemical structure (fluopicolide is not an hydrocarbon, primary alcohol or ketone) aspiration hazard is not expected.

10.13.2 Comparison with the CLP criteria

An aspiration hazard is indicated at a kinematic viscosity of ≤ 20.5 mm^2/s at 40 °C. Measurements for kinetmatic viscosity are not available for fluopicolide. However, on the basis of existing animal studies and expert judgment that takes into account surface tension, water solubility, boiling point, volatility and chemical structure (fluopicolide is not an hydrocarbon, primary alcohol or ketone) aspiration hazard is not expected.

10.13.3Conclusion on classification and labelling for aspiration hazard

A classification for aspiration hazard according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017) is **not warranted**.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Environmental hazards are **not** assessed in this dossier. Only health hazards are assessed.

11.1 Rapid degradability of organic substances

Please refer to Section 11.

11.1.1 Ready biodegradability

Please refer to Section 11.

11.1.2 BOD_5/COD

Please refer to Section 11.

11.1.3 Hydrolysis

Please refer to Section 11.

11.1.4 Other convincing scientific evidence

Please refer to Section 11.

11.1.4.1 Field investigations and monitoring data (if relevant for C&L)

Please refer to Section 11.

11.1.4.2 Inherent and enhanced ready biodegradability tests

Please refer to Section 11.

11.1.4.3 Water, water-sediment and soil degradation data (including simulation studies)

Please refer to Section 11.

11.1.4.4 Photochemical degradation

Please refer to Section 11.

11.2 Environmental transformation of metals or inorganic metals compounds

Please refer to Section 11.

11.2.1 Summary of data/information on environmental transformation

Please refer to Section 11.

11.3 Environmental fate and other relevant information

Please refer to Section 11.

11.4 Bioaccumulation

Please refer to Section 11.

11.4.1 Estimated bioaccumulation

Please refer to Section 11.

11.4.2 Measured partition coefficient and bioaccumulation test data

Please refer to Section 11.

11.5 Acute aquatic hazard

Please refer to Section 11.

11.5.1 Acute (short-term) toxicity to fish

Please refer to Section 11.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

Please refer to Section 11.

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

Please refer to Section 11.

11.5.4 Acute (short-term) toxicity to other aquatic organisms

Please refer to Section 11.

11.6 Long-term aquatic hazard

Please refer to Section 11.

11.6.1 Chronic toxicity to fish

Please refer to Section 11.

11.6.2 Chronic toxicity to aquatic invertebrates

Please refer to Section 11.

11.6.3 Chronic toxicity to algae or other aquatic plants

Please refer to Section 11.

11.6.4 Chronic toxicity to other aquatic organisms

Please refer to Section 11.

11.7 Comparison with the CLP criteria

Please refer to Section 11.

11.7.1 Acute aquatic hazard

Please refer to Section 11.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Please refer to Section 11.

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

Please refer to Section 11.

12 EVALUATION OF ADDITIONAL HAZARDS

Additional hazards are **not** assessed in this dossier. Only health hazards are assessed.

12.1 Hazardous to the ozone layer

Please refer to Section 12.

12.1.1 Short summary and overall relevance of the provided information on ozone layer hazard

Please refer to Section 12.

12.1.2 Comparison with the CLP criteria

Please refer to Section 12.

12.1.3 Conclusion on classification and labelling for hazardous to the ozone layer

Please refer to Section 12.

13 ADDITIONAL LABELLING

Not applicable

14 REFERENCES

A full reference list is provided in a separate confidential annex to this CLH report

Additional references

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15 ANNEXES

Annex I Annex I to CLH Report

Annex I to the CLH Report is provided separately.

Annex II Evaluation of the proposed MoA of fluopicolide according to the IPCS/human Framework method

Postulated MOA

The liver findings after fluopicolide treatment in mice were very similar to that demonstrated for phenobarbital and many other non-genotoxic compounds which cause liver tumours in rodents. *In vitro* studies in mouse and human hepatocytes showed that the key event of CAR/PXR activation which lead to liver cell proliferation and eventually to liver tumors in rodents, is not relevant to humans in which CAR induction and liver cell proliferation do not occur after exposure to fluopicolide. Thus, the postulated MOA for fluopicolide-induced liver tumours is mediated by CAR/PXR activation which in rodents leads to hyperplasia and eventually to liver tumours and is not relevant for humans.

Key events

The key and associative events as listed in Section 10.9.3.3 were demonstrated for the liver tumour MOA of fluopicolide.

Key event 1: Activation of CAR/PXR nuclear receptor

This MOA is starting with the activation of CAR and also PXR which was demonstrated in the described *in vivo* MOA study in which clear evidence of liver enzyme induction (AE 1) was seen. Total cytochrome P-450 content was markedly increased with the highest increases of enzymatic activity being PROD (+1143%) and BROD activities (+1785%) at 3,200 ppm (same dose as highest dose in chronic toxicity study).

This is further supported by the observed centrilobular hypertrophy (AE 2) in the liver of mice already observed at 400 ppm in the oncogenicity study, at 320 and 800 ppm in the subchronic studies and at 640 ppm in the subacute study. Therefore, it is assumed that liver enzyme induction starts at lower doses than the other effects of the MOA sequence. This supports the MOA as a consequence of liver-enzyme-mediated effects.

Key event 2: Altered gene expression secondary to CAR activation

The CAR/PXR activation subsequently leads to an increased activation of CYP2A and CYP3A enzymes, which was also demonstrated in the 28-day MOA study and the *in vitro* studies in mouse hepatocytes. The CAR/PXR activation is the first key event at an early time-point, with the subsequent events following later.

Key event 3: Increased hepatocellular proliferation

The activation of CAR and also PXR and subsequently increased activation of CYP2A and CYP3A enzymes was followed by cell proliferation as demonstrated in the *in vivo* and *in vitro* studies. A transiently increased cell proliferation was demonstrated after 7 days exposure, the mean BrdU labelling index was approximately 6.5-times higher in 3,200 ppm males and females than in controls. At terminal sacrifice afer 28-days, there was no increased hepatocellular proliferation in treated animals observed. The mean BrdU labeling index was even slightly lower in treated animals, when compared to controls. Moreover, the PCNA assessment on liver tissue from animals at 3,200 ppm in the second subchronic mouse study showed that fluopicolide did not produce hepatocellular proliferation on Day 90. This is completely consistent with the lack of cell proliferation

observed on Day 28 with the BrdU assessment. These findings emphasize that a transient liver cell proliferation followed by a return to control levels is a prerequisite for the development of hepatocellular adenoma following a long term exposure period to fluopicolide.

The *in vitro* studies supported the CAR-mediated action since hepatocellular proliferation was observed in WT cells but not in CARKO cells. Human hepatocytes did not display proliferation after fluopicolide thus demonstrating human non-relevance.

Key event 4: Increased clonal expansion, leading to altered foci

Markedly elevated incidences of altered foci (especially acidophilic) were observed in the chronic mouse study after 78 weeks at the highest tested dose of 3,200 ppm in males and females.

Key event 5: Increased incidence of hepatocellular tumours

This key event represents the occurrence of liver adenomas as observed in a mouse oncogenicity study due to the events described before.

In order to further corroborate the proposed liver tumour MOA the parameters dose and time concordance were evaluated.

Dose concordance

The repeated dose toxicity studies in mice demonstrated a clear dose-response relationship of the key events. In this regard the first key event was liver enzyme induction measured by PROD and BROD and/or liver hypertrophy and liverw eight increase, starting already at 400 ppm in the oncogenicity study, at 800 ppm in the subchronic study and at 640 ppm in the subacute study. This was followed by induction of altered liver foci and finally liver adenomas only at 3,200 ppm in the chronic study. Liver cell necrosis was never seen at any dose up to 6,400 ppm in the standard toxicity studies. An overview about the dose concordance of these events is given in the following table.

Table 15-1: Dose concordance of main liver effects

Study type / **Effects**	**Dose level [ppm]**										
	6	**32**	**50**	**64**	**200**	**320**	**400**	**640**	**800**	**3,200**	**6,400**
Subacute mouse toxicity											
Hepatocellular hypertrophy	NE	-	-	NE	-	-	-	↑ (m/f)	-	-	↑ (m/f)
Liver weight	NE	-	-	NE	-	-	-	↑ (f)	-	-	↑ (m/f)
Liver cell necrosis	NE	-	-	NE	-	-	-	NE	-	-	NE
Subchronic mouse toxicity (Crl: CD-1 (ICR) BR)											
Hepatocellular hypertrophy	-	NE	-	-	-	↑ (m/f)	-	-	-	↑ (m/f)	↑ (m/f)
Liver weight	-	NE	-	-	-	NE	-	-	-	↑ (m/f)	↑ (m/f)
Liver cell necrosis	-	NE	-	-	-	NE	-	-	-	↑ (f)	↑ (m/f)

Subchronic mouse toxicity (C57BL/6)											
Hepatocellular hypertrophy	-	-	NE	-	NE	-	-	-	↑ (m/f)	↑ (m/f)	-
Liver weight	-	-	NE	-	NE	-	-	-	↑ (m/f)	↑ (m/f)	-
Liver cell necrosis	-	-	NE	-	NE	-	-	-	NE	NE	-
Liver cell proliferation (PCNA)	-	-	-	-	-	-	-	-	-	NE	-
Mouse oncogencity study											
Hepatocellular hypertrophy (52 weeks)	-	-	NE	-	-	-	↑ (m/f)	-	-	↑ (m/f)	-
Hepatocellular hypertrophy (78 weeks)	-	-	NE	-	-	-	↑ (m/f)	-	-	↑ (m/f)	-
Altered foci (52 weeks)	-	-	NE	-	-	-	NE	-	-	NE	
Altered foci (78 weeks)	-	-	NE	-	-	-	NE	-	-	↑ (m/f)	-
Liver cell necrosis (52 weeks)	-	-	NE	-	-	-	NE	-	-	NE	-
Liver cell necrosis (78 weeks)	-	-	NE	-	-	-	NE	-	-	NE	-
Liver weight (52 weeks)	-	-	NE	-	-	-	↑ (m)	-	-	↑ (m/f)	-
Liver weight (78 weeks)	-	-	NE	-	-	-	↑ (m/f)	-	-	↑ (m/f)	-
Hepatocellular adenomas (52 weeks)	-	-	NE	-	-	-	NE	-	-	↑ (f)	-
Hepatocellular adenomas (78 weeks)	-	-	NE	-	-	-	NE	-	-	↑ (m/f)	-

m: male, f: female

- : dose not tested

NE: no effect

Temporal relationship

It is obvious from the fluopicolide study data that early key events occur before the formation of liver adenomas. Thus, activation of the nuclear CAR/PXR receptors and liver enzyme induction occurs in the studies as early as after already 7-day treatment as observed in the MOA study. Cell proliferation typically also occurs early in this MOA cascade, which is the case in the MOA study with fluopicolide in which liver cell proliferation and increased incidence of mitotic cells was seen after 7 days at 3,200 ppm. An increased incidence of mitotic cells was also seen after 28 days. However, at terminal sacrifice afer 28 days, there was no increased hepatocellular proliferation in treated animals observed. Moreover, the PCNA assessment on liver tissue from animals at 3,200 ppm in the second subchronic mouse study showed that fluopicolide did not produce hepatocellular proliferation on Day 90 which is in agreement with the transient induction of cell proliferation known from phenobarbital. The next step in this cascade of key events is the transition to hyperplasia and eventually to liver tumours. A significantly increased incidence of hepatocellular adenoma was observed at 3,200 ppm at 52 weeks in females only and at 78 weeks in both males and females in the mouse carcinogenesis study. Therefore, the time concordance observed for fluopicolide is in agreement with the postulated MOA. This can be seen in the following table which gives an overview of the time concordance of the relevant events and parameters.

Table 15-2: Time concordance of main events in liver

Time/ Effect	7 days (MOA study)	28 days (MOA study)	28 days (subacute study)	90 days (subchronic study)	90 days (subchronic study)	52 weeks (oncogenicity study)	78 weeks (oncogenicity study)
Cyp2b/Cyp3a (CAR/PXR activation)	↑ (f, 3200 ppm)	↑ (f, 3200 ppm)	-	-	-	-	-
Liver weight	↑ (f, 3200 ppm)	↑ (f, 3200 ppm)	↑ (f ≥640 ppm; m 6400 ppm)	↑ (m/f≥3200 ppm)	↑ (m/f ≥800 ppm)	↑ (m≥400 ppm; f 3200 ppm)	↑ (m/f≥400 ppm)
Liver cell hypertrophy	↑ (f, 3200 ppm)	↑ (f, 3200 ppm)	↑ (m/f≥ 640 ppm)	↑ (m/f≥320 ppm)	↑ (m/f ≥800 ppm)	↑ (m/f≥400 ppm)	↑ (m/f≥400 ppm)
Liver single cell necrosis	↑ (f, 3200 ppm)	NE	NE	↑ (f≥3200 ppm; m 6400 ppm)	NE	NE	NE
Liver increased mitotic cells	↑ (f, 3200 ppm)	↑ (f, 3200 ppm)	NE	NE	NE	NE	NE
Liver cell proliferation (PCNA/BrdU)	↑ (f, 3200 ppm)	NE	-	-	NE	-	-
Liver altered foci	NE	NE	NE	NE	NE	NE	↑ (m/f, 3200 ppm)
Liver adenomas	NE	NE	NE	NE	NE	↑ (f, 3200 ppm)	↑ (m/f, 3200 ppm)

m: male, f: female
- : dose not tested
NE: no effect

Strength, consistency and specificity of association of key events and tumour response

For a MOA to be accepted, it must be demonstrated that the key events are causally related to the formation of tumors, that the key events are actually required steps that lead to tumors, and that the data are reproducible. All of key events were observed in one or more studies. The early key events and subsequent events followed the dose and time concordance of the postulated MOA.

Biological plausibility and coherence

The liver is the most common target tissue in chronic toxicity and carcinogenicity studies since it is the first organ to be exposed after absorption and the major site of metabolization of xenobiotics. The MOA of liver tumour induction for fluopicolide is similar to the MOA of liver tumour induction of phenobarbital in rodents. Since this phenobarbital-type liver tumour MOA was intensively investigated and published it can be used as an example. Also phenobarbital leads to an activation of mainly CAR and CYP2B induction which is associated with liver weight increases, hepatocellular hypertrophy, proliferation of smooth endoplasmatic reticulum and cell proliferation as demonstrated by BrdU labelling and the eventual liver tumour formation. The importance of the CAR activation as the first key event in this cascade was proven in studies with fluopicolide and phenobarbital in CAR/PXR knockout mouse hepatocytes since in the CAR knockout cells no proliferation occurred. Moreover, neither fluopicolide nor phenobarbital induced replicative DNA synthesis in male or female human hepatocytes. Therefore, it can be assumed that the described MOA for liver tumour induction by fluopicolide and also the non-relevance for humans are well-supported.

Other modes of action

There was no evidence of a genotoxic potential of fluopicolide, so that this potential liver tumour MOA can be excluded.

Also PPARα or Arylhydrocarbon receptor mediated effects as possible alternative MOA could be excluded based on the results of the 28-day mechanistic study in mice since no relevant effects on EROD and Lauric acid, respectivel were noted.

Evidence of oxidative stress as possible MOA was not seen in the conducted toxicity studies with fluopicolide. Also liver cytotoxicity as a primary mode of action for the liver tumours can be excluded, since in the toxicity studies with fluopicolide in mice despite systemic effects at the highest doses, no typical signs of severe cytotoxicity in the liver, like inflammatory signs, broad hepatic necrosis, hepatocellular death, fibrosis, cirrhosis or severely increased transaminase activities were observed. Only some foci of single cell necrosis/apoptosis with minimal to moderate severity were seen in a few treated animals at interim sacrifice and at terminal sacrifice in the 28-day mechanistic study and in the first subchronic study at the highest tested dose levels.

Concordance table according to the 'International Programme on Chemical Safety (IPCS) Mode of Action Framework'

The described existing data were evaluated in this chapter according to the 'International Programme on Chemical Safety (IPCS) Mode of Action Framework' approach which leads to the conclusion that the liver adenomas after fluopicolide treatment in mice are not relevant to humans. A tabular summary is given in the following.

Table 15-3: Overview of fluopicolide liver tumour MOA

Key events	- CAR/PXR activation as demonstrated by increased PROD and BROD conversation activity (upregulated CYP2B and CYP3A protein/enzyme activity) - Transiently ncreased liver cell proliferation after CAR and PXR activation - CAR/PXR-mediated increased liver cell proliferation in rodents leads to regenerative hyperplasia and eventually to liver tumours
Concordance of dose-response relationship	- In repeated dose toxicity studies with fluopicolide, dose-dependent increases in liver weight and hepatocellular hypertrophy as evidence of enzyme induction - In the mouse oncogenicity study, liver adenomas occurred with increased incidences only at the highest dose at which a higher liver workload due to xenobiotic metabolism can be assumed
Temporal association	All phases of tumour development are in good agreement with the temporal succession of the MOA steps: - Early start of CAR/PXR activation after 7 days of treatment - Liver cell proliferation as demonstrated by BrdU incorporation or increased incidence of mitotic cells after 7- and 28-day treatment with fluopicolide - Some liver effects at doses of 3200 ppm already after 7 days and at later time points - altered liver foci and adenomas after longer treatment duration, after 52 weeks liver adenomas only in females and after 78 weeks in both male and female animals in the mouse oncogenicity study
Strength, consistency and specificity of association of tumour response with key events	- The importance of CAR/PXR activation as key event for the liver tumour induction was clearly demonstrated in special 28-day MOA studies in mice - In vitro studies in hepatocytes of wildtype mice and CAR/PXR-knockout mice showed cell proliferation in hepatocytes of wildtype mice only, but did not show a liver cell proliferating potential in hepatocytes of CAR/PXR KO mice clearly confirming that the liver tumour formation is CAR/PXR-mediated - In vitro studies in human hepatocytes with fluopicolide did not show a liver cell proliferating potential which confirms that human hepatocytes are not sensitive to this liver tumour MOA and thus that this MOA is not relevant to humans

Biological plausibility and coherence	- Succession of key events and liver tumour development in rodents is in agreement with knowledge about biological and morphological processes in the liver - The proposed MOA of rodent-specific liver tumour development is in agreement with broadly accepted knowledge that increased cell proliferation is the main underlying process which leads to development of tumours by non-genotoxic compounds - Well-documented support of the rodent-specific liver tumour MOA of fluopicolide by many other CAR/PXR-activating compounds, e.g. phenobarbital which cause liver tumours via a similar MOA, based on the literature - Since phenobarbital is used in human medicine since decades and did not lead to an increased liver tumour incidence in humans, this demonstrates that this MOA has no relevance to humans
Other possible MOAs	- The main other possible MOA, i.e. by genotoxicity, can be excluded since the genotoxicity testing of fluopicolide did not indicate a genotoxic potential - the 28-day mechanistic study demonstrated absence of a peroxisome-proliferating or AhR-mediating effect - Oxidative stress or severe liver cytotoxicity as a mode of action for the liver tumours alone can be excluded, since in the toxicity studies with fluopicolide in mice no signs of severe cytotoxicity, like inflammatory signs, broad hepatic necrosis, hepatocytic death, fibrosis, cirrhosis or severely increased transaminase activities were observed
Uncertainties, inconsistencies and data gaps	- No inconsistencies since there is a clear concordance between dose- and time-relationship of MOA key events and mouse liver adenoma increases - Although in vitro data from CAR/PXR -knockout hepatocytes are available which show clear involvement of CAR/PXR in the liver tumour MOA, in vivo studies in CAR/PXR-knockout animals would be additional information
Assessment of postulated mode of action	- High reliability of MOA since very good concordance between dose, temporality, and the expected sequence of events for tumourigenicity in the liver and agreement with broad database of published work in this area - Further support of high reliability of MOA due to very good agreement with the MOA of other compounds, like phenobarbital which showed the same concordance between dose, temporality, and the expected sequence of events for tumour induction in the rodent liver - This rodent-specific liver tumour MOA is not relevant to humans, based on the in vitro hepatocyte studies with human hepatocytes, which is also supported by published scientific research and on the fact that phenobarbital which has the same MOA and is used in human medicine since decades, epidemiologically did not show any effect on human tumour incidences

Annex I to the CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

International Chemical Identification:

Fluopicolide (ISO); 2,6-dichloro-N-[3-chloro-5-(trifluoromethyl)-2-pyridylmethyl]benzamide

EC Number: **not allocated**

CAS Number: **239110-15-7**

Index Number: **not allocated**

Contact details for dossier submitter:

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Physical hazards are **not** assessed in this dossier. Only health hazards are assessed.

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1.2 Flammable gases (including chemically unstable gases)

Please refer to Section 1.

1.3 Oxidising gases

Please refer to Section 1.

1.4 Gases under pressure

Please refer to Section 1.

1.5 Flammable liquid

Please refer to Section 1.

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1.12 Oxidising liquids

Please refer to Section 1.

1.13 Oxidising solids

Please refer to Section 1.

1.14 Organic peroxides

Please refer to Section 1.

1.15 Corrosive to metals

Please refer to Section 1.

2 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Toxicokinetics are **not** assessed in this dossier, only health hazards are assessed; however as it is relevant to the human health evaluation, a short summary of the available data is provided below.

Table 2- 1: Summary of toxicokinetic studies

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206: Single high & low dose rat A.D.E. study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The major elimination route was via faeces (82 to 88% of dose), while urine contained 5 to 13% dose. Almost complete excretion occurred within 48 hours for the low dose group and 24 hours for the high dose group. At 168 hours post dose tissues were low (0.75 to 1.25% of the dose). Liver and kidneys contained highest residues, and also skin & fur for females of the high dose group.	ADE report. 2 separate metabolism reports (see below). [Phenyl-U-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw.	Anonymous; 2001; M-204781-01-1
[Phenyl-U-^{14}C]-AE C638206: Rat metabolism following administration of a single oral low dose - (including Amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-fluopicolide was very extensively metabolised in low dose rats (10 mg/kg bw/day) with up to 55 metabolites in urine (9 to 13% dose in urine) and 52 in faecal extracts (81 to 82% dose eliminated in faeces). Biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were oxidised to both sulphones and sulphoxides. The formation of an acetylated version of AE C653711 (M-01, BAM), indicated that fluopicolide could be cleaved which is consistent with results from the other radiolabelled metabolism study with [Pyridyl-2,6 -^{14}C]-fluopicolide.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 4 male & 4 female rats at 10 mg/kg bw (see above).	Anonymous; 2004; M-227026-02-1

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206: Rat metabolism following administration of a single oral high dose - (including Amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]- fluopicolide was also extensively metabolised in high dose animals (100 mg/kg bw/day) with 46 metabolites detected in urine (4 to 6% dose in urine) and 14 in faecal extracts (86 to 87% dose eliminated in faeces). The same routes of metabolism as seen in the low dose group were observed in high dose animals.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 4 male & 4 female rats at 100 mg/kg bw (see above).	Anonymous; 2004; M-227025-02-1
[Pyridyl-2,6 - ^{14}C]-AE C638206 - Single oral low dose rat A.D.E. study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The major elimination route was via faeces (69 to 72% of dose), while urine contained 21 to 27% dose. Almost complete excretion occurred within 48 hours. At 168 hours post dose tissues contained between 0.7 to 0.5% of the dose. Liver, kidneys and blood consistently contained highest residues.	Separate ADE and Metabolism reports (see below). [Pyridyl-2,6-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw.	Anonymous; 2001; M-202609-02-1
[Pyridyl-2,6-14C]-AE C638206: Rat metabolism following administration of a single oral low dose US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Pyridyl-2,6-^{14}C]- fluopicolide was also very extensively metabolised in the rat with up to 28 metabolites in urine (17 to 21% dose in urine) and 31 in faecal extracts (63% dose eliminated in faeces). The same biotransformations as seen in rats dosed with [phenyl-U-^{14}C]-fluopicolide were observed in rats dosed with [pyridyl-2,6-^{14}C]-fluopicolide. The formation of AE C657188 (M-02, PCA), indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule.	[Pyridyl-2,6-^{14}C]-Fluopicolide: ADE: 4 male & 4 female rats at 10 mg/kg bw (see above).	Anonymous; 2004; M-227023-01-1

Method	Results	Remarks	Reference
[Phenyl-U-14C]-AE C638206: Repeat oral low dose A.D.M.E. study in the rat - (including amendment No. 1) US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	No evidence of accumulation was observed. Following 14 daily oral administrations of [phenyl-U-^{14}C]-fluopicolide the major route of elimination was via faeces (73 to 79% dose). Repeated dosing enhanced elimination via urine compared with the single oral dose (15 to 22% dose). Tissue levels were consistently low (mean 0.38%). Liver, kidneys (organs of excretion and metabolism) and blood contained the highest concentrations of radioactivity in both sexes. [Phenyl-U-^{14}C]- fluopicolide was also extensively metabolised in repeat dose animals with 46 metabolites detected in urine and 14 in faecal extracts. A large number of metabolites were observed in the excreta (up to 57 in the urine and 45 in the faeces). The observed routes of metabolism included glutathione conjugation and its subsequent biotransformation products, hydroxylation, conjugation with glucuronic acid, conjugation with sulphate and oxidative N-dealkylation.	[Phenyl-U-^{14}C]-Fluopicolide: Metabolism: 5 male & 5 female rats at 10 mg/kg bw	Anonymous; 2004; M-227027-02-1
[Phenyl-U-^{14}C]-AE C638206: Rat bile excretion study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	Biliary elimination was a major route in low dose animals (10 mg/kg bw/day). 77% of the low dose for males and 83% for the females (mean 80%) was detected in the bile of cannulated rats dosed with [phenyl-U-^{14}C]-fluopicolide. At the high dose level, the values were 34% for the males and 41% for the females (mean 37%), demonstrating absorption had been saturated by this dose level.	[Phenyl-U-^{14}C]-Fluopicolide: 4 male & 4 female bile-duct cannulated rats at 10 mg/kg bw; 4 male & 4 female bile-duct cannulated rats at 100 mg/kg bw.	Anonymous; 2002; M-212243-01-1
[Pyridyl-2,6-^{14}C]-AE C638206: Single oral low dose rat bile excretion study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	When dosed with [pyridyl-U-^{14}C]-fluopicolide 59% of the low dose for males and 64% for the females (mean 62%) was detected in the bile of cannulated rats. The difference between the two radiolabels likely indicates a portion of the fluopicolide dose is metabolised to form single ring metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA), which behave differently in the rat.	[Pyridyl-U-^{14}C]-Fluopicolide: 4 male & 4 female bile-duct cannulated rats at 10 mg/kg bw;	Anonymous; 2003; M-230976-01-1

Method	Results	Remarks	Reference
[Phenyl-U-^{14}C]-AE C638206 and [pyridyl-2,6-^{14}C]-AE C638206: Rat blood and plasma kinetics study US EPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	The general pharmacokinetic profiles were similar between radiolabels and sexes. Fluopicolide was absorbed relatively rapidly with maximal concentrations achieved between 7 and 10 hours post dose at 10 mg/kg bw.	[Phenyl-U-^{14}C]-Fluopicolide: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw. [Pyridyl-U-^{14}C]-Fluopicolide: 4 male & 4 female rats at 10 mg/kg bw; 4 male & 4 female rats at 100 mg/kg bw.	Anonymous; 2002; M-221902-01-1
[Phenyl-U-^{14}C]-AE C638206 rat tissue kinetic study	Fluopicolide was rapidly and widely distributed into the tissues. No significant sex difference was found. In rats dosed with [phenyl-U-^{14}C]-fluopicolide, highest tissue concentrations were in the intestine and contents with next highest concentrations observed in liver, kidneys and adrenals, which decreased with time. The compound was extensively metabolised with 13 metabolites detected in liver by 8 hours post dose, of which AE C653711 (M-01, BAM), AE 0717559, AE C643890 (M-06) and AE 0717560 were identified.	[Phenyl-U-^{14}C]-Fluopicolide: 16 male & 16 female rats at 10 mg/kg bw; 16 male & 16 female rats at 100 mg/kg bw. Sacrificed at 8, 24 or 30, 36 or 48, 72 (males) & 120 hours (females).	Anonymous; 2003; M-221892-01-1
[2,6-Pyridyl-^{14}C]-AE C638206: Rat tissue kinetic study	In rats dosed with [pyridyl-U-^{14}C]-fluopicolide, the compound was similarly distributed into tissues, followed by a significant and rapid decrease in tissue concentrations. Again, no significant sex difference was found. Highest radioactivity concentrations were in the intestine and contents presumably as a result of biliary excretion of radioactivity. The next highest concentrations were in the liver, kidneys, adrenals and cardiac blood which declined with time post dose.	[Pyridyl-U-^{14}C]-Fluopicolide: 16 male & 16 female rats at 10 mg/kg bw. Sacrificed at 6 or 7, 24, 36, 48 (males) & 120 hours (females).	Anonymous, 2003; M-221885-01-1

Method	Results	Remarks	Reference
(^{14}C)-AE C638206: Preliminary toxicokinetic studies in the rat Non guideline preliminary study GLP	The findings aer consistent with later studies. Fluopicolide was relatively rapid adsorped with blood C_{max} between 8 to 12 hours. At low dose residues were below 0.10 µg/g in all tissues except the liver, kidney and blood 168 h after dosing. The major metabolic reactions identified were aromatic hydroxylation of the phenyl ring, glucuronidation of the phase I hydroxyl products and sequential metabolism through the mercapturic acid pathway.	Combined ADME & kinetics preliminary study (25 & 500 mg/kg bw) [Phenyl-U-^{14}C]-Fluopicolide: ADME: 2 male & 2 female rats at 25 mg/kg bw; 2 male & 2 female rats at 500 mg/kg bw. Blood kinetic: 2 male & 2 female rats at 25 mg/kg bw; 2 male & 2 female rats at 500 mg/kg bw. [Pyridyl-2,6-^{14}C]-Fluopicolide: ADME: 2 male & 2 female rats at 25 mg/kg bw. Blood kinetic: 2 male & 2 female rats at 25 mg/kg bw.	Anonymous; 2000; M-197858-01-1
Interspecies comparison of in vitro metabolism of [phenyl-UL-^{14}C] fluopicolide using mouse, rat, dog and human liver microsomes Non guideline preliminary study GLP	[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from mice, rat, dog and humans, with a total of 8 metabolites detected. No human-specific metabolites were observed.	[Phenyl-U-^{14}C]-Fluopicolide: *In vitro* (1 and 10 µM) with liver microsomes from mice, rat, dog and humans.	Anonymous; 2019; M-653630-02-1

In vivo studies

Toxicokinetic studies on the absorption, distribution, metabolism and excretion of fluopicolide, were conducted in the rat. Studies were performed using two different radiolabels; [phenyl-U-^{14}C]-fluopicolide or [pyridyl-2,6-^{14}C]-fluopicolide.

The major route of elimination of fluopicolide was via the faeces for both the 10 and 100 mg/kg bw oral dose and for both pyridyl (69 to 72% of the administered dose) and phenyl (82 to 88%) ring radiolabels. No significant sex difference was observed. There was a tendency towards a higher urinary excretion level with the pyridyl radiolabel (19% in males and 22% in females for the 10 mg/kg bw dose) compared to the phenyl radiolabel (10% in males and 13% in females for the 10 mg/kg bw dose). This suggests that a proportion of the metabolites that were formed differed between the two radiolabels and were presumably linked to the formation of AE C657188 (M-02, PCA) from the pyridyl ring moiety and AE C653711 (M-01, BAM) from the phenyl ring.

Following repeated (14x) daily oral administrations of [phenyl-U-^{14}C]-fluopicolide the total recovery of radioactivity was approx. 96% of the administered dose; with the faeces, again, being found to be the major route of elimination representing 79% for the males and 72% for the females. The urine was found to represent 15% of the administered dose for the males and 21% for the females. It appeared that repeated dosing enhanced elimination via urine compared with the single oral dose.

Tissue radioactivity levels were consistently low and ranged between 0.46 to 1.25% of the administered dose for the single dose studies and a mean of 0.38% for the repeat dose study.

Investigations in bile-cannulated rats over 48 hours showed a large proportion of the radioactivity found in the faeces had been absorbed and then eliminated via the bile. The extent of oral absorption based on the biliary excretion study only for the 10 mg/kg bw oral dose, was 80% of the administered dose for the phenyl radiolabel and 62% for the pyridyl radiolabel. However, blood and plasma pharmacokinetic data show the systemic exposure was similar between both the radiolabels and the sexes. The bioavailability of fluopicolide, taking into account the material undergoing entero-hepatic recirculation, was calculated to be 75 to 88% of the administered dose.

Fluopicolide was well distributed into organs and tissues (blood T_{max} 5.5 to 7.5 hours and plasma T_{max} 6.5 to 8 hours for 10 mg/kg bw) followed by a moderately rapid elimination such that the majority was eliminated by 48 hours post dose followed by a slower terminal elimination phase with a mean half-life of approx. 99 hours for blood. A lower mean half-life of 16 hours was observed for plasma due to the difference in limits of quantification.

The highest tissue residues were found in the liver and kidney and (to a lesser extent) in the spleen and blood.

In tissue kinetic studies the highest tissue concentrations were observed in the intestine and contents, reflecting a combination of unabsorbed material and biliary excretion. The next highest concentrations were consistently observed in the liver, kidneys and adrenals albeit that the concentrations were decreasing with time post dosing. AE C653711 (M-01, BAM), AE 0717559, AE C643890 (M-06) and AE 0717560 were identified in the liver 8 hour post dosing with [phenyl-U-^{14}C]-fluopicolide.

Fluopicolide was extensively metabolised in the rat. The formation of the metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA) was confirmed during the course of the biotransformation investigations and indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule. Generally, the biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate and glutathione. The glutathione conjugates were seen to be further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were seen to be further metabolised by acetylation to form the mercapturic acids or to be dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were seen to be oxidised to both sulphones and sulphoxides.

In vitro study

The comparative *in-vitro* metabolism of fluopicolide was studied with liver microsomes from CD-1 mouse, Wistar rat, Beagle dog and human. Incubations were performed with [phenyl-U-^{14}C]-fluopicolide at two concentrations (1 and 10 µM) at 1, 60 and 120 minutes.

[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from all four species. Conversion of fluopicolide was 98% in dog, 82% in mouse, 68% in human and 54% in rat microsomes after 120 minutes of incubation. A total of 8 metabolites were detected, named Metabolite 1 to 8 based on their HPLC retention time. Metabolites accounting for ≥ 5% were considered as main metabolites. Overall, five main metabolites were detected: Metabolite 1 (mouse, rat and human) and Metabolite 2 (mouse and rat), Metabolite 3 and Metabolite 5 (mouse, dog and human) and Metabolite 6 which was detected as a main metabolite in the four species. Metabolite 2 was detected in the mouse and rat microsome incubations only. No human-specific fluopicolide metabolites were detected.

3 HEALTH HAZARDS

Table 3- 2: Summary of acute toxicity studies

Method **Guideline** **GLP compliance**	**Test substance** **Purity** **Solvent/Vehicle**	**Result**	**Classification ((EC) No 1272/2008)**	**Reference**
Rat acute oral toxicity 5 Sprague Dawley rats/sex OECD 423 (1996) GLP	Fluopicolide (purity 97.7%) in 1% w/v aqueous methylcellulose	LD_{50} > 5000 mg/kg bw in M/F	none	Anonymous; 2000; M-197224-01-1
Rat acute dermal toxicity 5 Sprague Dawley rats/sex OECD 402 (1987) GLP	Fluopicolide (purity 97.7%) in 1% w/v aqueous methylcellulose	LD_{50} > 5000 mg/kg bw in M/F	none	Anonymous; 2000; M-197225-01-1
Rat acute inhalation toxicity (4 hours) 5 Sprague Dawley rats/sex OECD 403 (1981) GLP	Fluopicolide (purity 98.3%) as dust	LC_{50} inhalation: > 5.16 mg/L	none	Anonymous; 2000; M-197229-01-1
Rabbit skin irritancy 3 female New Zealand White rabbits OECD 404 (1992) GLP	Fluopicolide (purity 97.7%) moisted with distilled water	The mean irritation score (24-72 hours) was 0.0	none	Anonymous; 2000; M-197226-01-1

Method **Guideline** **GLP compliance**	**Test substance** **Purity** **Solvent/Vehicle**	**Result**	**Classification ((EC) No 1272/2008)**	**Reference**
Rabbit eye irritancy 4 female New Zealand White rabbits OECD 405 (1987) GLP	Fluopicolide (purity 97.7%)	Mean scores for 24, 48 and 72 hours post instillation were 0.0 for all parameters, with the exception of conjunctival redness, which had a score of 0.33. Reactions had resolved in all instances two days after instillation.	none	Anonymous; 2000; M-197227-01-1
Guinea pig skin sensitization study Dunkin/Hartley guinea pigs 20 animals (treatment) + 10 animals (control) OECD 406 (1992) GLP	Fluopicolide (purity 97.7%) in sterile water	No specific skin responses compared to control	none	Anonymous; 2000; M-197228-01-1
Phototoxicity	-	Considered to be not required due to its ultraviolet/visible molar extinction/absorption properties	-	-

M = male F = female

The acute oral LD_{50} of fluopicolide in rats was > 5,000 mg/kg bw. Signs of toxicity included piloerection within 1 – 2½ hours after dosing in all rats. Later on Day 1 piloerection was accompanied only by hunched posture and abnormal gait. Recovery was completed by Day 3.

The acute dermal LD_{50} of fluopicolide in rats was > 5,000 mg/kg bw.

The 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

Fluopicolide was not irritating to rabbit skin. No dermal irritation was observed during the study. The mean irritation score over 24 – 72 hours was for erythema 0 and for oedema 0.

Fluopicolide was transiently slightly irritating to the rabbit eye. The ocular reactions were very slight and resolved in all instances within two days after instillation.

Fluopicolide was not a skin sensitizer in this guinea pig Magnusson and Kligman test.

Therefore, no classification is required for the acute toxicity of fluopicolide when comparing the study results / derived LD_{50} and LC_{50} values with CLP criteria according to the Regulation (EC) No 1272/2008[1].

[1] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006.

3.1 Acute toxicity - oral route

3.1.1 Animal data

3.1.1.1 Anonymous; 2000; M-197224-01-1

Study reference:

Anonymous; 2000; Rat acute oral toxicity Code: AE C638206 00 1C99 0005; M-197224-01-1

Deviations: Deviations from the current OECD guideline (423, 2002):

- Five animals of each sex, rather than three were used in the limit test

This deviation does not compromise the results and outcome of the study.

Executive Summary:

In an acute toxicity study by the acute toxic class method, five male and five female fasted Sprague-Dawley rats were each administered by gavage a single oral dose of 5,000 mg/kg bw of fluopicolide.

No mortality was observed. Clinical signs of reaction to treatment were confined to piloerection and hunched posture, seen in all female rats and in three male rats with abnormal gait notable in three females. Recovery of rats, as judged by external appearance and behavior, was complete by Day 3. All animals were considered to have achieved satisfactory body weight gains throughout the study.

In conclusion, the acute median lethal oral dose (LD_{50}) of fluopicolide in rats was greater than 5,000 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats of Sprague-Dawley origin (Hsd: Sprague-Dawley(CD))
Age:	8-11 weeks
Weight at start:	195 g (186 – 201 g) in males and 206 g (196 – 220 g) in females
Source:	Harlan U.K. Ltd, Bicester, Oxon, England
Acclimation period:	Yes
Diet:	Special Diet Services RM1(E) SQC expanded pellet
Water:	Water *ad libitum*
Housing:	In groups of up to five rats of the same sex in metal cages (RS Biotech Sub-Dividable Rodent Cages - polished stainless steel (20 cm high x 39 cm wide x 39 cm long)).
Temperature:	21 – 22 °C
Humidity:	30 – 50%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: April 3 to April 19, 2000

2. Animal assignment and treatment

A group of ten rats (five males and five females) was treated at 5000 mg/kg bw, a dosage selected after review of preliminary study results with administration of a dose of 4000 mg/kg bw. The appropriate dose volume of the test substance was administered to each rat by oral gavage using a plastic syringe and catheter of the appropriate gauge. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least twice daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed on two occasions during the day (once in the morning and again at the end of the experimental day, with the exception of the day of study termination – morning only). The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals in the preliminary and main study were observed for 7 or 14 days respectively after dosing.

The body weight of each rat in the preliminary study was recorded on Days 1 (prior to dosing) and 8 and in the main study on Days 1 (prior to dosing), 8 and 15.

2. Necropsy

All animals were killed by carbon dioxide asphyxiation at study termination.

Animals were subjected to a macroscopic examination, which consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in Table 3.1.1.1- 1.

LD_{50}: > 5,000 mg/kg bw

Table 3.1.1.1- 1: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
Prelim. 4,000	0/1/1	1/1	-	0
5,000	0/5/5	5/5	-	0
Females				
Prelim. 4,000	0/1/1	1/1	-	0
5,000	0/5/5	5/5	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs

Clinical signs of reaction to treatment were confined to piloerection and hunched posture, seen in all female rats and in 3/5 males with abnormal gait notable in 3/5 females. Recovery of rats was complete by Day 3.

3. Body weights

There were no treatment-related effects on body weight or body weight gain.

4. Necropsy findings

The necropsies performed at the end of the study did not reveal any findings.

III. Conclusion

The acute oral LD_{50} of fluopicolide in rats was greater than 5,000 mg/kg bw.

3.1.2 Human data

No human data.

3.1.3 Other data

No other data.

3.2 Acute toxicity - dermal route

3.2.1 Animal data

3.2.1.1 Anonymous; 2000; M-197225-01-1

Study reference:

Anonymous; 2000; Rat acute dermal toxicity Code: AE C638206 00 1C99 0005; M-197225-01-1

Deviations: Deviations from the current OECD guideline (402, 2017):
- Five animals of each sex, rather than three were used in the limit test
- All animals were treated concurrently instead of sequentially

These deviations do not compromise the results and outcome of the study.

Executive Summary:

A group of ten rats (five males and five females) received a single topical application of the test substance, administered as supplied at a dose level of 5,000 mg/kg bw of fluopicolide. The application site was occluded for 24 hours. All animals were observed daily for 14 days and body weights were recorded at weekly intervals post dosing. They were killed as scheduled at study termination (Day 15) and subjected to a macroscopic examination.

No mortality was observed. There were no clinical signs of reaction to treatment observed in any animal throughout the study.

There was no evidence of a dermal response to treatment observed in any animal throughout the study.

A slight reduced body weight was evident in 2/5 females on day 8. All other animals were considered to have achieved satisfactory body weight gains throughout the study.

No macroscopic abnormalities were observed for animals killed at study termination on Day 15.

The acute median lethal dermal dose (LD_{50}) to rats of fluopicolide was greater than 5,000 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats of Sprague-Dawley origin (Hsd: Sprague Dawley(CD))
Age:	8-11 weeks
Weight at start:	Males: 247 g (231 – 263 g), females: 228 g (220 – 233 g)
Source:	Harlan U.K. Ltd, Bicester, Oxon, England.
Acclimation period:	Yes
Diet:	Special Diet Services RM1(E) SQC expanded pellet
Water:	Water *ad libitum*
Housing:	In groups of up to five rats of the same sex in metal cages (RS Biotech Sub-Dividable Rodent Cages - polished stainless steel (20 cm high x 39 cm wide x 39 cm long)).
Temperature:	19 - 21 °C
Humidity:	24 – 50%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 1 to February 17, 2000

2. Animal assignment and treatment

In a preliminary study comprising of two rats (one male and one female) dosed at 3,200 mg/kg bw was conducted to help define the toxic potential of the test substance.

In the main study a group of ten rats (five males and five females) received a single dermal administration of the test substance at a dose level of 5,000 mg/kg bw.

In the main study the test substance was applied to the closely-clipped dorsum of each animal on day 1 at a dosage of 5,000 mg/kg bw, and was covered by an occlusive dressing for 24 hours. Fluopicolide was formulated at a maximum practical concentration of 100% w/v in 1% w/v aqueous methylcellulose and administered at a dose volume of 5 mL/kg bw in order to achieve the desired dose concentration. The test substance was prepared on the day of dosing. The test substance was applied by spreading it evenly over the prepared skin. The treatment area (approx. 50 mm x 50 mm) was covered with porous gauze held in place with a non-irritating dressing, and further covered by a waterproof dressing encircled firmly around the trunk of the animal. At the end of the 24 hours exposure period the dressings were carefully removed and the treated area of skin was washed with warm water (30-40 °C) to remove any residual test substance. The treated area was blotted dry with absorbent paper.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least twice daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed on two occasions during the day (once in the morning and again at the end of the experimental day, with the exception of the day of study termination – morning only). The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals in the preliminary and main study were observed for 7 or 14 days respectively after dosing.

Local dermal irritation at the treatment site was assessed daily.

The body weight of each rat in the preliminary study was recorded on Days 1 (prior to dosing) and 8 and in the main study on Days 1 (prior to dosing) 8 and 15 (or at death).

2. Necropsy

All animals were killed by carbon dioxide asphyxiation at study termination.

Animals were subjected to a macroscopic examination, which consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in Table 3.2.1.1- 1.

LD_{50} dermal: > 5,000 mg/kg bw

Table 3.2.1.1- 1: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
Prelim. 3,200	0/0/1	0/1	-	0
5,000	0/0/5	0/5	-	0
Females				
Prelim. 4,000	0/0/1	0/1	-	0
5,000	0/0/5	0/5	-	0

* Number of animals which died, and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs

There were no deaths following a single dermal application of fluopicolide to a group of ten rats (five males and five females) at a dose level of 5,000 mg/kg bw.

There were no clinical signs of reaction to treatment observed in any animal throughout the study.

There was no evidence of a dermal response to treatment observed in any animal throughout the study.

3. Body weights

A slight body weight loss was evident in two females on Day 8. All other animals were considered to have achieved satisfactory body weight gains throughout the study.

4. Necropsy findings

No macroscopic abnormalities were observed for animals killed at study termination on Day 15.

III. Conclusion

The acute dermal LD_{50} to rats of fluopicolide was greater than 5,000 mg/kg bw.

3.2.2 Human data

No human data.

3.2.3 Other data

No other data.

3.3 Acute toxicity - inhalation route

3.3.1 Animal data

3.3.1.1 Anonymous; 2000; M-197229-01-1

Study reference:

Anonymous; 2000; Rat acute inhalation toxicity Code: AE C638206 00 1C99 0005; M-197229-01-1

Deviations:	Deviations from the current OECD guideline (403, 2009): None.

Executive Summary:

The acute inhalation toxicity of fluopicolide was investigated by exposing a group of five male and five female Sprague-Dawley (CD) rats to a dust atmosphere of the limit concentration of test substance (5 mg/L). The test group was subjected to a single 4-hour continuous, snout only exposure. Signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were sacrificed at the end of the observation period and were subjected to detailed necropsy.

No mortality was recorded. Common observations noted both during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen. Animals recovered quickly to appear normal on the first day after exposure.

Normal body weight gain was noted during the study.

No macroscopic abnormalities were noted for 9/10 animals. One male showed dark foci on its lungs.

In conclusion, the 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	98.3%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	None

3. Test animals

Species:	Rat
Strain:	Sprague-Dawley Crl:CD®BR
Age:	8-10 weeks
Weight at start:	Males: 295 to 317 g, females: 230 to 243 g
Source:	Charles River (UK) Ltd, Margate, Kent
Acclimation period:	Yes
Diet:	Rat and Mouse Expanded Diet No. 1, Special Diets Services Limited, Witham, Essex, UK)
Water:	Water *ad libitum*
Housing:	In groups of five by sex in solid-floor polypropylene cages with stainless steel lids, furnished with softwood flakes (Dates and Ltd, Cheshire, UK).
Temperature:	21 ± 2 °C
Humidity:	55 ± 15%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 21 to March 23, 2000

2. Animal assignment and treatment

The acute inhalation toxicity of fluopicolide was investigated by exposing a group of five male and five female Sprague-Dawley (CD) rats to a dust atmosphere of the limit concentration (5 mg/L) of test substance. The test group was subjected to a single 4-hour continuous, snout only exposure. Signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were sacrificed at the end of the observation period and were subjected to detailed necropsy.

Table 3.3.1.1- 1: Actual concentration and atmosphere characteristics in chamber

Mean concentration ± SD (mg/L)	Nominal concentration (mg/L)	MMAD ± GSD (µm)	Resp. fraction (% < 4 µm)
5.16 ± 0.38	9.09	3.37 ± 2.09	59.1

C. Methods

1. Observations

All animals were observed for clinical signs at hourly intervals during exposure, immediately on removal from the restraining tubes at the end of exposure, one hour after termination of exposure and subsequently once daily for fourteen days. Any evidence of overt toxicity was recorded at each observation.

Individual body weights were recorded prior to treatment on the day of exposure and on Days 7 and 14.

2. Necropsy

At the end of the 14-day observation period, the animals were killed by intravenous overdose of sodium pentobarbitone. All animals were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded. The respiratory tract was subjected to a detailed macroscopic examination for signs of irritancy or local toxicity.

II. Results and Discussion

A. Results

1. Dose-response table (LC_{50})

The results of the study for acute inhalation toxicity in the fasted rat, including the LC_{50}, are summarized in Table 3.3.1.1- 2.

LC_{50} inhalation: > 5.16 mg/L

Table 3.3.1.1- 2: Dose response

Dose (mg/L)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
5.16	0/5/5	5/5	-	0
Females				
5.16	0/5/5	5/5	-	0

* Number of animals which died, and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs, mortality

There was no mortality during the exposure and observation period. Common observations noted both during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen. Animals recovered quickly to appear normal on the first day after exposure.

3. Body weights

Normal body weight gain was noted during the study.

4. Necropsy findings

No macroscopic abnormalities were noted for 9/10 animals. One male showed dark foci on its lungs.

III. Conclusion

The 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

3.3.2 Human data

No human data.

3.3.3 Other data

No other data.

3.4 Skin corrosion/irritation

3.4.1 Animal data

3.4.1.1 Anonymous; 2000; M-197226-01-1

Study reference:

Anonymous; 2000; AE C638206 - Rabbit skin irritancy; M-197226-01-1

Deviations:	Deviations from the current OECD guideline (404, 2015): None.

Executive Summary:

The potential of fluopicolide to cause inflammatory or corrosive changes upon first contact with skin was assessed by semi-occluded application of 0.5 g of the test substance to the closely-clipped dorsa of three New Zealand White rabbits for four hours. Dermal reactions were assessed 1, 24, 48 and 72 hours after removal of the dressings.

A single semi-occlusive application of fluopicolide to intact rabbit skin for four hours elicited no dermal irritation in any animal during the study. The mean irritation score (24-72 hours) was 0.0.

In conculsion, fluopicolide was not irritating to rabbit skin according to Regulation (EC) 1272/2008.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	None

3. Test animals

Species:	Rabbit, females
Strain:	New Zealand White strain
Age:	At least 11 weeks of age
Weight at start:	2.3 to 2.7 kg
Source:	Harlan UK Ltd, Bicester, Oxon, England
Acclimation period:	Yes
Diet:	Special Diet Services STANRAB (P) SQC pellet
Water:	Water *ad libitum*
Housing:	Individually in stainless steel cages with perforated floors
Temperature:	20.0 to 20.5 °C
Humidity:	26 to 36%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 15 to February 18, 2000

2. Animal assignment and treatment

Approximately 24 hours prior to application of the test substance, hair was removed with electric clippers from the dorso-lumbar region of each rabbit exposing an area of skin approx. 100 mm x 100 mm. Approximately 0.5 g of the test substance was applied under a 2-ply 25 mm x 25 mm gauze pad which had been moistened with 0.5 mL distilled water, to one intact skin site on each animal. Each treatment site was covered with "Elastoplast" elastic adhesive dressing for four hours. The animals were not restrained during the exposure period and were returned to their cages immediately after treatment. At the end of the exposure period, the semi-occlusive dressing and gauze pad were removed and the treatment site was washed with warm water (30 to 40 °C) to remove any residual test substance. The treated area was blotted dry with absorbent paper.

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity.

2. Dermal observations

Examination of the treated skin was made on Day 1 (i.e. approximately 60 minutes after removal of the dressings) and on Days 2, 3 and 4 (equivalent to approximately 24, 48 and 72 hours after exposure).

Local dermal irritation was assessed using the prescribed numerical system:

<u>Erythema and eschar formation:</u>

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) or eschar formation (injuries in depth) preventing erythema reading	4

<u>Oedema formation:</u>

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (edges raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health in any rabbit during the observation period.

2. Dermal reactions

The numerical values given to the dermal reactions elicited by fluopicolide are shown in Table 3.4.1.1-1.

A single semi-occlusive application of fluopicolide to intact rabbit skin for four hours elicited no dermal irritation in any animal during the study.

The mean irritation score (24-72 hours) was 0.0.

Table 3.4.1.1- 1: Dermal reactions

Rabbit no.	E = Erythema O = Oedema	Hours after treatment				Mean irritation score (24-72 hrs)
		1*	24	48	72	
156 female	E O	0 0	0 0	0 0	0 0	0 0
157 female	E O	0 0	0 0	0 0	0 0	0 0
158 female	E O	0 0	0 0	0 0	0 0	0 0

*: Approximately 1 hr after removal of the dressing

III. Conclusion

No dermal irritation was observed during the study. The mean irritation score over 24 – 72 hours was 0.0 for erythema and oedema 0. Therefore, fluopicolide was not irritating to rabbit skin.

3.4.2 Human data

No human data.

3.4.3 Other data

No other data.

3.5 Serious eye damage/eye irritation

3.5.1 Animal data

3.5.1.1 Anonymous; 2000; M-197227-01-1

Study reference:

Anonymous; 2000; AE C638206 - Rabbit eye irritancy; M-197227-01-1

Deviations: Deviations from the current OECD guideline (405, 2017):
- Topical and systemic analgesics were not applied as recommended by the current test method

The deviation(s) are considered not to compromise the results and outcome of the study.

Executive Summary:

The potential of fluopicolide to cause damage to the conjunctiva, iris or cornea was assessed in four New Zealand White rabbits. An amount of 0.1 mL of the test substance was instilled into the conjunctival sac of each of the test animals on Day 1. Ocular reactions were assessed at 1, 24, 48 and 72 hours and 7 days after treatment in accordance with the Draize scheme as provided in the OECD guideline No. 405. In one animal (screen animal) the eye was rinsed 30 seconds post instillation.

A single instillation of fluopicolide into the rinsed eye of the screen rabbit elicited slight conjunctival irritation at one hour post instillation only.

A single instillation of fluopicolide into the unrinsed eye of the rabbit elicited slight conjunctival irritation in all animals from one hour post instillation. The ocular reactions resolved in all instances within two days after instillation.

In conclusion, fluopicolide was transiently slightly irritating to the rabbit eye but does not warrant classification according to Regulation (EC 1272/2008).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	Not given

3. Test animals

Species:	Rabbit, females
Strain:	New Zealand White strain
Age:	At least 11 weeks of age
Weight at start:	2.3 to 2.7 kg
Source:	Harlan UK Ltd, Bicester, Oxon, England
Acclimation period:	Yes
Diet:	Special Diet Services STANRAB (P) SQC pellet
Water:	Water *ad libitum*
Housing:	Individually in stainless steel cages with perforated floors
Temperature:	19.5 to 21 °C
Humidity:	25 to 38%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 29 to March 6, 2000

2. Animal assignment and treatment

The eyes of each animal were examined prior to instillation of the test substance to ensure that there was no pre-existing corneal damage, iridial inflammation or conjunctival irritation.

Screen study - rinsed eye:

One animal was treated in advance of the others, to ensure that, if a severe response was produced, no further animals would be exposed (see Table 3.5.1.1- 2 for screen animal). The treated eye of this animal was rinsed with distilled water 30 seconds after instillation for duration of 30 seconds.

Main study - unrinsed eyes:

One animal was treated in advance of the other two, again to ensure that if a severe response was produced, no further animals would be exposed (see

Table 3.5.1.1- 3 for pilot animal). In compliance with the study guideline, the weight of the test substance which when gently compacted occupied a volume of 0.1 mL was measured (see Table 3.5.1.1- 1).

On all occasions, a volume of 0.1 mL of the test substance (mean weight 93 mg) was placed in the lower everted lid of one eye of each animal. The eyelids were then gently held together for one second before releasing. The contralateral eye remained untreated.

Table 3.5.1.1- 1: Weight of test substance

Syringe	Weight of 1 mL test substance (mg)
1 2 3 4	80 99 100 94
Mean weight of 0.1 mL test substance	93

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity.

2. Ocular observations

Examination of the eyes was made after one hour and 1, 2 and 3 days (equivalent to 24, 48 and 72 hours) after instillation. Observation of the eyes was aided by the use of a handheld light.

Ocular irritation was assessed using the prescribed numerical system:

Cornea Opacity: degree of density (area most dense taken for reading):

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Nacreous areas, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4

Area of cornea involved:

None	0
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4

Iris:

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

Conjunctivae Redness (refers to the most severe reading of palpebral and bulbar conjunctivae, as compared to the control eye):

Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3

Chemosis (lids and/or nictating membranes):

No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half-closed	3
Swelling with lids more than half-closed	4

Discharge:

No discharge	0
Any amount greater than normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health in any rabbit during the observation period.

2. Ocular reactions

The numerical values given to the ocular reactions elicited by fluopicolide are shown in Table 3.5.1.1-2 and

Table 3.5.1.1- 3.

Table 3.5.1.1- 2: Screen animal, ocular reactions (rinsed eye)

Readings	Animal	Cornea		Iris	Conjunctiva			Additional findings
		Area involved	Density		Redness	Chemosis	Discharge	
1 h	213	0	0	0	1	0	0	–
24 h		0	0	0	0	0	0	–
48 h		0	0	0	0	0	0	–
72 h		0	0	0	0	0	0	–
Individual 24-48-72 h means		0	0	0	0	0	0	–

Table 3.5.1.1- 3: Main study, ocular reactions (non-rinsed eyes)

Readings	Animal	Cornea		Iris	Conjunctiva			Additional findings
		Area involved	Density		Redness	Chemosis	Discharge	
1 h	214*	0	0	0	1	0	0	–
	215	0	0	0	1	0	0	–
	216	0	0	0	1	0	0	–
24 h	214	0	0	0	0	0	0	–
	215	0	0	0	1	0	0	–
	216	0	0	0	1	0	0	–
48 h	214	0	0	0	0	0	0	–
	215	0	0	0	0	0	0	–
	216	0	0	0	0	0	0	–
72 h	214	0	0	0	0	0	0	–
	215	0	0	0	0	0	0	–
	216	0	0	0	0	0	0	–
Individual 24-48-72 h means	214	0.0	0.0	0.0	0.0	0.0	0.0	–
	215	0.0	0.0	0.0	0.33	0.0	0.0	–
	216	0.0	0.0	0.0	0.33	0.0	0.0	–

* Pilot animal
\- Not applicable

Screen study - rinsed eye:

No corneal damage or iris inflammation was seen in the animal.

Injected blood vessels of the conjunctivae were seen in the animal at one hour post instillation. Reactions had resolved one day after instillation.

Main study - unrinsed eyes:

No corneal damage or iris inflammation was seen in any animal.

Injected blood vessels to a crimson coloration of the conjunctivae were seen in all three animals from one hour post instillation. Reactions had resolved in all instances two days after instillation.

Mean scores for 24, 48 and 72 hours post instillation were 0.0 for all parameters, with the exception of conjunctival redness, which had a score of 0.33 in two out of three animals.

III. Conclusion

Fluopicolide was transiently slightly irritant to the rabbit eye but does not warrant classification according to Regulation (EC 1272/2008).

3.5.2 Human data

No human data.

3.5.3 Other data

No other data.

3.6 Respiratory sensitisation

No data on respiratory sensitisation available. Fluopicolide was of low toxicity in an acute inhalation study (see Section 3.3.1) and was negative in a skin sensitisation study (see Section 3.7); therefore, it is unlikely that it would induce respiratory sensitisation. Furthermore, medical surveillance data on manufacturing personnel was obtained during the pilot-scale production of fluopicolide (2004-2005 in Lyon, France & Dormagen, Germany). No incidences of adverse reactions were reported during the pilot-scale manufacture/formulation of fluopicolide (fluopicolide DAR 2005)

3.6.1 Animal data

No animal data.

3.6.2 Human data

No human data.

3.6.3 Other data

No other data.

3.7 Skin sensitisation

3.7.1 Animal data

3.7.1.1 Anonymous; 2000; M-197228-01-1

Study reference:

Anonymous; 2000; Guinea pig skin sensitization study - AE C638206; M-197228-01-1

Deviations:	Deviations from the current OECD guideline (406, 1992): None.

Executive Summary:

The potential of fluopicolide to cause delayed contact hypersensitivity in guinea pigs was assessed by the Magnusson-Kligman maximisation test. Based on the findings of a preliminary study, the closely-clipped dorsa of twenty female Dunkin-Hartley guinea-pigs were subject to intradermal injections of Freund's Complete Adjuvant, 10% w/v fluopicolide in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund's complete adjuvant in sterile water on Day 1. Six days later, the same area of skin was treated by topical application of 100% w/v fluopicolide in sterile water and the test site was covered by an occlusive dressing for 48 hours. The same induction procedures were carried out on 10 control animals, except that the test substance was replaced by vehicle in all doses. Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank. The occlusive dressings were removed on the following day and the condition of the test sites was assessed approximately 24 and 48 hours later.

There were no deaths or signs of ill health or toxicity. Body weight changes were similar between control and treated animals.

During the induction phase, necrosis was observed at sites receiving Freund's Complete Adjuvant in all test and control animals following intradermal injections. Slight irritation was seen in six (out of 20) test animals at sites receiving fluopicolide, 10% w/v in sterile water and no irritation was observed in any control animal receiving sterile water. Following topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was also seen in one control guinea-pig.

The challenge application produced no dermal reactions indicative of skin sensitization in any of the test or control animals. Slight erythema was observed in two test animals at the 24 and 48 hour reading compared to slight to well-defined erythema for two control animals at the 48 hour reading only. The reactions observed were noted to be of similar incidence and severity and as no reactions were observed for any of the remaining test or control animals, the overall response was considered negative.

In conclusion, fluopicolide was not a skin sensitizer in this guinea pig Magnusson and Kligman test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	Sterile water

3. Test animals

Species:	Guinea pigs, females
Strain:	Albino guinea pigs of the Dunkin/Hartley strain
Age:	At least 11 weeks of age
Weight at start:	390 – 464 g
Source:	D. Hall, Newchurch, Staffs, UK
Acclimation period:	Yes
Diet:	Vitamin C enriched guinea-pig diet (Harlan Teklad 9600 FD2 SQC) ad libitum. Hay was given three times each week.
Water:	Water ad lib
Housing:	In groups of five in suspended plastic cages with solid floors and sawdust bedding
Temperature:	18 to 20 °C
Humidity:	43 to 59%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 17 to March 20, 2000

2. Animal assignment and treatment

The potential of fluopicolide to cause delayed contact hypersensitivity in guinea pigs was assessed by the Magnusson-Kligman Maximisation Test. Based on the findings of a preliminary study, the closely-clipped dorsa of twenty female Dunkin-Hartley guinea-pigs were subject to intradermal injections of Freund's Complete Adjuvant, 10% w/v fluopicolide in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund’s complete adjuvant in sterile water on day 1. Six days later, the same area of skin was treated by topical application of 100% w/v fluopicolide in sterile water and the test site was covered by an occlusive dressing for 48 hours. The same induction procedures were carried out on 10 control group animals, except that the test material was replaced by vehicle in all doses. Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank. The occlusive dressings were removed on the following day and the condition of the test sites was assessed approx. 24 and 48 hours later.

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity. The body weight of each guinea-pig on the main study was recorded on Day 1 (day of intradermal injections) and on the last day observations were made of dermal responses to the challenge application.

2. Dermal observations

The dermal reactions resulting from intradermal injection and topical application on the preliminary study, and topical application at the challenge were assessed using the following numerical system.

Erythema and eschar formation:

No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation:

No oedema	0
Slight oedema	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	4

The approximate diameter (mm) of the dermal response at the intradermal injection sites was recorded in the preliminary study only to assist in the choice of concentrations for the main study.

Any lesion not covered by this scoring system was described.

The challenge sites were evaluated approx. 24, 48 and 72 hours after removal of the patches. On completion of the study all animals were killed by cervical dislocation.

Dermal reactions in the test animals elicited by the challenge application were compared with the findings simultaneously obtained in the control animals.

A test animal was considered to show positive evidence of delayed contact hypersensitivity if the observed dermal reaction at challenge was definitely more marked and/or persistent than the maximum reaction seen in animals of the control group.

If the dermal reaction seen in a test animal at challenge was slightly more marked and/or persistent than (but not clearly distinguishable from) the maximum reaction seen in control animals, the result for that test animal was classified as inconclusive.

A test animal was considered to show no evidence of delayed contact hypersensitivity if the dermal reaction resulting from the challenge application was the same as, or less marked and/or persistent than the maximum reaction seen in animals of the control group.

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health.

2. Dermal reactions

Preliminary study

In the preliminary study, a dose response relationship for the endpoints of diameter, erythema and oedema was evident from doses ranging from 0.1 – 40% fluopicolide. Based upon these findings, the following concentrations of fluopicolide were selected:

Induction intradermal injection: 10% fluopicolide w/v in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund's complete adjuvant.

This was the highest concentration that caused irritation but did not adversely affect the animals.

Induction topical application: 100% fluopicolide w/v in sterile water.

Topical challenge: 100% w/v and 50% w/v in sterile water.

From preliminary investigations the test material applied topically at 100% w/v did not give rise to irritating effects.

Main study

Induction

After intradermal injections, necrosis was recorded at sites receiving Freund´s Complete Adjuvant in all test and control animals.

Slight irritation was seen in six (out of 20) test animals at sites receiving fluopicolide, 10% w/v in sterile water and no irritation was observed in any control animal receiving sterile water.

Following topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was also seen in one control guinea-pig.

Challenge

The numerical values given to the dermal reactions elicited by the challenge applications are shown in Table 3.7.1.1- 1.

Slight erythema was observed in two test animals at the 24 and 48 hour reading compared to slight to well-defined erythema for two control animals at the 48 hour reading only. As the reactions observed were of similar incidence and severity and no reactions were observed for any of the remaining test or control animals, all test animals gave negative responses.

Table 3.7.1.1- 1: Dermal reactions after challenge application with fluopicolide

Guinea pig no.	E=erythema O=Oedema	Score						Results
		24 hours		48 hours		72 hours		
		A	P	A	P	A	P	
Freund's treated controls								
815	E	0	0	1	0	0	0	-
	O	0	0	0	0	0	0	
816	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
817	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
818	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
819	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
820	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
821	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
822	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
823	E	0	0	2	0	0	0	-
	O	0	0	0*	0	0	0*	
824	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
Test animals								
825	E	1	0	1	0	0	0	-
	O	0	0	0	0	0*	0	
825	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
826	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
827	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
828	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
829	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
830	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
831	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
832	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
833	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
834	E	1	0	1	0	0	0	-
	O	0	0	0	0	0	0	
835	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
836	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
837	E	0	0	0	0	0	0	-
	O	0	0	0	0	0*	0*	
838	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	

Guinea pig no.	E=erythema O=Oedema	Score						Results
		24 hours		48 hours		72 hours		
		A	P	A	P	A	P	
839	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
840	E	0	0	0	0	0	0	-
	O	0	0	0*	0*	0*	0*	
841	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
842	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
843	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
844	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	

* Dryness and sloughing of the epidermis
A: Anterior site, exposed to fluopicolide, 100% w/v in sterile water
P: Posterior site, exposed to fluopicolide, 50% w/v in sterile water
Results: + : Positive; - : Negative; ± : Inconclusive

III. Conclusion

Fluopicolide was not a skin sensitizer in this guinea pig Magnusson and Kligman test.

3.7.2 Human data

No human data.

3.7.3 Other data

No other data.

3.8 Germ cell mutagenicity

Table 3.8- 1: Overview of genotoxicity studies performed with fluopicolide

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
***In vitro* studies**				
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 97.8% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9) Experiment II/III (TA98 only): 50; 160; 500; 1600; 2000; 3000; 4000 and 5000 µg/plate (+S9) <u>Pre-incubation</u> Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9)	**Positive** at precipitating dose levels	Anonymous; 2004; M-197259-02-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide Purity not reported DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202931-01-1

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 95.6% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202927-01-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 95.9% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202939-01-1

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 99.3% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) <u>Pre-incubation</u> Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	Anonymous; 2001; M-202935-01-1
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 98.2% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 TA 100 and TA 102 The following concentrations were tested: <u>Plate incorporation</u> Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (±S9) Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated (see experiment Ia). Experiment Ia (TA102 only): 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (+S9) <u>Pre-incubation</u> Experiment II: 10; 33; 100; 333; 1000; 2500 and 5000 µg/plate (±S9)	**Negative**	Anonymous; 2017; M-595228-01-1

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
In vitro chromosome aberration assay in Chinese hamster lung V79 cells OECD 473 (1997) GLP <u>Deviations:</u> Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide 97.8% DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 25; 50; 75 and 100 µg/mL (±S9) Experiment II: 1.6; 3.2 and 6.3 µg/mL (-S9)	**Positive** at cytotoxic concentrations	Anonymous; 2004; M-197260-02-1
In vitro chromosome aberration assay in human lymphocytes OECD 473 (1997) GLP <u>Deviations:</u> Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide 95.9% DMSO	Test system: Human lymphocytes The following concentrations were tested: Experiment I: 19.53; 78.13 and 156.25 µg/mL (-S9) 78.13; 312.5 and 625 µg/mL (+S9) Experiment II: 1.22; 9.77 and 19.53 µg/mL (-S9) 39.06; 156.25 and 312.5 µg/mL (+S9)	**Negative**	Anonymous; 2001; M-201582-01-1

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
In vitro HPRT mutation assay in Chinese hamster lung V79 cells OECD 476 (1997) GLP	Fluopicolide 97.8% DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 1.2; 3.8; 12.1; 38.2; 120.8; 382; 1208 and 3820 µg/mL (±S9) Experiment II: 0.4; 0.8; 1.6; 3.2; 6.3; 12.5; 25; 50; 75; 100 and 120 µg/mL (±S9) Experiment III: 0.313; 0.625; 1.25; 2.5; 5; 10; 20; 30; 40; 50 and 60 µg/mL (±S9)	**Negative**	Anonymous; 2005; M-210831-02-1
***In vivo* studies in somatic cells**				
Mouse micronucleus test OECD 474 (1997) GLP <u>Deviations:</u> Target organ exposure not measured. Only 2000 instead of 4000 erythrocytes were analysed. Only 200 instead of 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide 97.8% 1% (w/v) methylcellulose	Test system: Mouse (HsdWin:NMRI) The following concentrations were tested: 200, 600 and 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2005; M-197261-02-1
Mouse micronucleus test OECD 474 (1997) GLP <u>Deviations:</u> Target organ exposure not measured. Only 2000, not 4000 erythrocytes, were analysed. Only 200, not 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide 96.1% 1% (w/v) methylcellulose	Test system: Mouse (Crl:CD1) The following concentration was tested: 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2003; M-219364-01-1

Method **Guideline** **GLP compliance** **Deviations**	**Test substance** **Purity** **Solvent/Vehicle**	**Relevant study information**	**Result**	**Reference**
Mouse micronucleus test (i.p.) OECD 474 (1997) GLP Deviations: Altered NCE/PCE ratio. Only 2000, not 4000 erythrocytes, were analysed.	Fluopicolide 99.4% 0.5% cremophor	Test system: Mouse (Crl:CD1) The following concentrations were tested: 150, 300 and 600 mg/kg bw (i.p.)	**Negative**	Anonymous; 2003; M-223119-01-1
Rat UDS assay OECD 486 (1997) GLP	Fluopicolide 97.7% 1% (w/v) methylcellulose	Test system: Rat (Hsd/Ola SD) The following concentrations were tested: 600 and 2000 mg/kg bw (oral)	**Negative**	Anonymous; 2000; M-197230-02-1
Comet assay in mice OECD 489 (2016) GLP	Fluopicolide 98.2% 1% (w/v) methylcellulose	Test system: Mouse (Hsd:ICR (CD-1)) The following concentrations were tested: 500, 1000 and 2000 mg/kg bw (oral, gavage)	**Negative**	Anonymous; 2018; M-635020-01-1

Several genotoxicity studies were performed for fluopicolide: six reverse gene mutation tests in *Salmonella typhimurium* and *Escherichia coli* strains of bacteria, one of them recently (Anonymous.; 2017; M-595228-01-1), one chromosomal aberration assay in Chinese hamster V79 cells *in vitro*, one chromosomal aberration assay in human lymphocytes *in vitro*, one HPRT mutation assay in Chinese hamster V79 cells, two *in vivo* micronucleus assays in mouse bone marrow cells with oral administration, one *in vivo* micronucleus assay in mouse bone marrow cells with intraperitoneal administration, one *in vivo* UDS assay in rat hepatocytes by oral route and one *in vivo* Comet assay in male mice with oral gavage administration. Several deviations were reported from the current OECD test guidelines for a number of these studies, specifically, two *in vitro* chromosome abberation tests (OECD 473) and three *in vivo* micronucleus tests (OECD 474); however, the dossier submitter considers that these deviations do not affect the validity or results of the studies.

One of the earlier five bacterial reverse mutation assay showed a very slight increase in the number of revertant colonies in strain (TA 98) only with metabolic activation and to a lesser extent with TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation at the highest concentration of 5000 µg/plate where precipitation was observed. Therefore, this result was considered of doubtful biological significance and four additional assays were conducted to confirm/infirm this equivocal response. No evidence of mutagenic activity of fluopicolide was observed in the four additional bacterial reverse mutation assays performed with five *Salmonella typhymurium* strains and one *Escherichia coli* strain. In addition, a recently conducted bacterial reverse mutation assay (Anonymous.; 2017; M-595228-01-1) was also negative and also confirmed the overall negative outcome in this study type. Furthermore, a Comet assay was also recently (Anonymous.; 2018; M-635020-01-1) performed to confirm the negative profile for the endpoint gene mutation *in vivo*. In this

in vivo Comet assay, no statistically significant or dose related increases in % tail DNA were observed in liver or kidney cells of treated male mice (6/dose) up to doses of 2000 mg/kg bw/day. The positive control gave the expected response and the increase in % tail DNA observed in the vehicle control group was within the range of the laboratory historical control data, thus confirming the validity of the study (a detailed summary of this comet assay is provided in Annex I of this CLH report).

The chromosomal aberration assay performed in Chinese hamster V79 showed a positive response. However, the increase of aberrant cells occured at cytotoxic concentrations where mitotic indices were clearly below the limit of 50% indicating the doubtful biological signifiance of these data. This chromosome aberration assay was therefore repeated in human lymphocytes and gave a clear negative response. Moreover, two *in vivo* micronucleus assays were performed in mice by the oral route up to the limit dose of 2000 mg/kg bw. Both of these assays were negative. However, one was of questionable biological significance due to the slight increase of micronucleated polychromatic erythrocytes in bone marrow of some animals given 2000 mg/kg bw as well as in one control animal. As the ratio of polychromatic to normochromatic erythrocytes was not significantly affected and no clinical signs were observed in both assays, a third assay was performed in mice by the intraperitoneal route to increase the likelihood of bone marrow exposure. This assay gave a clear negative result for clastogencity in vivo at dose levels showing clear cytotoxicity of the bone marrow.

The HPRT mutation assay in Chinese hamster V79 cells was negative. Moreover, the *in vivo* rat hepatocyte UDS assay clearly showed that fluopicolide does not induce damage to DNA.

In summary, 2 out of 15 tests (one *in vitro* bacterial reverse mutation assay and one *in vitro* chromosome aberration assay) gave positive responses of doubtful biological significance. The *in vivo* mutagenicity data from micronucleus tests are reliable and it is clear that no mutagenic effects were seen in the tested animals. Clearly, chromosomal damage does not occur *in vivo*. Furthermore, in the carcinogenicity studies (see section 10.9) fluopicolide caused an increase in hepatocellular adenomas in male and female mice by a mechanism considered not relevant to humans, and increased neoplasms were observed only at or above the maximum tolerated dose (MTD). Altogether these findings clearly show that fluopicolide is devoid of any genotoxic potential in somatic cells when tested *in vitro* and *in vivo*. Therefore, and since no evidence of an effect on germ cells was seen in other studies, an *in vivo* genotoxicity study in germ cells was not regarded as necessary.

According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017), a mutagenicity classification of fluopicolide is not warranted.

3.8.1 *In vitro* data

3.8.1.1 Anonymous; 2004; M-197259-02-1

Study reference:

Anonymous; 2004; Bacterial reverse mutation test Code: AE C638206 00 1C99 0005; M-197259-02-1

Deviations:	Deviations from the current OECD guideline (471, 1997): None.

Executive Summary:

This study was designed to assess the mutagenic potential of fluopicolide in amino acid dependent strains of *Salmonella typhimurium* and a strain of *Escherichia coli*.

Technical fluopicolide was tested for mutagenicity with the strains TA 100, TA 1535, TA 1537 and TA 98 of *Salmonella typhimurium* and with *Escherichia coli* WP2uvrA.

Two independent mutagenicity studies were conducted with all tester strains (one plate incorporation test and one pre-incubation test), each in the absence and in the presence of an Aroclor-induced metabolizing system derived from a rat liver homogenate. To confirm a mutagenic response in the plate incorporation test with the tester strain TA 98 in the presence of S9-mix, two further plate incorporation tests were conducted with this strain using smaller dose intervals.

The test substance was dissolved in DMSO, and each bacterial strain was exposed to 5 to 8 dose levels. Doses ranged from 50 to 5000 µg/plate.

Control plates without mutagen showed that the number of spontaneous revertant colonies was within the laboratory's historical control range and similar to that described in the literature. All positive control compounds showed the expected increase in the number of revertant colonies.

The test substance was not toxic to the bacterial strains in either the presence and in the absence of metabolic activation. Visible precipitation on the plates was observed at 500 µg/plate and above.

In the plate incorporation test fluopicolide gave a dose-dependent increase in the number of revertant colonies with the bacterial strain TA 98. This response was confirmed in the additional tests with this tester strain. In the pre-incubation test an increase in the number of revertant colonies was found with the *Salmonella* strains TA 98 and to a lesser extent with TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation.

Fluopicolide was mutagenic in this bacterial mutation test at precipitating dose levels.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide), technical
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO (concentration not given)
Positive control:	Without S9 mix:
	Na-azide: TA 100, TA 1535
	9-aminoacridine: TA 1537
	2-nitrofluorene: TA 98
	4-nitroquinoline-N-oxide: WP2uvrA
	With S9 mix:
	2-Aminoanthracene: TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods, an exogenous metabolic activation system is necessary.

The S9 fraction was prepared by the department conducting the study according to Ames *et al.* (1975)[2]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann (33178 Borchen, Germany), received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) 5 days before killing.

[2] Ames BN, Mccann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res. 1975 Dec;31(6):347-64.

The livers were removed from at least 5-6 animals and, using cold sterile solutions at approx. 0 to 4 °C and glassware, were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 x g for 10 minutes. The supernatant was the S9 fraction. This was divided into small portions, rapidly frozen and stored at approx. -80 °C for not longer than six months.

The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

S9 Mix:

Sufficient S9 fraction was thawed at room temperature immediately before each test. One volume of S9 fraction (batch no. 99/9 for the first plate incorporation test, protein concentration 56.7 g/L; batch no. 99/10 for the second and third plate incorporation test and for the pre-incubation test, protein concentration 54.3 g/L) was mixed with 9 volumes of the S9 cofactor solution, which was kept on ice until used. This preparation is termed S9-mix. The concentrations of the different compounds in the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP
100 mM phosphate buffer pH 7.4.

4. Test organisms:

The strains of *Salmonella typhimurium* were obtained from Professor B.N. Ames, University of California, U.S.A. The strain of *Escherichia coli* was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

Bacteria were grown overnight in nutrient broth (25 g Oxoid Nutrient Broth No. 2 /liter) at approx. 37 °C. The amount of bacteria in the cell suspension was checked by nephelometry. Inoculation was performed with stock cultures which had been stored at approx. -80 °C. The different bacterial strains were checked half-yearly with regard to their respective biotin, histidine and/or tryptophan requirements, membrane permeability, ampicillin resistance, crystal violet sensitivity, UV resistance and response to diagnostic mutagens. All criteria for a valid assay were fulfilled.

Sterility checks and control plates:

Sterility of S9-mix and the test substance were indicated by the absence of contamination on the test substance and S9-mix sterility check plates. Control plates (background control and positive controls) gave the expected number of colonies, i.e. values were within the laboratory's historical control range.

5. Test substance concentrations used:

The test substance was dissolved in DMSO and a stock solution of 50 mg/mL was prepared for the highest concentration, which provided a final concentration of 5000 µg/plate. Further dilutions of 1600, 500,160 and 50 µg/plate were used in the first plate incorporation test.

To verify the findings in the first test with tester strain TA 98 in the presence of S9-mix, two further tests with this strain were conducted. They used smaller intervals between dose levels of 50, 160, 500, 1600, 2000, 3000, 4000 and 5000 µg/plate.

For the second (pre-incubation) test dose levels of 50, 160, 500, 1600 and 5000 µg/plate were chosen for all tester strains and additional concentrations of 2000, 3000 and 4000 µg/plate with the tester strain TA 98.

Visible precipitation of the test substance on the plates was observed at 500 µg/plate and above.

The test substance was not toxic to the bacterial strains.

Based on this, in the plate incorporation and pre-incubation test, the following concentrations were used:

Plate incorporation test: 50, 160, 500, 1600, 5000 µg/plate
Pre-incubation test: 50, 160, 500, 1600, 5000 µg/plate.

B. Test performance

Experimental phase: April 04 to April 28, 2000

1. Assay procedure

Two independent mutation tests were performed unless clearly positive or dose-related activity was observed in the first test. Where results were negative or equivocal, a second test was conducted. This included a pre-incubation step if the first test was clearly negative. Pre-incubation involved incubating the test substance, S9-mix and bacteria for a short period before pouring this mixture onto plates of minimal agar.

Each test was performed in both the presence and absence of S9-mix using all bacterial tester strains and a range of concentrations of the test substance. Positive and negative controls as well as solvent controls were included in each test. Triplicate plates were used.

The highest concentration in the first mutation experiment was 50 mg/mL of the test substance in the chosen solvent, which provided a final concentration of 5000 µg/plate.

Further dilutions of 1600, 500, 160 and 50 µg/plate were used. Dose levels used in the second experiment were based on findings, including toxicity, in the first experiment. A reduction in the number of spontaneously occurring colonies and visible thinning of the bacterial lawn were used as toxicity indicators. Thinning of the bacterial lawn was evaluated microscopically.

In both tests top agar was prepared which, for the *Salmonella* strains, contained 100 mL agar (0.6% (w/v) agar, 0.5% (w/v) NaCl) with 10 mL of a 0.5 mM histidine-biotin solution. For *E. coli* histidine was replaced by tryptophan (2.5 mL, 0.5 mM). The following ingredients were added (in the following order) to 2 mL of molten top agar at approx. 48 °C:

0.5 mL S9-mix (if required) or buffer
0.1 mL of an overnight nutrient broth culture of the bacterial tester strain
0.1 mL test substance solution (dissolved in DMSO).

In the second mutagenicity test if appropriate these top-agar ingredients were pre-incubated by shaking for approx. 20 minutes at approx. 30 °C. After mixing, and pre-incubation if appropriate, the liquid was poured into a petri dish containing a 25 mL layer of minimal agar (1.5% (w/v) agar, Vogel-Bonner E medium with 2% (w/v) glucose). After incubation for approx. 48 hours at approx. 37 °C in the dark, colonies (his* and trp* revertants) were counted by hand or by a suitable automatic colony counter. The counter was calibrated for each test by reading a test pattern plate to verify the manufacturer's requirements for the counter's sensitivity.

2. Statistics

Not given (according to the OECD guideline 471, a statistical analysis of the data is not mandatory).

3. Acceptance / assessment criteria:

The assay is considered valid if the following criteria are met:

- The solvent control data are within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induce increases in the mutation frequency which are significant and within the laboratory's normal range.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

a) It produces at least a 2-fold increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control at complete bacterial background lawn

b) It induces a dose-related increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control in at least two to three concentrations of the test compound at complete bacterial background lawn.

If the test substance does not achieve either of the above criteria, it is considered to show no evidence of mutagenic activity in this system.

II. Results and Discussion

A. Mutation assays

The test compound was not toxic to the bacterial strains in either the presence and in the absence of metabolic activation. Visible precipitation on the plates was observed at concentrations of ≥ 500 µg/plate.

In the plate incorporation test fluopicolide produced a significant increase (2.8x compared to solvent control) in the number of revertant colonies with the bacterial strain TA 98 with metabolic activation at a precipitating dose level of 5000 µg/plate. A slight increase in mutation frequency (1.4-2.5x compared to the respective solvent controls) was also observed in two further plate incorporation tests with narrower dose spacing with this tester strain at concentrations ≥ 3000 µg/plate with metabolic activation.

In the pre-incubation test an increase in the number of revertant colonies was also found with the *Salmonella* strain TA 98 with metabolic activation at precipitating concentrations ≥ 2000 µg/plate (2.2-2.7x compared to solvent control) and additionally to a lesser extent with TA 1537 in the presence of metabolic activation (2.2x compared to solvent control) and with the tester strain TA 1535 in the absence of exogenous metabolic activation (2.0x compared to solvent control, covered by historical control range) at 5000 µg/plate.

All positive controls produced significant increases in the number of revertant colonies. Thus, the sensitivity of the assay and the efficacy of the exogenous metabolic activation system were demonstrated.

An overview of the results is given in Table 3.8.1.1- 1 to Table 3.8.1.1- 3.

Table 3.8.1.1- 1: Revertant colony counts obtained in the plate incorporation test – Experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	22.0p	110.0p	10.7p	7.0p	20.0p
	1600	22.3p	138.7p	13.0p	5.3p	19.3p
	500	18.7p	137.7p	7.3p	5.7p	19.0p
	160	26.0	134.0	10.7	5.0	28.7
	50	24.0	124.7	9.3	4.7	30.7
Historical solvent control mean	-	20.1 ± 4.6	123.4 ± 26.3	8.4 ± 2.5	6.3 ± 2.1	23.8 ± 5.9
Historical control range	-	8.3-40.3	57.7-223.0	3.0-23.3	3.0-14.3	10.7-42.7
Solvent control	-	23.7	116.3	8.0	5.7	27.7
Negative control	-	27.3	163.0	9.7	6.0	26.3
Positive control						
Sodium azide	1	NA	579.7	565.3	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	447.3
2-nitrofluorene	2.5	342.3	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	113.3	NA
With metabolic activation (+S9)						
Fluopicolide	5000	69.0p (2.8x)	150.7p	8.3p	10.7p	33.0
	1600	33.0p	118.7p	12.0p	8.3p	29.0
	500	24.0p	128.7p	8.7p	7.3p	30.0
	160	25.0	147.7	8.7	7.0	30.3
	50	25.3	138.0	10.0	8.3	28.7
Historical solvent control mean	-	23.3 ± 5.0	134.4 ± 27.7	8.9 ± 2.1	6.8 ± 2.2	25.0 ± 6.4
Historical control range	-	8.3-38.3	73.0-244.3	4.0-17.7	2.3-14.0	13.0-48.0
Solvent control	-	24.3	139.0	9.3	7.7	34.0
Negative control	-	28.3	154.7	12.0	6.0	30.7
Positive control 2-aminoanthracene	0.5 - 10 #	1060.0	1284.7	169.3	177.7	194.3

NA: not applicable
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
p: precipitated
Number of experiments for historical control data:
Plate incorporation test: n=186 (TA 100, ± S9); n= 177 and 179 (TA 1535, +S9 and –S9, respectively); n= 176 and 175 (TA 1537, +S9 and –S9, respectively); n= 179 and 177 (TA 98, +S9 and –S9, respectively); n= 97 (WP2uvrA, ±S9)

Table 3.8.1.1- 2: Revertant colony counts obtained in the plate incorporation test – confirmation of mutagenic response in strain TA98 with metabolic activation (+S9)

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strain TA 98	
		Experiment II	Experiment III
Fluopicolide	5000	42.0p (1.4x)	74.0p (2.5x)
	4000	60.3p (2.0x)	62.3p (2.1x)
	3000	48.3p (1.6x)	61.3p (2.1x)
	2000	42.3p (1.4x)	41.3p (1.4x)
	1600	37.3p	42.3p (1.4x)
	500	28.3p	29.7p
	160	30.7	24.3
	50	22.7	23.0
Historical solvent control mean	-	23.3 ± 5.0	23.3 ± 5.0
Historical control range	-	8.3-38.3	8.3-38.3
Solvent control	-	30.3	29.3
Negative control	-	26.3	30.0
Positive control 2-aminoanthracene	0.5 - 10 #	712.0	917.7

p: precipitated
#: 0.5 µg/plate for TA 98
Number of experiments for historical control data:
Plate incorporation test: n= 179 (TA 98, +S9)

Table 3.8.1.1- 3: Revertant colony counts obtained in the pre-incubation test

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	18.0p	90.3p	8.0p (2.0x)	5.3p	27.7p
	1600	22.7p	88.0p	6.3p	5.0p	28.3p
	500	17.7p	80.3p	3.0p	3.7p	29.3p
	160	17.0	81.0	7.7	4.3	27.3
	50	20.0	82.3	5.0	3.7	26.0
Historical solvent control mean	-	20.5 ± 4.6	116.6 ±20.9	7.3 ± 1.6	6.0 ± 1.6	22.0 ± 5.6
Historical control range	-	12.0-32.0	84.3-196.0	3.3-11.0	3.3-12.3	10.0-34.3
Solvent control	-	17.7	81.7	4.0	6.3	22.0
Negative control	-	25.7	114.0	9.7	5.3	24.3

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Positive control						
Sodium azide	1	NA	329.0	283.3	NA	NA
4-nitroquinoline-N- oxide	2	NA	NA	NA	NA	179.7
2-nitrofluorene	2.5	465.7	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	153.0	NA
With metabolic activation (+S9)						
Fluopicolide	5000	70.7p (2.7x)	141.7p	9.7p	12.3p (2.2x)	31.7p
	4000	62.3p (2.3x)	NA	NA	NA	NA
	3000	70.0p (2.6x)	NA	NA	NA	NA
	2000	57.7p (2.2x)	NA	NA	NA	NA
	1600	43.7p	120.3p	8.7p	5.3p	33.7p
	500	31.3p	135.7p	7.0p	7.7p	26.7p
	160	32.3	111.3	9.3	5.0	29.0
	50	25.7	111.7	10.0	4.3	31.3
Historical solvent control mean	-	24.9 ± 5.0	128.4 ±19.8	8.1 ± 1.6	6.5 ± 1.6	24.6 ± 6.1
Historical control range	-	15.3-40.3	94.7-177.7	4.3-12.0	3.0-11.0	14.3-37.7
Solvent control	-	26.7	115.7	9.3	5.7	31.0
Negative control	-	30.3	141.0	9.3	4.3	33.0
Positive control 2-aminoanthracene	0.5 - 10 #	829.0	717.0	123.3	120.0	151.0

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Number of experiments for historical control data:
Pre-incubation test: n= 52 and 61 (TA 100, +S9 and –S9, respectively); n= 65 and 72 (TA 1535, +S9 and –S9, respectively); n= 59 and 71 (TA 1537, +S9 and –S9, respectively); n= 67 and 69 (TA 98, +S9 and –S9, respectively); n= 62 (WP2uvrA, ±S9)

III. Conclusion

Fluopicolide was mutagenic in this bacterial mutation test in TA 98 and to a lesser extent in TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation at precipitating dose levels.

3.8.1.2 Anonymous; 2001; M-202931-01-1

Study reference:

Anonymous; 2001; Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206 00 1C99 0005; M-202931-01-1

Deviations:	Deviations from the current OECD guideline (471, 1997): None.

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver postmitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was observed on all plates treated at 1000 µg/plate, except those of strain WP2 uvrA in the presence of S9.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges, and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1C99 0005 (fluopicolide)
Purity:	Not given in report
Batch no.:	OP2050190

2. Vehicle and/or positive control

Vehicle:	DMSO (concentration not given)
Positive control:	Without S9 mix:
	Na-azide: TA 100, TA 1535
	9-aminoacridine: TA 1537
	2-nitrofluorene: TA 98
	4-nitroquinoline-N-oxide: WP2uvrA
	With S9 mix:
	Benzo[a]pyrene: TA 98
	2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in in vitro methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalyzed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[3] and De Serres and Shelby[4]. All experimentation commenced within 2 hours of the end of the period of incubation.

[3] Maron D M and Ames B N (1983) Revised methods for the Salmonella mutagenicity test. Mutation Research 113, 173-215.5

[4] De Serres F J and Shelby M D (1979) Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. Mutation Research 64, 159-165.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050190, was a beige powder, which was received on 21 July 2000. It was stored at room temperature in the dark. Purity and certificate of analysis was not supplied from the sponsor. The expiry date of the test substance was stated to be July 2002. Determinations of the stability and characteristics of the test substance were the responsibility of the Sponsor.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), with the aid of vortexing in experiment I, immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized (Gelman Acrodisc CR filter, 0.2 µm pore size) and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approx. 5 hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: July 25 to August 21, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9. Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1 °C:

• 0.1 mL bacterial culture
• 0.1 mL test article solution or control
• 0.5 mL 10 % S9 mix or buffer solution

followed by rapid mixing and pouring on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37±1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46±1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This and some other organic solvents are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of precipitate affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[4]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers. Precipitation of the test article was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test article within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step. Following all treatments in experiment II, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was recorded during the treatment of all plates at the maximum test dose of 1000 µg/plate, but after incubation of the test plates observations of precipitation at the time of revertant colony scoring excluded strain WP2 uvrA plates treated in the presence of S9. Due to the observations made at the time of plate treatment, and those in experiment I, the maximum test dose of these treatments was considered to have been very close to a precipitating dose level.

The individual plate counts were averaged to give mean values. From the data it can be seen that mean solvent control counts were comparable with the normal historical ranges as shown in the following tables. The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The mutation data were evaluated as follows. Fluopicolide treatments of all the test strains resulted in only a single increase in revertant numbers that was statistically significant when the data were analysed at the 1% level using Dunnett's test. This increase occurred following experiment II treatments of strain TA 1535 in the absence of S9, but failed to demonstrate any clear dose-relationship, occurring at an intermediate dose only. Furthermore, the data within this study indicated that this increase was not reproducible, as no similar increases were observed following comparable strain treatments in experiment I. The observed increase in revertant numbers was therefore attributed to chance, and this study was considered to have provided no clear evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 3.8.1.2- 1 and Table 3.8.1.2- 2 .

Table 3.8.1.2- 1: Mean revertant colony counts/plate in experiment I

Treatment	**Concentration (µg/plate)**	**Mean revertant colony counts in strains**				
		TA 98	**TA 100**	**TA 1535**	**TA 1537**	**WP2uvrA/ pKM101**
Without metabolic activation (-S9)						
Fluopicolide	5000	52p	109p	28p	17p	18p
	1000	45p	102p	31p	12p	19p
	200	50	108	26	18	19
	40	38	105	30	16	13
	8	55	103	28	22	15
	1.6	42	114	29	15	21
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	1 – 34	1 – 23
Solvent control	-	44	114	24	14	20
Positive control						
Sodium azide	2	NA	626	573	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	657
2-nitrofluorene	5	1107	NA	NA	NA	NA
Benzo[a]pyrene	10	300	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	224	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9)						
Fluopicolide	5000	65p	107p	17p	26p	25p
	1000	50p	114p	26p	14p	15p
	200	51	102	20	20	20
	40	42	97	21	23	25
	8	52	113	18	21	20
	1.6	49	111	20	18	23
Historical solvent control mean	-	38.2 ± 9.6	104.8 ±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	51	98	21	18	25
Positive control 2-aminoanthracene	5 - 10 #	NA	1757	187	266	242

NA: not applicable
p: precipitation
#: 5 µg/plate for TA 100, TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 3.8.1.2- 2: Mean revertant colony counts/plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	42p	127p	21p	13p	16p
	500	38	136	24	14	19
	250	36	145	35	14	16
	125	40	143	43**	14	13
	62.5	34	144	30	16	17
	31.25	36	138	29	13	9
Solvent control	-	35 ± 15	122 ± 11	28 ± 2	14 ± 2	16 ± 2
Positive control						
Sodium azide	2	NA	757	735	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	925
2-nitrofluorene	5	1444	NA	NA	NA	NA
Benzo[a]pyrene	10	300	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	242	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	45p	128p	27p	21p	33
	500	44	116	23	15	35
	250	40	122	23	17	23
	125	34	122	19	13	21
	62.5	40	126	22	18	17
	31.25	41	130	25	18	21
Solvent control	-	39	128	23	15	23
Positive control 2-aminoanthracene	5 - 10 #	NA	1871	220	428	38

**: $p \leq 0.01$, statistically significant compared to control (Dunnett´s test)
NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide was not genotoxic in the bacterial gene mutation assay under the conditions of this assay. It did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

3.8.1.3 Anonymous; 2001; M-202927-01-1

Study reference:

Anonymous; 2001; Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206 00 1C96 0002; M-202927-01-1

Deviations: Deviations from the current OECD guideline (471, 1997):
None.

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was observed on all plates treated at the maximum test dose of 1000 µg/plate.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1C96 0002 (OP 2050045) (fluopicolide)
Purity:	95.6%
Batch no.:	OP2050045

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	<u>Without S9 mix:</u> Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide: WP2uvrA <u>With S9 mix:</u> Benzo[a]pyrene : TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[3] and De Serres and Shelby[4]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050045, was a fine beige powder, which was received on 7 August 2000. It was stored at room temperature in the dark. Purity was stated as 95.6%. Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approximately 5½ hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: August 10 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9. Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1 °C:

• 0.1 mL bacterial culture
• 0.1 mL test article solution or control
• 0.5 mL 10 % S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37±1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46 ± 1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This and some other organic solvents are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter or manually where confounding factors such as split agar or the presence of precipitate affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[4]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step. Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was observed on all plates treated at 1000 µg/plate.

The individual plate counts were averaged to give mean values, which are presented in the following tables. From the data it can be seen that mean solvent control counts were comparable with the normal historical ranges.

The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

No fluopicolide treatments of any of the test strains, either in the absence or in the presence of S9, resulted in any increases in revertant numbers that were statistically significant when the data were analysed at the 1% level using Dunnett's test. This study was therefore considered to have provided no evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 3.8.1.3- 1 and Table 3.8.1.3- 2.

Table 3.8.1.3- 1: Summary of the incidence of revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	27p	89p	20p	9p	21p
	1000	39p	97p	25p	19p	25p
	200	35	92	30	16	21
	40	31	116	34	20	24
	8	38	101	24	15	22
	1.6	37	108	28	16	25
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	1 – 34	1 – 23
Solvent control	-	34	112	26	18	23
Positive control						
Sodium azide	2	NA	654	613	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	616
2-nitrofluorene	5	1032	NA	NA	NA	NA
Benzo[a]pyrene	10	260	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	159	NA
With metabolic activation (+S9)						
Fluopicolide	5000	48p	108p	23p	13p	23p
	1000	44p	135p	28p	12p	29p
	200	37	134	27	18	24
	40	37	131	26	13	24
	8	47	135	21	18	24
	1.6	39	136	19	13	19
Historical solvent control mean	-	38.2 ± 9.6	104.8 ±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	39	128	25	21	24
Positive control 2-aminoanthracene	5 - 10 #	NA	2652	278	398	293

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 3.8.1.3- 2: Summary of the revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	36p	151p	15p	10p	14p
	500	44	143	22	10	18
	250	41	140	25	13	19
	125	37	144	21	13	15
	62.5	33	151	22	14	16
	31.25	30	146	21	17	16
Solvent control	-	31	138	25	16	17
Positive control						
Sodium azide	2	NA	836	548	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	522
2-nitrofluorene	5	1445	NA	NA	NA	NA
Benzo[a]pyrene	10	301	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	188	NA
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	32p	121p	25p	20p	36p
	500	25	111	24	15	29
	250	35	123	22	13	27
	125	35	124	23	15	20
	62.5	39	112	23	16	19
	31.25	30	112	33	12	23
Solvent control	-	32	116	23	14	25
Positive control 2-aminoanthracene	5 - 10 #	NA	1764	200	394	41

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

3.8.1.4 Anonymous; 2001; M-202939-01-1

Study reference:

Anonymous; 2001; AE C638206 00 1C96 0001 (OP2050046): Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one trytophan-requiring strain of Escherichia coli; M-202939-01-1

Deviations:	Deviations from the current OECD guideline (471, 1997): None.

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Some reductions in revertant numbers at the highest test dose in some tester strains may have been the result of test article toxicity.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, clear evidence of toxicity was only observed at the maximum test dose in strain TA 98 in the absence of S9. Precipitation of test agent was observed on all plates treated at concentrations of 500 µg/plate and above.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1C96 0001 (OP 2050046) (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide: WP2uvrA With S9 mix: Benzo[a]pyrene : TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[3] and De Serres and Shelby[4]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050046, is a fine beige powder, which was received on 7 August 2000. It was stored at room temperature in the dark. Purity was stated as 95.9%. The expiry date of the test substance was stated to be 12 October 2000 according to the Sponsor's certificates of analysis. Determinations of the stability and characteristics of the test substance were the responsibility of the Sponsor.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approximately 5½ hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: July 25 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9.

Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46 ± l °C:

• 0.1 mL bacterial culture
• 0.1 mL test article solution or control
• 0.5 mL 10 % S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37 ± 1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

Initial experiment I treatments were performed using a supply of test substance that due to inconsistencies with labelling and associated paper work, had some doubt over whether the sample was the correct and intended test substance. These data were therefore invalidated and are not presented in this report. A further supply of test substance was therefore obtained and the experiment I treatments repeated in order to provide the valid data that are presented in this report.

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test substance or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37 ± 1 °C, with shaking, before the addition of 2.5 mL molten agar at 46 ± 1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This, and some other organic solvents, are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of excessive precipitate affected the accuracy of the automated counter. The background lawn of each plate was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[4]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above)
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. However, some small reductions in revertant numbers were observed at the maximum test dose treatments with several test strains, and these may have been due to test substance toxicity. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose-limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step.

Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains with the exception of strain TA 98 treatments in the absence of S9. Here a slight thinning of the background bacterial lawn was observed at the maximum test dose only. Precipitation of test substance was again observed, on this occasion on all plates treated at 500 µg/plate and above.

The individual plate counts were averaged to give mean values. From the following tables it can be seen that mean solvent control counts were comparable with the normal historical ranges.

The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The mutation data were evaluated as follows. Following fluopicolide treatments of all the test strains, both in the absence and in the presence of a rat liver metabolic activation system (S9), only experiment II treatments of strain TA 100 in the absence of S9 (see Table 3.8.1.4- 2) provided a statistically significant increase in revertant numbers when the data were analysed at the 1% level using Dunnett's test. This increase was very small in magnitude, failed to show a clear dose-relationship, occurring at an intermediate test dose, and also failed to demonstrate any reproducibility within this study, as comparable experiment I treatments failed to provide any similar increases in revertant numbers. The observed increase was therefore attributed to chance, and this study was considered to have provided no clear evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 3.8.1.4- 1 and Table 3.8.1.4- 2.

Table 3.8.1.4- 1: Revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	28p	94p	16p	14p	18p
	1000	34p	100p	24p	14p	21p
	200	33	117	30	11	24
	40	32	100	33	16	19
	8	31	107	22	9	20
	1.6	33	108	28	17	16
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	1 – 34	1 – 23
Solvent control	-	33	114	25	24	24
Positive control						
Sodium azide	2	NA	660	615	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	641
2-nitrofluorene	5	1041	NA	NA	NA	NA
Benzo[a]pyrene	10	261	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	152	NA
With metabolic activation (+S9)						
Fluopicolide	5000	43p	115p	17p	22p	19p
	1000	33p	116p	21p	17p	29p
	200	29	114	22	12	27
	40	31	130	22	14	28
	8	32	123	25	10	27
	1.6	38	129	28	21	26
Historical solvent control mean	-	38.2 ± 9.6	104.8±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	42	128	25	23	24
Positive control 2-aminoanthracene	5 - 10 #	NA	2532	280	396	305

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 3.8.1.4- 2: Revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	33p	151p	22p	10p	15p
	500	26p	149p	26p	16p	19p
	250	28	156**	23	12	17
	125	35	131	30	15	19
	62.5	34	134	30	18	19
	31.25	30	127	27	9	17
Solvent control	-	31	128	28	13	15
Positive control						
Sodium azide	2	NA	826	790	NA	NA
4-nitroquinoline-N- oxide	2	NA	NA	NA	NA	997
2-nitrofluorene	5	1520	NA	NA	NA	NA
Benzo[a]pyrene	10	325	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	283	NA
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	41p	142p	25p	15p	24p
	500	41p	132p	22p	17p	28p
	250	34	143	17	13	18
	125	37	129	22	12	20
	62.5	43	127	24	14	21
	31.25	37	139	18	17	20
Solvent control	-	35	128	21	16	21
Positive control 2-aminoanthracene	5 - 10 #	NA	2435	254	459	51

**: $p \leq 0.01$, statistically significant compared to control (Dunnett´s test)
NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

3.8.1.5 Anonymous; 2001; M-202935-01-1

Study reference:

Anonymous; 2001; Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206 00 1B99 0002; M-202935-01-1

Deviations:	Deviations from the current OECD guideline (471, 1997): None.

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, clear evidence of toxicity was only observed at the maximum test dose in strain TA 98 in the absence of S9. Precipitation of test substance was observed on all plates treated at concentrations of 500 µg/plate and above.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges, and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1B99 0002 (R001737) (fluopicolide)
Purity:	99.3%
Batch no.:	R001737

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide : WP2uvrA With S9 mix: Benzo[a]pyrene : TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[3] and De Serres and Shelby[4]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number R001737, was a beige powder, which was received on 10 July 2000. It was stored at room temperature in the dark. Purity was stated as 99.3%. The expiry date of the test substance was stated to be 8 December 2000 according to the Sponsor's certificate of analysis.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), with warming at 37 °C and vortexing in experiment I, immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilised (Gelman Acrodisc CR filter, 0.2 µm pore size) and further dilutions were made using DMSO. The test article solutions were protected from light and used within approx. 3½ hours of the initial formulation of the test substance.

Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: July 25 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9.

Platings were achieved by the following sequence of additions to 2.5 mL molten agar at46±l °C:

• 0.1 mL bacterial culture
• 0.1 mL test substance solution or control
• 0.5 mL 10% S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37±1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46±1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay. Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This, and some other organic solvents, are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of excessive precipitate affected the accuracy of the automated counter. The background lawn of each plate was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[4]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ng/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step.

Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains, with the exception of strain TA 98 treatments in the absence of S9. Here a slight thinning of the background bacterial lawn was observed at the maximum test dose only. Precipitation of test substance was again observed, on this occasion on all plates treated at 500 µg/plate and above.

The individual plate counts were averaged to give mean values. From the following tables it can be seen that mean solvent control counts fell within the normal historical ranges. The positive control chemicals all induced large increases in revertant numbers in the appropriate strains. Less than 5% of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The evaluation of the mutation data revealed that no fluopicolide treatments of any of the test strains, either in the absence or in the presence of S9, resulted in any increases in revertant numbers that were statistically significant when the data were analysed at the 1% level using Dunnett's test.

This study was therefore considered to have provided no evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 3.8.1.5- 1 and Table 3.8.1.5- 2.

Table 3.8.1.5- 1: Revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	42p	91p	20p	14p	13p
	1000	50p	103p	34p	17p	25p
	200	48	95	22	14	18
	40	48	97	26	19	21
	8	49	108	26	21	17
	1.6	50	105	22	16	20
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	1 – 34	1 - 23
Solvent control	-	44	109	23	14	20
Positive control						
Sodium azide	2	NA	622	574	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	645
2-nitrofluorene	5	1051	NA	NA	NA	NA
Benzo[a]pyrene	10	296	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	227	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9)						
Fluopicolide	5000	37p	108p	21p	19p	17p
	1000	50p	118p	22p	18p	19p
	200	49	111	25	19	19
	40	50	104	18	17	18
	8	56	110	20	21	22
	1.6	60	114	22	20	20
Historical solvent control mean	-	38.2 ± 9.6	104.8±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 - 32
Solvent control	-	50	99	20	18	23
Positive control 2-aminoanthracene	5 - 10 #	NA	1764	184	266	241

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 3.8.1.5- 2: Revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	26p	130p	25p	14p	10p
	500	24p	131p	26p	8p	14p
	250	26	129	21	18	16
	125	28	133	24	13	12
	62.5	29	149	25	12	15
	31.25	30	129	26	9	12
Solvent control	-	31	128	29	15	16
Positive control						
Sodium azide	2	NA	654	700	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	854
2-nitrofluorene	5	1362	NA	NA	NA	NA
Benzo[a]pyrene	10	330	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	240	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	32p	133p	18p	15p	17p
	500	32p	123p	21p	15p	22p
	250	42	141	22	20	21
	125	33	127	25	17	20
	62.5	39	133	21	15	17
	31.25	36	131	24	12	12
Solvent control	-	33	129	21	14	22
Positive control 2-aminoanthracene	5 - 10 #	NA	2074	246	444	49

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

3.8.1.6 Anonymous; 2017; M-595228-01-1

Study reference:

Anonymous; 2017; Fluopicolide, technical: Salmonella typhimurium reverse mutation assay; M-595228-01-1

Deviations: Deviations from the current OECD guideline (471, 1997):
None.

Executive Summary:

In this study the potential of technical fluopicolide, to induce gene mutations according to the plate incorporation test (experiment I and Ia) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 was investigated.

The assay was performed in three independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test substance was tested at the following concentrations:

Experiment I & Ia: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Precipitation of the test substance in the overlay agar in the test tubes as well as on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording. The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with fluopicolide, at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, fluopicolide is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (BCS-AM59797) (fluopicolide)
Purity:	98.2%
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	DMSO (purity >99%, concentration not given)
Positive control:	<u>Without S9 mix:</u> Na-azide: TA 100, TA 1535 4-nitro-o-phenylene-diamine (4-NOPD): TA 1537, TA 98 Methyl methane sulfonate (MMS) : TA 102 <u>With S9 mix:</u> 2-Aminoanthracene: TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. Furthermore for each S9 batch a sterility test and the determination of the protein concentration were performed.

The protein concentration of the S9 preparation was 32.3 mg/mL (Lot. No.: 090217B) in all experiments.

S9 mix:

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP
in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

The S9 cofactor solution was prepared freshly and sterile-filtrated before the S9 supernatant was added.

During the experiment, the S9 mix is stored in an ice bath. The S9 mix preparation is performed according to Ames *et al* .(1977)[5].

4. Test organisms:

The histidine dependent strains are derived from *S. typhimurium* strain LT2 through mutations in the histidine locus. Additionally due to the "deep rough" (rfa-) mutation they possess a faulty lipopolysaccharide envelope, which enables substances to penetrate the cell wall more easily. A further mutation (deletion of the uvrB gene) causes an inactivation of the excision repair system. The latter alteration also includes a deletion in the nitrate reductase and biotin genes. In the strains TA 98, TA 100, and TA 102 the R-factor plasmid pKM 101 carries umu DC analogous genes that are involved in error-prone repair and the ampicillin resistance marker. The strain TA 102 does not contain the uvrB-- mutation. Additionally, TA 102 contains the multicopy plasmid pAQ1, which carries the hisG428 mutation and a tetracycline resistance gene. TA 102 contains the ochre mutation in the hisG gene.

The mutations of the bacterial strains used in this study are described in Table 3.8.1.6- 1.

Table 3.8.1.6- 1: Salmonella typhimurium strains

Salmonella typhimurium		
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa^-; $uvrB^-$	frame shift mutations
TA 98	his D 3052; rfa^-; $uvrB^-$; R-factor	" "
TA 1535	his G 46; rfa^-; $uvrB^-$	base-pair substitutions
TA 100	his G 46; rfa^-; $uvrB^-$; R-factor	" "
TA 102	his G 428; rfa^-; $uvrB^+$; R-factor	" "

Regular checking of the properties of the *Salmonella typhimurium* strains regarding the membrane permeability, ampicillin resistance; UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates is performed at Envigo CRS GmbH according to Ames *et al.* (1977)[5] and Maron and Ames (1983)[3]. In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

5 Ames, B.N., J. McCann, and E. Yamasaki (1977) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test In: B.J. Kilbey et al. (Eds.) Handbook of Mutagenicity Test Procedures Elsevier, Amsterdam, 1-17

5. Test substance concentrations used:

In the pre-experiment and the repeated experiment Ia the concentration range of the test item was 3 – 5000 µg/plate. The pre-experiment is reported as experiment I. Since no toxic effects, but precipitation of the test substance was observed in experiment I, seven concentrations were tested in experiment II. 5000 µg/plate were chosen as maximal concentration.

The concentration range included two logarithmic decades. The following concentrations were tested:

Experiment I & Ia: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate.

B. Test performance

Experimental phase: April 25 to May 23, 2017

1. Pre-experiment for toxicity

To evaluate the toxicity of the test substance a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each three plates. The experimental conditions in this pre-experiment were the same as described for the experiment I below (plate incorporation test).

The pre-experiment is reported as main experiment I, since the acceptance criteria are met.

Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated. The experimental conditions in the repeated experiment Ia were the same as in the pre-experiment.

2. Mutagenicity test

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Experiment I (Plate Incorporation):

- 100 µL test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL overlay agar.

Experiment II (pre-incubation):

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark (De Serres and Shelby[4]).

In parallel to each test a sterile control of the test substance was performed and documented in the raw data. Therefore, 100 µL of the stock solution, 500 µl S9 mix / S9 mix substitution buffer were mixed with 2.0 mL overlay agar and poured on minimal agar plates.

Data recording:

The colonies were counted using a validated computer system (cf. 3.8, major computerized systems), which was connected to a PC with printer to print out the individual values, the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results).

Due to precipitation of the test substance the colonies were partly counted manually.

3. Statistics

According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

4. Acceptance / assessment criteria:

The *Salmonella typhimurium* reverse mutation assay is considered acceptable if it meets the following criteria:

- Regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control are in the range of our historical data.
- The positive control substances should produce an increase above the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control.
- A minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

5. Evaluation of results

A test substance is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed.

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and Discussion

A. Mutation assays

The test substance fluopicolide was assessed for its potential to induce gene mutations according to the plate incorporation test (experiment I and Ia) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in three independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

The test substance was tested at the following concentrations:

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Precipitation of the test substance in the overlay agar in the test tubes as well as on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording. The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with fluopicolide at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

An overview of the results is given in Table 3.8.1.6- 2 to Table 3.8.1.6- 3.

Table 3.8.1.6- 2: Summary of experiment I

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD)				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
Without metabolic activation (-S9)						
Fluopicolide	3	14±3	6±3	23±4	168±10	526±20
	10	12±5	9±5	24±6	163±12	553±20
	33	13±3	10±3	26±9	166±16	496±6
	100	10±1	8±1	24±3	164±12	499±10
	333	12±2 P	9±3 P	30±2 P	163±10 P	503±11 P
	1000	12±2 P	9±2 P	23±3 P	177±9 P	516±14 P
	2500	9±1 PM	5±2 PM	21±2 PM	162±8 PM	421±29 PM
	5000	8±3 PM	6±1 PM	18±4 PM	163±18 PM	471±48 PM
DMSO	-	11±5	8±2	24±10	166±11	525±10
Untreated	-	11±3	6±1	24±4	198±14	536±9
Positive control						
NaN_3	10	1290±93	NA	NA	2240±115	NA
4-NOPD	10	NA	NA	248±7	NA	NA
	50	NA	76±9	NA	NA	NA
MMS	2.0 µL	NA	NA	NA	NA	4330±251
Historical solvent control mean	-	12±2.5	10±2.2	25±4.4	156±26.0	463±51.0
Historical control range	-	6-25	6-19	13-43	78-209	320-534
With metabolic activation (+S9)						
Fluopicolide	3	15±6	9±3	33±8	128±1	691±6
	10	9±1	9±7	29±5	146±24	693±25
	33	14±5	11±4	31±4	158±25	682±63
	100	12±4	12±6	30±8	152±4	630±40
	333	12±6 P	15±3 P	34±4	141±10 P	679±27 P
	1000	12±5 P	12±3 P	31±6 P	151±1 P	670±18 P
	2500	10±3 P	12±3 P	40±2 P	139±9 P	613±11 P
	5000	13±3 P	8±4 PM	31±5 PM	144±9 PM	581±45 PM
DMSO	-	11±6	10±3	33±4	136±5	632±3
Untreated	-	12±3	13±6	36±4	158±19	667±7
Positive control						
2-AA	2.5	443±23	138±29	4507±509	4072±348	NA
	10.0	NA	NA	NA	NA	1135±38
Historical solvent control mean	-	12±2.5	13±3.5	34±6.2	148±32.3	571±71.1
Historical control range	-	7-26	7-30	15-58	73-208	325-652

NaN_3 = sodium azide; 2-AA = 2-aminoanthracene; MMS = methyl methane sulfonate; 4-NOPD = 4-nitro-o-phenylene-diamine

NA: not applicable

P: precipitate

M: manual count

Historical control data based on approx. 600 experiments (in case of TA 102 the historical data are based on approx. 150 experiments) from November 2014 until November 2016.

Table 3.8.1.6- 3: Summary of experiment Ia (with metabolic activation; +S9)

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD) TA 102
Fluopicolide	3	591±42
	10	536±30
	33	548±31
	100	578±37
	333	570±24[P]
	1000	581±5[P]
	2500	546±4[P]
	5000	576±35[PM]
DMSO	-	603±12
Untreated	-	627±23
Positive control 2-AA	10.0 µg	1409±92
Historical solvent control mean	-	571±71.1
Historical control range	-	325-652

2-AA = 2-aminoanthracene
P: precipitate
M: manual count
Historical control data based on approx. 150 experiments from November 2014 until November 2016.

Table 3.8.1.6- 4: Summary of experiment II (pre-incubation test)

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (mean±SD) TA 1535	TA 1537	TA 98	TA 100	TA 102
Without metabolic activation (-S9)						
Fluopicolide	10	10±1	11±3	25±6	135±8	470±43
	33	11±2	10±4	27±3	135±11	422±21
	100	13±3	10±1	21±1	133±6	476±29
	333	11±2[P]	10±1[P]	26±6[P]	138±20[P]	448±3[P]
	1000	11±3[PM]	11±4[P]	22±2[PM]	147±10[P]	445±17[P]
	2500	13±3[PM]	8±2[PM]	29±3[PM]	139±10[PM]	435±6[PM]
	5000	14±3[PM]	9±1[PM]	22±5[PM]	148±24[PM]	410±17[PM]
DMSO	-	13±1	11±4	25±4	151±18	458±47
Untreated	-	9±4	11±1	24±5	209±5	458±23
Positive control						
NaN3	10	1261±35	NA	NA	2031±132	NA
4-NOPD	10	NA	NA	328±15	NA	NA
	50	NA	107±11	NA	NA	NA
MMS	2.0 µL	NA	NA	NA	NA	2887±129

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (mean±SD)				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
With metabolic activation (+S9)						
Fluopicolide	10	14±2	15±1	43±10	112±10	690±64
	33	12±4	14±1	39±9	112±3	628±27
	100	14±3	12±4	40±3	122±27	620±85
	333	15±1 P	15±3 P	44±7 P	115±18 P	680±8 P
	1000	13±2 PM	12±2 PM	46±4 P	122±8 P	691±15 P
	2500	12±2 PM	14±4 PM	29±5 PM	111±14 PM	703±14 PM
	5000	12±4 PM	8±2 PM	25±4 PM	118±11 PM	706±9 PM
DMSO	-	13±3	14±4	45±2	112±3	646±7
Untreated	-	17±3	23±5	40±6	110±10	659±30
Positive control 2-AA	2.5	348±33	176±18	4733±364	3083±322	NA
	10.0	NA	NA	NA	NA	1467±30

NaN3 = sodium azide; 2-AA = 2-aminoanthracene; MMS = methyl methane sulfonate; 4-NOPD = 4-nitro-o-phenylene-diamine
NA: not applicable
P: precipitate
M: manual count

III. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, fluopicolide is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

3.8.1.7 Anonymous; 2004; M-197260-02-1

Study reference:

Anonymous.; Graeser, H.; 2004; In vitro Chinese hamster lung V79 cells chromosome aberration assay Code: AE C638206 00 1C99 0005; M-197260-02-1

Deviations: Deviations from the current OECD guideline (473, 2016):

- Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC)
- Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed

The deviation(s) are considered not to compromise the results and outcome of the study.

Executive Summary:

This study assessed the potential of technical fluopicolide (mixture of Batch No. PP/241024/2 & PP241067/1) to induce chromosome aberrations in Chinese hamster lung V79 cells *in vitro*. For each experiment two cell cultures were used.

Following preliminary toxicity testing, two independent chromosome aberration tests were conducted in the absence and one in the presence of an exogenous metabolic activation system (S9-mix) using a range of concentrations of the test compound. Positive and vehicle (DMSO) cultures were included in each assay. Duplicate cell cultures per dose level were used. The cells sampled 20 hours after the start of treatment and examined for chromosome aberrations.

The test compound was dissolved in DMSO and tested at the following concentrations:

First experiment with 3 h treatment time:

without S9-mix: 3.2, 6.3,12.5, 25.0*. 50.0*. 75.0* and 100.0* µg/mL
with S9-mix: 3.2, 6.3, 12.5, 25.0*, 50.0*, 75.0* and 100.0* µg/mL

Second experiment with 20 h treatment time:

without S9-mix: 0.1, 0.2, 0.4, 0.8,1.6*, 3.2* and 6.3* µg/mL
* = slides evaluated

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The test substance produced a distinct lowering of the mitotic index in the first main experiment at a concentration of 100 µg/mL in the absence of metabolic activation and at the dose levels of 75 and 100 µg/mL in the presence of metabolic activation. In the second main experiment cytotoxicity as a reduction of the mitotic index was observed at dose levels of 3.2 µg/mL and above.

In the first main experiment the test substance induced a statistically significant increase in the number of chromosome aberrations at a concentration of 100 µg/mL in the absence and in the presence of metabolic activation (mitotic indices 38.5% and 48.7% respectively). In the second main experiment without S9-mix a statistically significant increase of the aberration rate was observed at a dose level of 3.2 µg/mL (corresponding to a mitotic index of 63.8%) and at a concentration of 6.3 µg/mL (mitotic index of 22.4%).

Because of the clear positive results at one concentration with a mitotic index higher than 50% of the solvent control value and considering the low cytotoxicity in the microwell plates, fluopicolide was judged to be clastogenic. Appropriate reference mutagens used as positive controls showed a significant increase in chromosome aberrations, thus indicating the sensitivity of the assay, and the efficacy of the S9-mix.

Fluopicolide was clastogenic in this *in vitro* chromosome aberration assay with V79 Chinese hamster lung cells in both the presence and absence of metabolic activation.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide), technical
Purity:	97.8% (w/w)
Batch no.:	mixture of PP/241024/2 & PP241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Non-activation: Ethyl methane sulfonate, dissolved in cell culture medium on the day of treatment: final concentration: 1.5 mg/mL (3 h treatment) final concentration: 0.4 mg/mL (20 h treatment) Activation: Cyclophosphamide, dissolved in cell culture medium on the day of treatment, final concentration in cell culture medium: 2.5 µg/mL

3. Activation:

The S9 fraction was prepared by the testing facility according to Ames *et al.* (1975)[2]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann, Gartenstrasse 27, 33178 Borchen, Germany, received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) 5 days before killing. The livers were removed from at least 5-6 animals and, using cold sterile solutions at approx. 0 to 4 °C and glassware, were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 g for 10 minutes. The supernatant, the S9 fraction, was divided into small portions, rapidly frozen and stored at approx. –80 °C for not longer than six months. The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

Preparation of S9-mix:

Sufficient S9 fraction was thawed to room temperature immediately before each test. An appropriate quantity of S9 fraction (batch no. 99/6, protein concentration 27.5 g/L) was mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/mL in the cultures which was kept on ice until used. This preparation is termed S9-mix. The concentrations of the different cofactors of the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
100 mM phosphate buffer pH 7.4

4. Cell cultures:

Large stocks of the mycoplasma-free V79 cell line are stored in liquid nitrogen in the cell bank of "Genetic Toxicology", thus permitting repeated use of the same cell culture batch for numerous experiments. The identical characteristics of the cells ensure comparability of the experimental parameters.

5. Culture Medium:

Thawed stock cultures were kept at approx. 37 °C and approx. 4% CO_2 in 175 cm^2 plastic flasks. About 5 x 10^5 to 1 x 10^6 cells were seeded into each flask in 30 mL of MEM-medium supplement with approx. 10% (v/v) FCS (foetal calf serum) containing approx. 2 mM L-glutamine and approx. 0.1% (w/v) neomycinsulfate. The cells were subcultured twice a week.

6. Test substance concentrations used:

Table 3.8.1.7- 1: Test substance concentrations

Group	1st experiment			2nd experiment		
	S9 mix	Concentration (µg/mL)	Treatment time (h)	S9 mix	Concentration (µg/mL)	Treatment time (h)
Solvent control	- / +	0	3	-	0	20
Fluopicolide	- / +	3.2	3	-	0.1	20
	- / +	6.3	3	-	0.2	20
	- / +	12.5	3	-	0.4	20
	- / +	25.0*	3	-	0.8	20
	- / +	50.0*	3	-	1.6*	20
	- / +	75.0*	3	-	3.2*	20
	- / +	100	3	-	6.3*	20
Ethyl methane	-	1500	3	-	400	20
Cyclophospha-mide	+	2.5	3	/	/	/

* = slides evaluated

B. Test performance

Experimental phase: April 17 to May 19, 2000

1. Preliminary assay

The concentrations for the mutagenicity assay are based on the results of the toxicity experiment.

For non-toxic, freely soluble test substances, the top dose is either 10 mM or 5000 µg/mL according to international testing guidelines. For relatively insoluble test substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used is a concentration above the limit of solubility in the final culture medium after the end of the treatment period. For toxic compounds, the highest dose level is selected to reduce survival to approx. 20-50%, and/or the mitotic index to approx. 50% of the corresponding solvent control.

A preliminary toxicity test was undertaken in order to select appropriate dose levels for the cytogenetic assay. Cell cultures were subjected to the same treatment conditions as in the main experiment. Cytotoxic effects were determined by photometric measurement of V79 cell cultures grown in microwell plates (approx. 3125 cells/cm^3) and stained with crystal violet. The relative cell density in the microwell plates was similar to that in the Quadriperm® dishes.

The test included the following treatments:

Solvent control: the maximum final concentration of organic solvents was approx. 1% (v/v).

Test substance: the highest dose level for the preliminary toxicity test was determined by the solubility of the test compound and equated to the international maximum of 10 mM.

Treatments were performed both in the presence and absence of the S9-mix metabolic activation system using a single cell culture at each test point.

2. Cytogenetic Assay

Two independent experiments were conducted in the presence of S9 mix and one test in its absence using duplicate cultures of cells seeded onto slides (i.e. 2 per dose level) and at least three dose levels.

In the first experiment, cells were treated for 3 hours in both the presence and absence of S9-mix whereas in the second assay, they were treated for 20 hours in the absence of S9-mix alone. In both experiments the cells were sampled 20 hours after the start of treatment as were the concurrent solvent and positive control cultures. Colcemide was added to each culture 2 hours before sampling in order to arrest cell division.

Chromosome preparations were made, fixed, stained and examined. However, where clearly positive results were obtained in the first experiment, those from the second assay were not examined. Where equivocal or negative results were obtained in the first experiment, modifications to the testing procedure were included in order to clarify the result.

Before treatment, the pH values and osmolality of the treatment medium were determined. If necessary the pH was adjusted to pH 7.3 with NaOH or HCl. Any effects on the osmolality during the study were described in the study report.

Exponentially growing cultures which were more than 50% confluent were trypsinated by an approx. 0.25% (v/v) trypsin solution ready for use (supplied by Gibco) and a single cell suspension (culture) was prepared. The trypsin concentration was approx. 0.25% (v/v) in Ca-Mg-free salt solution. Two slides were placed in Quadriperm® dishes which were then seeded with cells to yield 3-4 x 10^4 cells/slide. Thus for each dose level and treatment time, duplicate cultures slides are used. The Quadriperm® dishes contain 6 mL MEM with approx. 10% (v/v) FCS.

After 48 h, the medium was replaced with one containing approx. 10% (v/v) FCS and the test substance, or positive control, or solvent and in the presence of metabolic activation additionally 2% (v/v) S9-mix. For the 3 hour treatment time, the medium was replaced by normal medium following two rinses. In the second experiment the cells were exposed to the treatment medium without S9-mix for 20 h.

18 h after the start of the treatment, Colcemide was added (approx. 0.05 µg/mL/culture medium) to the cultures to arrest mitosis and 2 h later (20 h after the start of treatment) metaphase spreads were prepared as follows:

The cultures were made hypotonic by adding about 5 mL of approx. 0.075 M potassium chloride solution at around 37 °C. The cells were then incubated for 20 minutes at approx. 37 °C. The next step was the addition of 2 mL fixative.

Then the liquid was replaced by 6 mL fixative (methanol: glacial acetic acid, 3:1). After 10 minutes the procedure was repeated. After at least another 10 minutes, the slides were taken out and air-dried for 24 hours. The chromosomes were stained as follows:

- staining for 10 minutes in approx. 2% (w/v) orcein solution
- rinsing 3 times in distilled water
- rinsing twice in acetone
- brief rinsing in acetone/xylene
- 2 minutes in acetone/xylene
- 5 minutes in xylene
- 10 minutes in xylene
- embedding in Entellan® or Corbit®

3. Evaluation of data:

Analysis of metaphases:

The slides were coded and 25-100 metaphases per experimental group and cell culture were examined. The set of chromosomes was examined for completeness and the various chromosomal aberrations were assessed and classified. Only metaphases with 22 +/- 2 chromosomes are included in the analysis. The metaphases were examined for the following aberrations: chromatid gap, chromosome gap, chromatid break, chromosome break, minute, double minute, chromatid deletion, chromosome deletion, chromatid exchanges including intra-changes, chromosome exchanges including intra-changes, dicentrics, pulverization and ring formation. Furthermore the incidence of polyploid metaphases was determined in 1000 cells of each cell culture. Additionally the mitotic index was determined by counting the number of cells undergoing mitosis in a total of 1000 cells. The mitotic index is expressed as a percentage.

After the metaphases had been evaluated, the code was broken. For each experiment the results from the dose groups were compared with those of the control group and the positive control at each sampling time.

4. Criteria for a valid assay:

The assay was considered valid if the following criteria are met:

- The solvent control data were within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induced increases in the mutation frequency which were both statistically significant and within the laboratory's normal range.

5. Criteria for a positive response:

The evaluation of the results was performed as follows:

- The test substance is classified as clastogenic if it induces a statistically significant increase in the aberration rate (without gaps) with one or more of the concentrations tested as compared with the solvent controls.
- The test substance is classified as clastogenic if there is a reproducible concentration-related increase in the aberration rate (without gaps).
- The test substance is classified as not clastogenic if the tests are negative both with and without metabolic activation.

6. Statistics

The Biometry of the results was performed with a one-sided Fisher-Exact test.

II. Results and Discussion

A. Preliminary cytotoxicity assay

Fluopicolide was suspended in DMSO at a stock concentration of 382 mg/mL. Evaluation of the solubility of that suspension in cell culture medium showed that 3820 µg/mL was the highest practicable concentration and produced precipitate. This concentration corresponds to 10 mM, which is the international top dose level for these studies.

Accordingly, the preliminary toxicity study was carried out using a maximum concentration of 3820 µg/mL and a range of lower dose levels down to 10 µg/mL.

Following treatment in the absence of S9 metabolic activation survival declined in a dose-dependent manner reaching 44.5% of the solvent control value (after a treatment time of 3 h) and 20.4% of the solvent control value (after a treatment time of 20 h) at the highest dose level, 3820 µg/mL.

In the presence of S9 metabolic activation survival was dose-dependently reduced after a treatment time of 3 h to 55.4% of the solvent control value at the highest dose level, 3820 µg/mL.

In a dose range of 120 to 3820 µg/mL at a treatment time of 3 hours and in a dose range of 12.1 to 3820 µg/mL at the treatment time of 20 hours the cells survived, but so extremely damaged, that no scorable metaphases were found.

Based in these results a concentration of 100 µg/mL (stock solution in DMSO of 10 mg/mL) were chosen as maximum dose level for a treatment time of 3 hours in the absence and in the presence of S9-mix in the first main experiment. For the fixation interval of 20 hours 6.3 µg/mL were determined as the top dose level in the second main experiment.

Before treatment, the pH values and osmolality of the treatment media were determined. The addition of test substance solutions did not have any effect on these parameters.

B. Cytogenetic assay

Mitotic index:

In the main experiments cytotoxicity was also evaluated by treatment of cells seeded in microwell plates. At the 3 hours treatment time survival was reduced in a dose-dependent manner reaching 68.4% of the solvent control value without S9-mix and 73.1% of the solvent control with S9-mix at a dose level of 100 µg/mL. At 20 hours treatment time in the absence of S9-mix 58.9% of the solvent control survived after treatment with at the highest concentration tested, 6.3 µg/mL.

The test substance produced a distinct lowering of the mitotic index in the first main experiment at a concentration of 100 µg/mL in the absence of metabolic activation and at the dose levels of 75 and 100 µg/mL in the presence of metabolic activation. In the second main experiment cytotoxicity as a reduction of the mitotic index was observed at dose levels of 3.2 µg/ml and above.

Treatment with the test substance did not cause any relevant increase in the number of polyploid cells as compared with the solvent controls.

Chromosome aberrations:

The test substance fluopicolide was assessed for its potential to induce chromosome aberrations (clastogenicity) in two independent *in vitro* experiments without metabolic activation and one experiment with metabolic activation. There was a statistically significant increase in the absolute aberration numbers 3 h after the start of the treatment with 100 µg/mL in the absence and in the presence of S9-mix (data not shown). Because of a distinct lowering of the mitotic index at these dose levels (38.3% without S9-mix and 48.7% with S9-mix), the slides of the second main experiment were also evaluated. At the sampling time of 20 h without S9-mix at the concentrations 3.2 µg/mL (corresponding to a mitotic index of 63.8%) and at a concentration of 6.3 µg/mL (mitotic index of 22.4%) the absolute aberration numbers were statistically significantly increased.

Because of the clear positive results at one concentration (3.2 µg/mL) with a mitotic index higher than 50% of the solvent control value and considering the low cytotoxicity in the microwell plates, fluopicolide was judged to be clastogenic.

The sensitivity of the test system was also demonstrated by the enhanced mutation frequency in the cell cultures treated with the positive control compounds.

Table 3.8.1.7- 2 and Table 3.8.1.7- 3 give an overview of the study results.

Table 3.8.1.7- 2: Relative mitotic indices and mean percentage of aberrant V79 cells, including and excluding gaps – Experiment I

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps	Percent aberrant cells exchanges
Without metabolic activation (-S9)						
Fluopicolide	100	3	38.3	10.5	8.0	0.5
	75	3	76.6	2.5	0.5	0.0
	50	3	81.3	1.5	0.0	0.0
	25	3	110.3	1.0	0.5	0.0
Solvent control	-	3	100.0	1.5	1.0	0.0
Positive control Ethyl methane sulfonate	1500	3	92.5	22.0	20.0	17.0
With metabolic activation (+S9)						
Fluopicolide	100	3	48.7	10.5	7.0	1.0
	75	3	43.6	6.5	3.0	0.5
	50	3	114.1	2.0	0.0	0.0
	25	3	106.4	2.5	2.5	0.5
Solvent control	-	3	100.0	2.5	1.5	0.0
Positive control Cyclophosphamide	2.5	3	123.1	26.0	19.0	8.0

No statistical analyses were performed for the relative / percentage values.

Table 3.8.1.7- 3: Relative mitotic indices and mean percentage of aberrant V79 cells, including and excluding gaps – Experiment II, without metabolic activation (-S9)

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps	Percent aberrant cells exchanges
Fluopicolide	6.3	20	22.4	17.0	12.5	0.0
	3.2	20	63.8	13.5	6.5	2.0
	1.6	20	105.2	0.5	0.0	0.0
Solvent control	-	20	100.0	1.0	0.0	0.0
Positive control Ethyl methane sulfonate	400	20	72.4	11.0	10.0	7.5

III. Conclusion

Fluopicolide was clastogenic in both the presence and absence of metabolic activation in this in vitro chromosome aberration assay with V79 Chinese hamster lung cells.

3.8.1.8 Anonymous; 2001; M-201582-01-1

Study reference:

Anonymous; 2001; AE C638206: In vitro mammalian chromosome aberration test in human lymphocytes; M-201582-01-1

Deviations: Deviations from the current OECD guideline (473, 2016):

- Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC)
- Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed

The deviation(s) are considered not to compromise the results and outcome of the study.

Executive Summary:

This study was performed to assess the ability of fluopicolide to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes, in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of fluopicolide to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test:

- Without S9 mix - 3 hours treatment, 18 hours recovery: 19.53, 78.13 and 156.25 µg/mL.
- With S9 mix - 3 hours treatment, 18 hours recovery: 78.13, 312.5 and 625 µg/mL.

Second test:

- Without S9 mix - 21 hours continuous treatment: 1.22, 9.77 and 19.53 µg/mL.
- With S9 mix - 3 hours treatment, 18 hours recovery: 39.06, 156.25 and 312.5 µg/mL.

In both the absence and presence of S9 mix, fluopicolide caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No increases in the proportion of polyploid cells were seen in either test.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that fluopicolide has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	DMSO (highest final concentration used for subsequent testing was 625 mg/mL)
Positive control:	Non-activation: Mitomycin (0.1 µg/mL) Activation: Cyclophosphamide (6 µg/mL)

3. Activation:

S9 fraction was prepared from a group of ca. 10 animals (male rat, Sprague-Dawley derived, Charles River UK, 7-8 weeks old, <300 g). Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bw. On the fifth day after injection, following an overnight fasting, the rats were killed and their livers aseptically removed.

The following steps were carried out at 0-4 °C under aseptic conditions. The livers were placed in 0.15 M KCl (3 mL KCl: 1 g liver) before being transferred to a homogeniser. Following preparation, the homogenates were centrifuged at 9000 x *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80 °C or below until required.

Preparation of S9-mix:

S9 mix contained: S9 fraction (10 % v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

4. Cell cultures and medium:

Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium (Sigma) supplemented with 10% foetal calf serum (Globepharm), 1 unit/mL Heparin (CP Pharmaceuticals), 20 I.U./ml penicillin/20 µg/mL streptomycin (Imperial) and 2.0 mM glutamine (Imperial). Aliquots (0.4 mL blood: 4.5 mL medium: 0.1 mL phytohaemagglutinin (Gibco)) of the cell suspension were placed in sterile universal containers and incubated at 37 °C for approx. 48 hours. The cultures were gently shaken daily to re-suspend the cells.

5. Test substance concentrations used:

Test concentrations for the first experiment were 0 (solvent control), 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL in tests with and without metabolic activation.

In the second experiment a continuous treatment was used in the absence of S9 mix at test concentrations of 1.22, 2.44, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL and in the presence of S9 mix, a three hour treatment was used, as in the first test at concentrations of 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL. The harvest time was at 21 hours for both parts of the test.

On the basis of the toxicity data, the following concentrations were selected for metaphase analysis:

Experiment I without S9 mix - 3 hours treatment, 18 hours recovery: 19.53, 78.13 and 156.25 µg/mL.
Experiment I with S9 mix - 3 hours treatment, 18 hours recovery: 78.13, 312.5 and 625 µg/mL.

Experiment II without S9 mix - 21 hours continuous treatment: 1.22, 9.77 and 19.53 µg/mL.
Experiment II with S9 mix - 3 hours treatment, 18 hours recovery: 39.06, 156.25 and 312.5 µg/mL.

B. Test performance

Experimental phase: September 19 to November 3, 2000

1. Preliminary Assay

After approx. 48 hours, 50 µL aliquots of fluopicolide were added to one set of duplicate cultures to give final concentrations of 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL. DMSO, the solvent control, in 50 µL aliquots, was added to two cultures. Mitomycin C, at a final concentration of 0.1 mg/mL, was added to duplicate cultures.

Immediately before treatment of the second set of cultures, 1 mL of medium was removed from each culture and discarded. This was replaced with 1 ml of S9 mix, followed by 50 µL aliquots of the various dilutions of fluopicolide, giving the same series of final concentrations as above. DMSO (50 µL) was added to two cultures. Cyclophosphamide was added to duplicate cultures at a final concentration of 6 µg/mL.

Three hours after dosing, the cultures were centrifuged at 500 x *g* for 5 minutes. The cell pellets were rinsed and re-suspended in fresh medium. They were then incubated for a further 18 hours.

Harvesting and fixation:

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid (Sigma) to each culture at a final concentration of 0.1 ng/ml. After 2 hours incubation, each cell suspension was transferred to a conical centrifuge tube and centrifuged for 5 minutes at 500 *g*. The cell pellets were treated with a hypotonic solution (0.075 M KCl pre-warmed at 37 °C). After a 10 minute period of hypotonic incubation at 37 °C, the suspensions were centrifuged at 500 *g* for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced twice.

Slide preparation:

The pellets were resuspended, then centrifuged at 500 *g* for 5 minutes and finally re-suspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10 % Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX.

Microscopic examination:

The prepared slides were examined by light microscopy using a low power objective. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures.

From these results the dose level causing a decrease in mitotic index of approx. 50% of the solvent control value or, if there was no decrease, the maximum achievable concentration was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

The concentration of each positive control compound selected for analysis was the lowest concentration dosed unless a preliminary scan of metaphase figures indicated an insufficient level of aberrant cells. The selected slides were then coded. Metaphase cells were identified using a low power objective and examined at a magnification of x1000 using an oil immersion objective. One hundred metaphase figures were examined, where possible, from each culture. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44-48 chromosomes were analysed.

Polyploid and endoreduplicated cells were noted when seen. The vernier readings of all aberrant metaphase figures were recorded. The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations.

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test.

2. Second test

Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 21 hours for both parts of the test. Concentrations of fluopicolide were as follows:

Without S9 mix: 1.22, 2.44, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 mg/mL.
With S9 mix: 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 mg/mL.

Duplicate cultures were used for each treatment and two cultures were treated with the solvent control.

Positive control cultures were treated as in the first test.

Three hours after dosing, the cultures containing S9 mix were centrifuged. The cell pellets were rinsed and resuspended in fresh medium. They were then incubated for a further 18 hours. Cultures treated in the absence of S9 mix were incubated for 21 hours.

All cultures were treated with Colcemid, at a final concentration of 0.1 µg/mL, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically as previously described.

3. Assessment of results

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

- Statistically significant increases ($p<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.
- The increases exceed the negative control range of this laboratory, taken at the 99 % confidence limit.
- The increases are reproducible between replicate cultures.
- The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.
- Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

4. Statistics

Not mentioned, but under references: 'FISHER, R.A. (1973) The Exact Treatment of 2 x 2 Table in: Statistical Methods for Research Workers. Hafner Publishing Company, New York.' cited.

II. Results and Discussion

1. First test

In the absence of S9 mix, fluopicolide caused a reduction in the mitotic index to 53% of the solvent control value at 156.25 µg/mL. The dose levels selected for the metaphase analysis were 19.53, 78.13 and 156.25 µg/mL.

In the presence of S9 mix, fluopicolide caused a reduction in the mitotic index to 32% of the solvent control value at 625 µg/mL. The dose levels selected for the metaphase analysis were 78.13, 312.5 and 625 µg/mL.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase figures when compared to the solvent control.

The effects of fluopicolide on the chromosomes of cultured human lymphocytes are shown in the tables below.

In both the absence and the presence of S9 mix, fluopicolide caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($p < 0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

2. Second test

In the absence of S9 mix, fluopicolide caused a reduction in the mitotic index to 48% of the solvent control value at 19.53 µg/mL. The dose levels selected for the metaphase analysis were 1.22, 9.77 and 19.53 µg/mL.

In the presence of S9 mix, fluopicolide caused a reduction in the mitotic index to 50% of the solvent control value at 312.5 µg/mL. The dose levels selected for the metaphase analysis were 39.06, 156.25 and 312.5 µg/mL.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase cells when compared to the solvent control.

The effects of fluopicolide on the chromosomes of cultured human lymphocytes are shown in the following tables.

In both the absence and the presence of S9 mix, fluopicolide caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($p < 0.001$) in the proportion of aberrant cells.

Table 3.8.1.8- 1 and Table 3.8.1.8- 2 give an overview of the study results.

Table 3.8.1.8- 1: Relative mitotic indices and mean percentage of aberrant human lymphocytes, including and excluding gaps – Experiment I

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
Fluopicolide	156.25	3	53	3.0	2.5
	78.13	3	71	2.5	1.5
	19.53	3	96	1.5	0.0
Solvent control	-	3	100	1.0	0.5
Positive control Mitomycin C	0.1	3	-	15.0***	8.5***
With metabolic activation (+S9)					
Fluopicolide	625	3	32	8.0	6.5
	312.5	3	63	5.5	5.0
	78.13	3	92	7.0	4.5
Solvent control	-	3	100	5.5	3.0
Positive control Cyclophosphamide	6	3	-	22.0***	18.5***

*** $p < 0.001$ statistically signifcantly different from controls Fisher's test

Table 3.8.1.8- 2: Relative mitotic indices and mean percentage of aberrant human lymphocytes, including and excluding gaps – Experiment II

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent. (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
Fluopicolide	19.53	21	48	2.0	0.5
	9.77	21	69	2.0	1.0
	1.22	21	100	1.5	0.5
Solvent control	-	21	100	1.0	0.0
Positive control Mitomycin C	0.1	21	-	19.5***	17.0***
With metabolic activation (+S9)					
Fluopicolide	312.50	3	50	2.0	1.0
	156.25	3	70	1.5	1.5
	39.06	3	90	1.5	1.0
Solvent control	-	3	700	2.0	1.5
Positive control Cyclophosphamide	6	3	-	18.5***	17.0***

*** $p < 0.001$ statistically signifcantly different from controls Fisher's test

III. Conclusion

It is concluded that fluopicolide has shown no evidence of clastogenic activity in this in vitro cytogenetic test system, under the experimental conditions described.

3.8.1.9 Anonymous; 2005; M-210831-02-1

Study reference:

Anonymous; 2005; In vitro chinese hamster Lung V79 cell HPTR mutation test AE C6308206 Code: AE C6308206 00 1C99 0005; M-210831-02-1

Deviations: Deviations from the current OECD guideline (476, 2016):
None.

Executive Summary:

The study was performed to investigate the potential of fluopicolide (Batch No. PP/241024/2 & PP241067/1) to induce gene mutations at the HPRT locus in V 79 cells of the Chinese hamster *in vitro*.

The assay was performed in three independent experiments, using identical procedures, both with and without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at the following concentrations:

First study: 3820.0*. 1208.0, 382.0, 120.8, 38.2, 12.1, 3.8 and 1.2 µg/mL

Second study: 120.0, 100.0, 75.0, 50.0, 25.0, 12.5, 6.3, 3.2, 1.6, 0.8 and 0.4 µg/mL

Third study: 60.0, 50.0, 40.0, 30.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625 and 0.313 µg/mL

* = 10 mM, the international regulatory limit dose

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The highest concentration was toxic both with and without metabolic activation.

No relevant increase in mutant colony numbers was obtained in three independent experiments. The statistically significant increase observed in the presence of S9-mix was caused by a low mutation frequency of the solvent control values and therefore this increase was considered to be without any relevance. In the second main study a statistically significant increase in the number of mutant colonies was observed at the concentrations of 0.8 and 12.5 µg/mL without metabolic activation. This was considered to be without biological relevance because at the dose of 12.5 µg/mL it was not three-fold higher than the solvent control value, no dose-dependency was obtained and the findings were not reproduced in the third experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay.

Based on these results, fluopicolide was not mutagenic in this HPRT test with V79 Chinese hamster cells.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP/241024/2 & PP241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Non-activation:
	Ethyl methane sulfonate (EMS)
	Activation:
	9,10-dimethyl-1,2-benzanthracene (DMBA)

3. Activation:

The S9 fraction was prepared by the department conducting the study according to Ames *et al.* (1975)[2]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann (33178 Borchen, Germany), received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) five days before killing.

The livers were removed from at least 5-6 animals using cold sterile solutions at approx. 0 to 4 °C and glassware, and were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 x *g* for 10 minutes. The supernatant is the S9 fraction. This was divided into small portions, rapidly frozen and stored at approx. -80 °C for not longer than six months.

The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

The preparation of S9-mix was s follows. Sufficient S9 fraction was thawed immediately at room temperature before each test. An appropriate quantity of S9 fraction (batch no. 99/6 for all experiments, protein concentration 55.0 g/L) was mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/mL in the cultures which was kept on ice until used. This preparation is termed S9-mix.

The concentrations of the different compounds in the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
100 mM phosphate buffer pH 7.4

4. Cell cultures and medium:

Large stocks of the mycoplasma-free V79 cell line are stored in liquid nitrogen in the cell bank of "Genetic Toxicology", thus permitting repeated use of the same cell culture batch for numerous experiments. The identical characteristics of the cells ensure comparability of the experimental parameters.

Thawed stock cultures are kept at approx. 37 °C and approx. 4% CO_2 in 175 cm^2 plastic flasks. About $5x10^5$ to $1x10^6$ cells were seeded into each flask in 30 mL of MEM-medium supplement with approx. 10% (v/v) FCS (fetal calf serum) containing approx. 2 mM L-glutamine and approx. 0.1% (w/v) neomycinsulfate. The cells were sub-cultured twice a week. For the selection of mutants the medium was supplemented with approx. 11 µg/mL thioguanine.

5. Test substance concentrations used:

The following test substance concentrations were used.

Table 3.8.1.9- 1: Test substance concentrations

Experiment	S9 mix	Concentration in culture medium (µg/mL)
1st Experiment	+/-	1.2, 3.8, 12.1, 38.2, 120.8*, 382.0*, 1208.0*, 3820.0*
2nd Experiment	+/-	0.4, 0.8, 1.6, 3.2, 6.3, 12.5, 25.0, 50.0**, 75.0, 100.0*, 120.0*
3rd Experiment	+/-	0.313, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0**, 50.0**, 60*

* because of high toxicity no mutant selection was performed with and without S9 mix
** because of high toxicity no mutant selection was performed with S9 mix

B. Test performance

Experimental phase: April 4 to May 19, 2000

1. Preliminary assay

A preliminary toxicity test was undertaken in order to select appropriate dose levels for the mutation assay. In the test a wide range of dose levels of test substance was used. Cell cultures were subjected to the same treatment conditions as in mutation assays, and the survival of the cells was subsequently determined.

The test included the following treatments:

Solvent control: The maximum final concentration of organic solvents will not exceed approx. 1% (v/v).
Test substance: The highest dose level for the preliminary cytotoxicity test was determined by the solubility of the test substance up to the maximum of 10 mM or 5000 µg/mL.

Treatments were performed both in the presence and absence of S9 metabolic activation system using a single cell culture.

2. Main assay

In preliminary toxicity experiments approximately 4500 cells were seeded in each well of a microtiter plate, allowed to attach overnight and exposed to the test and control compound for four hours.

For each concentration at least 6 wells were used. Approximately 24 hours after treatment, the cells were fixed and stained with crystal violet. Survival was determined by measurement of the crystal violet extinction.

In the main experiments the cultures were prepared and treated with the test substance in the same way as for the preliminary experiment. 24 hours after seeding of approx. 4500 cells per well in a microtiter plate, the medium was replaced with serum-reduced (5% v/v) medium containing the test substance, either without S9-mix or with S9-mix. After 4 hours the treatment medium was removed and the cells were rinsed twice with normal medium. Thereafter normal medium was added to the wells. The cultures were stained with crystal violet and survival was determined after an incubation period of approx. 24 hours.

As rationale for dose selection, for non-toxic, freely soluble test substances, the top dose is 10 mM or 5000 µg/mL according to international testing guidelines. For non-toxic, poorly soluble test substances, the top dose is the highest evaluable dose. For toxic compounds a percentage survival rate relative to the solvent control was calculated for each treatment. The dose level which results in a predicted survival of about 30% was estimated from the results obtained. This dose was chosen as the highest dose level. At least eleven single dose levels or four duplicated doses are included in the treatment series.

Three independent mutation tests were performed.

Exponentially growing cultures which were more than 50% confluent were trypsinated by an approx. 0.25% (v/v) trypsin ready for use (mfr. Gibco). A single cell suspension was prepared. Subsequently the cells were replated to determine the mutation frequency and plating efficiency.

The treatment schedule of the mutagenicity test is described below:

Day 1: Sub-culturing of an exponentially growing culture

a) Approx. 4500 cells in each well of a microtiter plate for determination of the plating efficiency.
b) $6x10^5 – 1x10^6$ cells in 175 cm^2 flasks with 30 mL medium for the mutagenicity test, one flask per experimental point.

Day 2: Treatment of a) and b) with the test substance in the presence and absence of S9-mix (final protein concentration: approx. 0.3 mg/mL) for 4 hours.

Day 3: Fixation and staining of the cells in a) microtiter plate for the determination of the plating efficiency.

Day 5 or 6: Subculturing of b) in 175 cm^2 flasks.

Day 9: Subculturing of b) in five 75 cm^2 flasks with culture medium containing 6-thioguanine: Mutant selection (about 300,000 cells/flask); Subculturing of b) in two 25 cm^2 flasks for plating efficiency (about 400 cells per flask).

Day 16: Fixation and staining of colonies of b) – from subcultures seeded on day 9.

All incubations were carried out at approx. 37 °C and 4 % CO_2. Staining was performed with approx. 10% (v/v) methylene blue in approx. 0.01% KOH solution. Only colonies with more than 50 cells were counted.

3. Acceptance Criteria

This assay was considered valid if the following criteria were met:

- The spontaneous control data were within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induced increases in the mutation frequency which were both statistically significant and within the laboratory's normal range.
- The plating efficacy for the solvent control was greater than 50%.

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- It reproducibly induces with one of the test compound concentrations a mutation frequency that is three times higher than the spontaneous mutant frequency in this experiment.
- There is a reproducible concentration-related increase in the mutation frequency. Such an evaluation may be considered independently from the enhancement factor for induced mutants.
- Survival of the responding dose group is at least 30%.

However, in a case by case evaluation both decisions depend on the level of the corresponding negative control data.

5. Statistical analysis

The biometry of the results was performed off-line with the Mann-Whitney-U-Test.

II. Results and Discussion

A. Preliminary cytotoxicity assay

Fluopicolide was suspended in DMSO. Evaluation of the solubility of that suspension in cell culture medium showed that 3820 µg/mL was the highest practicable concentration and produced a heavy precipitate. This concentration corresponds to 10 mM, which is the highest dose level tolerated to be tested and the recommended international regulatory limit dose.

Accordingly, the preliminary toxicity study was carried out in microtiter plates using a maximum concentration of 3820 µg/mL and a wide range of lower dose levels down to 10 µg/mL.

Following treatment in the absence of S9 metabolic activation, high toxicity was observed. Survival declined in a dose-related manner reaching 36.9% of the solvent control value at the highest dose level. In the presence of S9-mix a broadly similar response was seen. Survival reached 56.3% of the solvent control value at the highest dose level. Macroscopic precipitation of the test substance in the medium was observed at 250 µg/mL and above, whilst microscopic precipitation was obtained at 50 µg/mL and higher.

On the basis of these results, a concentration of 3820 µg/mL was used for the first main assay and seven lower dose levels down to 1.2 µg/mL were included in the treatment series.

B. Main assay

Plating efficiency (microtiter plates)

In the absence of S9 metabolic activation in all mutation experiments a dose-related decrease in survival was observed in the microtiter plates. In the first assay survival declined reaching 45.9% of the solvent control value at the highest dose level (3820 µg/mL). In the second mutation test survival was reduced to 74.4% at the concentration of 120 µg/mL, in the third experiment survival declined to 56.7% at the highest dose level of 60 µg/mL. In the presence of S9 metabolic activation survival in microtiter plates also decreased in a dose-related manner reaching 44.5% in the first study at a dose level of 3820 µg/mL. In the second and third experiment survival declined reaching 57.4%, and 62.6% of the solvent control value in the microtiter plates at 120.0 µg/mL and 60.0 µg/mL, respectively.

Macroscopic precipitation of the test substance in the medium was observed at 382.0 µg/mL and above, whilst microscopic precipitation was obtained at 30 µg/mL and higher.

In all assays survival in microtiter plates, which were stained 24 hours after treatment, was not comparable with the mass cultures, which were subcultured five days after treatment.

Mutagenicity test (mass culture)

In the first main experiment marked decrease in the mass culture was observed at the concentration of 120.8 µg/mL with and without S9-mix, where survival was reduced to 2.9% and 2.5% of the solvent control value, respectively. Therefore, the mutation selection was possible only up to a dose level of 38.2 µg/mL .

Based on these results the second main study was carried out using a maximum concentration of 120 µg/mL.

In the second main study survival in the mass culture was markedly decreased to 1.5% of the solvent control value at the concentration of 75 µg/ml without S9-mix. In the presence of S9 metabolic activation high toxicity was also observed, reaching 4.3% at the dose level of 50 µg/mL. Therefore, the mutation selection could be performed only up to dose levels of 50 µg/mL (without metabolic activation) and 25 µg/mL (with metabolic activation).

Based on these results the third main study was carried out using a maximum concentration of 60 µg/mL.

In the third mutation assay without S9-mix, survival in mass culture was reduced to 1.6% of the solvent control value at the highest concentration (60 µg/mL). In the presence of a metabolic activation high toxicity was obtained reaching 1.4% of the solvent control value at the dose level of 40 µg/mL. Therefore, the mutation selection was possible only up to a dose level of 50 µg/mL (without metabolic activation) and 30 µg/mL (with metabolic activation).

Fluopicolide was assessed for its mutagenic potential in vitro in this HPRT-test in three independent experiments without metabolic activation and three independent experiments with metabolic activation. The results of these experiments are presented in the following tables.

No relevant reproducible increase of the mutant colonies or mutant frequency over the range of the solvent control was found with any of the concentrations used with metabolic activation by S9-mix.

The statistically significant increase observed in the first main study in the presence of S9-mix was caused by a low mutation frequency of the solvent control values and therefore were without any relevance. In the second main assay, there was a statistically significant increase of the mutation rate over the range of the solvent controls at 0.8 and 12.5 µg/mL without metabolic activation. These findings were without biological relevance because at the dose level of 12.5 µg/mL the increase was not three-fold higher than the solvent control value, no dose related response was observed and the results were not reproduced in the third experiment.

The sensitivity of the test system was demonstrated by the enhanced mutation frequency in the cell cultures treated with the positive control compounds.

Table 3.8.1.9- 2: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) – Experiment I

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	3820.0	2.5	NA
	1208.0	1.7	NA
	382.0	2.1	NA
	120.8	2.9	NA
	38.2	129.5	12.6
	12.1	144.0	15.7
	3.8	120.3	14.5
	1.2	99.6	13.5
Negative control	-	100.0	6.5
Solvent control	-	100.0	17.1
Positive control EMS	1000	90.9	742.6*
With metabolic activation (+S9)			
Fluopicolide	3820.0	2.5	NA
	1208.0	1.7	NA
	382.0	2.1	NA
	120.8	2.9	NA
	38.2	129.5	12.8
	12.1	144.0	14.2
	3.8	120.3	25.1*
	1.2	99.6	23.7*
Negative control	-	100.0	21.4
Solvent control	-	100.0	11.2
Positive control			
DMBA	7.7	74.8	128.4*

NA: not applicable as % relative survival < 50 %
*: $p < 0.05$ statistically significant Mann-Whitney-U-Test

Table 3.8.1.9- 3: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) –Experiment II

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	120.0	2.2	NA
	100.0	1.5	NA
	75.0	1.5	NA
	50.0	108.2	11.4
	25.0	121.7	11.1
	12.5	106.7	26.2*
	6.3	104.9	19.8
	3.2	113.1	21.2
	1.6	98.5	9.7
	0.8	98.9	55.9*
	0.4	115.0	17.2
Negative control	-	100.0	13.2
Solvent control	-	100.0	13.3
Positive control EMS	1000	70.0	1025.2*
With metabolic activation (+S9)			
Fluopicolide	120.0	2.4	NA
	100.0	1.9	NA
	75.0	1.9	NA
	50.0	4.3	NA
	25.0	118.4	31.6
	12.5	143.5	12.8
	6.3	155.1	24.3
	3.2	121.3	20.5
	1.6	108.7	12.3
	0.8	157.0	20.4
	0.4	163.3	23.8
Negative control	-	100.0	18.1
Solvent control	-	100.0	27.7
Positive control DMBA	7.7	86.0	169.2*

NA: not applicable as % relative survival < 50 %
*: p<0.05 statistically significant Mann-Whitney-U-Test

Table 3.8.1.9- 4: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) – Experiment III

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	60.0	1.6	NA
	50.0	65.5	19.0
	40.0	108.1	25.1
	30.0	112.1	14.9
	20.0	146.0	22.7
	10.0	102.5	9.0
	5.0	113.7	11.8
	2.5	112.4	18.9
	1.25	89.1	30.8
	0.625	141.9	7.0
	0.313	134.8	31.6
Negative control	-	100.0	12.1
Solvent control	-	100.0	16.9
Positive control EMS	1000	113.7	889.1*
With metabolic activation (+S9)			
Fluopicolide	60.0	0.8	NA
	50.0	0.8	NA
	40.0	1.4	NA
	30.0	104.2	17.7
	20.0	106.4	27.0
	10.0	99.7	20.4
	5.0	111.7	10.0
	2.5	105.3	27.5
	1.25	108.4	27.6
	0.625	112.8	10.9
	0.313	95.5	15.4
Negative control	-	100.0	12.0
Solvent control	-	100.0	20.3
Positive control DMBA	7.7	96.7	93.3*

NA: not applicable as % relative survival < 50 %
*: $p<0.05$ statistically significant Mann-Whitney-U-Test

III. Conclusion

Fluopicolide did not induce gene mutation in either the presence or absence of metabolic activation, i.e. was not mutagenic, in this HPRT test with V79 Chinese hamster cells.

3.8.2 Animal data

3.8.2.1 Anonymous; 2005; M-197261-02-1

Study reference:

Anonymous; 2005; Mouse erythrocyte micronucleus test Code: AE C638206 00 1C99 0005; M-197261-02-1

Deviations:

Deviations from the current OECD guideline (474, 2016):

- Exposure of the target organ, i.e. bone marrow, was not verified
- 2000 instead of the currently required 4000 immature erythrocytes were analysed
- For the assessment of the ratio of polychromatic to total erythrocytes, 200 instead of the required 500 erythrocytes were analysed.

However, bone marrow exposure was shown in an additional micronucleus test *in vivo* (Herbold, B.; 2003; M-223119-01-1 (i.p. administration)) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.

Executive Summary:

In this study the potential of fluopicolide to induce chromosomal damage (clastogenicity) in mouse bone marrow erythrocytes as evidenced by micronuclei formation was assessed.

Groups of five male and five female NMRI mice were used. They were 7 weeks of age and had mean body weights of 33.0 g (males) and 26.4 g (females) at the start of the study (first dose). Each mouse was given two gavage doses separated by an interval of 24 hours of either 200, 600 or 2000 mg/kg bw of fluopicolide in aqueous methylcellulose (l% w/v). The highest dose level was the international regulatory limit dose. The animals were killed 24 hours after the second dose.

Cyclophosphamide (Endoxan), the positive control substance, was administered as a single oral (gavage) dose of 50 mg/kg bw to five male and five female mice which were killed 24 hours after dosing.

Bone marrow smears were prepared from each animal and one from each was examined for the presence of micronuclei in 2000 polychromatic erythrocytes. The ratio of polychromatic to 200 erythrocytes was assessed.

Treatment with fluopicolide did not increase the number of polychromatic erythrocytes containing micronuclei. The ratio of polychromatic to norrnochromatic erythrocytes was not significantly affected.

Thus, fluopicolide was not clastogenic in this mouse erythrocyte micronucleus test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP124102412 & PPl24106711

2. Vehicle and/or positive control

Vehicle:	Aqueous methylcellulose 1 % (w/v)
Positive control:	Cyclophosphamide

3. Test animals

Species:	Mouse
Strain:	HsdWin:NMRI
Age:	Approx. 7 weeks
Weight at start:	30-36 g (males), 23-30 g (females)
Source:	Harlan Winkelmann GmbH, Gartenstrasse 27,331 78 Borchen
Acclimation period:	Yes
Diet:	Rat/mouse diet ssniff RIM-H (V 1534), ad libitum ssniff GmbH, Postbox 2039, 59480 Soest
Water:	Potable water taken from the public supply was freely available via polycarbonate bottles fitted with sipper tubes, except when urine was being collected
Housing:	Fully air-conditioned rooms in makrolon cages type 4 (five animals of the same sex per cage) on soft wood granulate
Temperature:	22 ± 3 °C
Humidity:	50 ± 20%
Air changes:	Fully air-conditioned
Photoperiod:	12 hours

4. Test substance doses

Gavage administrations of 200, 600, 2000 mg/kg bw, in two doses, separated by 24 hours.

B. Test performance

Experimental phase: April 3 to April 28, 2000

1. Treatment and sampling times

The study design of the main study was as follows.

Table 3.8.2.1- 1: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals per sex	Route and number of applications	
Negative control	0	5	oral	2
Fluopicolide	200	5	oral	2
	600	5	oral	2
	2000	5	oral	2
Positive control (Cyclophosphamide)	50	5	oral	1

The test substance was administered two doses separated by an interval of 24 hours orally by gavage to the test animals at doses of 200, 600 and 2000 mg/kg bw. The vehicle, aqueous methylcellulose 1 % (w/v), was administered in the same way to the negative control groups. The study included a concurrent positive control using cyclophosphamide (Endoxan) which was administered once orally by gavage at a dose of 50 mg/kg bw.

Following dosing, the animals were examined regularly for mortality and clinical signs of toxicity.

2. Tissues and cells examined

For the extraction of the bone marrow, the animals were killed by carbon dioxide asphyxiation 24 hours after the last dose. Two femora were removed and the bones freed of muscle tissue. The proximal ends of the femora were opened, the bone marrow flushed into a centrifuge tube containing approx. 3 mL of fetal bovine serum and a suspension is prepared. The mixture was then centrifuged for 5 minutes at approx. 1200 rpm, after which almost all the supernatant was discarded. One drop of the thoroughly mixed sediment was smeared onto a cleaned slide, identified by project code and animal number and air-dried for approx. 12 hours.

Subsequently the slides are stained as follows:

- 5 minutes in methanol
- 5 minutes in May-Grünwald's solution brief rinsing twice in distilled water
- 10 minutes staining in 1 part Giemsa solution to 6 parts buffer solution, pH 7.2 (Weise)
- rinsing in distilled water
- drying
- coating with Entellan.

3. Scoring

2000 polychromatic erythrocytes were counted for each animal. The number of cells with micronuclei was recorded, not the number of individual micronuclei. In addition, the ratio of polychromatic erythrocytes to 200 erythrocytes was determined. Main parameter for the statistical analysis, i.e. validity assessment of the study and mutagenicity of the test substance, was the proportion of polychromatic erythrocytes with micronuclei out of the 2000 counted erythrocytes. All bone marrow smears for evaluation were coded to ensure that the group from which they were taken remained unknown to the investigator.

4. Evaluation criteria

A one-sided Wilcoxon-Test (Hollander, M.; Wolfe, D.A.: Nonparametric statistical methods; Wiley Seriesin Probability and Mathematical Statistics; John Wiley & Sons, Inc.; New York (1973) Streitberg, B.; Rdhmel, J.: Exakte Verteilung fur Rang- und Randornisierungstests irn allgerneinen c-Stichprobenproblem; EDV in Medizin und Biologie 18; 12 - 19; Verlag Eugen Ulmer GmbH & Co., Stuttgart; Gustav Fischer Verlag KG, Stuttgart (1987) was used to check the validity of the study. The study was considered as valid if the proportion of polychromatic erythrocytes with micronuclei in the positive control was significantly higher than in the negative control ($p = 0.05$).

5. Criteria for a positive response

Both biological and statistical significances were considered together for evaluation purposes.

A substance is considered as positive if there is a significant dose-related increase in the number of micronucleated polychromatic erythrocytes compared with the concurrent negative control group. A test substance producing no significant dose-related increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system.

6. Statistical methods

Assuming the study is valid based on a monotone dose-relationship, one-sided Wilcoxon tests were performed initially comparing control values with those of the highest dose group. Tests on lower dose groups were only performed if all higher dose groups were significantly different from the control[6]. A significance level of 5% is adopted for all tests.

II. Results and Discussion

A. Micronucleus assay

Mice were given 2 doses of 200, 600 and 2000 mg fluopicolide per kg body weight separated by an interval of 24 hours to study the induction of micronuclei in bone marrow cells.

All animals survived after treatment. No signs of toxicity were observed. The dissection of the animals revealed no test substance related macroscopic findings.

The bone marrow smears were examined for the occurrence of micronuclei in red blood cells. The results are summarized in Table 3.8.2.1- 2.

The incidence of micronucleated polychromatic erythrocytes in the dose groups of fluopicolide was within the normal range of the negative control groups. No statistically significant increase of micronucleated polychromatic erythrocytes was observed.

The ratio of polychromatic erythrocytes to total erythrocytes (PCE/Ery ratio) remained essentially unaffected by the test compound and was not less than 20% of the control values.

Cyclophosphamide (Endoxan B) induced a marked and statistically significant increase in the number of polychromatic erythrocytes with micronuclei, thus indicating the sensitivity of the test system.

An overview is given in Table 3.8.2.1- 2.

[6] Hothom, L.; Lehmacher, W.: A Simple Testing Procedure "Control versus k Treatments" for One-sided Ordered Alternatives, with Application in Toxicology; Biom. J. 33,179 - 189; Akademie Verlag (1991)

Table 3.8.2.1- 2: Group mean PCE/NCE ratios and incidences of micronucleated PCE

Treatment	Dose (mg/kg bw)	No. of animals	Total no. PCE scored	PCE/Ery ratio ± SD	Mean number micronucleated PCE ± SD
Males					
Fluopicolide	2000	5	10000	0.47 ± 0.06	1.6 ± 0.04
	600	5	10000	0.45 ± 0.02	1.6 ± 0.04
	200	5	10000	0.44 ± 0.06	1.8 ± 004
	0	5	10000	0.48 ± 0.07	1.0 ± 0.04
Positive control (Cyclophosphamide)	50	5	10000	0.46 ± 0.04	72.2 ± 1.03
Females					
Positive control (Cyclophosphamide)	2000	5	10000	0.48 ± 0.05	1.0 ± 0.04
	600	5	10000	0.46 ± 0.06	2.0 ± 0.04
	200	5	10000	0.53 ± 0.03	1.8 ± 0.07
	0	5	10000	0.47 ± 0.06	1.6 ± 0.04
Positive control (Cyclophosphamide)	50	5	10000	0.55 ± 0.03	55.8 ± 1.13

PCE: polychromatic erythrocytes
NCE: normochromatic erythrocytes
Ery: total erythrocytes (PCE+NCE)

III. Conclusion

Fluopicolide did not induce clastogenicity. It was not mutagenic in this micronucleus test.

3.8.2.2 Anonymous; 2003; M-219364-01-1

Study reference:

Anonymous; 2003; AE C638206: Induction of micronuclei in the bone marrow of treated mice; M-219364-01-1

Deviations: Deviations from the current OECD guideline (474, 2016):
- Exposure of the target organ, i.e. bone marrow, was not verified

However, bone marrow exposure was shown in an additional micronucleus test in vivo (Herbold, B.; 2003; M-223119-01-1 (i.p. administration) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.

Executive Summary:

Fluopicolide was assayed *in vivo* in a mouse bone marrow micronucleus test at a single limit dose.

For the range-finder experiment, fluopicolide was formulated in 1% (w/v) aqueous methylcellulose (1% MC) and administered once daily on two consecutive days to a group of three male mice at a dose of 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines).

Observations were made over a two-day period following the second administration and signs of toxicity recorded. As no clinical signs of toxicity were observed, so the main experiment was conducted using at a single limit concentration (2000 mg/kg bw/day).

In the main study, fluopicolide was formulated as described and administered at 2000 mg/kg bw/day to a group of six male mice killed 24 hours after the second administration.

The negative (vehicle) control in the study was 1% MC also administered orally by gavage once daily on two consecutive days to a group of six male mice. These animals were killed 24 hours after the second administration.

Cyclophosphamide (CPA), the positive control, was dissolved in saline and administered orally by gavage as a single dose of 40 mg/kg bw to a group of six male mice which were killed after 24 hours. Positive control animals exhibited increased numbers of micronucleated polychromatic erythrocytes (PCE) such that the micronucleus frequency in the positive control group was significantly greater than in concurrent controls.

Negative (vehicle) control mice exhibited a group mean ratio of PCE to NCE (normochromatic erythrocytes) which was within the historical negative control range. However, it was noted that one animal showed a high number of micronucleated PCE (2.25 micronucleated PCE/1000 cells) that clearly exceeds the historical negative control data frequency (0.40 micronucleated PCE/1000 cells).

However, this was observed in just one animal in a total of six and all other vehicle control animals exhibited numbers of micronucleated PCE that were similar to the expected distribution. The vehicle control data was therefore considered valid.

Mice treated with fluopicolide at 2000 mg/kg bw/day exhibited a group mean ratio of PCE to NCE which was similar to the value for the vehicle control group and which lay within the historical control data range. The group mean frequency of micronucleated PCE in the test substance treated group was slightly increased compared to the concurrent negative control group value (1.50 ± 0.8 and 0.88 ± 0.70, respectively), but without any statistical significance. As such the protocol criteria for a positive result were not met. This is supported by the fact that an incidentally high number of MN PCE (9 MN PCE per 4000 analysed) was also detected in one control animal (see above) and the group mean value of the positive control was significantly higher than the mean value of fluopicolide treatment group.

It is concluded that treatment of mice with fluopicolide at 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines) resulted in a group mean frequency of micronucleated PCE in the test group that was slightly increased compared to the concurrent negative control group value. This result was statistically non-significant and of questionable biological significance.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	96.1% (w/w)
Batch no.:	OP 2050046

2. Vehicle and/or positive control

Vehicle:	Aqueous methylcellulose 1% (w/v)
Positive control:	Cyclophosphamide

3. Test animals

Species:	Mouse
Strain:	Male out bred CD-I Crl:CD-l™(ICR) BR mice
Age:	4-6 weeks
Weight at start:	22-32 g
Source:	Charles River UK Ltd, Margate, UK
Acclimation period:	Yes
Diet:	Special Diets Services Ltd, RM1.(E).SQC.)
Water:	Bottled water (public supply)
Housing:	Groups of no more than three animals in solid-floored cages, cleaned and dried before use with wood shavings for bedding.
Temperature:	19.7-21.5 °C
Humidity:	40-70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

4. Test substance doses

Gavage administrations of 2000 mg/kg bw, in two doses, separated by 24 hours (based on a range finding study).

B. Test performance

Experimental phase: March 20 to June 12, 2003

1. Treatment and sampling times

The study design of the main study was as follows.

Table 3.8.2.2- 1: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Range finding study				
Fluopicolide	2000	3	oral	2
Main study				
Negative control	0	6	oral	2
Fluopicolide	2000	6	oral	2
Positive control (Cyclophosphamide)	40	6	oral	1

Animals were dosed once daily for two consecutive days with the test substance or vehicle. The positive control was given as a single administration at 40 mg/kg bw, on the second day of dosing (24 hours prior to harvest).

2. Tissues and cells examined

Test substance and vehicle treated mice were killed in groups, 24 hours after the second administration; CPA-treated mice were killed 24 hours after the single dose. Mice were killed by asphyxiation with carbon dioxide (subsequently ensured by cervical dislocation) in the same order as they were dosed.

Both femurs from each animal were exposed, removed, cleaned of adherent tissue and the ends removed from the shanks. Using a syringe and needle, bone marrows were flushed from the marrow cavity with 1 mL foetal bovine serum into appropriately labelled centrifuge tubes (one per animal).

The tubes were centrifuged (1250 x *g*, 2-3 minutes) and the serum was aspirated to leave one or two drops and the cell pellet. The pellet was mixed into this small volume of serum in each tube and from each tube a small volume of suspension was placed on the end of each of two slides labelled with the appropriate study number, sampling time, sex, date of preparation and animal number. The latter served as a code so analysis could be conducted "blind". A smear was made from the drop by drawing the end of a clean slide along the labelled slide.

Slides were allowed to air-dry and were fixed for 5 minutes in absolute methanol, followed by rinsing several times in water. One slide from each set of two was then taken, the other was kept in reserve. After a second fixing/rinsing procedure, slides were stained according to the modification of Gollapudi and Kamra[7]. Slides were stained for 10 minutes in filtered Giemsa stain diluted 1:6 (v/v) in distilled water. Stained slides were rinsed, and allowed to dry thoroughly before clearing in xylene for 3 minutes. When dry, the slides were mounted with coverslips.

In this study, a reserve set of slides were stained (as detailed above) and analysed in order to generate additional data.

3. Scoring

Slides from the CPA-treated mice were initially checked at Covance Laboratories Limited to ensure the system was operating satisfactorily. The slides from all control and dose groups were arranged in numerical order by sampling time and analysed by a person not connected with the dosing phase of the study. Initially the relative proportions of polychromatic erythrocytes (PCE), seen as pale blue or blue/grey enucleate cells, and normochromatic erythrocytes (NCE), seen as smaller yellow/orange-stained enucleate cells, were determined until a total of at least 1000 cells (PCE plus NCE) had been analysed. Counting continued (but of PCE only) until at least 2000 PCE per animal had been observed (where possible). All PCE containing rnicronuclei observed during these two phases of counting were recorded. The vernier coordinates of all cells containing rnicronuclei were recorded to a maximum of six per 2000 cells scored. In order to obtain more data for each animal additional scoring was performed to provide a total of 2000 PCE + NCE and a total of 4000 PCE per animal had been examined for rnicronuclei (where possible).

Slide analysis was performed by an analyst trained in accordance with Covance Laboratories Limited Standard Operating Procedures. Details of the analyst are included in the responsible personnel list. All slides and raw data have been retained at Covance Laboratories Limited for archiving in accordance with the archive statement in this report.

[7] G. Gollapudi B. and Kamra O.P. (1979): Application of a simple Giemsa staining method in the micronucleus test. Mutation Res 64, 45-46

4. Evaluation criteria

After completion of microscopic analysis and decoding of the data, the ratio of PCE/NCE for each animal and the mean for each group was calculated. The individual and group mean frequency of micronucleated PCE/1000 cells (+ standard deviation) were also determined.

PCE/NCE ratios were examined to see if there was any decrease in groups of treated animals that could be taken as evidence of bone marrow toxicity. The group mean frequencies of micronucleated PCE in vehicle control animals were compared with the historical negative control range to determine whether or not the assay was acceptable.

5. Acceptance criteria

The assay is considered valid if the following criteria are met:

1. The incidence of micronucleated PCE in the vehicle control group falls within or close to the historical vehicle control range, and
2. At least five animals (males) out of each group are available for analysis, and
3. The positive control chemical (CPA) induced a statistically significant increase in the frequency of micronucleated PCE.

6. Criteria for a positive response

A test substance is considered as positive in this assay if:

1. A statistically significant increase in the frequency of micronucleated PCE occurs at least at one dose, and
2. The frequency of micronucleated PCE at such a point exceeds the historical vehicle control range.

7. Statistical methods

For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity chi-square test.

The numbers of micronucleated PCE in each treated group were then compared with the numbers in vehicle control groups by using a 2 x 2 contingency table to determine chi-square. Probability values of $p < 0.05$ were to be accepted as significant. A further statistical test (for linear trend) was used to evaluate possible dose-response relationships.

If the heterogeneity chi-square test provides evidence of significant ($p < 0.05$) variability between animals within at least one group, non-parametric analysis is more appropriate. Provision was made to use the Wilcoxon rank sum test under these circumstances.

In this study, small, but statistically significant heterogeneity was observed within the vehicle control group and as such additional statistical analysis using non parametric analysis was considered appropriate. The Wilcoxon rank sum test was performed to compare the vehicle against the test article treated group.

II. Results and Discussion

A. Micronucleus assay

As no clinical signs of toxicity were observed in the range finding study, a single limit dose of 2000 mg/kg bw/day (the recommended maximum dose for *in vivo* cytogenetic assays, according to current regulatory guidelines) was chosen for the main study.

In the main study, no clinical signs of toxicity were observed in any treated animal.

The results of the formulations analysis indicated that the achieved concentrations were above 10% of nominal on all occasions. However, it was noted that percentage nominal results were similar on all formulation days for the range finding and main study experiments, the percentage nominal reading of all formulations was close to 120% of nominal indicating consistency of formulation. As no clinical signs were observed at the limit dose of 2000 mg/kg bw/day and the formulations analysis indicated achieved concentrations above 100%, these data were considered acceptable and the study data valid.

The results of the stability and homogeneity analysis indicated that the samples were homogenous and stable following storage at room temperature for a 24 hour period.

Mice treated with fluopicolide at 2000 mg/kg bw/day exhibited a group mean ratio of PCE to NCE which was similar to the value for the vehicle control group and which lay within the historical control data range (historical control data range: 0.38–1.67).
A higher number of micronucleated PCE as expected were noted in one control animal (animal no. 119: 2.25 micronucleated PCE/1000 cells). However, this was observed in just one animal in a total of six and all other vehicle control animals exhibited numbers of micronucleated PCE that fell within the expected distribution. The vehicle control data was therefore considered valid.

The group mean frequency of micronucleated PCE (MN PCE) in the test substance treated group was slightly increased compared to the concurrent negative control group value (1.50 ± 0.84 and 0.88 ± 0.70, respectively), but without any statistical significance. As such the protocol criteria for a positive result were not met. This is supported by the fact that a high number of MN PCE (9 MN PCE per 4000 analysed) was also detected in one control animal (see above) and that the group mean value of the positive control was significantly higher than the mean value of the fluopicolide treatment group (approx. 19 MN PCE compared to 1.5 MN PCE in the fluopicolide group).

An overview is given in Table 3.8.2.2- 2.

Table 3.8.2.2- 2: Individual and group mean PCE/NCE ratios and incidences of micronucleated PCE

Treatment group	Animal No.	PCE count	NCE count	Ratio PCE/NCE	No. MN PCE	No. MN PCE / 1000
Negative control (vehicle)	115	1089	991	1.10	3	0.75
	119	1132	959	1.18	9	2.25
	109	1045	1055	0.99	1	0.25
	101	1063	998	1.07	2	0.52
	102	980	1140	0.86	3	0.75
	112	898	1139	0.79	3	0.75
	Mean (± SD)	-	-	**1.00**	-	**0.88 ± 0.70**
Fluopicolide (2000 mg/kg bw/day)	106	1127	955	1.18	2	0.50
	116	1100	960	1.15	5	1.25
	103	986	1085	0.91	11	2.75
	108	1049	1012	1.04	3	0.75
	105	1049	1116	0.94	7	1.75
	104	1080	997	1.08	8	2.00
	Mean (± SD)	-	-	**1.05**	-	**1.50 ± 0.84**

Treatment group	Animal No.	PCE count	NCE count	Ratio PCE/NCE	No. MN PCE	No. MN PCE / 1000
Positive control (Cyclophosphamide, 40 mg/kg bw)	117	1116	1012	1.10	65	16.25
	107	1025	1037	0.99	84	21.00
	114	1087	1142	0.95	91	22.75
	118	1155	1101	1.05	71	17.75
	111	1005	1004	1.00	98	24.50
	113	1103	1064	1.04	46	11.50
	Mean (± SD)	**-**	**-**	**1.02**	**-**	**18.96± 4.77****
Historical negative control data (range)[#]		-	-	0.99 (0.38–1.67)	-	0.40

PCE: polychromatic erythrocytes
NCE: normochromatic erythrocytes
MN: micronucleated
**: $p \leq 0.01$
#: Historical control data based on results from 72 males from 11 studies

III. Conclusion

It is concluded that treatment of mice with fluopicolide at 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines) resulted in a group mean frequency of micronucleated PCE in the test substance treated group that was slightly increased compared to the concurrent negative control group value, but was not statistically different. As such the protocol criteria for a positive result were not met which is supported by the fact that a high number of MN PCE was also detected in one control animal.

3.8.2.3 Anonymous; 2003; M-223119-01-1

Study reference:

Anonymous; 2003; Micronucleus-test on the male mouse Code: AE C638206; M-223119-01-1

Deviations: Deviations from the current OECD guideline (474, 2016):
- 2000, instead of the currently required 4000 immature erythrocytes were analysed

The deviation(s) are considered not to compromise the results and outcome of the study.

Executive Summary:

The micronucleus test was employed to investigate fluopicolide in male NMRI mice for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts.

The known clastogen and cytostatic agent cyclophosphamide served as positive control.

Male mice treated with fluopicolide received two intraperitoneal administrations of 150, 300 or 600 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide. Intraperitioneal administration as application route was chosen because bone marrow exposure could not be verified after oral administration (see Roth, T.; 2005; M-197261-02-1 and Whitwell, J.; 2003; M-219364-01-1).

The femoral marrow of all groups was prepared 24 hours after the last administration.

Males treated twice with fluopicolide in doses up to and including 600 mg/kg bw showed symptoms of toxicity after administration starting at 150 mg/kg. These symptoms demonstrate relevant systemic exposure of males to fluopicolide. However, all males survived until the end of the test.

There was an altered ratio between polychromatic and normochromatic erythrocytes. This finding demonstrates relevant systemic exposure of the animals to fluopicolide.

After two intraperitoneal treatments of males with doses up to and including 600 mg/kg bw no indications of a clastogenic effect of fluopicolide were found.

Cyclophosphamide, the positive control, had a clear clastogenic effect, as is shown by the biologically relevant increase in polychromatic erythrocytes with micronuclei. The ratio of polychromatic to normochromatic erythrocytes was not altered.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	99.4%
Batch no.:	OP2350005

2. Vehicle and/or positive control

Vehicle:	0.5% Cremophor
Positive control:	Cyclophosphamide

3. Test animals

Species:	Male mice
Strain:	Hsd/Win: NMRI
Age:	Approx. 6-12 weeks
Weight at start:	37-42 g
Source:	Harlan Winkelmann GmbH, Borchen
Acclimation period:	Yes
Diet:	Fixed formula feed 3883 (10 mm cubes), produced according to specification by Provimi Kliba SA, CH-4303 Kaiseraugst
Water:	Tap water in polycarbonate bottles, 300 ml volume
Housing:	Singly in type I cages, bedding of soft wood granules, type BK8/15 (J. Rettenmaier & Söhne, Fullstoff-Fabriken, 73494 Ellwangen-Holzmühle)
Temperature:	22±1.5 °C
Humidity:	40-70%
Air changes:	10/hour
Photoperiod:	12 hours

4. Test substance doses

Intraperitioneal administration as application route was chosen because bone marrow exposure could not be verified after oral administration (see Roth, T.; 2005; M-197261-02-1 and Whitwell, J.; 2003; M-219364-01-1). The selection of the fluopicolide doses was based on a pilot test. This pilot test was performed in the laboratory which conducted the main study using animals of the same source, strain and age. Groups consisting each of three males and three females received two intraperitoneally injections separated by 24 hours. The following doses were used: 400 and 1000 mg/kg bw fluopicolide. In males the following symptoms were recorded for up to at least 48 hours after the second application, starting at 400 mg/kg bw: apathy, roughened fur, loss of weight, reduced body temperature, sternal recumbency, spasm, periodically stretching of body, difficulty in breathing and slitted eyes. In addition, 2 of 3 males died in the 1000 mg/kg bw group. In females the following symptoms were recorded for up to at least 48 hours after the second application, starting at 400 mg/kg bw: apathy, uncoordinated movement, roughened fur, loss of weight, reduced body temperature, sternal recumbency, spasm, twitching, periodically stretching of body, difficulty in breathing and slitted eyes. In addition, 2 of 3 females died in the 1000 mg/kg bw group.

Based on these findings, a dose of 600 mg/kg bw fluopicolide was chosen as MTD for males. Due to the results of the dose range finder it is concluded, that there are no substantial differences between sexes in toxicity. Therefore, no females were used.

B. Test performance

Experimental phase: September 3 to October 17, 2003

1. Treatment and sampling times

The study design of the main study was as follows.

Table 3.8.2.3- 1: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	5	i.p.	2
Fluopicolide	150	5	i.p.	2
	300	5	i.p.	2
	600	5	i.p.	2
Positive control (Cyclophosphamide)	20	5	i.p.	1

Male mice (five animals/group) treated with fluopicolide (batch OP235005, purity 99.4%) received two intraperitoneal administrations of 150, 300 or 600 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide.

2. Tissues and cells examined

At least one intact femur was prepared from each sacrificed animal (not pretreated with a spindle inhibitor) (Schmid's method). A suitable instrument was used to sever the pelvic bones and lower leg.

The femur was separated from muscular tissue. The lower-leg stump, including the knee and all attached soft parts, was separated in the distal epiphyseal cartilage by a gentle pull at the distal end.

The proximal end of the femur was opened at its extreme end with a suitable instrument, e.g. fine scissors, making visible a small opening in the bone-marrow channel.

A suitable tube was filled with sufficient fetal calf serum. A small amount of serum was drawn from the tube into a suitable syringe with a thin cannula. The cannula was pushed into the open end of the marrow cavity. The femur was then completely immersed in the calf serum and pressed against the wall of the tube, to prevent its slipping off. The contents were then flushed several times and the bone marrow was passed into the serum as a fine suspension. Finally, the flushing might be repeated from the other end, after it had been opened. The tube containing the serum and bone marrow was centrifuged in a suitable centrifuge at approximately 1000 rpm for five minutes. The supernatant was removed with a suitable pipette (e.g. Pasteur pipette), leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

One drop of the viscous suspension was placed on a well-cleaned slide and spread with a suitable object, to allow proper evaluation of the smear. The labeled slides were dried overnight. If fresh smears needed to be stained, they needed to be dried with heat for a short period.

The smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry.

Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly (e.g. with tweezers) to be covered. A small amount of covering agent was taken from a bottle with a suitable object (e.g. glass rod) and applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

3. Evaluation

Coded slides were evaluated using a light microscope at a magnification of about 1000. Micronuclei appear as stained chromatin particles in the anucleated erythrocytes. They can be distinguished from artifacts by varying the focus. Normally, 2000 polychromatic erythrocytes were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

It is expedient to establish the ratio of polychromatic to normochromatic erythrocytes for two reasons:

1. Individual animals with pathological bone marrow depressions may be identified and excluded from the evaluation.
2. An alteration of this ratio may show that the test compound actually reaches the target.

Therefore, the number of normochromatic erythrocytes per 2000 polychromatic ones was noted. If the ratio for a single animal amounts to distinctly more than 6000 normochromatic erythrocytes per 2000 polychromatic ones, or if such a ratio seems likely without other animals in the group showing similar effects, then the case may be regarded as pathological and unrelated to treatment, and the animal may be omitted from the evaluation. A relevant, treatment-related alteration of the ratio polychromatic to normochromatic erythrocytes can only be concluded if it is clearly lower for a majority of the animals in the treated group than in the negative control.

In addition to the number of normochromatic erythrocytes per 2000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways. Firstly, it permits the detection of individuals already subject to damage before the start of the test. Secondly, combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

An increase in the number of micronucleated normochromatic erythrocytes, without a preceding increase in micronucleated polychromatic erythrocytes, is irrelevant to the assessment of a clastogenic effect, since normochromatic erythrocytes originate from polychromatic ones. Before an effect can be observed in normochromatic erythrocytes, there must be a much greater increase in micronucleated polychromatic erythrocytes, due to the "dilution effect" of the "old" cells, i.e. normochromatic erythrocytes already present at the start of the test, and this effect would have been observed previously.

4. Evaluation criteria

An assay was considered acceptable if the figures of negative and positive controls were within the expected range, in accordance with the laboratory's experience and/or the available literature data.

5. Criteria for a positive response

A test is considered positive if there was a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

A test was considered negative if there was no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes. A test was also considered negative if there was a significant increase in that rate which, according to the laboratory's experience was within the range of historical negative controls.

In addition, a test was considered equivocal if there was an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls, provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group. In this case, normally a second test will be performed.

6. Statistical methods

The fluopicolide group(s) with the highest mean (provided this superceded the negative control mean) and the positive control were checked by Wilcoxon's nonparametric rank sum test with respect to the number of polychromatic erythrocytes having micronuclei and the number of normochromatic erythrocytes. A variation was considered statistically significant if its error probability was below 5 % and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi^2-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control. In addition, standard deviations (1s ranges) were calculated for all the means.

II. Results and Discussion

A. Micronucleus assay

After two intraperitoneal administrations of 150, 300 or 600 mg/kg bw fluopicolide, treated males showed the following compound-related symptoms until sacrifice: apathy, roughened fur, loss of weight, spasm, periodically stretching of body and difficulty in breathing. These symptoms demonstrate relevant systemic exposure of males to fluopicolide. There was no substance-induced mortality. No symptoms were recorded for the control groups. No animals died in these groups.

An overview of the genotoxicity evaluation is given in Table 3.8.2.3- 2.

Table 3.8.2.3- 2: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group	Dose (mg/kg bw)	No. of animals	Total no. PCE scored	No. NCE/ 2000 PCE ± SD	No. micronucleated cells/2000 ± SD	
					NCE	PCE
Fluopicolide	600	5	10000	4948 ± 1782*	2.1 ± 1.8	5.0 ± 2.9
	300	5	10000	3523 ± 1189	0.9 ± 0.3	3.8 ± 1.3
	150	5	10000	2085 ± 1217	2.9 ± 3.1	1.8 ± 0.8
	0	5	10000	2356 ± 929	4.2 ± 2.4	2.8 ± 1.1
Positive control (Cyclophosphamide)	20	5	10000	1815 ± 614	3.4 ± 2.0	14.4 ± 5.2**

* $p \leq 0.05$; ** $p \leq 0.01$ (non-parametric Wilcoxon ranking test)

As can be seen in Table 3.8.2.3- 2, the ratio of polychromatic to normochromatic erythrocytes in males was altered by the treatment with fluopicolide, being 2000:2356 (1s = 929) in the negative control, 2000:2085 (1s = 1217) in the 150 mg/kg bw group, 2000:3523 (1s = 1189) in the 300 mg/kg bw group and 2000:4948 (1s = 1782) in the 600 mg/kg bw group. Relevant variations were thus noted for males. This finding demonstrates bone marrow exposure of the males to fluopicolide.

No biologically important or statistically significant variations existed for males between the negative control and the groups treated intraperitoneally with fluopicolide, with respect to the incidence of micronucleated polychromatic erythrocytes. The incidence of these micronucleated PCE was 2.8/2000 (1s = 1.1) in the negative control group, and 1.8/2000 (1s = 0.8), 3.8/2000 (1s = 1.3) and 5.0/2000 (1s = 2.9) in the fluopicolide groups at 150, 300 and 600 mg/kg bw, respectively.

Similarly, no biologically significant variation between the negative control and fluopicolide groups in the number of micronucleated normochromatic erythrocytes was observed, since normochromatic erythrocytes originated from polychromatic ones.

The positive control cyclophosphamide caused a clear, statistically significant increase in the number of polychromatic erythrocytes with micronuclei. The incidence of micronucleated PCE was 14.4/2000 (1s = 5.2), which represents biologically relevant increases in comparison to the negative control and thus confirms the sensitivity of this study.

III. Conclusion

The results with fluopicolide gave no relevant indications of clastogenic effects for male mice after two intraperitoneal treatments with doses of up to and including 600 mg/kg bw.

The ratio of polychromatic to normochromatic erythrocytes was altered by treatment and thus confirmed relevant systemic bone marrow exposure.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered fluopicolide in the micronucleus test in male mice, i.e. in a somatic test system *in vivo*.

3.8.2.4 Anonymous; 2000; M-197230-02-1

Study references:

Anonymous; 2000; In vivo rat liver unscheduled DNA synthesis (DNA repair) test Code: AE C638206 00 1C99 0005; M-197230-02-1

Deviations:	Deviations from the current OECD guideline (486, 1997): None.

Executive Summary:

The potential of technical fluopicolide to induce DNA damage and repair as evidenced by unscheduled DNA synthesis in rat hepatocytes following a single oral dose was tested.

Two groups of four male Sprague Dawley rats were given a single oral dose of either 0, 600 or 2000 mg/kg bw fluopicolide in 1% w/v aqueous methyl cellulose. The higher dose level corresponded to the international regulatory limit dose for such tests. A concurrent negative control group was treated with the vehicle (1% w/v aqueous methylcellulose) and a positive control group was treated with dimethylnitrosamine at 4 mg/kg bw (for the 2 hour expression) or 2-acetylaminofluorene at 50 mg/kg bw (for the 14 hour expression). Hepatocytes were isolated by enzymatic dissociation at 2 or 14 hours after exposure of the animals to the test substance. Four animals were assessed at each experimental point with the exception that only two animals from the positive control group were assessed at each expression time.

The isolated hepatocytes were allowed to attach to glass coverslips and were cultured *in vitro* with (methyl-^{3}H)thymidine at 10 µCi/mL for four hours to 'radiolabel' DNA undergoing repair replication. The hepatocytes were 'chased' for 24 hours with unlabelled thymidine then they were fixed and processed for autoradiography. DNA repair was assessed by comparing the labelling levels of hepatocyte nuclei from treated animals with control values and with the accompanying cytoplasmic labelling levels (usually a total of 150 cells per animal were examined).

Fluopicolide did not cause any significant increases in either the gross nuclear grain count or the net nuclear grain count (i.e. the gross nuclear grain count minus the cytoplasmic grain count) at any dose level at either sampling time.

Positive control group animals showed a large statistically significant increase in the net nuclear grain count, which was accompanied by a large increase in the gross nuclear grain count.

Thus, it is concluded that fluopicolide did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241067/1 & PP/241024/2

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose
Positive control:	Dimethylnitrosamine (4 mg/kg bw for the 2 hour expression) 2- acetylaminofluorene (50 mg/kg bw for the 14 hour expression)

3. Test animals

Species:	Male rat
Strain:	Outbred albino Hsd/Ola Sprague-Dawley rats
Age:	Approx. 6 weeks
Weight at start:	140-160 g
Source:	Harlan Olac UK Ltd, Bicester, Oxon, England.
Acclimation period:	Yes
Diet:	Special Diet Services rat and mouse maintenance diet No. 1 (RM1(E) SQC expanded pellet)
Water:	Tap water
Housing:	Plastic disposable cages with a stainless steel grid top and maintained in a controlled environment
Temperature:	19-25 °C
Humidity:	30-70%
Air changes:	20 times/hour
Photoperiod:	12 hours

4. Test substance doses

Information received from a previous experiment indicated that 2000 mg/kg bw, the international regulatory limit dose for the rat liver DNA repair (UDS) test, was tolerated. Therefore, a preliminary toxicity test was not performed.

Doses of 0, 600 and 2000 mg/kg bw were chosen for use in the DNA repair test.

B. Test performance

Experimental phase: May 25 to August 15, 2000

1. Treatment and sampling times

Doses of 0, 600 and 2000 mg/kg bw were chosen for use in the DNA repair test. More than the required minimum of four animals (two for positive controls) were treated at each experimental point to allow for possible mortalities or technical difficulties encountered during perfusion. Two expression times were utilised in order to allow for variations in the rate of absorption, metabolism and accumulation of DNA damage.

The experimental design is shown below.

Table 3.8.2.4- 1: UDS *in vivo* test design

Experimental group	Dose in mg/kg bw	No. of animals	
		2 hour*	14 hour*
Negative control	0	4 (5)	4 (5)
Fluopicolide	600	4 (5)	4 (5)
	2000	4 (5)	4 (5)
Positive control			
Dimethylnitrosamine	4	2 (3)	Not done
2-Acetylamino-fluorene	50	Not done	2 (3)

* No. of animals for hepatocyte cultures (no. of treated animals)

Solutions of the test substance were freshly prepared on the day of use (using identical methods for each phase of the test) and were diluted to the required concentration in 1% w/v aqueous methylcellulose obtained from Colorcon, batch number MK02012N01.

Stability and homogeneity of the test substance in the vehicle at concentrations of 60 and 200 mg/mL were determined via an HPLC method using UV detection. Mean results for concentration were within the range 87.3 – 108.8% (acceptable range: ± 20% of nominal), with homogeneity at both concentrations on day 1 being within the range of 80 – 120% of nominal and stability declining by less than 12 % over 14 hours storage at room temperature.

All animals in all groups were dosed orally by gastric intubation with the standard dose volume of 10 mL/kg bw. Animals in the negative control group were treated with the vehicle, 1% w/v aqueous methylcellulose. Animals in the positive control group were treated orally with dimethylnitrosamine at 4 mg/kg bw for the 2 hour expression or 2-acetyIaminofluorene at 50 mg/kg bw for the 14 hour expression.

2. UDS test

Hepatocyte isolation and culture:

At the appropriate time after exposure (2 or 14 hours) each animal was killed by exposure to an increasing concentration of carbon dioxide. The liver was exposed and the hepatic portal vein was cannulated using a 18 gauge 1¼" Angiocath intravenous catheter placement unit (B-D 3828721). The liver was perfused by this cannulation and via a bubble trap using a peristaltic pump set at a flow rate of 10 mL/min.

Perfusing media were held in a water bath at approx. 42 °C to give a temperature of approx. 37 °C at the outlet. The liver was initially perfused with EGTA solution for 5 minutes to deplete the liver of calcium ions and reduce cellular adhesion. Excess pressure on the liver was avoided by making a small puncture in the subhepatic vena cava just below the right renal vein. The liver was allowed to drain freely throughout the perfusion. It was then perfused with collagenase solution for 10 minutes. The liver was then excised and placed in a petri dish with a further aliquot of collagenase solution. Liver cells were combed into suspension using forceps and scissors then filtered through nylon bolting cloth (200 µm mesh). The hepatocytes were partially purified by differential centrifugation and finally resuspended in Williams' medium E, complete (WEC).

A viable cell count was performed after diluting an aliquot of the cells with an equal volume of trypan blue solution. Normally, mean viability values of about 85% are routinely obtained in this laboratory.

The viability of the cultures is not an absolute determinant of the validity of the experiment, subsequent attachment and washing stages tend to remove non-viable cells. Results are largely independent of the initial viability of the cultures. The viable cell yield was also calculated.

The isolated cells were suspended in WEC at a density of approximately 0.2 x 10^6 cells/mL. This cell suspension was dispensed in 2 mL aliquots into the 35 mm diameter wells of multi-well tissue culture plates, each well containing a sterile 22 mm diameter No. 1½ glass coverslip. Twelve replicate cultures were initiated per animal. The cultures were incubated at 37 °C in a humid atmosphere containing 5% carbon dioxide for 90 minutes to allow hepatocytes to attach to the coverslips. After this attachment period the supernatant medium was removed and the cells were gently rinsed with one wash of Williams' medium E, incomplete (WEI).

The medium was then replaced with WEI containing high specific activity (methyl-^{3}H)thymidine (Amersham International TRK 686, batch number 200; specific activity 79.0 Ci/mmol) at a final activity of 10 µCi/mL. The cultures were incubated in this medium for a period of 4 hours. After this labelling period, the supernatant medium was removed and replaced by WEI containing 250 µM cold (unlabelled) thymidine (TdR). The cultures were then incubated for a 'chase' period of 24 hours. This additional culture period helps to wash out excess radiolabel and improves cell morphology thus facilitating subsequent grain count analysis of autoradiographs.

Cell harvest:

After the 24 hour cold chase with thymidine, coverslips with attached cells were removed from the culture medium, given three 5 minute washes in Hanks' balanced salts solution then fixed in 2.5 % v/v acetic acid in ethanol (2 washes each of 5 minutes) and allowed to air dry. They were mounted on glass microscope slides, with the cell layer uppermost, using DPX mountant. The mountant was allowed to harden at approx. 37 °C.

Autoradiography:

Autoradiographs were prepared from six cultures per animal; slides from the remaining six cultures per animal were held temporarily in reserve in case of any technical problems with the first set of autoradiographs. Any unused slides were discarded on completion of the study.

Ilford K2 emulsion was applied to the slides in the dark room working under a 25 watt Kodak Number 1 red safelight. The emulsion was melted then diluted with an equal volume of water containing 4% v/v glycerol. The melted emulsion was placed in a dipping chamber and held at approx. 43 °C. Each slide was in turn dipped into the emulsion, withdrawn and held vertically for a few seconds, then excess emulsion was wiped off the back of the slide which was placed on a chilled metal plate for a few minutes to allow title emulsion to gel. The slides were partially dried in a gentle stream of air for approximately one hour then they were sealed in a light-tight box containing desiccated silica gel and allowed to dry overnight at room temperature. The silica gel was renewed and the autoradiographs exposed for a further 13 days at approx. 4 °C.

After the total exposure period of 14 days the autoradiographs were allowed to warm to room temperature for several hours then developed:

- Kodak D-19 developer 5 minutes at 15 °C
- 0.5 % v/v acetic acid 1 minute at 15-20 °C
- Kodak T-max 5 minutes at 20 °C
- Running tap water 20 minutes at 15-20 °C
- Distilled water 5 minutes at 15-20 °C

The slides were stained in Mayers' Haemalum (BDH 35060 4T) for 1 minute, rinsed in distilled water, washed in running tap water and then allowed to air dry.

Examination of the slides:

The stained autoradiographs were examined under code using a Zeiss Photomicroscope II connected to a dedicated Sorcerer (Perceptive Instruments) image analysis system via a solid state video camera.

Initially, autoradiographs were examined for signs of test substance-induced toxicity (e.g. pyknosis, reduced levels of radiolabelling). Three slides per animal were examined using high-magnification, oil-immersion optics; the remaining autoradiographs prepared from each animal were held as reserves in case of any technical problems with the three slides initially examined.

The image analyser was used in the area count mode and the count obtained was automatically converted to an equivalent grain count using a constant conversion factor of 0.1655 grains per pixel. This method is believed to give the most accurate assessment of labelling levels because actual grain counting methods do not take into account variation in grain size or overlapping of grains at the high density seen in the hepatocyte UDS system. Usually 50 hepatocytes over several widely separated randomly chosen fields of view from each of three cultures per animal were analysed. Only results from hepatocytes not in S-phase with a normal morphology (i.e. not pyknotic or lysed) without staining artifacts or debris were recorded. For each cell the number of silver grains overlying the nucleus was estimated using the image analysis system, then the number of silver grains in an equivalent and most heavily-grained, adjacent area of cytoplasm was estimated. The cytoplasmic grain count was subtracted from the gross

nuclear grain count to give the net nuclear grain count. Mean grain counts were calculated for each slide examined. For slides snowing a strong response, i.e. where the mean net grain count was in excess of 10, only 25 cells were examined. The number of cells with a net gram count of greater than or equal to five was recorded in the raw data.

3. Evaluation

Both gross and net nuclear grain counts for treated animals were compared with vehicle control counts using classical one-way analysis of variance followed by a Student's t test with an appropriate transformation of values if indicated by excessive variance (Snedecor and Cochran[8]).

A positive response is normally indicated by a substantial dose-associated statistically significant increase in the net nuclear grain count which is accompanied by a substantial increase in the gross nuclear grain count over concurrent control values.

A negative response is indicated by a mean net nuclear grain count which is not significantly greater than the concurrent control.

An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

4. Statistical methods

No details given in the report, only a publication given under References: Snedecor and Cochran[8].

II. Results and Discussion

A. DNA repair test

No mortalities or clinical signs of toxicity were obtained after treatment of the animals with fluopicolide, the vehicle control or the positive controls.

The results of the DNA repair test using the 2 hour and 14 hour expression periods are presented in Table 3.8.2.4- 2 and Table 3.8.2.4- 3, respectively.

Table 3.8.2.4- 2: Mean nuclear and cytoplasmic grain counts at the 2 hour expression

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count	Mean cytoplasmic grain count	Mean net nuclear grain count
Fluopicolide	2000	12.8	20.6	-7.8
	600	13.1	20.5	-7.4
	0	14.9	23.6	-8.7
Positive control (Dimethylnitrosamine)	4	45.7***	13.7	32.0***
Historical vehicle control mean	-	13.2	-	-1.7

*** $p < 0.001$ statistically significant (Student's t test)

Results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments.

[8] Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th ed., Iowa State University Press

Table 3.8.2.4- 3: Mean nuclear and cytoplasmic grain counts at the 14 hour expression

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count	Mean cytoplasmic grain count	Mean net nuclear grain count
Fluopicolide	2000	16.7	27.1	-10.4
	600	13.4	23.4	-10.0
	0	16.2	24.8	-8.6
Positive control (2-Acetylamino-fluorene)	4	41.9***	13.4	28.6***
Historical vehicle control mean [#]	-	13.2	-	-1.7

*** $p \leq 0.001$ statistically significant (Student's t test)

[#] Results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments.

None of the slides from any of the animals showed any obvious signs of toxicity. For animals treated with the vehicle control or the test substance a total of 150 hepatocytes were scored per animal. Since a positive response was obvious for animals treated with dimethylnitrosamine and 2-acetylaminofluorene, it was only necessary to score 75 hepatocytes per animal for the positive control group.

Animals treated with the test substance did not show any significant increase in the gross or net nuclear grain count at any dose level at either the 2 or 14 hour expression time. Grain counts were similar to vehicle control values and were within the range of historical control values (see Table 3.8.2.4- 2, Table 3.8.2.4- 3 and Figure 3.8.2.4- 1).

Animals treated with dimethylnitrosamine or 2-acetylaminofluorene showed a significant increase in the net nuclear grain count which was accompanied by a substantial increase in the gross nuclear grain count.

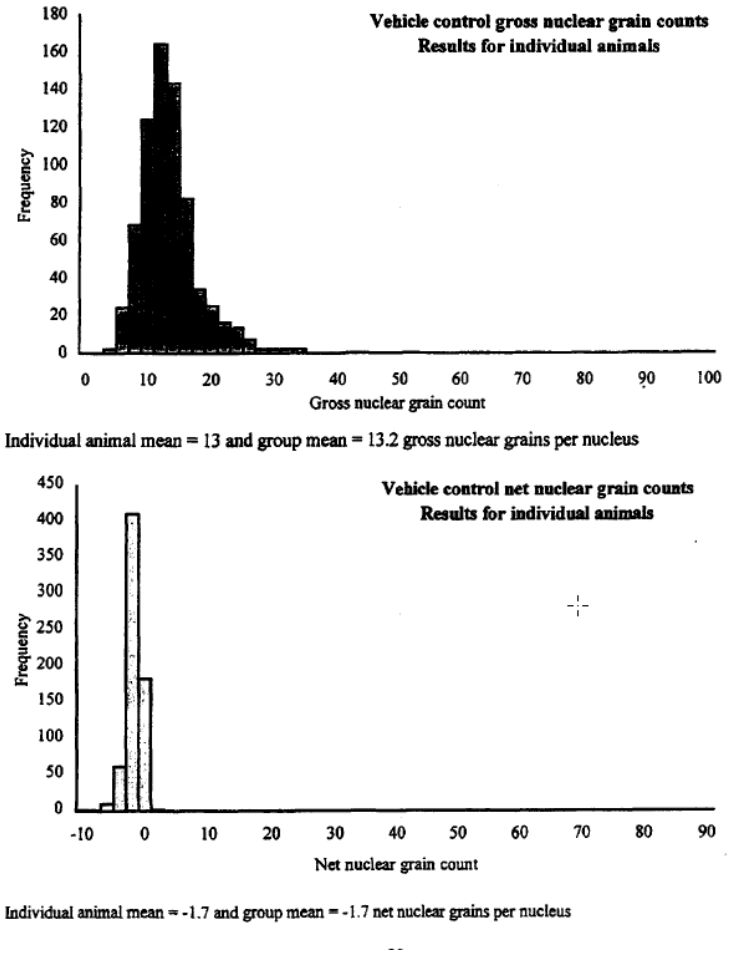


Figure 3.8.2.4- 1: Frequency distribution of historical vehicle control data (results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments).

III. Conclusion

Fluopicolide did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

3.8.2.5 Anonymous; 2018; M-635020-01-1

Study reference:

Anonymous; 2018; In vivo mammalian alkaline comet assay - Fluopicolide, technical; M-635020-01-1

Deviations:	Deviations from the current OECD guideline (489, 2016): None.

Executive Summary:

The test substance fluopicolide was evaluated for its genotoxic potential in the Comet assay to induce DNA damage in liver and kidney cells of male mice. 1% (w/v) methylcellulose (400 to 800 cPs) in deionized water was selected as the vehicle. Test and/or control substance formulations were administered at a dose volume of 10 mL/kg/dose by oral gavage. The dose levels tested were 500, 1000 and 2000 mg/kg bw.

The test substance gave a negative (non-DNA damaging) response in this assay in liver and kidney for males in % Tail DNA. None of the test substance-treated animal slides had significant increases in the % Tail DNA compared to the respective vehicle controls. The vehicle control % Tail DNA was within the testing facility's historical range, and the positive control showed a statistically significant increase in % Tail DNA compared to the vehicle control. Thus, all criteria for a valid assay were met for liver and kidney.

Under the conditions of this study, the administration of fluopicolide at doses up to and including 2000 mg/kg bw did not cause any significant increase in DNA damage in liver and kidney relative to the concurrent vehicle control.

Therefore, fluopicolide was concluded to be negative in the *in vivo* Comet Assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	98.2%
Batch no.:	AE C638206-01-29

2. Vehicle and/or positive control

Vehicle:	1% (w/v) methylcellulose (400 to 800 cPs) in deionized water
Positive control:	Methyl methanesulfonate (MMS)

3. Test animals

Species:	Mice (male)
Strain:	Hsd:ICR (CD-1) mice
Age:	Approx. 6 weeks
Weight at start:	23.2 – 36.5 g
Source:	Envigo RMS, Inc., Frederick, MD
Acclimation period:	Yes
Diet:	Certified laboratory rodent chow (Envigo 2018C Teklad Global 18% Protein Rodent Diet) ad libitum.
Water:	Tap water (U.S. EPA drinking water standards)
Housing:	Animals of the same sex were housed up to five per Micro-Barrier cage. Cages were placed on racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system
Temperature:	~22 °C (calculated from °F)
Humidity:	50 ± 20%
Air changes:	10 times/hour
Photoperiod:	12 hours

4. Test substance doses

Doses of 0, 500, 1000 and 2000 mg/kg bw were chosen for this *in vivo* study. Dose formulation analysis demonstrated that the prepared samples were 91.2, 86.9, and 82.0-82.9% of target, respectively, with ≤ 10.0% RSD; indicating that the formulations were accurately prepared and homogenous. No test item was detected in the vehicle control sample.

Methyl methanesulfonate (MMS) was administered at a dose level of 40 mg/kg/day.

B. Test performance

Experimental phase: May 30 to June 27, 2017.

1. Treatment times

All animals of the control and treatment groups were dosed on two consecutive days (Study Days 1 and 2) with the vehicle / with test substance. The second dose occurred approx. 21 hours after the first dose.

Animals of the positive control group were dosed with the positive control (Methyl methanesulfonate) once approx. 3 to 4 hours prior to euthanasia on day 2. Animals initially were treated (on Day 1 at T=0) with the test or control substances and euthanized at the appropriate time as described in Table 3.8.2.5-1.

Table 3.8.2.5- 1: Comet assay design

Treatment group	Dose level (mg/kg bw/day)	Dose volume[A] (mL/kg/	Route of application[B]	No. of animals per sex	Euthanasia time (hours after treatment)
Negative control (vehicle)	0	10	Oral, gavage	6	3-4
Fluopicolide	500	10	Oral, gavage	6	3-4
	1000	10	Oral, gavage	6	3-4
	2000	10	Oral, gavage	6	3-4
Positive control (MMS)[C]	40	10	Oral, gavage	3	3-4

A Based upon individual body weight

B Using appropriately sized disposable polypropylene syringes with gastric intubation tubes (needles). The route has been routinely used and is widely-accepted for use in the mammalian alkaline comet assay

C Animals dosed only once on day 2.

2. Tissue collection for Comet assay:

All animals were euthanized 3 to 4 hours after the last dose (Study Day 2) by CO_2 asphyxiation, and then, the following was performed:

- Animals were dissected and the liver and one kidney were removed and collected.
- A section of the liver and kidney were cut and placed in formalin for possible histopathology analysis.
- Another section of the liver and remaining kidney was placed in chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO) and was used in preparation of cell suspensions and Comet slides.

3. Preparation of cell suspensions and Comet slides:

A portion of each dissected liver and kidney were placed in 3 mL of cold mincing buffer, then the liver and kidney were finely cut (minced) with a pair of fine scissors to release the cells. Each cell suspension was strained through a Cell Strainer into a pre-labeled 50 mL polypropylene conical tube and the resulting liver and kidney cell suspensions were placed on wet-ice. An aliquot of the suspensions were used to prepare the Comet slides.

Preparation of slides:

From each liver and kidney suspensions, an aliquot of 2.5 µL was mixed with 75 µL (0.5 %) of low melting agarose. The cell/agrose suspension was applied to microscope slides commercially available pre-treated multi-well slides. Commercially purchased multi-well slides were used and these slides have 3 individual circular areas, referred to as wells in the text below. The slides were kept at 2-8 °C for at least 15 minutes to allow the gel to solidify. Slides will be identified with a random code that reflects the study number, group, animal number, and organ/tissue. At least two Trevigen, Inc 3-well slides were prepared per animal per tissue. Three wells were used in scoring and the other wells were designated as a backup. Following solidification of agarose, the slides were placed in jars containing lysis solution.

Lysis:

Following solidification of agarose, the slides were submerged in a commercially available lysis solution supplemented with 10% DMSO on the day of use. The slides were kept in this solution at least overnight at 2-8 °C.

Unwinding:

After cell lysis, slides/wells were washed with neutralization buffer (0.4 M tris hydroxymethyl aminomethane in purified water, pH ~7.5) and placed in the electrophoresis chamber. The chamber reservoirs were slowly filled with alkaline buffer composed of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water. The pH was > 13. All slides remained in the buffer for ~20 minutes at 2-10 °C and protected from light, allowing DNA to unwind.

Electrophoresis:

Using the same buffer, electrophoresis was conducted for 30 minutes at 0.7 V/cm, at 2-10 °C and protected from light. The electrophoresis time was constant for all slides.

Neutralization:

After completion of electrophoresis, the slides were removed from the electrophoresis chamber and washed with neutralization buffer for at least 10 minutes. The slides (gels) were then dehydrated with 200-proof ethanol for at least 5 minutes, then air dried for at least 4 hours and stored at room temperature with desiccant.

Staining:

Slides were stained with a DNA stain (i.e., Sybr-gold™) prior to scoring. The stain solution was prepared by diluting 1 µL of Sybr-gold™ stain in 15 mL of 1xTBE (tris-boric acid EDTA buffer solution).

4. Evaluation of DNA damage:

Three slides/wells per organ/animal were used. Fifty randomly selected, non-overlapping cells per slide/well were scored resulting in a total of 150 cells evaluated per animal for DNA damage using the fully validated automated scoring system Comet Assay IV from Perceptive Instruments Ltd. (UK).

The following endpoints of DNA damage were assessed and measured:

- Comet Tail Migration; defined as the distance from the perimeter of the Comet head to the last visible point in the tail.
- % Tail DNA; (also known as % tail intensity or % DNA in tail); defined as the percentage of DNA fragments present in the tail.
- Tail Moment (also known as Olive Tail moment); defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length) / 100)].

Each slide was also examined for indications of cytotoxicity. The rough estimate of the percentage of "clouds" was determined by scanning 150 cells per animal, when possible (percentage of "clouds" was calculated by adding the total number of clouds for all slides scored, dividing by the total number of cells scored and multiplying by 100). The "clouds", also known as "hedgehogs", are a morphological indication of highly damaged cells often associated with severe genotoxicity, necrosis or apoptosis. A "cloud" is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost nonexistent. "Clouds" with visible gaps between the nuclei and the comet tail were excluded from comet image analysis.

The Comet slides, which are not permanent (the slides can be affected/damaged by environmental storage conditions), will be discarded prior to report finalization.

5. Histopathology Evaluation

A portion of each dissected tissue was placed in formalin (10% neutral-buffered formalin) for possible histopathology analysis. Per the study protocol, histopathology evaluation was not performed since biologically significant increases in DNA damage were not observed.

All unused tissues/slides/blocks and tissue samples saved for histopathology will be discarded prior to finalization of the report.

6. Statistical Analysis

The median value of 150 counts of % Tail DNA, Tail moment and Tail migration were determined and presented for each animal in each treatment group for each organ. The mean and standard deviation of the median values only for % Tail DNA were presented for each treatment group. Statistical analysis was performed only for % Tail DNA.

In order to quantify the test substance effects on DNA damage, the following statistical analysis was performed:

- The use of parametric or non-parametric statistical methods in evaluation of data was based on the variation between groups. The group variances for % Tail DNA generated for the vehicle and test substance groups were compared using Levene's test (significant level of $p \leq 0.05$). If the differences and variations between groups were found not to be significant, a parametric one-way ANOVA followed by a Dunnett's post-hoc test was performed (significant level of $p < 0.05$).
- A linear regression analysis was conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$).
- A pair-wise comparison (Student's T-test, $p \leq 0.05$) was used to compare the positive control group to the concurrent vehicle control group.

7. Criteria for Determination of a Valid Assay

The group mean for the % Tail DNA for each tissue analyzed should ideally be within the distribution of the historical negative control database for that tissue, and the positive control group must be significantly greater than the concurrent vehicle control group ($p \leq 0.05$) and should be compatible with those observed in the historical positive control data base.

At least three test substance doses were tested for at least one sampling time. At least five animals per group were available for analysis (with the exception of the Comet positive control animal group).

At least 150 cells/organ/animal were scored for % Tail DNA. In addition, at least 150 cells per organ per animal were scored to determine the proportion of hedgehogs as an indication of cytotoxicity.

The maximum dose evaluated for Comets must

a) be the MTD or MFD, or

b) in the absence of cytotoxicity or MFD, a dose of 2000 mg/kg bw/day (limit dose) was used.

8. Evaluation of Test Results

The test substance was considered to have induced a positive response if

a) at least one of the group mean for the % Tail DNA of the test substance doses exhibited a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and

b) when multiple doses were examined at a particular sampling time, the increase was dose-related ($p \leq 0.01$) and

c) results of the group mean or of the individual animals of at least one group were outside the distribution of the historical negative control database for that tissue.

The test substance was considered to have induced a clear negative response if none of the criteria for a positive response were met and there was direct or indirect evidence supportive of exposure of, or toxicity to, the target tissue.

If the response was neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data were evaluated by expert judgment and/or further investigations. Any additional work was only carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set precluded making a conclusion of positive or negative, at which time the response was concluded to be equivocal. In such cases, the Study Director used sound scientific judgment and reported and described all considerations.

Biological significance of a positive, negative and equivocal result was based on the information on cytotoxicity at the target tissue. Where positive or equivocal findings were observed solely in the presence of clear evidence of cytotoxicity (e.g. histopathology evaluation, changes in clinical chemistry measures), the study was concluded as equivocal for genotoxicity unless there was enough information that was supportive of a definitive conclusion. In the case of a negative study outcome where there were signs of toxicity at all doses tested, further study at non-toxic doses may be advisable.

9. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data included but not limited to the following (version numbers are maintained in the system documentation):

Table 3.8.2.5- 2: Electronic Data Collection Systems

System	Purpose
- LIMS Labware System	- Test Substance Tracking
- Excel (Microsoft Corporation)	- Calculations/Randomization
- Minitab	- Statistics
- Kaye Lab Watch Monitoring System (Kaye GE)	- Environmental Monitoring
- BRIQS	- Deviations and audit reporting
- Comet Assay IV(Perceptive Instruments)	- Scoring slides

II. Results and Discussion

A. Comet assay

No mortalities occurred and no clinical sign of toxicity were obtained at doses of 500 and 1000 mg/kg bw. At 2000 mg/kg bw piloerection was observed. No effect on the body weight development was seen.

Liver:

The mean % Tail DNA in liver cells are summarized for each treatment group and presented in the following table.

Table 3.8.2.5- 3: % tail DNA (group mean) in liver cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose			
Treatment (mg/kg bw)	**No. of animals**	**Group mean (% of Clouds)**	**Tail DNA (%)[A] (Mean±SD)**
Negative control	6	0.3	0.68±0.42
Fluopicolide (500)	6	4.0	1.16±0.68
Fluopicolide (1000)	6	2.5	1.32±0.42
Fluopicolide (2000)	6	2.8	1.62±1.08
MMS (40)[B]	3	23.7	28.84±1.42*
HCD – negative control	-	-	0.012 – 3.51

[A] Mean of 3 or 6 animals

[B] Methyl methanesulfonate (MMS), positive control for Comet assay, orally administered only once at 3 to 4 hours prior to organ collection on day 2.

SD = Standard Deviation

HCD: Range of studies performed 2011 to 2015

* $p \leq 0.05$ (Student's t-test)

Median values for the % Tail DNA, Tail moment and Tail migration (µm) for liver cells are calculated per 150 cells for each animal and are presented in Table 3.8.2.5- 4.

Table 3.8.2.5- 4: DNA damage data in liver cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose								
Treatment	**Animal No.**	**Cloud %**	**Individual Comet Assay Data**			**Mean Comet Assay Data[A]**		
			Tail Moment	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Negative control	51	0	0.13	24.67	0.52	0.12	19.87	0.49
			0.13	20.97	0.50			
			0.11	13.98	0.44			
	52	0	0.04	12.75	0.19	0.05	14.39	0.26
			0.08	16.86	0.39			
			0.03	13.57	0.21			
	53	0	0.14	26.32	0.55	0.16	27.00	0.70
			0.14	30.02	0.63			
			0.19	24.67	0.92			
	54	1	0.05	8.63	0.25	0.05	12.75	0.27
			0.04	12.75	0.23			
			0.06	16.86	0.32			
	55	0	0.31	17.27	1.68	0.22	18.09	1.24
			0.17	16.04	1.03			
			0.19	20.97	1.00			
	56	1	0.27	31.66	1.63	0.22	25.63	1.14
			0.18	24.26	0.72			
			0.21	20.97	1.06			
Fluopicolide (500 mg/kg bw)	57	3	0.18	31.25	0.92	0.18	25.90	0.93
			0.20	25.49	0.98			
			0.15	20.97	0.89			
	58	3	0.13	19.33	0.76	0.11	19.19	0.65
			0.08	19.33	0.45			
			0.14	18.91	0.73			
	59	7	0.20	23.85	0.85	0.21	22.20	1.02
			0.21	22.62	1.14			
			0.23	20.15	1.06			
	60	3	0.28	27.96	1.87	0.19	21.11	1.18
			0.16	16.86	0.74			
			0.14	18.50	0.92			
	61	5	0.28	25.08	1.39	0.46	26.45	2.49
			0.52	28.78	2.97			
			0.57	25.49	3.11			
	62	3	0.14	18.50	0.71	0.14	18.37	0.69
			0.09	17.68	0.44			
			0.20	18.91	0.92			

Samples collected 3 to 4 hours post-last dose								
Treatment	**Animal No.**	**Cloud %**	**Individual Comet Assay Data**			**Mean Comet Assay Data[A]**		
			Tail Moment	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Fluopicolide (1000 mg/kg bw)	63	2	0.32	18.50	2.17	0.38	17.13	2.58
			0.35	17.68	1.80			
			0.46	15.21	3.77			
	64	1	0.20	11.92	1.27	0.22	14.12	1.36
			0.19	13.98	1.30			
			0.28	16.45	1.50			
	65	2	0.08	14.39	0.55	0.10	16.31	0.57
			0.11	16.45	0.58			
			0.12	18.09	0.59			
	66	3	0.24	20.56	1.37	0.18	17.96	1.04
			0.16	18.09	0.95			
			0.12	15.21	0.79			
	67	3	0.12	17.68	0.91	0.24	19.33	1.68
			0.27	19.33	1.98			
			0.34	20.97	2.15			
	68	4	0.07	9.46	0.60	0.12	15.35	0.70
			0.13	16.04	0.78			
			0.17	20.56	0.72			
Fluopicolide (2000 mg/kg bw)	69	3	0.23	15.21	1.37	0.19	15.90	1.17
			0.19	14.39	1.25			
			0.15	18.09	0.90			
	70	3	0.01	11.10	0.05	0.03	11.38	0.20
			0.04	10.28	0.27			
			0.04	12.75	0.27			
	71	3	0.16	16.86	0.69	0.19	19.74	1.00
			0.19	23.44	1.08			
			0.22	18.91	1.22			
	72	4	0.44	22.20	2.20	0.42	19.74	2.53
			0.25	14.80	2.15			
			0.55	22.20	3.23			
	73	3	0.23	17.68	1.20	0.28	17.54	1.69
			0.20	16.45	1.46			
			0.42	18.50	2.40			
	74	1	0.44	23.44	2.37	0.55	22.07	3.16
			0.51	19.33	3.47			
			0.69	23.44	3.63			

Samples collected 3 to 4 hours post-last dose								
Treatment	**Animal No.**	**Cloud %**	**Individual Comet Assay Data**			**Mean Comet Assay Data[A]**		
			Tail Moment	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Positive control (MMS)[B]	75	15	6.03	42.35	30.21	6.33	43.31	29.77
			6.60	41.53	28.92			
			6.36	46.05	30.17			
	76	27	5.91	37.01	28.27	5.98	38.51	29.55
			5.70	38.65	29.31			
			6.33	39.88	31.09			
	77	29	4.00	39.47	21.64	5.42	43.72	27.20
			5.52	45.64	28.54			
			6.73	46.05	31.43			

[A] Mean of median of 150 cells scored per animal

[B] Orally administered only once at 3 to 4 hours prior to organ collection on day 2.

The scoring results and a statistical analysis of data indicated the following:

The presence of 'clouds' in the test substance groups was ≤ 4.0%, which was higher than the % of clouds in the vehicle control group (0.3%).

Group variances for mean of medians of the % Tail DNA in the vehicle and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p > 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

No statistically significant response in the % Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent vehicle control group (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$).

No dose-dependent increase in the % Tail DNA was observed across three test substance doses (regression analysis, $p > 0.01$).

The positive control (Methyl methanesulfonate) induced a statistically significant increase in the mean % Tail DNA in liver cells as compared to the vehicle control groups (Student's t test, $p \leq 0.05$).

In the vehicle control group, % Tail DNA was within the historical vehicle control range for the liver.

Kidney:

The mean % Tail DNA in kidney cells are summarized for each treatment group and presented in Table 3.8.2.5- 5.

Table 3.8.2.5- 5: % Tail DNA (group mean) in kidney cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose			
Treatment (mg/kg bw)	**No. of animals**	**Group mean (% of Clouds)**	**Tail DNA (%)[A] (Mean±SD)**
Negative control	6	3.8	0.43±0.18
Fluopicolide (500)	6	1.5	0.29±0.08
Fluopicolide (1000)	6	1.2	0.27±0.17
Fluopicolide (2000)	6	2.3	0.24±0.11
MMS (40)[B]	3	5.0	19.62±4.20*
HCD – negative control	-	-	0.21 – 1.04

[A] Mean of 3 or 6 animals
[B] Methyl methanesulfonate (MMS), positive control for Comet assay, orally administered only once at 3 to 4 hours prior to organ collection on day 2.
SD: Standard deviation
HCD: Range of studies performed 2014 to 2015
* $p \leq 0.05$ (Student's t-test)

Median values for the % Tail DNA, Tail moment and Tail migration (µm) for kidney cells are calculated per 150 cells for each animal and are presented in Table 3.8.2.5- 6.

Table 3.8.2.5- 6: DNA damage data in kidney cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose								
Treatment	**Animal No.**	**Cloud %**	**Individual Comet Assay Data**			**Mean Comet Assay Data[A]**		
			Tail Moment	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Negative control (vehicle)	51	6	0.06	10.11	0.27	0.09	13.63	0.43
			0.08	13.32	0.51			
			0.12	17.46	0.51			
	52	5	0.11	15.62	0.51	0.10	13.17	0.51
			0.06	10.57	0.35			
			0.13	13.32	0.67			
	53	1	0.02	11.03	0.10	0.04	11.33	0.18
			0.05	13.32	0.26			
			0.04	9.65	0.19			
	54	9	0.05	14.24	0.21	0.05	15.31	0.23
			0.06	16.54	0.25			
			0.04	15.16	0.22			
	55	1	0.08	17.92	0.35	0.12	19.91	0.62
			0.15	23.89	0.84			
			0.14	17.92	0.67			
	56	1	0.07	11.49	0.38	0.10	11.94	0.58
			0.06	11.03	0.26			
			0.18	13.32	1.10			

Samples collected 3 to 4 hours post-last dose								
			Individual Comet Assay Data			Mean Comet Assay Data[A]		
Treatment	**Animal No.**	**Cloud %**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Fluopicolide (500 mg/kg bw)	57	0	0.03	7.81	0.14	0.03	8.58	0.15
			0.01	11.03	0.06			
			0.04	6.89	0.24			
	58	2	0.07	15.16	0.33	0.06	13.32	0.32
			0.04	11.49	0.21			
			0.07	13.32	0.41			
	59	1	0.09	15.16	0.37	0.06	15.62	0.27
			0.05	16.54	0.23			
			0.04	15.16	0.20			
	60	2	0.04	15.16	0.20	0.05	12.10	0.26
			0.02	5.05	0.12			
			0.09	16.08	0.45			
	61	1	0.06	10.11	0.29	0.06	9.95	0.36
			0.06	7.35	0.44			
			0.06	12.40	0.35			
	62	3	0.06	6.89	0.28	0.08	12.10	0.35
			0.13	14.70	0.54			
			0.04	14.70	0.22			
Fluopicolide (1000 mg/kg bw)	63	0	0.06	14.70	0.33	0.11	14.70	0.57
			0.09	13.32	0.52			
			0.19	16.08	0.87			
	64	2	0.03	4.13	0.14	0.05	9.65	0.26
			0.04	9.65	0.18			
			0.09	15.16	0.47			
	65	0	0.02	6.43	0.13	0.05	8.27	0.29
			0.09	11.03	0.50			
			0.05	7.35	0.25			
	66	2	0.01	5.05	0.08	0.02	7.20	0.10
			0.02	6.89	0.12			
			0.02	9.65	0.10			
	67	2	0.03	9.65	0.23	0.05	11.64	0.25
			0.05	11.03	0.25			
			0.05	14.24	0.26			
	68	1	0.02	5.97	0.10	0.02	9.19	0.12
			0.03	12.40	0.13			

Samples collected 3 to 4 hours post-last dose								
			Individual Comet Assay Data			Mean Comet Assay Data[A]		
Treatment	**Animal No.**	**Cloud %**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Fluopicolide (2000 mg/kg bw)	69	5	0.02	9.65	0.13	0.03	11.64	0.15
			0.05	14.24	0.25			
			0.01	11.03	0.05			
	70	1	0.05	8.73	0.25	0.05	11.33	0.32
			0.07	12.40	0.49			
			0.04	12.86	0.21			
	71	0	0.01	7.81	0.05	0.01	6.43	0.07
			0.01	6.89	0.08			
			0.01	4.59	0.07			
	72	6	0.05	16.08	0.23	0.04	13.48	0.20
			0.04	11.94	0.19			
			0.04	12.40	0.17			
	73	1	0.10	15.62	0.43	0.08	16.31	0.36
			0.06	17.00	0.28			
	74	1	0.04	15.62	0.24	0.06	14.24	0.32
			0.04	12.86	0.18			
			0.09	14.24	0.54			
Positive control (MMS)[B]	75	11	2.77	33.54	17.17	2.94	35.38	16.93
			2.78	35.83	15.17			
			3.27	36.75	18.46			
	76	3	4.60	39.05	23.29	4.98	41.50	24.46
			5.26	40.43	25.75			
			5.07	45.02	24.34			
	77	1	2.84	32.62	15.74	3.14	34.46	17.46
			3.43	36.29	19.17			

[A] Mean of median of 150 cells scored per animal
[B] Orally administered only once at 3 to 4 hours prior to organ collection on day 2.

The scoring results and a statistical analysis of data indicated the following:

The presence of 'clouds' in the test substance groups was ≤ 2.3%, which was lower than the % of clouds in the vehicle control group (3.8%).

Group variances for mean of medians of the % Tail DNA in the vehicle and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p > 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

No statistically significant response in the % Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent vehicle control group (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$).

No dose-dependent increase in the % Tail DNA was observed across three test substance doses (regression analysis, $p > 0.01$).

The positive control (Methyl methanesulfonate) induced a statistically significant increase in the mean % Tail DNA in kidney cells as compared to the vehicle control groups (Student's t test, $p \leq 0.05$).

In the vehicle control group, % Tail DNA was within the historical vehicle control range for the kidney.

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

The differences in the incidences of "clouds" between vehicle control and fluopicolide treatment group in liver and kidney are considered incidental artefacts because there was no dose-response and no consistent trend (increased presence of clouds after fluopicolide exposure in liver, decreased presence of clouds after fluopicolide exposure in kidney).

III. Conclusion

Under the conditions of the assay described in this report, fluopicolide was concluded to be negative for the induction of DNA damage in liver and kidney.

3.8.3 Human data

No human data.

3.8.4 Other data

No other data.

3.9 Carcinogenicity

Table 3.9- 1: Summary of long term studies

Study	NOAEL	LOAEL	Findings at LOAEL	Referemce
2 year combined toxicity and carcinogenicity study in rats 0, 50, 200, 750 or 2,500 ppm (equivalent to 0, 2.1, 8.4, 31.5, 109.4 / 0 2.8, 10.8, 41.0, 142.2 mg/kg bw/day (M/F))	200 ppm (8.4/10.8 mg/kg bw/day in M/F)	750 ppm (31.5/41.0 mg/kg bw/day in M/F)	↑ total protein concentration and ↓ A/G ratio (M/F) ↑ liver and kidney weights (M/F) ↓ incidence of mammary masses (F) ↑ increased incidence and/or severity of centrilobular hepatocyte hypertrophy (M/F), incidence and/or severity of cystic degeneration and foci of alteration (M) and ↑ increased incidence of eosinophilic foci of alteration (F) in the liver ↑ incidence of cortical tubular basophilia and hyperplasia of the papillary epithelium (M/F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids week 104 (M)	Anonymous; 2003; M-225616-01-1 Anonymous; 2005; M-263575-01-1
Carcinogenicity study in mice 0, 50, 400, or 3,200 ppm (equivalent to 0, 7.9, 64.5 , 551.0 / 0, 11.5, 91.9, 772.3 mg/kg bw/day M/F)	50 ppm (7.9/11.5 mg/kg bw/day in M/F)	400 ppm (64.5/91.9 mg/kg bw/day in M/F)	↑ liver weights (M/F) ↑ increased incidence of animals bearing liver masses and nodules (M) ↑ increased incidence of hepatocyte hypertrophy (M/F)	Anonymous; 2003; M-225595-01-1 Anonymous; 2005; M-263591-01-1
Fluopicolide - Assessment of hepatocellular proliferation and lack of carcinogenic potential	N/A	N/A	This position paper draws together the mechanistic studies and data available to demonstrate that the increased hepatic tumours in mice are of no relevance to humans.	Anonymous; 2006; M-275342-01-1

M = male F = female

Fluopicolide was administered to Sprague Dawley **rats** in the diet at concentrations of 0, 50, 200, 750 or 2,500 ppm for **2 years**. After a 1-year treatment period, 20 animals/sex/group were killed for assessment of chronic toxicity. In addition, the recovery of any effects seen during the 52-week toxicity phase was assessed in a subsequent 13-week recovery period. After the 2-year treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (60 animals/sex/group). The main target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm after 52 and/or 104 weeks of treatment in males and/or females. Histopathological examination after 52 weeks of treatment indicated a dose related increased incidence and severity (slight to moderate) of centrilobular hepatocyte hypertrophy and an increased incidence and/or severity of cortical tubular basophilia in the kidneys in males at 2500 ppm and to a lesser extend at 750 ppm. At 2,500 ppm, the change in the kidneys was associated with increased incidences of other degenerative changes, including hyaline droplets within the cortical tubules, hyaline tubular casts and granular medullary casts especially in the male animals. After completion of the 13-week recovery period, there was still a slight increase in the severity of cortical tubular basophilia in the kidneys of the males, with all other changes showing full recovery. After 104 weeks an increased incidence and/or severity of cystic degeneration and foci of alteration in males and an increased incidence of eosinophilic foci of alteration in females was additionally detected in the liver at 750 and 2,500 ppm. Secondary to the induced

metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was observed at these dose levels. Secondary to the increased metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was recorded. In addition degenerative changes with increased severties, proliferative changes were recorded in the kidneys after 104 weeks. Hyperplasia of the papillary epithelium at 2,500 and 750 ppm was present at an increased incidence and severity in females and this was usually associated with mineralisation of the papillary/pelvic epithelium (high dose level only). No treatment-related adverse changes were observed at 200 ppm; at this dose a slight increase in hepatocellular hypertrophy in males was the only non-neoplastic finding (an adaptive response secondary to liver enzyme induction), whilst the only neoplastic finding was a decrease in mammary and/or adrenal masses in females (which is not toxicologically relevant). There were no findings at 50 ppm. Therefore, the No Observed Adverse Effect Level (NOAEL) for toxicity was 200 ppm in both males and females (equivalent to 8.4 and 10.8 mg/kg bw/day in males and females, respectively). Furthermore, there was no evidence of carcinogenicity with fluopicolide up to and including the dose level of 2,500 ppm (equivalent to 109.4 and 142.2 mg/kg bw/day, in males and females, respectively).

In a **mouse oncogencity** study, fluopicolide was administered to C57/BL6 mice in the diet at concentrations of 0 (control), 50, 400 and 3,200 ppm for **78 weeks**. After 52-week treatment period, 10 animals/sex/group were killed for assessment of chronic toxicity. After 78-week treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (50 animals/sex/group). Fluopicolide administered daily for 78 weeks produced severe reduction of the body weight gain (-45% in males and –35% in females) at 3,200 ppm indicating that the Maximal Tolerated Dose (MTD) was reached. The target organ identified was the liver. Higher liver weights, enlarged liver, increased number of masses and nodules in the liver were observed at 400 and 3,200 ppm at 52 and 78 weeks. These changes were associated with hepatocellular hypertrophy at 52 and 78 weeks, and high incidence of altered cell foci at 3,200 ppm at 78 weeks. A high incidence of hepatocellular adenoma was observed at 3,200 ppm at 78 weeks in both males and females and to a lesser extend at 52 weeks in females. Therefore, the NOAELs are 50 ppm for toxicity (equivalent to 7.9 mg/kg bw/day and 11.5 mg/kg bw/day in males and females, respectively) and 400 ppm for carcinogenicity (equivalent to 64.5 mg/kg bw/day and 91.9 mg/kg bw/day in males and females, respectively). These benign liver tumors occurred in only one sex, at the highest dose reaching the MTD (severe body weight gain reduction in high dose animals). Moreover, no tumors were observed in other mouse tissues and these tumors did not progress into malignant neoplasia during the lifespan of these animals, no increased incidence of hepatocellular carcinoma was observed in any groups after a 78-week treatment period. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus, secondary to liver enzyme induction, like that of phenobarbital (see Anonymous.; 2006; M-275342-01-1 and study summaries under Section 3.9.4). This MoA is considered of no relevance in humans.

According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017), a carcinogenicity classification of fluopicolide is **not warranted**.

3.9.1 Animal data

3.9.1.1 Anonymou.; 2003; M-225616-01-1 + Anonymou.; 2005; M-263575-01-1

Study references:

Anonymou.; 2003; Combined carcinogenicity and toxicity study by dietary administration to CD rats for 104 weeks AE C638206; M-225616-01-1

+

Anonymou.; 2005; Historical control data for neoplasms in long-term studies in CD rats; M-263575-01-1

Deviations: Deviations from the current OECD guideline (453, 2018):
- Coagulating gland, Harderian gland, vagina and bone marrow were not sampled, fixed or examined histopathologically

These minor deviation(s) are considered not to compromise the results and outcome of the study.

Executive Summary:

The carcinogenic and toxic potential of fluopicolide was assessed in a 104-week dietary study in CrlCD* (SD)IGS BR rats. Groups of 60 male and 60 female rats received in the diet at concentrations of 0, 50, 200, 750 or 2,500 ppm for 104 weeks. An additional 20 male and 20 female rats assigned to each group were sacrificed after completion of 52 weeks of treatment and comprised the Toxicity phase of the study. A further 10 male and 10 female rats assigned to each group were treated for 52-weeks, followed by a 13-week period without treatment to assess the reversibility of any treatment-related findings; these animals constituted the Recovery phase of the study.

The overall achieved dosages for the 52-week toxicity phase were 2.5, 9.8, 37.0 and 125.5 mg/kg bw/day for males and 3.3, 12.9, 48.7 and 163.6 mg/kg bw/day for females receiving 50, 200, 750 and 2,500 ppm, respectively. The overall achieved dosages for the 104-week carcinogenicity phase were 2.1, 8.4, 31.5 and 109.4 mg/kg bw/day for males and 2.8, 10.8, 41.0 and 142.2 mg/kg bw/day for females receiving 50, 200,750 and 2,500 ppm, respectively.

There was no effect of treatment upon mortality. At 2,500 ppm, there was a marked reduction in body weight gain and a reduction in food consumption during week one, in both sexes, with body weight gain continuing to be low in week 2. Subsequent body weight gain was generally lower than controls, particularly in females where food intake continued to be slightly low. At dose levels of 750 and 200 ppm, body weight gain was reduced in week 1 only, but there was no measured effect on food consumption.

There were no ophthalmoscopic changes that were attributable to treatment and no alteration in urinary composition. Low haemoglobin concentrations were recorded throughout the majority of the treatment period in males and females at 2,500 ppm. Occasionally associated with this was a reduction of haematocrit in males and in females and low erythrocyte counts in females. As a consequence of these changes, mean cell haemoglobin was consistently low in males and mean cell haemoglobin concentration and mean cell volumes were also low on most occasions in these animals. No toxicological significant changes in haematological parameters were observed at lower dose levels.

Treatment related biochemical changes in the blood plasma that were detected at 750 and 2,500 ppm comprised high total protein concentrations and low albumin to globulin ratios up to Week 52 in males and females and high albumin concentrations in Week 13 in males; high creatinine concentrations (2,500 ppm only) and a trend towards marginally high total cholesterol concentrations in males; high potassium and calcium concentrations in both sexes in Week 52 and 104. At 200 ppm, biochemical changes in the blood plasma were confined to high total protein concentrations in Week 13 and 26, associated in Week 26 with low albumin to globulin ratio, in males. None of the haemtologic or clinical chemistry changes were present at the end of the recovery period.

The main target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm after 52 and/or 104 weeks of treatment in males and/or females. None of the changes apparent after 52 weeks of treatment were present after completion of the recovery period. After 104 weeks of treatment there was an increase of the thyroid weight at 2,500 ppm in males only. No relevant organ weight changes were detected at lower dose levels.

There were no treatment-related macroscopic changes after 52 weeks of treatment. In the carcinogenicity phase animals, when all animals were considered together, there was a lower incidence of mammary masses in females at ≥ 200 ppm.

Histopathological examination after 52 weeks of treatment indicated a dose related increased incidence and severity (slight to moderate) of centrilobular hepatocyte hypertrophy and an increased incidence and/or severity of cortical tubular basophilia in the kidneys in males at 2,500 ppm and to a lesser extent at 750 ppm. At 2,500 ppm, the change in the kidneys was associated with increased incidences of other degenerative changes, including hyaline droplets within the cortical tubules, hyaline tubular casts and granular medullary casts especially in the male animals. After completion of the 13 week recovery period, there was still a slight increase in the severity of cortical tubular basophilia in the kidneys of the males at 750 and 2,500 ppm, with all other changes showing full recovery. After 104 weeks in addition to centrilobular hepatocyte hypertrophy an increased incidence and/or severity of cystic degeneration and foci of alteration in males and an increased incidence of eosinophilic foci of alteration in females were detected in the liver at 750 and 2,500 ppm. Secondary to the induced metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was observed at these dose levels. Furthermore, in addition to degenerative changes in the kidneys described after 52 weeks, proliferative changes were recorded in the kidneys after 104 weeks. Hyperplasia of the papillary epithelium at 2,500 and 750 ppm was present at an increased incidence and severity in females and this was usually associated with mineralisation of the papillary/pelvic epithelium (high dose level only). At the highest dose level there was an increased incidence and/or severity of acinar atrophic change in the pancreas in both sexes, evident in males as acinar atrophy and in females as acinar replacement by adipose tissue. An increased incidence of acinar atrophy, often associated with reduced colloid, was present in the prostate of males at 2,500 ppm only and is considered secondary to the decreased body weight at this dose level. There were no adverse treatment-related histopathological changes after 52 and 104 weeks of treatment at ≤ 200 ppm. The centrilobular hepatocytic hypertrophy in the livers of 2/60 males at 200 ppm observed during the histopathological examination of the carcinogenicity phase animals at 200 ppm is considered to be adaptive and not an adverse effect of treatment.

The administration of fluopicolide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential.

It is concluded that the administration of fluopicolide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential. The liver and kidneys were identified as target organs. A small number of findings were recorded for animals receiving 200 ppm. However, these were considered non-adverse. This dietary concentration is therefore assumed to be the No Observed Adverse Effect Level (NOAEL) of this study, equivalent to 8.4 mg/kg bw/day in males and 10.8 mg/kg bw/day in females. No treatment-related changes were detected in animals receiving 50 ppm and this dietary concentration is considered the No Observed Effect Level (NOEL) in this study, equivalent to 2.1 mg/kg bw/day in males and 2.8 mg/kg bw/day in females.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rat
Strain:	Crl:CD® (SD)IGS BR
Age:	40 to 44 days for males and 41 to 45 days for females
Weight at start:	159 to 234 g for males and 137 to 196 g for females
Source:	Charles River (UK) Ltd, Margate, Kent, England.
Acclimation period:	Yes
Diet:	Standard rodent diet (Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England), except when urine was being collected and overnight before routine blood sampling
Water:	Potable water taken from the public supply was freely available via polycarbonate bottles fitted with sipper tubes, except when urine was being collected
Housing:	The animals were housed four per sex per cage (Toxicity and Carcinogenicity phase animals) and three or four per sex per cage (Recovery phase animals), unless this number was reduced by mortality or isolation. The cages used were from RS Biotech, Finedon, Northamptonshire, England and were made of a stainless steel body with a stainless steel mesh lid and floor, and were suspended above absorbent paper, which was changed at appropriate intervals.
Temperature:	19 – 25 °C
Humidity:	40 to 70%
Air changes:	Each animal room was kept at positive pressure with respect to the outside by its own supply of filtered fresh air, which was passed to atmosphere and not re-circulated.
Photoperiod:	12 hours

B. Study design

1. In-life dates: August 23, 2000 to April 16, 2003

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 3.9.1.1- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Toxicity phase			
1	0	20	20
2	50	20	20
3	200	20	20
4	750	20	20
5	2,500	20	20
Recovery phase (52 weeks + 13 weeks recovery)			
1	0	10	10
2	50	10	10
3	200	10	10
4	750	10	10
5	2,500	10	10
Carcinogenicity phase (104 weeks)			
1	0	60	60
2	50	60	60
3	200	60	60
4	750	60	60
5	2,500	60	60

The test substance fluopicolide was administered over a period of 52 weeks to animals in the Toxicity and Recovery phases and 104 consecutive weeks to animals in the Carcinogenicity phase.

Animals assigned to the Recovery phase completed a further thirteen weeks without treatment.

Treatment, and the recording of serial observations, continued for all surviving animals throughout the respective necropsy periods.

3. Diet preparation and analysis

Before treatment commenced, a reserve sample of the test substance was taken and placed in a well closed container and stored in the archives.

In order to demonstrate the integrity of the test substance under the storage conditions used at these laboratories, samples were returned to the Sponsor, for re-analysis, at the following intervals: October 2000, April 2001, September 2001, December 2001 and June 2002. The results of these analyses show that the quality of the test substance was satisfactory throughout the study.

Fluopicolide was prepared for administration as a series of graded concentrations in the diet. A pre-mix of a suitable dietary concentration was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equaled this mixture was then added and stirred into the mixture. This doubling-up procedure was followed until a visibly homogenous pre-mix of the required weight was achieved, and the pre-mix blended in a Turbula Mixer. A second pre-mix was formulated from this first pre-mix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,500 and 750 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 200 and 50 ppm formulations were prepared by direct dilution of the second pre-mix with plain diet. Blending was achieved by mixing in a Turbula Mixer. Blending in the Turbula Mixer was set at 100 cycles (approx. six minutes duration).

All dietary concentrations were expressed in terms of the test material as supplied.

Before treatment commenced, the suitability of the proposed mixing procedure was determined and specimen formulations were analyzed to assess the homogeneity and stability of the test substance in the diet. The homogeneity and stability were confirmed, with respect to the level of concentration, for fluopicolide in SDS Rat and Mouse No. 1 maintenance diet at 50 and 2,500 ppm.

Samples of each formulation prepared for administration in Weeks 1, 13, 26, 39, 52, 65, 79, 91 and 103 of treatment were analysed for achieved concentration of the test substance. The treated diets were mixed in batches for each group; different batches for each sex. Achieved concentration samples were taken alternately from batches designated for males and females commencing with males in Week 1. Samples were originally scheduled to be taken in Week 78 but were taken in Week 79. The method of analysis was an adaptation of a method supplied by the Sponsor.

All formulations were shown to be homogenous in the diet and stable at ambient temperature for up to 22 days. The mean concentrations of fluopicolide in Weeks 1, 13, 26, 39, 52, 65, 79, 91 and 103 were within 7% of intended and were therefore considered satisfactory.

4. Statistics

All statistical analyses were carried out separately for males and females. The analyses were carried out using the individual animal as the basic experimental unit.

The following data types were analysed at each time point separately:

Bodyweight, using gains over appropriate study periods
Blood chemistry, hematology and urinalysis
Organ weights, both absolute and adjusted for terminal bodyweight
Pathological findings, for the number of animals with and without each finding

For categorical data, including pathological findings, the proportion of animals was analysed using Fisher's Exact test for each treated group versus the control.

For continuous data, Bartlett's test was first applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared with the Control group, incorporating adjustment for multiple comparisons where necessary.

For body weight gains and organ weights, whenever Bartlett's test was found to be statistically significant, a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

The following sequence of statistical tests was used for clinical pathology data:

If 75% of the data (across all groups) were the same value, for example c, then a frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the control both for i) values <c versus values >=c, and for ii) values <=c versus values >c, as applicable.

If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.

If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Steel's test was performed instead.

Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), l% ($p<0.01$) or 0.1% ($p<0.001$) level. The following statistical cyphers were used throughout the report:

* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$ - using categorical or parametric tests

Details of the methods used are presented in the Statisticians Report.

C. Methods

1. Observations

Animals were inspected visually at least twice daily for evidence of ill-health or reaction to treatment.

Cages and cage-trays were inspected daily for evidence of ill-health amongst the occupants, such as loose faeces. Any deviation from normal was recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

In addition, a more detailed weekly physical examination, which included palpation, was performed on each animal to monitor general health. Particular attention was paid to superficial or palpable swellings, for which the location, size, consistency, time of first observation and subsequent history were recorded.

During the acclimatisation and recovery periods, observations of the animals and their cages were made at least once per day.

Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism.

Animals judged to be in extremis were killed. Animals were also killed to prevent unnecessary or prolonged suffering. A complete necropsy was performed in all cases.

2. Body weight

The weight of each rat was recorded on the day that treatment commenced (Week 0), each week for the first 16 weeks, then once every four weeks (to coincide with the end of a food consumption measurement period) and before necropsy. An exception to this was the body-weight recording for males that was scheduled originally for Week 68 but was re-scheduled to Week 67.

More frequent weight determinations were instituted, when appropriate, for animals displaying ill-health, so that the progress of the observed condition could be monitored.

3. Food intake

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded each week for the first 16 weeks, then for one week in every four. An exception to this was the recording of food consumption for males that was scheduled originally for Week 68 but was re-scheduled to Week 67. From these records the mean weekly consumption per animal (g/rat/week) was calculated for each cage.

4. Ophthalmoscopic examination

Before treatment commenced, the eyes of all animals allocated to the study (including spare animals) were examined by means of a binocular indirect ophthalmoscope. Rejected animals were replaced with animals with no adverse ocular abnormality, selected from the spare animals for the study.

During Week 51 of treatment, the eyes of all surviving Toxicity phase animals from Groups 1 (control) and 5 (2,500 ppm) were examined. During Weeks 78 and 104, 20 male and 20 female animals from the Carcinogenicity phase (with the lowest surviving animal numbers) from Groups 1 and 5 were also examined.

Prior to each examination, the pupils of each animal were dilated using 0.5% tropicamide ophthalmic solution (Mydriacyl, Alcon Laboratories Ltd.). The adnexae, conjunctiva, comea, sclera, anterior chamber, iris (pupil dilated), lens, vitreous and Hindus were examined.

As no treatment-related changes were observed, the examination was not performed during the Recovery phase, nor was it extended to include animals of Groups 2, 3 and 4 (50, 200 and 750 ppm).

5. Laboratory investigations

Haematology, peripheral blood

During Weeks 13, 26 and 52, blood samples were obtained from all surviving Toxicity phase animals.

During Week 13 of recovery, blood samples were obtained from all surviving Recovery phase animals. During Weeks 78 and 104, blood samples were obtained from the 20 male and 20 female Carcinogenicity phase animals with the highest animal numbers remaining in each group. After overnight fasting, animals were held under light general anaesthesia induced by isoflurane and blood samples were withdrawn from the retro-orbital sinus. Blood samples (nominally 0.5 mL) were collected into EDTA as anticoagulant and examined.

The following parameters were measured using a Technicon H-1 haematology analyser:

Table 3.9.1.1- 2: Haematology

Haematocrit (Hct)	Differential leucocyte count
Haemoglobin concentration (Hb)	Neutrophils (N)
Erythrocyte count (RBC)	Lymphocytes (L)
Mean cell haemoglobin (MCH)	Eosinophils (E)
Mean cell haemoglobin concentration (MCHC)	Basophils (B)
	Monocytes (M)
Mean cell volume (MCV)	Large unstained cells (LUC)
Leucocyte count (WBC)	Platelet count (Plt)

Abnormal morphology was flagged by the Technicon analyser. The most common morphological changes, anisocytosis, micro/macrocytosis and hypo/hyperchromasia were recorded as slight, moderate or marked.

Blood film (prepared for all samples) - Romanowsky stain, examined for abnormalities by light microscopy, in the case of flags from the Technicon analyser. Confirmation or a written description from the blood film was made where appropriate.

Additional blood samples (nominally 0.5 mL) were taken into citrate anticoagulant and examined for prothrombin time (PT) - using an ACL 1000 Analyser and IL PT-Fibrinogen reagent, and Activated partial thromboplastin time (APTT) - using an ACL 1000 Analyser and IL APTT reagent.

Haematology, blood smears

During Weeks 52, 78 and 104 blood smears were prepared from samples obtained without the use of anaesthesia from the tail veins of all Carcinogenicity phase animals not subject to the peripheral hematology sampling described above. In Week 104, due to poor survival, smears were only obtained from a small number of females. Animals were not starved prior to sampling.

The smears prepared from animals in Groups 1 (control) and 5 (2,500 ppm) were examined as follows:

Differential leucocyte count - Romanowsky stain and direct visual count, differentiating among the following parameters:

Neutrophils
Lymphocytes
Eosinophils
Basophils
Monocytes
Abnormalities of the blood film

The smears obtained from the lower dose group animals were not examined as no blood disorder was suspected.

Blood chemistry

During Weeks 13, 26 and 52, blood samples were obtained from 10 male and 10 female Toxicity phase animals with the highest animal numbers remaining in each group.

During Week 13 of recovery, blood samples were obtained from all surviving Recovery phase animals. During Weeks 78 and 104, blood samples were obtained from 10 male and 10 female Carcinogenicity phase animals with the highest animal numbers remaining in each group.

Samples were taken, after overnight starvation, from the retro-orbital sinus with the animals held under light general anaesthesia induced by isoflurane. The blood samples (nominally 0.7 ml) were collected into lithium heparin as anticoagulant. All tubes were mechanically agitated for at least two minutes and the sample subsequently centrifuged at 3000 rpm for 10 minutes in order to separate the plasma. After separation, the plasma was examined using a Hitachi 917 Clinical Chemistry Analyser on the following parameters.

Table 3.9.1.1- 3: Blood chemistry

Alkaline phosphatase (ALP) Alanine aminotransferase (ALT) Aspartate aminotransferase (AST) Gamma-glutamyl transpeptidase (gGT) Creatinine phosphokinase (CPK) Total bilirubin (Bili) Urea Creatinine (Creat) Glucose (Glue) Total cholesterol (Chol) Trigtycerides (Trig)	Sodium (Na) Potassium (K) Chloride (Cl) Calcium (Ca) Inorganic phosphorus (Phos) Total protein (Total Prot) Albumin (Alb) Albumin/globulin ratio (A/G Ratio) - calculated from total protein concentration and analyzed albumin concentration

Urinalysis

During Weeks 12, 25 and 51, overnight urine samples were collected from 10 male and 10 female Toxicity phase animals with the highest animal numbers remaining in each group. During Week 13 of recovery, samples were collected from all surviving Recovery phase animals. During Weeks 77 and 103, samples were collected from 10 male and 10 female Carcinogenicity phase animals with the highest animal numbers remaining in each group.

Animals were placed in an individual metabolism cage without food or water at approx. 16.00 hours; urine was collected until approx. 08.30 hours the following day.

The individual samples were examined for the following characteristics:

Table 3.9.1.1- 4: Urinalysis

Appearance (App) - by visual assessment
Volume (Vol)
pH - using a Radiometer PHM 92 pH meter
Specific gravity (SG) - using Atago UR-1 digital refractometer
Protein (Prot) - using Hitachi 917 Clinical Chemistry Analyser
Glucose (Glue), ketones (Keto), bile pigments (Bill), blood pigments (Blood) by Multistix.

A microscopic examination of the urine sediment was performed. An aliquot of the urine sample was centrifuged and the resulting deposit spread on a microscope slide. The deposit was examined for the presence of the following:

Table 3.9.1.1- 5: Urine sediment analysis

Epithelial cells (Epi)
Leucocytes (Leuc)
Erythrocytes (RBC)
Crystals (Cryst)
Spermatozoa and precursors (Sperm)
Casts
Other abnormal components (Abn)

Urinalysis for investigation of metabolite(s)

During Week 58 of treatment urine samples were obtained from 10 male and 10 female Carcinogenicity phase animals with the lowest animal numbers remaining in Groups 1 and 5. Each animal was placed in an individual metabolism cage without food or water at approx. 16.00 hours; urine was collected until approx. 08.30 hours the following day. The urine was collected over dry ice and stored deep frozen (approx. -20 °C) until analysis.

6. Sacrifice and pathology

Animals killed during the study and those surviving until the end of their scheduled treatment or recovery period were killed by carbon dioxide asphyxiation. The sequence in which the animals were killed after completion of treatment or recovery was selected to allow satisfactory inter-group comparison.

Macroscopic pathology

All animals were subject to a detailed necropsy. After a review of the history of each animal, a full macroscopic examination of the tissues was performed. The cranial roof was removed to allow observation of the brain, pituitary gland and cranial nerves. After ventral mid-line incision, the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were exposed and examined in situ. Any abnormal position, morphology or interaction was recorded.

The requisite organs were weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Any abnormality in the appearance or size of any organ and tissue was recorded and the required tissue samples preserved in appropriate fixative.

Photographs of unusual findings were taken at the discretion of the necropsy supervisor. These photographs are not presented in this report but are retained in the archives. The retained tissues were checked before disposal of the carcass.

Organ weights

The following organs, taken from each animal killed after the scheduled treatment or recovery periods, were dissected free of adjacent fat and other contiguous tissue and the weights recorded:

Table 3.9.1.1- 6: Organ weights

Adrenals	Ovaries
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Thyroid with parathyroids*
Liver	Uterus with cervix

* Weighed after partial fixation

Bilateral organs were weighed together. The weight of each organ was also expressed as a percentage of the body weight recorded immediately before necropsy.

Tissue sampling and fixation

Testes and epididymides were fixed in Bouin's fluid prior to transfer to 70% industrial methylated spirit and eyes were fixed in Davidson's fluid. The urinary bladder was initially inflated with Bouin's fluid. Samples (or the whole) of the other tissues listed below from all animals were preserved in 10% neutral buffered formalin.

The following organs/tissues were sampled.

Table 3.9.1.1- 7: Sampled organs and tissues

Adrenals	Oesophagus
Aorta - thoracic	Ovaries
Brain	Pancreas
Caecum	Pituitary
Colon	Pharynx #
Duodenum	Prostate
Epididymides	Rectum
Eyes	Salivary glands +
Femurs +	Sciatic nerves +
Head #	Seminal vesicles
Heart	Skeletal muscle – thighs -
Ileum	Skin
Jejunum	Spinal cord
Kidneys	Spleen
Lachrymal glands	Sternum
Larynx #	Stomach
Liver	Testes
Lungs	Thymus
Lymph nodes	Thyroid with parathyroids
- mandibular	Trachea
- mesenteric	Urinary bladder
- regional to masses	Uterus and cervix
Mammary area - caudal	
Nose #	

\+ Only one processed for examination
\# Not processed for examination

Samples of any abnormal tissues were also retained and processed for examination. In those cases where a lesion was not clearly delineated, contiguous tissue was fixed with the grossly affected region and sectioned as appropriate.

Samples of the head (including nasal cavity, paranasal sinuses and nasopharynx), the larynx, nose and pharynx and the remaining femur, salivary gland, sciatic nerve and skeletal muscle (thigh) were not examined histologically, but are retained against any future requirement for microscopic examination.

7. Histopathology

Tissue processing

Relevant tissues were subject to histological processing. Tissue samples were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin, except the testes which were stained using a standard periodic acid/Schiff (PAS) method.

Those tissues subject to histological processing included the following regions:

Adrenals - cortex and medulla
Brain - cerebellum, cerebrum and midbrain
Femur with joint - longitudinal section including articular surface, epiphyseal plate and bone marrow
Heart - included auricular and ventricular regions
Kidneys - included cortex, medulla and papilla regions
Liver - section from all main lobes
Lungs - section from two major lobes, to include bronchi
Spinal cord - transverse and longitudinal section at the cervical, lumbar and thoracic levels
Sternum - included bone marrow
Stomach - included keratinised, glandular and antrum in sections
Thyroid - including parathyroids in section where possible
Uterus - uterus section separate from cervix section

For bilateral organs, sections of both organs were prepared. A single section was prepared from each of the remaining tissues required for microscopic pathology.

Microscopic examination

Microscopic examination was performed as follows:

- All tissues preserved for examination (as specified above) were examined for all animals killed or dying during the study.
- All tissues preserved for examination (as specified above) were examined for all animals of Groups 1 (control) and 5 (2,500 ppm) sacrificed on completion of the Toxicity and Carcinogenicity phases.
- The kidney, liver and lungs were examined for all animals of Groups 2, 3 and 4 (50, 200 or 750 ppm) sacrificed on completion of the Toxicity and Carcinogenicity phases and from all animals killed on completion of the Recovery phase.
- Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals.

Findings were either reported as "present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe. A reviewing pathologist undertook a peer review of the microscopic findings.

II. Results and Discussion

A. Results

1. Clinical results

There was no effect of treatment upon mortality.

In the Toxicity/Recovery phases there was a total of seven males and 12 females killed or dying during the treatment period, and a total of one male and two females killed or dying during the recovery period.

Table 3.9.1.1- 8: Cumulative mortality in toxicity/recovery group animals at selected time points

Week	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Toxicity phase										
13	0	0	2	0	0	0	1	1	0	0
26	0	0	2	0	0	2	2	2	2	0
27	0	0	3	0	0	4	2	2	2	0
38	0	0	4	0	0	4	2	2	2	0
51	0	0	4	0	0	4	2	3	2	0
52	0	0	5	0	0	4	2	3	2	1
Recovery phase										
41	0	0	0	0	1	0	0	0	0	0
45	1	0	0	0	1	0	0	0	0	0
R2	1	0	0	0	1	0	0	1	0	0
R8	1	0	0	0	1	0	0	1	0	1
R9	1	1	0	0	1	0	0	1	0	1

R: Recovery

In the carcinogenicity phase there was a total of 161 males and 192 females killed or dying during the treatment period. Statistical analysis of mortality among carcinogenicity phase animals indicated that there was no statistically significant difference between the control and treated groups ($p > 0.05$).

Table 3.9.1.1- 9: Cumulative mortality in carcinogenicity group animals at selected time points

Week	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
3	0	1	0	0	0	0	0	0	0	0
19	2	1	0	0	0	0	1	0	0	0
27	2	1	0	2	0	0	1	0	0	0
39	2	2	0	2	0	0	2	0	1	1
53	4	2	4	3	2	0	4	2	2	1
65	6	2	5	5	5	3	10	7	4	1
78	13	9	9	11	12	12	19	10	13	8
91	23	18	18	20	22	24	37	22	24	21
104	36	32	32	28	31	39	45	37	36	35

During the treatment periods (toxicity, recovery and carcinogenicity animals), there were incidences of perogenital staining brown staining on the dorsal body surface and brown staining of the pinnae in females at 750 and 2,500 ppm, but there was no a clear dose relationship. These signs tended to resolve during the recovery period, though in each case, only a low number of recovery phase animals had these signs on completion of the 52-week treatment period. This observation of a generalized reduction in grooming is not indicative of significant toxicity and is not considered adverse. There were no treatment-related signs amongst animals receiving 50 or 200 ppm.

In the carcinogenicity phase a total of 137 males and 208 females bore one or more palpable swellings during the treatment period. In females the number of animals with swellings and the total number of swellings were lower in treated groups than in the controls in a broadly dosage-related manner; this was particularly marked in females receiving 750 or 2,500 ppm, 77 and 79 swellings respectively, compared with 111 in the controls. However, this is not considered an adverse effect.

Table 3.9.1.1- 10: Group distribution, multiplicity and mean time of onset of palpable swellings in the carcinogenicity phase animals

Dose level (ppm)	Multiplicity[#]					No. of animals with swellings	Total no. of swellings	Mean time of onset (weeks)
	0	1	2	3	≥ 4			
Males								
0	36	13	10	1	0	24	36	73
50	36	14	8	0	2	24	38	73
200	31	12	9	6	2	29	56	72
750	32	15	5	5	3	28	55	69
2,500	28	18	10	3	1	32	52	71
Females								
0	9	21	14	7	9	51	111	74
50	15	18	15	6	6	45	102	72
200	19	18	11	4	8	41	90	78
750	25	14	11	7	3	35	77	79
2,500	24	11	13	7	5	36	79	79

[#] Expressed as number of animals bearing the indicated number of swellings

2. Body weights

Absolute body weight was consistently lower compared to control at the highest tested dose in both sexes throughout treatment (week 104: -9% and -12% for males and females, respectively; Table 3.9.1.1-11).

Table 3.9.1.1- 11 provides information regarding absolute body weight measurements during the study.

Table 3.9.1.1- 11: Mean body weight at selected time points (g) *(% difference to control)*

	Dose level (ppm)								
	0	50		200		750		2,500	
Males									
Toxicity phase animals									
Wk 0	196	195	*(-0.5)*	196	*(±0.0)*	199	*(+1.5)*	197	*(+0.5)*
Wk 13	536	538	*(+0.4)*	534	*(-0.4)*	531	*(-0.9)*	503	*(-6.2)*
Wk 28	652	657	*(+0.8)*	658	*(+0.9)*	653	*(+0.2)*	625	*(-4.1)*
Wk 36	695	697	*(+0.3)*	698	*(+0.4)*	694	*(-0.1)*	664	*(-4.5)*
Wk 52	751	754	*(+0.4)*	756	*(+0.7)*	752	*(+0.1)*	723	*(-3.7)*
Recovery phase animals									
Wk R0	765	783	*(+2.4)*	773	*(+1.0)*	791	*(+3.4)*	733	*(-4.2)*
Wk R4	781	801	*(+2.6)*	788	*(+0.9)*	807	*(+3.3)*	743	*(-4.9)*
Wk R8	796	823	*(+3.4)*	800	*(+0.5)*	829	*(+4.1)*	767	*(-3.6)*
Wk R12	800	831	*(+3.9)*	813	*(+1.6)*	841	*(+5.1)*	778	*(-2.8)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Carcinogenicity Phase									
Wk 0	198	197	*(-0.5)*	198	*(±0.0)*	197	*(-0.5)*	194	*(-2.0)*
Wk 13	553	559	*(+1.1)*	566	*(+2.4)*	548	*(-0.9)*	509	*(-8.0)*
Wk 28	667	671	*(+0.6)*	681	*(+2.1)*	651	*(-2.4)*	624	*(-6.4)*
Wk 36	712	721	*(+1.3)*	731	*(+2.7)*	696	*(-2.2)*	669	*(-6.0)*
Wk 52	778	788	*(+1.3)*	787	*(+1.2)*	754	*(-3.1)*	729	*(-6.3)*
Wk 64	820	826	*(+0.7)*	824	*(+0.5)*	799	*(-2.6)*	758	*(-7.6)*
Wk 76	834	857	*(+2.8)*	847	*(+1.6)*	829	*(-0.6)*	772	*(-7.4)*
Wk 96	832	862	*(+3.6)*	866	*(+4.1)*	839	*(+0.8)*	787	*(-5.4)*
Wk 104	817	825	*(+1.0)*	861	*(+5.4)*	795	*(-2.7)*	746	*(-8.7)*
Females									
Toxicity phase animals									
Wk 0	169	164	*(-3.0)*	165	*(-2.4)*	164	*(-3.0)*	165	*(-2.4)*
Wk 13	314	313	*(-0.3)*	308	*(-1.9)*	297	*(-5.4)*	281	*(-11.1)*
Wk 28	375	360	*(-4.1)*	357	*(-4.8)*	348	*(-7.2)*	324	*(-13.6)*
Wk 36	406	383	*(-5.7)*	381	*(-6.2)*	372	*(-8.4)*	343	*(-15.5)*
Wk 52	465	438	*(-5.8)*	438	*(-5.8)*	421	*(-9.5)*	387	*(-16.8)*
Recovery phase animals									
Wk R0	462	455	*(-1.5)*	438	*(-5.2)*	432	*(-6.5)*	389	*(-15.8)*
Wk R4	481	467	*(-2.9)*	460	*(-4.4)*	450	*(-6.4)*	418	*(-13.1)*
Wk R8	497	478	*(-3.8)*	472	*(-5.0)*	463	*(-6.8)*	437	*(-12.1)*
Wk R12	491	477	*(-2.9)*	475	*(-3.3)*	460	*(-6.3)*	442	*(-10.0)*
Carcinogenicity Phase									
Wk 0	165	166	*(+0.6)*	163	*(-1.2)*	164	*(-0.6)*	167	*(+1.2)*
Wk 13	318	312	*(-1.9)*	309	*(-2.8)*	310	*(-3.1)*	296	*(-6.9)*
Wk 28	373	358	*(-4.0)*	360	*(-3.5)*	362	*(-2.9)*	338	*(-9.4)*
Wk 36	398	384	*(-3.5)*	387	*(-2.8)*	390	*(-2.0)*	362	*(-9.0)*
Wk 52	461	443	*(-3.9)*	440	*(-4.6)*	451	*(-2.2)*	411	*(-10.8)*
Wk 64	497	488	*(-1.8)*	478	*(-3.8)*	493	*(-0.8)*	446	*(-10.3)*
Wk 76	538	502	*(-6.7)*	517	*(-3.9)*	527	*(-1.7)*	481	*(-10.6)*
Wk 96	556	513	*(-7.7)*	540	*(-2.9)*	577	*(+3.8)*	505	*(-9.2)*
Wk 104	554	508	*(-8.3)*	542	*(-2.2)*	558	*(+0.7)*	486	*(-12.3)*

Wk: Week; R: Recovery

According to study report, statistical analyses were not performed for absolute body weights (only for body weight gains, see following table)

When compared with the controls there was a statistically significant reduction in body weight gain in the first week of treatment in animals of both sexes receiving 2,500 and 750 ppm and in females receiving 200 ppm. When compared with the control gains, the Week 1 gains in animals receiving 2,500 ppm were reduced markedly in both sexes and this persisted to Week 2 in the Carcinogenicity phase animals. Subsequent weight gain by animals receiving 2,500 ppm tended to be lower than that of the controls, though the difference was less than was seen during the first two weeks of treatment. For males and females given 2,500 ppm, the overall weight gain was 5 and 25% lower than controls, respectively, at the end of the Toxicity phase (week 52), and 11 and 17% lower than controls at the end of the Carcinogenicity phase (week 104). At 200 and 750 ppm, the subsequent body weight gain of both sexes was similar to that of the controls.

In the recovery group, following the cessation of treatment at Week 52, females previously given 2,500 ppm and all male treatment groups gained more weight than the controls. However, a dose-relation was only seen for high-dose females.

The body weight gain of animals receiving 50 ppm and of males receiving 200 ppm were considered unaffected by treatment. The variations of weight gains in Week 1 for males receiving 200 ppm were small and inconsistent between the two phases of the study and were, therefore, not considered toxicologically significant.

Table 3.9.1.1- 12 gives an overview about the body weight gains.

Table 3.9.1.1- 12: Mean body weight gain (g) *(% difference to control)*

	Dose level (ppm)								
	0	**50**		**200**		**750**		**2,500**	
Males									
Toxicity phase animals									
Gain Week 0-1	57	56	*(-1.8)*	53	*(-7.0)*	50**	*(-12.3)*	35**	*(-38.6)*
Gain Week 1-2	50	53	*(+6.0)*	51	*(+2.0)*	51	*(+2.0)*	49	*(-2.0)*
Gain Week 0-52	556	559	*(+0.5)*	559	*(+0.5)*	554	*(-0.4)*	527	*(-5.2)*
Recovery phase animals									
Gain Week R0-R12	35	44	*(+25.7)*	40	*(+14.3)*	50*	*(+42.9)*	44	*(+25.7)*
Carcinogenicity phase animals									
Gain Week 0-1	52	53	*(+1.9)*	56*	*(+7.7)*	49	*(-6.1)*	35**	*(-32.7)*
Gain Week 1-2	54	52	*(-3.7)*	51	*(-5.6)*	51	*(-5.6)*	48**	*(-11.1)*
Gain Week 0-80	622	661	*(+6.3)*	657	*(+5.6)*	637	*(+2.4)*	573	*(-7.9)*
Gain Week 0-104	623	629	*(+1.0)*	665	*(+6.7)*	602	*(+3.4)*	555	*(-10.9)*
Females									
Toxicity phase animals									
Gain Week 0-1	28	32*	*(+14.3)*	18**	*(-35.7)*	17**	*(-39.3)*	13**	*(-53.6)*
Gain Week 1-2	18	17	*(+5.6)*	26**	*(+44.4)*	24**	*(+33.3)*	20	*(+11.1)*
Gain Week 0-52	297	273	*(-8.1)*	273	*(-8.1)*	257	*(-13.5)*	222**	*(-25.3)*
Recovery phase animals									
Gain Week R0-R12	29	22	*(-24.1)*	31	*(+6.9)*	28	*(-3.4)*	49	*(+69.0)*
Carcinogenicity phase animals									
Gain Week 0-1	25	23	*(-8.0)*	20**	*(-20.0)*	7**	*(-72.0)*	18**	*(-28.0)*
Gain Week 1-2	26	22**	*(-15.4)*	27	*(+3.8)*	25	*(-3.8)*	15**	*(-42.3)*
Gain Week 0-80	376	337	*(-10.4)*	353	*(-6.1)*	369	*(-1.9)*	316**	*(-16.0)*
Gain Week 0-104	390	346	*(-11.3)*	378	*(-3.1)*	396	*(+1.5)*	322	*(-17.4)*

R: Recovery
* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

3. Food intake

Males and females receiving 2,500 ppm consumed less food than the controls in the first week of treatment (-13% and -7% for the toxicity phase males and females, respectively, and -20% and -7% for the carcinogenicity phase males and females, respectively). Thereafter, females receiving 2,500 ppm, particularly those in the toxicity phase, tended to consume slightly less food than the control animals in the majority of the weeks and, consequently, the total food intake of these animals was slightly low (-8% for the toxicity phase females and -5% for the carcinogenicity phase females).

In the recovery phase of the study all groups of previously treated animals consumed similar amounts of food as the control animals with the exception of females which had received 2500 ppm where the food intake remained slightly low.

Animals receiving 750 or 2,500 ppm scattered more food than the controls in the first week of treatment and in the carcinogenicity phase females this continued into Week 2 and 3 when it was also apparent in females receiving 200 ppm. From Week 4 of treatment the food scatter values for treated animals were similar to those of the controls.

Table 3.9.1.1- 13 gives an overview of the food consumption.

Table 3.9.1.1- 13: Mean food consumption in g/animal *(% difference to control)*

	Dose level (ppm)								
	0	**50**		**200**		**750**		**2,500**	
Males									
Toxicity phase animals									
Wk 1	199	200	*(+0.5)*	195	*(-2.0)*	192	*(-4.0)*	174	*(-12.6)*
Wk 13	179	177	*(-1.1)*	175	*(-2.2)*	177	*(-1.1)*	172	*(-3.9)*
Wk 28	198	199	*(+0.5)*	196	*(-1.0)*	198	*(±0.0)*	195	*(-1.5)*
Wk 36	192	196	*(+2.1)*	192	*(±0.0)*	194	*(+1.0)*	190	*(-1.0)*
Wk 52	180	194	*(+7.8)*	186	*(+3.3)*	183	*(+1.7)*	178	*(-1.1)*
Wk 1-52	10123	10227	*(+1.0)*	10170	*(+0.5)*	10162	*(+0.4)*	9890	*(-2.3)*
Recovery phase animals									
Wk 4	192	200	*(+4.2)*	188	*(-2.1)*	202	*(+5.2)*	198	*(+3.1)*
Wk 8	195	203	*(+4.1)*	193	*(-1.0)*	208	*(+6.7)*	213	*(+9.2)*
Wk 12	188	202	*(+7.4)*	188	*(±0.0)*	201	*(+6.9)*	196	*(+4.3)*
Wk 1-12	2300	2420	*(+5.2)*	2276	*(-1.0)*	2444	*(+6.3)*	2428	*(+5.6)*
Carcinogenicity phase animals									
Wk 1	205	207	*(+1.0)*	208	*(+1.5)*	197	*(-3.9)*	165	*(-19.5)*
Wk 13	197	196	*(-0.5)*	195	*(-1.0)*	190	*(-3.6)*	191	*(-3.0)*
Wk 28	198	191	*(-3.5)*	194	*(-2.0)*	190	*(-4.0)*	189	*(-4.5)*
Wk 36	194	196	*(+1.0)*	199	*(+2.6)*	185	*(-4.6)*	185	*(-4.6)*
Wk 52	196	196	*(±0.0)*	197	*(+0.5)*	183	*(-6.6)*	183	*(-6.6)*
Wk 64	191	199	*(+4.2)*	199	*(+4.2)*	192	*(+0.5)*	184	*(-3.7)*
Wk 76	200	200	*(±0.0)*	199	*(-0.5)*	202	*(+1.0)*	196	*(-2.0)*
Wk 96	204	209	*(+2.5)*	197	*(-3.4)*	213	*(+4.4)*	209	*(+2.5)*
Wk 104	197	192	*(-2.5)*	203	*(+3.0)*	192	*(-2.5)*	197	*(±0.0)*
Wk 1-104	20679	2101	*(+1.6)*	20975	*(+1.4)*	20487	*(-0.9)*	20255	*(-2.1)*
Females									
Toxicity phase animals									
Wk 1	144	146	*(+1.4)*	146	*(+1.4)*	147	*(+2.1)*	134	*(-6.9)*
Wk 13	132	137	*(+3.8)*	134	*(+1.5)*	132	*(±0.0)*	122	*(-7.6)*
Wk 28	151	154	*(+2.0)*	143	*(-5.3)*	142	*(-6.0)*	137	*(-9.3)*
Wk 36	156	151	*(-3.2)*	148	*(-5.1)*	148	*(-5.1)*	139	*(-10.9)*
Wk 52	150	148	*(-1.3)*	145	*(-3.3)*	138	*(-8.0)*	134	*(-10.7)*
Wk 1-52	7952	8083	*(+1.6)*	7854	*(-1.2)*	7700	*(-3.2)*	7290	*(-8.3)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Recovery phase animals									
Wk 4	159	169	*(+6.3)*	161	*(+1.3)*	158	*(-0.6)*	147	*(-7.5)*
Wk 8	172	179	*(+4.1)*	175	*(+1.7)*	164	*(-4.7)*	159	*(-7.6)*
Wk 12	151	153	*(+1.3)*	154	*(+2.0)*	144	*(-4.6)*	143	*(-5.3)*
Wk 1-12	1928	2004	*(+3.9)*	1960	*(+1.7)*	1864	*(-3.3)*	1796	*(-6.8)*
Carcinogenicity phase animals									
Wk 1	142	143	*(+0.7)*	141	*(-0.7)*	142	*(±0.0)*	132	*(-7.0)*
Wk 13	144	139	*(-3.5)*	139	*(-3.5)*	142	*(-1.4)*	134	*(-6.9)*
Wk 28	150	144	*(-4.0)*	143	*(-4.7)*	145	*(-3.3)*	143	*(-4.7)*
Wk 36	149	145	*(-2.7)*	145	*(-2.7)*	154	*(+3.4)*	149	*(±0.0)*
Wk 52	161	152	*(-6.0)*	150	*(-6.8)*	155	*(-3.7)*	146	*(-9.3)*
Wk 64	164	165	*(+0.6)*	155	*(-5.5)*	167	*(+1.8)*	156	*(-4.9)*
Wk 76	173	159	*(-8.1)*	160	*(-7.5)*	169	*(-2.3)*	158	*(-8.7)*
Wk 96	171	176	*(+2.9)*	169	*(-1.2)*	173	*(+1.2)*	158	*(-7.6)*
Wk 104	161	150	*(-6.8)*	142	*(-11.8)*	145	*(-9.9)*	155	*(-3.7)*
Wk 1-104	16791	16547	*(-1.5)*	15937	*(-5.1)*	16441	*(-2.1)*	15886	*(-5.4)*

Wk: Week
Statistical analyses were not performed for food consumption data.

Low food conversion efficiencies were recorded during Week 1 in animals receiving 2,500 ppm and in females receiving 200 or 750 ppm. The overall food conversion efficiencies during the first 16 weeks of treatment (week 1-16) were low in females receiving 2,500 ppm (-13% and -12% in the Toxicity phase and Carcinogenicity phase females, respectively).

The overall food conversion efficiencies (week 1-16) of animals receiving up to 750 ppm were similar to those of the controls.

Table 3.9.1.1- 14 gives an overview of the food conversion efficiencies.

Table 3.9.1.1- 14: Food conversion efficiency in % *(% difference to control)*

	Dose level (ppm)								
	0	**50**		**200**		**750**		**2,500**	
Males									
Toxicity phase									
Week 1	28.7	28.1	*(-2.1)*	27.3	*(-4.9)*	25.9	*(-9.8)*	20.3	*(-29.3)*
Week 8	8.5	9.9	*(+16.5)*	9.0	*(+5.9)*	9.3	*(+9.4)*	9.6	*(+12.9)*
Week 16	4.9	4.7	*(-4.1)*	5.6	*(+14.3)*	5.3	*(+8.2)*	5.8	*(+18.4)*
Weeks 1-16	11.9	11.9	*(±0.0)*	11.9	*(±0.0)*	11.6	*(-2.5)*	11.3	*(-5.0)*
Carcinogenicity phase									
Week 1	25.4	25.8	*(+1.6)*	26.9	*(+5.9)*	24.8	*(-2.4)*	20.9	*(-17.7)*
Week 8	7.8	8.2	*(+5.1*	7.9	*(+1.3)*	8.3	*(-6.4)*	9.1	*(+16.7)*
Week 16	4.6	5.0	*(+8.7)*	5.9	*(+28.3)*	6.1	*(+32.6)*	6.6	*(+43.5)*
Weeks 1-16	12.1	12.1	*(±0.0)*	12.2	*(+0.8)*	11.8	*(-2.5)*	11.4	*(-5.8)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Females									
Toxicity phase									
Week 1	19.6	22.2	*(+13.3)*	12.0	*(-38.8)*	11.3	*(-42.3)*	9.5	*(-51.5)*
Week 8	4.0	5.3	*(+32.5)*	4.0	*(±0.0)*	4.5	*(+12.5)*	4.6	*(+15.0)*
Week 16	4.7	3.8	*(-19.1)*	2.4	*(-48.9)*	3.3	*(-29.8)*	3.4	*(-27.7)*
Weeks 1-16	6.7	6.6	*(-1.5)*	6.4	*(-4.5)*	6.3	*(-6.0)*	5.8	*(-13.4)*
Carcinogenicity phase									
Week 1	17.7	16.2	*(-8.5)*	14.0	*(-20.9)*	12.0	*(-32.2)*	13.7	*(-22.6)*
Week 8	4.3	5.2	*(+20.9)*	4.8	*(+11.6)*	4.0	*(-7.0)*	5.3	*(-23.3)*
Week 16	2.6	2.1	*(-19.2)*	2.5	*(-3.8)*	3.9	*(-50.0)*	3.4	*(+30.8)*
Weeks 1-16	6.9	6.5	*(-5.8)*	6.8	*(-1.4)*	6.7	*(-2.9)*	6.1	*(-11.6)*

Statistical analyses were not performed for food conversion data.

The overall achieved doses in mg/kg bw/day were as follows.

Table 3.9.1.1- 15: Overall achieved doses (mg/kg bw/day)

	Dose level (ppm)									
	Males					**Females**				
	0	**50**	**200**	**750**	**2,500**	**0**	**50**	**200**	**750**	**2,500**
Toxicity phase										
Week 1-52	-	2.5	9.8	37.0	125.5	-	3.3	12.9	48.7	163.6
Carcinogenicity phase										
Week 1-104	-	2.1	8.4	31.5	109.4	-	2.8	10.8	41.0	142.2

4. Ophthalmoscopic examinations

During Week 51 (Toxicity phase), 78 and 104 (Carcinogenicity phase) of treatment, there were no ophthalmoscopic abnormalities detected which were considered to be a result of treatment with fluopicolide.

5. Laboratory investigations

Haematology:

Changes seen in the blood during the study were confined, in general, to animals receiving 2,500 ppm, with males being affected more consistently than the females.

When compared with the controls, low haemoglobin concentrations were recorded throughout the treatment period in males and females receiving 2,500 ppm, except in Week 52 in males and Week 26 in females. Associated with this was a reduction of haematocrit (packed cell volumes) in males in Week 26 and 78 and in females in Week 13, 78 and 104 and low erythrocyte counts in females in Week 13 and 78. An initial reduction of haematocrit, haemoglobin concentration and erythrocyte count was evident in Week 13 in females receiving 750 ppm. No similar effect was observed subsequently and these changes were, therefore, considered to be of no toxicological significance. As a result of these inter-group differences there were several changes in the calculated red blood cell parameters in both sexes at 2,500 ppm. Mean cell haemoglobin was consistently low in males and mean cell haemoglobin concentration was low on all occasions in males, with the exception of Week 26. The slightly low mean cell haemoglobin concentrations in Week 13, 26 and 52 and in females receiving 2,500 ppm were not

attributed to treatment since the difference from controls was small and there was no other effect upon erythrocytes at these examinations. Mean cell volumes were low in Week 26, 52 and 104 in males.

Inter-group differences in erythrocytic parameters at the lower dietary concentrations of fluopicolide were minor and less consistent and, in view of this, were not considered toxicologically significant. These included slightly low mean cell haemoglobin concentrations in Week 13, 52 and 78 in males receiving 750 ppm, slightly low mean cell haemoglobin concentrations in Week 26 in females receiving 750 ppm and marginally low mean cell haemoglobin concentrations in Week 13 and 52 in males at 200 ppm.

None of these changes was evident at the end of the recovery period following 52-weeks of treatment.

At 50 ppm there were no treatment-related findings in the peripheral blood recorded for either sex.

All other inter-group differences that attained statistical significance were minor, lacked dosage-relationship or were inconsistent between examinations. Such changes included inter-group differences in clotting times in animals receiving 2,500 ppm, where activated partial thromboplastin times were high in Week 26 in males and low in females, whilst in Week 104, prothrombin times were shortened in males. They also included the occasional variations of lymphocyte count in females receiving 750 or 2,500 ppm since these were evident only in Weeks 13, 52 and 104. In view of the absence of these findings at Week 26 and 78 they could not, with any confidence, be attributed to treatment.

Examination of the tail vein smears did not indicate any treatment-related abnormalities in either sex. Statistically significant changes occurred in females receiving 2,500 ppm where lymphocyte counts were marginally low and eosinophil counts were slightly high in Week 52, whilst in Week 104 neutrophil counts were low and lymphocyte counts were high in males. Since these differences were not reflected in animals sampled at the same time for the main haematological examination, it is considered that these minor changes arose by chance and are not related to treatment.

Table 3.9.1.1- 16 gives an overview of the most relevant findings.

Table 3.9.1.1- 16: Haematological results *(% difference to control)*

Parameter	Dose level (ppm)								
	0	50		200		750		2,500	
Males									
Week 13									
Hct (L/L)	0.446	0.451	*(+1.1)*	0.456	*(+2.2)*	0.445	*(-0.2)*	0.437	*(-2.0)*
Hb (g/dL)	15.3	15.4	*(+0.7)*	15.4	*(+0.7)*	15.1	*(-1.3)*	14.7**	*(-3.9)*
RBC ($x10^{12}$/L)	8.17	8.35	*(+2.2)*	8.38	*(+2.6)*	8.24	*(+0.9)*	8.24	*(+0.9)*
MCH (pg)	18.5	18.4	*(-0.5)*	18.5	*(±0.0)*	18.3	*(-1.1)*	17.9**	*(-3.2)*
MCHC (g/dL)	34.3	34.1	*(-0.6)*	33.9**	*(-1.2)*	33.9**	*(-1.2)*	33.7**	*(-1.7)*
MCV (fL)	54.0	54.0	*(±0.0)*	54.5	*(+0.9)*	54.1	*(+0.2)*	53.1	*(-1.7)*
Week 26									
Hct (L/L)	0.459	0.468	*(+1.5)*	0.465	*(+1.3)*	0.460	*(+0.2)*	0.442*	*(-3.7)*
Hb (g/dL)	15.4	15.7	*(+1.9)*	15.6	*(+1.3)*	15.5	*(+0.6)*	14.8**	*(-3.9)*
RBC ($x10^{12}$/L)	8.57	8.72	*(+1.8)*	8.67	*(+1.2)*	8.65	*(+0.9)*	8.50	*(-0.8)*
MCH (pg)	18.0	18.0	*(±0.0)*	18.0	*(±0.0)*	17.9	*(-0.6)*	17.5*	*(-2.8)*
MCHC (g/dL)	33.6	33.6	*(±0.0)*	33.5	*(-0.3)*	33.6	*(±0.0)*	33.5	*(-0.3)*
MCV (fL)	53.7	53.7	*(±0.0)*	53.7	*(±0.0)*	53.3	*(-0.7)*	52.1**	*(-3.0)*

Parameter	Dose level (ppm)								
	0	50		200		750		2,500	
Week 52									
Hct (L/L)	0.437	0.441	*(+0.9)*	0.451	*(+3.2)*	0.442	*(+1.1)*	0.428	*(-2.1)*
Hb (g/dL)	15.0	15.1	*(+0.6)*	15.3	*(+2.0)*	15.2	*(+1.3)*	14.6	*(-2.7)*
RBC ($x10^{12}$/L)	8.26	8.20	*(-0.7)*	8.45	*(+2.3)*	8.48	*(+2.7)*	8.41	*(+1.8)*
MCH (pg)	18.2	18.6	*(+2.2)*	18.2	*(±0.0)*	17.9	*(-1.6)*	17.4**	*(-4.4)*
MCHC (g/dL)	34.4	34.3	*(-0.3)*	34.0*	*(-1.2)*	34.3*	*(-0.3)*	34.1*	*(-0.9)*
MCV (fL)	52.9	54.2	*(+2.5)*	53.5	*(+1.1)*	52.2	*(-1.3)*	51.0**	*(-3.6)*
Week 13 of recovery									
Hct (L/L)	0.423	0.425	*(+0.5)*	0.423	*(±0.0)*	0.439	*(+3.8)*	0.412	*(-2.6)*
Hb (g/dL)	14.9	15.3	*(+2.7)*	15.3	*(+2.7)*	15.4	*(+3.4)*	14.7	*(-1.3)*
RBC ($x10^{12}$/L)	8.17	8.10	*(-0.9)*	8.22	*(+0.6)*	8.15	*(-0.2)*	7.89	*(-3.4)*
MCH (pg)	18.3	18.9	*(+3.3)*	18.6	*(+1.6)*	19.0	*(+3.8)*	18.6	*(+1.6)*
MCHC (g/dL)	35.1	36.0**	*(+2.6)*	35.4	*(+0.9)*	35.2	*(+0.3)*	35.7*	*(+1.7)*
MCV (fL)	52.1	52.5	*(+0.8)*	52.5	*(+0.8)*	53.9	*(+3.5)*	52.2	*(+0.2)*
Week 78 (Carcinogenicity phase)									
Hct (L/L)	0.416	0.403	*(-3.1)*	0.425	*(+2.2)*	0.427	*(+2.6)*	0.389*	*(-6.5)*
Hb (g/dL)	15.0	14.5	*(-3.3)*	15.2	*(+1.3)*	15.2	*(+1.3)*	13.8**	*(-8.0)*
RBC ($x10^{12}$/L)	8.09	7.94	*(-1.9)*	8.43	*(+4.2)*	8.24	*(+1.9)*	7.80	*(-3.6)*
MCH (pg)	18.6	18.2	*(-2.2)*	18.0	*(-3.2)*	18.4	*(-1.1)*	17.7**	*(-4.8)*
MCHC (g/dL)	36.0	35.9	*(-0.3)*	35.7	*(-0.8)*	35.5**	*(-1.4)*	35.4**	*(-1.7)*
MCV (fL)	51.5	50.7	*(-1.6)*	50.5	*(-1.9)*	51.9	*(+0.8)*	50.0	*(-2.9)*
Week 104 (Carcinogenicity phase)									
Hct (L/L)	0.425	0.425	*(±0.0)*	0.439	*(+3.3)*	0.420	*(-1.2)*	0.409	*(-3.8)*
Hb (g/dL)	14.4	14.4	*(±0.0)*	14.9	*(+3.5)*	14.1	*(-2.1)*	13.6	*(-5.6)*
RBC ($x10^{12}$/L)	7.68	7.71	*(+0.4)*	7.98	*(+3.9)*	7.38	*(-3.9)*	7.68	*(±0.0)*
MCH (pg)	18.8	18.8	*(±0.0)*	18.7	*(-0.5)*	19.2	*(+2.1)*	17.7**	*(-4.8)*
MCHC (g/dL)	33.8	33.9	*(+0.3)*	33.9	*(+0.3)*	33.4	*(-1.2)*	33.2**	*(-1.8)*
MCV (fL)	55.6	55.3	*(-0.5)*	55.0	*(-1.1)*	57.5	*(+3.4)*	53.2*	*(-4.3)*
Females									
Week 13									
Hct (L/L)	0.439	0.438	*(-0.2)*	0.438	*(-0.2)*	0.429*	*(-2.3)*	0.426*	*(-3.0)*
Hb (g/dL)	15.2	15.1	*(-0.7)*	15.2	*(±0.0)*	14.8**	*(-2.6)*	14.6**	*(-3.9)*
RBC ($x10^{12}$/L)	7.92	7.80	*(-1.5)*	7.85	*(-0.9)*	7.63*	*(-3.7)*	7.68*	*(-3.0)*
MCH (pg)	19.2	19.3	*(+0.5)*	19.3	*(+0.5)*	19.4	*(+1.0)*	19.0	*(-0.5)*
MCHC (g/dL)	34.7	34.5	*(-0.6)*	34.6	*(-0.3)*	34.5	*(-0.6)*	34.3**	*(-1.2)*
MCV (fL)	55.5	56.1	*(+1.1)*	55.8	*(+0.5)*	56.2	*(+1.3)*	55.5	*(0.0)*

Parameter	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Week 26								
Hct (L/L)	0.423	0.428	*(+1.2)*	0.427	*(+0.9)*	0.427	*(+0.9)*	0.416	*(-1.7)*
Hb (g/dL)	14.8	14.8	*(±0.0)*	14.8	*(±0.0)*	14.7	*(-0.7)*	14.4	*(-2.7)*
RBC ($x10^{12}$/L)	7.64	7.57	*(-0.9)*	7.62	*(-0.3)*	7.59	*(-0.7)*	7.50	*(-1.8)*
MCH (pg)	19.3	19.6	*(+1.6)*	19.4	*(+0.5)*	19.4	*(+0.5)*	19.2	*(-0.5)*
MCHC (g/dL)	34.9	34.7	*(-0.6)*	34.6	*(-0.9)*	34.5*	*(-1.4)*	34.5*	*(-1.4)*
MCV (fL)	55.4	56.5	*(+2.0)*	56.1	*(+1.3)*	56.3	*(+1.6)*	55.5	*(+0.2)*
Week 52									
Hct (L/L)	0.422	0.415	*(-1.7)*	0.418	*(-0.9)*	0.417	*(-1.2)*	0.409	*(-3.1)*
Hb (g/dL)	14.7	14.3	*(-2.7)*	14.5	*(-1.4)*	14.4	*(-2.0)*	14.0*	*(-4.8)*
RBC ($x10^{12}$/L)	7.44	7.24	*(-2.7)*	7.36	*(-1.1)*	7.30	*(-1.9)*	7.23	*(-2.8)*
MCH (pg)	19.8	19.8	*(±0.0)*	19.6	*(-1.0)*	19.7	*(-0.5)*	19.4	*(-2.0)*
MCHC (g/dL)	34.8	34.6	*(-0.6)*	34.6	*(-0.6)*	34.5	*(-0.9)*	34.2**	*(-1.7)*
MCV (fL)	56.8	57.3	*(-0.9)*	56.7	*(-0.2)*	57.2	*(-0.7)*	56.6	*(-0.4)*
Week 13 of recovery									
Hct (L/L)	0.403	0.397	*(-1.5)*	0.399	*(-1.0)*	0.403	*(±0.0)*	0.414	*(+2.7)*
Hb (g/dL)	14.4	14.2	*(-1.4)*	14.3	*(-0.7)*	14.3	*(-0.7)*	14.6	*(+1.4)*
RBC ($x10^{12}$/L)	7.25	7.16	*(+1.2)*	7.03	*(-1.7)*	6.96	*(-4.0)*	7.30	*(+0.7)*
MCH (pg)	19.9	19.8	*(-0.5)*	20.4	*(+2.5)*	20.5	*(+3.0)*	20.1	*(+1.0)*
MCHC (g/dL)	35.8	35.7	*(-0.3)*	35.9	*(+0.3)*	35.5	*(+0.8)*	35.4*	*(-1.1)*
MCV (fL)	55.7	55.5	*(-0.4)*	56.8	*(+2.0)*	57.8	*(+3.8)*	56.8	*(+2.0)*
Week 78 (Carcinogenicity phase)									
Hct (L/L)	0.417	0.400	*(-4.1)*	0.406	*(-2.6)*	0.406	*(-2.6)*	0.395*	*(-5.3)*
Hb (g/dL)	14.6	14.0	*(-4.1)*	14.4	*(-1.4)*	14.3	*(-2.1)*	13.9**	*(-4.8)*
RBC ($x10^{12}$/L)	7.39	7.03	*(-4.9)*	7.26	*(-1.8)*	7.17	*(-3.0)*	6.96**	*(-6.0)*
MCH (pg)	19.7	20.0	*(-1.5)*	19.9	*(+1.0)*	19.9	*(+1.0)*	19.9	*(+1.0)*
MCHC (g/dL)	35.3	35.1	*(-0.6)*	35.5	*(+0.6)*	35.2	*(-0.3)*	35.1	*(-0.6)*
MCV (fL)	55.8	57.0	*(+2.2)*	55.9	*(+0.2)*	56.6	*(+1.4)*	56.9	*(+2.0)*
Week 104 (Carcinogenicity phase)									
Hct (L/L)	0.404	0.408	*(+1.0)*	0.415	*(+2.7)*	0.399	*(-1.2)*	0.382*	*(-5.4)*
Hb (g/dL)	14.0	14.2	*(+1.4)*	14.4	*(+2.9)*	13.8	*(-1.4)*	13.1*	*(-6.4)*
RBC ($x10^{12}$/L)	6.97	7.09	*(+1.7)*	7.14	*(+2.4)*	6.87	*(-1.4)*	6.62	*(-5.0)*
MCH (pg)	20.1	20.1	*(±0.0)*	20.2	*(+0.5)*	20.2	*(+0.5)*	20.0	*(-0.5)*
MCHC (g/dL)	34.6	34.8	*(+0.6)*	34.7	*(+0.3)*	34.7	*(+0.3)*	34.4	*(-0.6)*
MCV (fL)	58.1	57.7	*(-0.7)*	58.2	*(+0.2)*	58.1	*(±0.0)*	58.1	*(±0.0)*

* $p \leq 0.05$; ** $p \leq 0.01$, significantly different from controls

Clinical chemistry:

Biochemical examination of the blood plasma at Week 13, 26 and 52 indicated, when compared with the controls, high total protein concentrations and low albumin to globulin ratios in males and females receiving 2,500 ppm. Males receiving 200 or 750 ppm had high total protein concentrations in Week 13 and 26 which, on the latter occasion, were associated with low albumin to globulin ratio.

There was no consistent effect upon plasma albumin concentration, with an increase being recorded only on Week 13 in males receiving 2,500 ppm. None of these differences was evident at the end of the recovery period, nor were they present at subsequent examinations during the remainder of the treatment period.

Males receiving 2,500 ppm had high plasma creatinine concentrations, compared with the controls, in Week 13, 26, 78 and 104, but not in Week 52, and there was no evidence for this trend being present at the end of the recovery period.

There was a trend throughout the treatment period towards high total plasma cholesterol concentrations in males receiving 2,500 ppm. This was observed at all investigations, with the exception of that performed in Week 78, and was also evident in Week 26 and 104 in males receiving 750 ppm. This was not present at the end of the 13-week recovery period. Statistical significance was only attained, when compared with the controls, at the Week 13 investigation.

There were some disturbances of plasma electrolyte concentrations on many of the sampling occasions. Plasma potassium concentrations were high in Week 52 and 104 in males receiving 750 ppm and in males and females receiving 2,500 ppm, and in Week 52 in females receiving 750 ppm. Plasma calcium concentrations were high in Week 52 and 104 in males and females receiving 2,500 ppm, with animals receiving 750 ppm also being similarly affected in Week 104. These changes were not apparent at the end of the recovery period. All other inter-group differences in electrolyte levels were minor or lacked dosage-relationship and were, therefore, not considered toxicologically significant.

All other inter-group differences that attained statistical significance when compared with the controls were minor, lacked dosage-relationship or were not observed consistently during the treatment period. These differences were, therefore, not clearly attributable to treatment and are likely to represent normal biological variation.

Table 3.9.1.1- 17: Selected clinical chemistry results *(% difference to control)*

Parameter	Dose level (ppm)								
	0	**50**		**200**		**750**		**2,500**	
Males									
Week 13									
Total protein (g/L)	64	64	*(±0.0)*	66*	*(+3.1)*	66*	*(+3.1)*	68**	*(+6.2)*
A/G ratio	1.15	1.15	*(±0.0)*	1.13	*(+1.7)*	1.10	*(-4.3)*	1.08*	*(-6.1)*
Cholesterol (mmol/L)	1.82	1.80	*(-1.1)*	1.90	*(+4.4)*	1.98	*(+8.8)*	2.14*	*(+17.6)*
Calcium (nmol/L)	2.70	2.74	*(+1.5)*	2.69	*(+0.4)*	2.74	*(+1.5)*	2.77	*(+2.6)*
Potassium (mmol/L)	4.5	4.3	*(-4.4)*	4.3	*(-4.4)*	4.2	*(-6.7)*	4.3	*(-4.4)*
Creatinine (µmol/L)	45	47	*(+4.4)*	49	*(+8.9)*	47	*(+4.4)*	50*	*(+11.1)*
Albumin (g/L)	34	34	*(±0.0)*	35	*(+2.9)*	35	*(+2.9)*	35**	*(+2.9)*

Parameter	Dose level (ppm)								
	0	**50**		**200**		**750**		**2,500**	
Week 26									
Total protein (g/L)	66	68	*(+3.0)*	68*	*(+3.0)*	68*	*(+3.0)*	68*	*(+3.0)*
A/G ratio	1.08	1.10	*(+1.9)*	1.03*	*(-4.6)*	1.02*	*(-5.6)*	1.04*	*(-3.7)*
Cholesterol (mmol/L)	1.82	1.57	*(-13.7)*	1.94	*(+6.6)*	2.07	*(+13.7)*	2.06	*(+13.2)*
Calcium (nmol/L)	2.80	2.81	*(+0.4)*	2.85	*(+1.8)*	2.82	*(+0.7)*	2.79	*(-0.4)*
Potassium (mmol/L)	4.1	4.1	*(±0.0)*	4.1	*(±0.0)*	4.1	*(±0.0)*	4.2	*(+2.4)*
Creatinine (µmol/L)	45	47	*(+4.4)*	44	*(-2.2)*	46	*(+2.2)*	50**	*(+11.1)*
Albumin (g/L)	34	34	*(±0.0)*	35	*(+2.9)*	34	*(±0.0)*	35	*(+2.9)*
Week 52									
Total protein (g/L)	69	67	*(-2.9)*	68	*(-1.4)*	69	*(±0.0)*	71*	*(+2.9)*
A/G ratio	0.97	1.01	*(+4.1)*	0.96	*(-1.0)*	0.95	*(-2.1)*	0.93	*(-4.1)*
Cholesterol (mmol/L)	2.46	2.30	*(-6.5)*	2.28	*(-7.3)*	2.66	*(+8.1)*	2.80	*(+13.8)*
Calcium (nmol/L)	2.83	2.84	*(+0.4)*	2.85	*(+0.7)*	2.88	*(+1.8)*	2.93**	*(+19.1)*
Potassium (mmol/L)	3.9	4.0	*(+2.6)*	4.0	*(+2.6)*	4.3**	*(+10.3)*	4.5**	*(+15.4)*
Creatinine (µmol/L)	50	50	*(±0.0)*	48	*(-4.0)*	50	*(±0.0)*	52	*(+4.0)*
Albumin (g/L)	34	34	*(±0.0)*	33	*(-2.9)*	34	*(±0.0)*	34	*(±0.0)*
Week 13 of recovery									
Total protein (g/L)	70	70	*(±0.0)*	69	*(-1.4)*	69	*(-1.4)*	69	*(-1.4)*
A/G ratio	2.34	2.64	*(+12.8)*	2.40	*(+2.6)*	2.89	*(+23.5)*	2.46	*(+5.1)*
Cholesterol (mmol/L)	2.90	2.89	*(-0.3)*	2.91	*(+0.3)*	2.90	*(±0.0)*	2.85	*(-1.7)*
Calcium (nmol/L)	3.8	3.8	*(±0.0)*	4.0	*(+5.3)*	3.9	*(+2.6)*	4.1**	*(+7.9)*
Potassium (mmol/L)	50	46	*(-8.0)*	46	*(-8.0)*	46	*(-8.0)*	50	*(±0.0)*
Creatinine (µmol/L)	35	34	*(-2.9)*	34	*(-2.9)*	34	*(-2.9)*	34	*(-2.9)*
Albumin (g/L)	0.98	0.96	*(-2.0)*	0.98	*(±0.0)*	0.96	*(-2.0)*	0.99	*(+1.0)*

Parameter	Dose level (ppm)							
	0	50		200		750		2,500	
Week 78 (Carcinogenicity phase)									
Total protein (g/L)	71	68	*(-4.2)*	69	*(-2.8)*	71	*(±0.0)*	73	*(+2.8)*
A/G ratio	0.97	0.97	*(±0.0)*	0.96	*(-1.0)*	0.95	*(-2.1)*	0.96	*(-1.0)*
Cholesterol (mmol/L)	3.18	2.51	*(-21.1)*	3.66	*(+15.1)*	3.53	*(+11.0)*	3.52	*(+10.7)*
Calcium (nmol/L)	2.97	2.93	*(-1.3)*	2.93	*(-1.3)*	3.01	*(+1.3)*	3.04	*(+2.4)*
Potassium (mmol/L)	4.0	4.0	*(±0.0)*	4.0	*(±0.0)*	4.2	*(+10.0)*	4.2	*(+10.0)*
Creatinine (µmol/L)	46	45	*(-2.2)*	47	*(+2.2)*	48	*(+4.3)*	50*	*(+8.7)*
Albumin (g/L)	35	33	*(-5.7)*	34	*(-2.9)*	35	*(±0.0)*	35	*(±0.0)*
Week 104 (Carcinogenicity phase)									
Total protein (g/L)	71	69	*(-2.8)*	71	*(±0.0)*	71	*(±0.0)*	73	*(+2.8)*
A/G ratio	0.91	0.87	*(-4.4)*	0.92	*(+1.1)*	0.88	*(-3.3)*	0.85	*(-6.6)*
Cholesterol (mmol/L)	3.34	3.24	*(-3.0)*	4.08	*(+22.2)*	4.49	*(+34.4)*	4.39	*(+31.4)*
Calcium (nmol/L)	2.85	2.88	*(+1.1)*	2.87	*(+0.7)*	2.95*	*(+3.5)*	3.07**	*(+7.7)*
Potassium (mmol/L)	3.5	3.6	*(+2.9)*	3.6	*(+2.9)*	4.1**	*(+17.1)*	4.0**	*(+11.4)*
Creatinine (µmol/L)	48	50	*(+4.2)*	50	*(+4.2)*	52	*(+8.3)*	58**	*(+20.8)*
Albumin (g/L)	34	32	*(-5.9)*	34	*(±0.0)*	33	*(-2.9)*	33	*(-2.9)*
Females									
Week 13									
Total protein (g/L)	70	69	*(-1.4)*	68	*(-2.9)*	70	*(±0.0)*	74*	*(-5.7)*
A/G ratio	1.20	1.17	*(-2.5)*	1.15	*(-4.2)*	1.17	*(-2.5)*	1.10*	*(-8.3)*
Cholesterol (mmol/L)	2.35	2.02	*(-14.0)*	2.24	*(-4.7)*	2.44	*(+3.8)*	2.44	*(+3.8)*
Calcium (nmol/L)	2.81	2.79	*(-0.7)*	2.78	*(-1.1)*	2.83	*(+0.7)*	2.88	*(+2.5)*
Potassium (mmol/L)	3.8	3.8	*(±0.0)*	3.9	*(+2.6)*	3.6	*(-5.3)*	3.8	*(±0.0)*
Creatinine (µmol/L)	50	52	*(+4.0)*	51	*(+2.0)*	50	*(±0.0)*	52	*(+4.0)*
Albumin (g/L)	38	37	*(-2.6)*	36	*(-5.3)*	38	*(±0.0)*	39	*(+2.6)*

Parameter	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Week 26									
Total protein (g/L)	71	72	*(+1.4)*	73	*(+2.8)*	73	*(+2.8)*	79**	*(+11.3)*
A/G ratio	1.20	1.21	*(+0.8)*	1.15	*(-4.2)*	1.14	*(-5.0)*	1.07*	*(-10.8)*
Cholesterol (mmol/L)	2.55	2.14	*(-16.1)*	2.72	*(+6.7)*	2.58	*(+1.2)*	2.81	*(+10.2)*
Calcium (nmol/L)	2.89	2.83	*(-2.1)*	2.90	*(+0.3)*	2.90	*(+0.3)*	2.98	*(+3.1)*
Potassium (mmol/L)	3.8	3.7	*(-2.6)*	3.7	*(+2.6)*	4.0	*(+5.3)*	3.9	*(+2.6)*
Creatinine (µmol/L)	49	52	*(+6.1)*	50	*(+2.0)*	47	*(-4.1)*	50	*(+2.0)*
Albumin (g/L)	39	39	*(±0.0)*	39	*(±0.0)*	39	*(±0.0)*	41	*(+5.1)*
Week 52									
Total protein (g/L)	77	78	*(+1.3)*	79	*(+2.6)*	77	*(±0.0)*	82*	*(+6.5)*
A/G ratio	1.12	1.16	*(+3.6)*	1.08	*(-3.6)*	1.12	*(±0.0)*	1.01**	*(-9.8)*
Cholesterol (mmol/L)	3.16	2.94	*(-7.0)*	3.30	*(+4.4)*	3.38	*(+7.0)*	3.60	*(+13.9)*
Calcium (nmol/L)	2.93	2.90	*(-1.0)*	2.97	*(+1.4)*	2.96	*(+1.0)*	3.00*	*(+2.4)*
Potassium (mmol/L)	3.1	2.9	*(-6.5)*	3.2	*(+3.2)*	3.6**	*(+16.1)*	3.6**	*(+16.1)*
Creatinine (µmol/L)	56	59	*(+5.4)*	54	*(-3.7)*	54	*(-3.6)*	58	*(+3.6)*
Albumin (g/L)	41	42	*(+2.4)*	41	*(±0.0)*	41	*(±0.0)*	41	*(±0.0)*
Week 13 of recovery									
Total protein (g/L)	73	75	*(+2.7)*	75	*(+2.7)*	75	*(+2.7)*	73	*(±0.0)*
A/G ratio	1.14	1.14	*(±0.0)*	1.14	*(±0.0)*	1.14	*(±0.0)*	1.10	*(-3.5)*
Cholesterol (mmol/L)	2.59	2.85	*(+10.0)*	2.96	*(+14.3)*	2.67	*(+3.1)*	2.60	*(+0.4)*
Calcium (nmol/L)	2.88	2.93	*(+1.7)*	2.88	*(±0.0)*	2.88	*(±0.0)*	2.83	*(-1.7)*
Potassium (mmol/L)	3.5	3.5	*(±0.0)*	3.5	*(±0.0)*	3.5	*(±0.0)*	3.6	*(+2.9)*
Creatinine (µmol/L)	51	53	*(+3.9)*	53	*(+3.9)*	54	*(+5.9)*	55	*(+7.8)*
Albumin (g/L)	39	40	*(+2.6)*	40	*(+2.6)*	40	*(+2.6)*	38	*(-2.6)*

Parameter	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Week 78 (Carcinogenicity phase)									
Total protein (g/L)	74	73	*(-1.4)*	76	*(+2.7)*	74	*(±0.0)*	77	*(+4.1)*
A/G ratio	1.13	1.09	*(-3.5)*	1.08	*(-4.4)*	1.14	*(+0.9)*	1.10	*(-2.6)*
Cholesterol (mmol/L)	3.42	2.76	*(-19.3)*	3.08	*(-9.9)*	3.35	*(-2.0)*	3.26	*(-4.7)*
Calcium (nmol/L)	2.88	2.81	*(-2.4)*	2.82	*(-2.1)*	2.85	*(-1.0)*	2.90	*(+0.7)*
Potassium (mmol/L)	3.5	3.6	*(+2.9)*	3.3	*(-5.7)*	3.6	*(+2.9)*	3.6	*(+2.9)*
Creatinine (µmol/L)	55	56	*(+1.8)*	50	*(-9.1)*	50	*(-9.1)*	53	*(-3.6)*
Albumin (g/L)	39	38	*(-2.6)*	39	*(±0.0)*	40	*(+2.6)*	40	*(+2.6)*
Week 104 (Carcinogenicity phase)									
Total protein (g/L)	76	77	*(+1.3)*	76	*(±0.0)*	75	*(-1.3)*	80	*(+5.3)*
A/G ratio	1.03	1.01	*(-1.9)*	1.04	*(+1.0)*	1.07	*(+3.9)*	0.97	*(-5.8)*
Cholesterol (mmol/L)	3.53	3.52	*(-0.3)*	3.20	*(-9.3)*	3.61	*(+2.3)*	4.15	*(+22.7)*
Calcium (nmol/L)	2.77	2.83	*(+2.2)*	2.87*	*(+3.6)*	2.96**	*(+6.9)*	2.95**	*(+6.5)*
Potassium (mmol/L)	3.3	3.1	*(-6.1)*	3.2	*(-3.0)*	3.6	*(+9.1)*	3.9*	*(+18.2)*
Creatinine (µmol/L)	49	48	*(-2.0)*	47	*(-4.1)*	45	*(-8.2)*	49	*(±0.0)*
Albumin (g/L)	38	38	*(±0.0)*	39	*(+2.6)*	39	*(+2.6)*	39	*(+2.6)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control using Fisher's Exact test

Urinalysis:

There was no clear effect of treatment upon urinary composition. There were occasional variations in urinary composition during the treatment period but they were minor, lacked dosage-relationship or were seen on only one occasion. In view of the absence of a consistent difference in any of the measured urinalysis parameters, the differences that were observed are considered to represent normal biological variation.

6. Sacrifice and pathology

Necropsy:

Macroscopic examination of the toxicity phase animals killed after 52 weeks of treatment or after 13 weeks of recovery revealed no findings related to the administration of fluopicolide in the diet.

In the carcinogenicity phase animals which were killed or dying during the treatment period lower incidences of mammary masses were reported in females that had received 200, 750 or 2,500 ppm.

In Carcinogenicity phase animals killed after 104 weeks of treatment there were several findings attributed to treatment in males that had received 2,500 ppm. These findings included a high incidence of skin masses (see histopathology chapter for further discussion) and a low incidence of scabs on the tail. When all animals of the carcinogenicity phase were considered together and comparisons made with the controls, slightly high incidences of enlarged kidneys and thyroids and a low incidence of scabs on the tail were evident in males which had received 2,500 ppm. In females given 2,500 ppm there was a lower incidence of adrenal masses whilst in those given 200, 750 or 2,500 ppm there were lower incidences of mammary masses which match with the clinical observation that in females the number of animals with swellings and the total number of swellings were lower in treated groups than in the controls. However, this finding is not considered an adverse effect.

Organ weights:

Evaluation of the organ weight data indicated increased relative kidney weights after 52 and 104 weeks of treatment in males given 750 or 2,500 ppm, with females given 2,500 ppm being similarly affected after 52 weeks of treatment only. In addition, there was a statistically significant increase in absolute kidney weights in males treated ≥ 200 ppm after 104 weeks; however, the effect at the 200 ppm is considered an artefact secondary to the increased body weight compared to controls (+6%) at this dose level (relative kidney weight was comparable to control). This is supported by the absence of histopathological kidney effects at 200 ppm.

Absolute and relative liver weights were increased after 52 and 104 weeks of treatment in males given 2,500 ppm, with relative liver weights also being higher after 52 weeks in males given 750 ppm and in females given 2,500 ppm compared to control. None of the changes apparent after 52 weeks of treatment were present after completion of the 13-week recovery period, indicating that full recovery had occurred.

After 104 weeks of treatment there was a slight increase of the absolute and bodyweight-relative thyroid weight, compared with the controls, in males given 2,500 ppm. This was not evident at the interim kill after completion of 52 weeks of treatment.

Several other organ weight difference attained statistical significance when compared with the controls but these were confined to one sex, not dosage-related or, in females given 2,500 ppm, considered to be associated with low bodyweight gains. Consequently, they are considered to represent normal biological variation.

The organ weights of both sexes given 50 or 200 ppm and females at 750 ppm were not affected by treatment.

Table 3.9.1.1- 18 gives an overview of selected organ weight results.

Table 3.9.1.1- 18: Selected organ weights after 52 weeks of treatment *(% difference to control)*

	Dose level (ppm)									
	0	**50**		**200**		**750**		**2,500**		
Males										
Terminal body weight	740.2	735.3	*(-0.7)*	738.9	*(-0.2)*	727.7	*(-1.7)*	711.6	*(-3.9)*	
Kidney										
Absolute weight (g)	3.87	4.00	*(+3.4)*	3.98	*(+2.8)*	4.13	*(+6.7)*	4.20	*(+8.5)*	
Relative weight to body weight (%)	0.526	0.546	*(+3.8)*	0.541	*(+2.9)*	0.571*	*(+8.6)*	0.592**	*(+12.5)*	
Liver										
Absolute weight (g)	21.51	22.17	*(+3.1)*	22.86	*(+6.3)*	23.17	*(+7.7)*	24.81*	*(+15.3)*	
Relative weight to body weight (%)	2.917	3.015	*(+3.4)*	3.075	*(+5.4)*	3.183*	*(+9.1)*	3.491**	*(+19.7)*	
Thyroid (+ para)										
Absolute weight (g)	0.029	0.027	*(-6.9)*	0.028	*(-3.4)*	0.033	*(+13.8)*	0.029	*(±0.0)*	
Relative weight to body weight (%)	0.0039	0.0037	*(-5.1)*	0.0038	*(-2.6)*	0.0046*	*(+17.9)*	0.0041	*(+5.1)*	
Females										
Terminal body weight	464.1	427.9	*(-7.8)*	438.8	*(-3.9)*	413.9	*(-10.8)*	386.0**	*(-16.8)*	
Kidney										
Absolute weight (g)	2.78	2.60	*(-6.5)*	2.72	*(-2.2)*	2.64	*(-5.0)*	2.67	*(-4.0)*	
Relative weight to body weight (%)	0.604	0.617	*(+2.2)*	0.622	*+(2.9)*	0.643	*(+6.5)*	0.694**	*(+14.9)*	
Liver										
Absolute weight (g)	14.47	14.07	*(-2.8)*	14.51	*(+0.3)*	13.94	*(-3.7)*	14.31	*(-1.1)*	
Relative weight to body weight (%)	3.130	3.307	*(+5.7)*	3.319	*(+6.0)*	3.384	*(+8.1)*	3.719**	*(+18.8)*	
Thyroid (+ para)										
Absolute weight (g)	0.023	0.022	*(-4.3)*	0.022	*(-4.3)*	0.022	*(-4.3)*	0.023	*(±0.0)*	
Relative weight to body weight (%)	0.0050	0.0051	*(+2.0)*	0.0051	*(+2.0)*	0.0053	*(+6.0)*	0.0058	*(+16.0)*	

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control using Fisher's Exact test

Table 3.9.1.1- 19: Selected organ weights after 104 weeks of treatment *(% difference to control)*

	Dose level (ppm)									
	0	**50**		**200**		**750**		**2500**		
Males										
Terminal body weight	810.7	820.6	*(+1.2)*	856.6	*(+5.7)*	791.8	*(-2.3)*	744.9	*(-8.1)*	
Kidney										
Absolute weight (g)	4.67	4.83	*(+3.4)*	5.10*	*(+9.2)*	5.08*	*(+8.8)*	5.69**	*(+21.8)*	
Relative weight to body weight (%)	0.586	0.592	*(+1.0)*	0.603	*(+2.9)*	0.655*	*(+11.8)*	0.800**	*(+36.5)*	
Liver										
Absolute weight (g)	22.49	23.03	*(+2.4)*	24.18	*(+7.5)*	24.21	*(+7.6)*	27.42**	*(+21.9)*	
Relative weight to body weight (%)	2.803	2.843	*(+1.4)*	2.850	*(+1.7)*	3.100	*(+10.6)*	3.757**	*(+34.0)*	
Thyroid (+ para)										
Absolute weight (g)	0.039	0.041	*(+5.1)*	0.047	*(+20.5)*	0.041	*(+5.1)*	0.060*	*(+53.8)*	
Relative weight to body weight (%)	0.0049	0.0050	*(+2.0)*	0.0055	*(+12.2)*	0.0053	*(+8.2)*	0.0080*	*(+63.3)*	

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2500**	
Females								
Terminal body weight	511.1	504.3	*(-1.3)*	540.2	*(+5.7)*	554.6	*(+8.5)*	484.1	*(-5.3)*
Kidney									
Absolute weight (g)	3.41	3.39	*(-0.6)*	3.18	*(-6.7)*	3.32	*(-2.6)*	3.23	*(-5.3)*
Relative weight to body weight (%)	0.641	0.700	*(+9.2)*	0.613	*(-4.4)*	0.631	*(-1.6)*	0.694	*(+8.3)*
Liver									
Absolute weight (g)	18.97	17.22	*(-9.2)*	16.79	*(-11.5)*	18.64	*(-1.7)*	17.28	*(-8.9)*
Relative weight to body weight (%)	3.541	3.456	*(-2.4)*	3.148	*(-11.1)*	3.440	*(-2.9)*	3.657	*(+3.3)*
Thyroid (+ para)									
Absolute weight (g)	0.034	0.029	*(-14.7)*	0.032	*(-5.9)*	0.035	*(+2.9)*	0.032	*(-5.9)*
Relative weight to body weight (%)	0.0063	0.0056	*(-11.1)*	0.0060	*(-4.8)*	0.0065	*(+3.2)*	0.0067	*(+6.3)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control using Fisher's Exact test

7. Histopathology

Animals killed after 52 weeks of treatment:

Findings considered related to treatment were seen in the liver and kidneys. There were no neoplastic findings considered related to treatment with fluopicolide.

An increased incidence of centrilobular hepatocytic hypertrophy was observed in males given fluopicolide at 750 and 2,500 ppm.

Table 3.9.1.1- 20: Incidences of liver findings after 52 weeks in males – Number of animals affected

Findings	**Dose level (ppm)**				
	0	**50**	**200**	**750**	**2,500**
Liver					
Centrilobular hypertrophy	0/20	0/20	0/15	14/20***	19/20***

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control using Fisher's Exact test

An increased incidence and/or severity of cortical tubular basophilia was observed in the kidneys of males given fluopicolide at 750 or 2,500 ppm. This was associated with increased incidences of other degenerative changes in the kidneys, as shown in Table 3.9.1.1- 21. There was an increased incidence of hyperplasia of the papillary epithelium in the kidneys of female animals receiving 200 ppm or above (see Table 3.9.1.1- 21). This finding occurred at a minimal or slight level and was usually associated with mineralisation of the papillary/pelvic epithelium, the incidence of which did not show a similar relationship to treatment.

Table 3.9.1.1- 21: Selected kidney findings after 52 weeks – Number of animals affected

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Males					
Cortical tubular basophilia	7/20	10/20	9/15	20/20***	20/20***
Cortical tubules with hyaline droplets	0/20	1/20	0/15	2/20	13/20***
Granular medullary casts	0/20	0/20	0/15	0/20	7/20**
Hyaline tubular casts	6/20	5/20	4/15	8/20	17/20**
Females					
Hyperplasia, papillary epithelium	2/16	3/18	8/17	7/18	9/19*

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control using Fisher's Exact test

Findings indirectly related to treatment included a reduced incidence and severity of chronic myocarditis in the heart of males given fluopicolide at 2,500 ppm. This is a common age-related degenerative change and the reduced incidence of this finding is considered to be secondary to the treatment-related body weight changes observed in these animals. As such, it is considered a non adverse finding.

Reduced incidences of a number of findings achieved statistical significance compared with control animals. These findings comprised reduced incidences of periportal hepatocyte vacuolation in the liver, sinus erythrocytes/erythrophagocytosis in the mandibular lymph nodes, dilated glands in the stomach and thymic haemorrhage. It is doubtful that any of these findings are related to treatment, and even if reduced incidences of these findings are not considered indicative of an adverse effect.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Animals killed after 13 weeks of the recovery period:

The majority of changes reported as related to treatment at the end of the 52-week treatment period were no longer apparent at the end of the 13-week recovery period. Only a slight increase in the severity of cortical tubular basophilia in the kidneys of males was still apparent, as shown in Table 3.9.1.1- 22.

Table 3.9.1.1- 22: Incidences of kidney findings after 13-week recovery in males – Number of animals affected

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Number examined	9	9	10	10	9
Kidney					
Cortical tubular basophilia					
Minimal	3	3	6	1	2
Slight	5	5	4	5	4
Moderate	0	1	0	2	3
Total incidence	8	9	10	10	9

Carcinogenicity phase:

Neoplastic findings

There were no neoplastic findings that were considered related to treatment with fluopicolide, especially no liver adenomas or carcinomas. The statistical analysis of the tumours in carcinogenicity phase animals similarly did not detect any statistically significant differences.

A non-statistically significant increase in the incidence of benign skin tumours was recorded in male rats given fluopicolide at 2,500 ppm (see Table 3.9.1.1- 23). The incidence was above the reported historical control data range given in a supplementary document (Fowles, J.; 2005; M-263575-01-1). Unexpectedly, the control and lower dose incidences were also clearly above the presented historical control data range.

The animal number per group was generally 60 animals, however referring to the numbers given in the study report only for 24, 28, 25, 26 and 34 animals the skin was histopathologiocally examined in the control and the different treatment groups. However, in the pathology part of the material and methods section in the study report it is described that "Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals" and that "The absence of a comment for a tissue scheduled for examination therefore indicates that the tissue was examined and found to be normal".Therefore, it is assumed that all 60 animals per group were examined for skin tumours, but only for animals with lesions a full histopathologic examination of the affected area was performed (see revised Table 3.9.1.1- 24). After recalculation most incidences of the control and lower treatment groups are within the reported HCD ranges and the tumour incidence for males at 2,500 ppm is 13.3% compared to a HCD range of 0-10.8% for keratocanthoma and 8.3% compared to a HCD range of 0-3.1% for squamous cell papilloma. This approach is supported by the statement of the study report author considering the incidence of these tumours as only slightly higher than the background inicidence in rats of this strain in this laboratory.

Skin tumours are relatively common tumours that are often associated with previous damage to the skin, and since there was no statistical significance, no clear-dose dependency at the lower dose levels and only males were affected, the slightly higher incidences seen at 2,500 ppm are considered to have arisen by chance and are not considered to be of any toxicological importance in the oncogenic assessment of fluopicolide.

Table 3.9.1.1- 23: Incidences of skin tumours in carcinogenicity phase animals *(%)*

Findings	Dose level (ppm)					HCD[#]
	0	50	200	750	2,500	
Males						
Keratoacanthoma	3/24 *(12.5)*	3/28 *(10.7)*	5/25 *(20)*	3/26 *(11.5)*	8/34 *(23.5)*	0-10.8%
Squamous cell papilloma	2/24 *(8.3)*	0/28 *(0.0)*	1/25 *(4.0)*	2/26 *(7.7)*	5/34 *(14.7)*	0-3.1%
Females						
Keratoacanthoma	1/20 *(5.0)*	0/15 *(0.0)*	0/13 *(0.0)*	0/10 *(0.0)*	0/13 *(0.0)*	0-1.7%
Squamous cell papilloma	1/20 *(5.0)*	0/15 *(0.0)*	0/13 *(0.0)*	0/10 *(0.0)*	0/13 *(0.0)*	0-1.5%

[#] Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks (Fowles, J.; 2005; M-263575-01-1).

Table 3.9.1.1- 24: Incidences of skin tumours in carcinogenicity phase animals (%) - revised

Findings	Dose level (ppm)					HCD[#]
	0	50	200	750	2,500	
Males						
Keratoacanthoma	3/60 *(5.0)*	3/60 *(5.0)*	5/60 *(8.3)*	3/60 *(5.0)*	8/60 *(13.3)*	0-10.8%
Squamous cell papilloma	2/60 *(3.3)*	0/60 *(0.0)*	1/60 *(1.7)*	2/60 *(3.3)*	5/60 *(8.3)*	0-3.1%
Females						
Keratoacanthoma	1/60 *(1.7)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0-1.7%
Squamous cell papilloma	1/60 *(1.7)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0-1.5%

[#] Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks (Fowles, J.; 2005; M-263575-01-1).

Non-neoplastic findings

Findings considered related to treatment were seen in the liver, kidneys, pancreas and prostate.

An increased incidence of centrilobular hepatocytic hypertrophy was observed in males given floupicolide at 200 ppm or above. An increased incidence and/or severity of cystic degeneration and foci of alteration was seen in male rats and an increased incidence of eosinophilic foci of alteration in females given 750 or 2,500 ppm (see Table 3.9.1.1- 25).

Table 3.9.1.1- 25: Incidences of liver findings of carcinogenicity phase animals – Number of animals affected

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Non neoplastic findings										
Number examined	60	60	60	60	60	60	60	60	60	60
Hepatocyte hypertrophy	0	0	2	9**	18***	0	0	0	0	0
Cystic degeneration	13	17	18	23	32***	0	1	0	0	0
Clear cell foci	22	29	28	36*	35*	21	12	20	17	23
Eosinophilic foci	13	17	15	19	25*	4	6	5	11	15*

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

An increased incidence and/or severity of a number of degenerative and proliferative changes were observed in the kidneys of males and females (see Table 3.9.1.1- 26). The majority of these changes were limited to male animals given 2,500 ppm. The occurrence of hyperplasia of the papillary epithelium showed an increased incidence and/or severity in females given 750 ppm or above. This finding was usually associated with mineralisation of the papillary/pelvic epithelium. There was no clear relationship to treatment in the incidence or severity of these findings in males.

Table 3.9.1.1- 26: Incidence and severity of kidney findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	**50**	**200**	**750**	**2,500**	**0**	**50**	**200**	**750**	**2,500**
Number examined	60	60	60	60	60	60	60	60	60	60
Tubular casts										
Minimal	10	15	19	19	10	14	11	12	11	16
Slight	13	14	12	9	27	5	4	6	11	4
Moderate	1	1	1	4	8	4	2	2	2	5
Total incidence	24	30	32	32	45***	23	17	20	24	25
Pelvic calculi										
Minimal	2	0	0	0	0	2	1	0	1	0
Slight	3	2	5	5	2	3	4	1	2	5
Moderate	1	0	2	3	6	0	0	0	1	3
Total incidence	6	2	7	8	8	5	5	1	4	8
Cortical tubular basophilia										
Minimal	5	12	6	12	3	15	11	18	17	16
Slight	32	22	32	30	22	36	36	33	32	24
Moderate	10	13	15	10	22	5	3	6	7	13
Marked	1	3	1	1	1	0	1	0	0	1
Total incidence	48	50	54	53	48	56	51	57	56	54
Cortical tubular dilation										
Minimal	3	2	1	3	6	3	2	4	6	0
Slight	6	5	7	3	17	4	4	4	5	12
Moderate	1	2	1	2	3	1	1	1	1	2
Marked	0	0	0	0	1	0	0	0	0	0
Total incidence	10	9	9	8	27**	8	7	9	12	14
Cortical tubules with hyaline droplets										
Minimal	2	1	0	2	4	0	0	0	0	0
Slight	0	1	1	2	7	1	0	0	1	0
Moderate	0	1	2	0	1	0	2	0	0	0
Marked	1	0	0	0	2	0	0	0	0	0
Total incidence	3	3	3	4	14**	1	2	0	1	0
Hyperplasia, papillary epithelium										
Minimal	4	6	7	6	3	20	17	15	23	16
Slight	10	8	8	12	6	15	7	14	17	17
Moderate	0	0	3	2	1	1	4	3	7	10
Marked	0	0	0	0	1	0	0	0	0	0
Total incidence	14	14	18	20	11	36	28	32	47*	43
Interstitial inflammation										
Minimal	3	4	4	5	3	1	0	0	1	0
Slight	9	3	9	10	15	2	4	2	5	11
Moderate	2	1	1	2	5	1	1	1	0	0
Total incidence	14	8	14	17	23	4	5	3	6	11

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Cortical cysts										
Minimal	1	1	1	2	1	2	1	0	0	0
Slight	1	0	3	4	4	1	3	1	1	2
Moderate	1	1	1	3	5	0	0	0	1	0
Marked	0	0	1	0	1	0	0	0	0	0
Total incidence	3	2	6	9	11*	3	4	1	2	2
Mineralization papillary/pelvic epithelium										
Minimal	11	3	4	5	4	27	27	27	27	21
Slight	4	0	3	1	1	12	9	14	18	15
Moderate	0	0	1	0	1	1	4	1	4	8
Total incidence	15	3**	8	6	6	40	40	42	49	44
Mineralization papilla										
Minimal	0	1	0	2	7	4	3	2	0	3
Slight	0	0	2	0	5	0	0	0	0	0
Total incidence	0	1	2	2	12***	4	3	2	0	3

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001, statistically different to control

An increased incidence and/or severity of atrophic changes was observed in the pancreas of animals given 2,500 ppm (see Table 3.9.1.1- 27), evident in males as acinar atrophy and in females as acinar replacement by adipose tissue.

Table 3.9.1.1- 27: Incidences of pancreas findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Pancreas, number examined	60	33	33	32	60	60	45	38	37	59
Acinar atrophy	17	13	12	19	26	8	2	8	12	11
Acinar replace-ment by adipose tissues	14	6	4	7	16	6	3	0	6	16*

* p ≤ 0.05, statistically different to control

An increased incidence of acinar atrophy, often associated with reduced colloid, was present in the prostate of males at 2,500 ppm only and is considered secondary to the decreased body weight at this dose level.

Table 3.9.1.1- 28: Incidences of prostate findings of carcinogenicity phase animals

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Acinar cell atrophy	19/60	12/35	7/34	6/31	31/60
Reduced colloid	8/60	3/35	7/34	3/31	17/60

* p ≤ 0.05, statistically different to control

As findings indirectly related to treatment, an increased incidence of cystic follicular cell hyperplasia was present in the thyroids of males given fluopicolide at 750 ppm or above. This finding was considered to be secondary to the increased metabolic activity of the liver at these dose levels indicated by increased liver weights at ≥ 750 ppm and an increased incidence of centrilobular hepatocytic hypertrophy at ≥ 200 ppm in males.

Table 3.9.1.1- 29: Incidences of thyroid findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
For animals killed or dying during the treatment period										
Thyroid, number examined	37	32	29	35	31	39	45	36	36	34
Cystic follicular cell hyperplasia	0	1	0	3	0	0	0	1	0	0
For animals killed after 104 weeks of treatment										
Thyroid, number examined	23	7	7	6	29	21	3	2	3	25
Cystic follicular cell hyperplasia	0	0	0	1	7*	1	0	0	0	1

* $p \leq 0.05$, statistically different to control

An increased incidence of sinus erythrocytosis/erythrophagocytosis, which attained statistical significance in the highest dietary concentration group, was seen in the mandibular lymph nodes of females. This is a relatively common finding in rats of all ages and is considered to have arisen by chance.

An increased incidence of interstitial cell hyperplasia of the testes of rats at 750 and 2,500 ppm was recorded, associated with a slightly increased incidence of benign interstitial (Leydig) cell adenoma. However, neither incidence attained statistical significance. Since the incidences of adenomas were additionally within the background range seen in this laboratory and there was no dose-response relationship the slightly increased adenoma incidences are considered incidental and reflective of the normal biological variability.Furthermore, no treatment-related effect on testes histopathology was observed in animals killed after 52 weeks.

Table 3.9.1.1- 30: Incidences of testes findings of carcinogenicity phase animals (%)

Findings	Dose level (ppm)					HCD[#]
	0	50	200	750	2,500	
Testes: Interstitial cell hyperplasia	6/60 (10%)	4/39 (10.3%)	3/38 (7.9%)	7/35 (20%)	14/60 (23.3%)	-
Benign interstitial (Leydig) cell adenoma	1/60 (1.6%)	2/39 (5.1%)	2/38 (5.3%)	3/35 (8.6%)	4/60 (6.7%)	0.0-9.2%

* $p \leq 0.05$, statistically different to control

\# Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks (Fowles, J.; 2005; M-263575-01-1).

A number of other findings showed statistically significant changes in incidence compared with the control groups in one or both of the sexes (mainly decreased incidences). These findings were generally of low incidence and lacked dosage-relationship and were, therefore, considered to be of no toxicological significance.

III. Conclusion

It is concluded that the administration of fluopicoplide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential. The liver and kidneys were identified as target organs. A small number of findings were recorded for animals receiving 200 ppm. However, these were considered non adverse, e.g. adaptive liver changes in 2/60 males. This dietary concentration is therefore considered the No Observed Adverse Effect Level (NOAEL) in this study, equivalent to 8.4 mg/kg bw/day in males and 10.8 mg/kg bw/day in females. No treatment-related changes were detected in animals receiving 50 ppm and this dietary concentration is considered the No Observed Effect Level (NOEL) in this study, equivalent to 2.1 mg/kg bw/day in males and 2.8 mg/kg bw/day in females.

3.9.1.2 Anonymous; 2003; M-225595-01-1 + Anonymous; 2005; M-263591-01-1

Study reference:

Anonymous; 2003; AE C638206 - Carcinogenicity study by oral route (dietary admixutre) in C57BL/6 mice; M-225595-01-1

+

Anonymous; 2005; Historical control data for long-term studies in C57bl/6 mice; M-263591-01-1

Deviations:	Deviations from the current OECD guideline (451, 2018): - Coagulating glands were not sampled, fixed or examined histopathologically This minor deviation(s) is considered not to compromise the results and outcome of the study.

Executive Summary:

The objective of this study was to evaluate the potential carcinogenicity of floupicolide (batch number: OP2050046, purity: 95.9% at study start) following daily oral administration (dietary admixture) to C57BL/6 mice for 78 weeks.

Four principal treated groups, each composed of 50 male and 50 female C57BL/6 mice received the test substance mixed with the diet at constant concentrations of 0 (control), 50, 400 or 3,200 ppm for 78 weeks. The concentrations of 50, 400 and 3,200 ppm corresponded on average to 7.9, 64.5 and 551.0 mg/kg bw/day for the males and to 11.5, 91.9 and 772.3 mg/kg bw/day for the females. For evaluation of toxic effects, 10 satellite males and 10 satellite females per group were sacrificed after a one-year period of administration.

Throughout the study, clinical signs and mortality were checked daily, and careful examination was carried out before the beginning of the treatment period and weekly thereafter to assess possible neurotoxic effects.

Palpation of possible masses was carried out every 4 weeks from Weeks 4 to 52 and every 2 weeks thereafter. Body weight and food consumption were measured at weekly intervals during the first 13 weeks of the study, every 4 weeks until Weeks 31/32 and every 2 weeks thereafter. Achieved dosages were calculated. Before the sacrifice of satellite animals in Week 53, blood was taken for the determination of liver enzyme activities (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase). At the end of the appropriate scheduled treatment period (52 or 78 weeks), animals were sacrificed and were submitted to a macroscopic post-mortem examination. A complete range of organs and any masses or macroscopic lesions were sampled. A microscopic examination was performed on all principal animals as well as on the liver and macroscopic abnormalities from the satellite animals (all groups).

The distribution of mortality, as well as the factors contributing to mortality or premature sacrifice, was similar in the control and treated groups. The incidence, nature and onset of the clinical signs were similar in the control and treated groups. No signs of neurotoxicity were observed during the study. The frequency, time of onset and size of the few palpable masses recorded were similar in the control and treated groups.

Food consumption and body weight of treated animals at 50 or 400 ppm were similar to that of controls.

When compared to the controls, the body weight and the body weight gain of the 3,200 ppm group was severely affected (body weight gain vs. controls in Week 78 vs. Week 1: males: -45%; females: -35%) by the treatment with the test substance. This effect correlated with a slight reduction of the food consumption.

There was no relevant difference in the liver enzyme activities between the treated and control groups.

Absolute and relative liver weights were higher in animals given 400 and 3,200 ppm at the end of 52-week and 78-week treatment periods. These changes were associated with hepatocellular hypertrophy noted among these animals.

After 52 weeks, liver enlargement was seen at 400 or 3,200 ppm in males only, and presence of masses and nodules in the liver in females treated at 3,200 ppm. After 78 weeks, a marked increase of liver enlargement was seen at 3,200 ppm, and the number of animals bearing masses and nodules in the liver in treated groups at 400 and 3,200 ppm was higher when compared with controls.

Microscopic examination at both 52- and 78-week revealed a dose-related hepatocellular hypertrophy at 400 ppm and 3,200 ppm; and a higher incidence of altered cell foci and hepatocellular adenoma at 3,200 ppm in both sexes at week 78.

In conclusion, as treatment-related effects, severe reductions of body weight gain and food consumption at 3,200 ppm were noted, thus indicating that the Maximal Tolerated Dose (MTD) was reached. Further observations were higher liver weights, enlarged liver, masses and nodules in the liver at 400 and 3,200 ppm, hepatocellular hypertrophy at 400 and 3,200 ppm, and high incidence of altered cell foci at 3,200 ppm and increased hepatocellular adenoma incidences at 3,200 ppm. This finding in the liver tissue might be attributed, at least in part, to the fact that 3,200 ppm reached the MTD. Additionally, mechanistic studies have demonstrated that the mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital (see Langrand-Lerche, C.; 2004; M-229594-01-1). This MoA is considered of no relevance in humans.

Therefore, under the experimental conditions, the No Observed Adverse Effect level (NOAEL) was 50 ppm (corresponding to 7.9 mg/kg bw/day for the males and 11.5 mg/kg bw/day for the females) for toxicity and 400 ppm (corresponding to 64.5 mg/kg bw/day for the males and 91.9 mg/kg bw/day for the females) for carcinogenicity.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mouse
Strain:	C57BL/6 Crl:BR
Age:	7 weeks old
Weight at start:	23.3 g (range: 21.5 g to 26.2 g) for the males and 19.5 g (range: 17.7 g to 22.3 g) for the females.
Source:	Charles River, Saint-Aubin les Elbeuf, France
Acclimation period:	Yes
Diet:	Powdered diet batch Nos. 1351, 1763 and 2373 (type: M20 EXTRALABO controlled, irradiated diet; supplier: SDS, 95450 Vigny, France)
Water:	Tap water (filtered with a 0.22 µm filter) contained in bottles
Housing:	The animals were individually housed in polycarbonate cages (24.0 x 13.5 x 13.0 cm) containing autoclaved sawdust (SICSA, Alfortville, France). The cages were placed in numerical order on the racks. On a monthly basis, all the racks were moved clockwise around the room, rack by rack
Temperature:	22± 2 °C
Humidity:	50± 20%
Air changes:	Approximately 12 cycles/hour of filtered, non-recycled air
Photoperiod:	12 hours

B. Study design

1. In-life dates: April 19, 2001 to November 15, 2002

2. Animal assignment and treatment

The mice were randomized and assigned to the following test groups.

Table 3.9.1.2- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Chronic phase (53-week)			
1	0	10	10
2	50	10	10
3	400	10	10
4	3,200	10	10
Oncogenicity phase (78-80 weeks)			
1	0	50	50
2	50	50	50
3	400	50	50
4	3,200	50	50

The dose-levels were specified based on the results of a preliminary 90-day toxicity study in C57BL/6 mice (Anonymous.; 2006; M-205579-02-1, Section 3.12.1.5). In this study, male and female C57BL/6 mice received 50, 200, 800 and 3200 ppm of fluopicolide in diet for at least 90 days. Body weight gains were slightly reduced (around -10%) at 3,200 ppm while higher liver weights associated with hepatocellular hypertrophy were observed in almost all the animals at 800 ppm and above. The NOEL was set at 50 ppm. Therefore, in the current carcinogenicity study, the dose-level of 3,200 ppm was selected as the highest dose-level which should produce signs of systemic toxicity without altering the normal life span of the animals. The dose-level of 50 ppm was selected as the lowest dose which should not produce any signs of toxicity and the dose-level of 400 ppm as the mid-range between the high and low dose-levels.

The oncogenicity phase animals were used for the evaluation of carcinogenic effects. The chronic phase animals were used to evaluate effects on target organs such as the liver after a one-year administration period.

The study duration was 53 weeks (i.e. 364 or 365 days) for the Chronic phase groups and 78 to 80 weeks (i.e. 546 to 557 days) according to the necropsy schedule for the Oncogenicity groups.

3. Diet preparation and analysis

The oral route was selected since it is a potential route of exposure in man. The test substance mixed with the diet was supplied to principal animals ad libitum for a period of 78 to 80 weeks (or 53 weeks for the satellite animals).

The HPLC analysis of the test substance carried out every 6 months confirmed that the purity remained the same throughout the treatment period.

The results of the analyses demonstrated the satisfactory homogeneity of each dietary admixture analyzed during the study.

Furthermore, there was a good correspondence between the nominal and the measured concentrations of the test substance in the diet.

The results of the stability analyses of the dietary admixtures were summarized as follows:

Table 3.9.1.2- 2: Stability results

Concentration (ppm)	50	3,200
Open feeders (Animal room conditions)	Stable 10 days	Stable 16 days
Closed bags (room temperature)	Stable 13 days	Stable 35 days

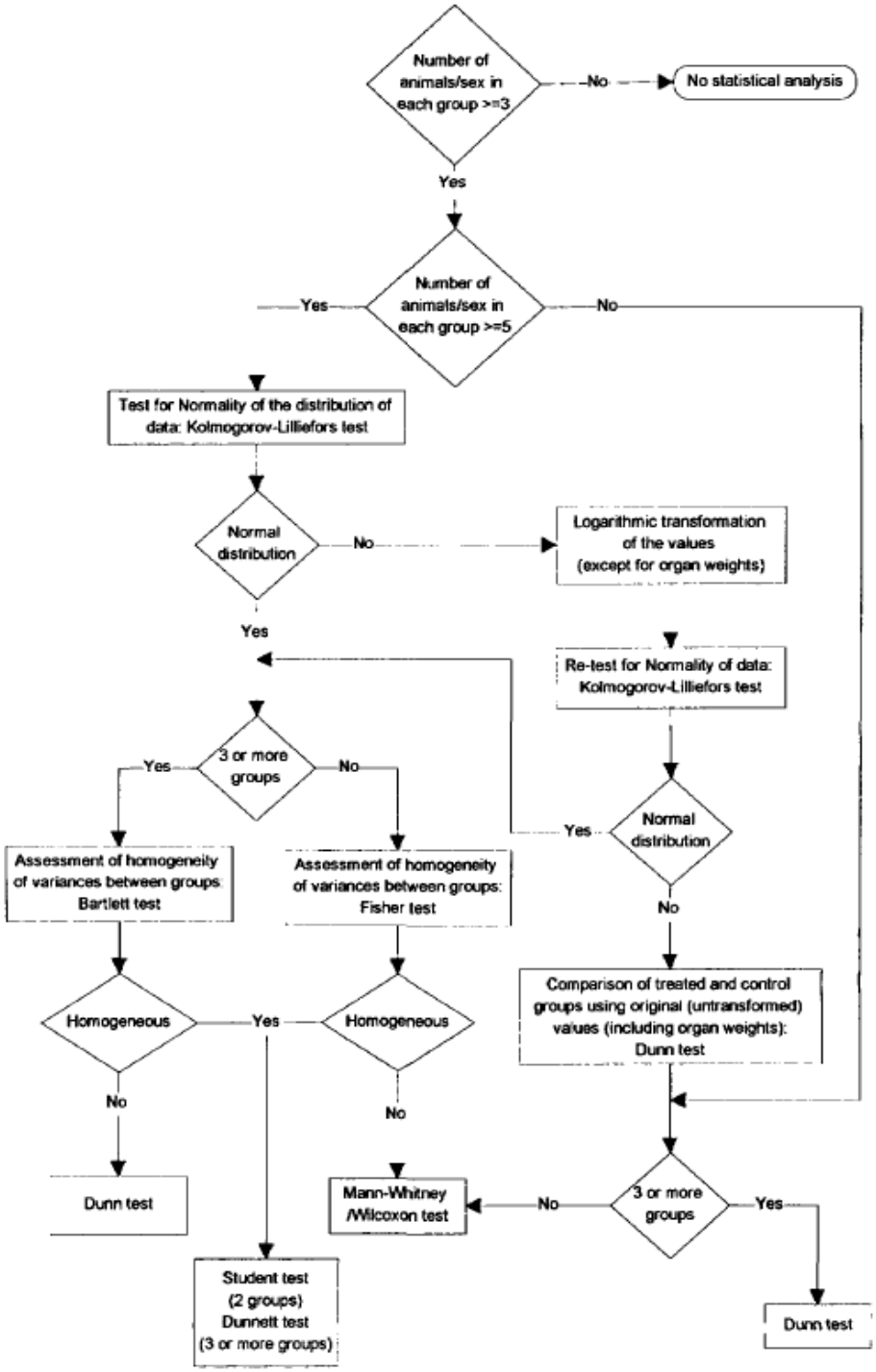
Throughout the study, a satisfactory agreement was observed between the nominal and actual concentrations of the test substance in the dietary admixtures administered since the deviations from nominal concentrations were in an acceptable range of ± 10%.

4. Statistics

Principal group animals and satellite animals were evaluated separately.

In vivo and organ weight data:

The following sequence was used for the statistical analyses of body weight, food consumption, haematology, blood biochemistry and organ weight data:



Analysis of survival and tumour data:

Survival rates were compared using the Chi-squared test.

The number of neoplasms (per group and per organ) were compared by Peto's test.

C. Methods

1. Observations

Each animal was checked at least twice a day (except on weekends and public holidays: at least once a day), for mortality or signs of morbidity. Any animal showing signs of poor clinical condition was humanely killed. When possible, a blood sample for the preparation of a blood smear was taken ante mortem for hematological examination. A macroscopic post-mortem examination was performed on all animals and the required tissues preserved for a microscopic examination.

Each animal was observed at least once a day, at approximately the same time, for the recording of clinical signs.

In addition to this routine check, a careful examination was carried out at least once a week. This observation included, but was not limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects (such as salivation), central nervous system (including tremors and convulsions), changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength and stereotypies or bizarre behavior (e.g., self mutilation, walking backwards).

From Week 4, all animals were palpated every 4 weeks, and then, from Week 52, every 2 weeks for the remainder of the study in order to record the date of detection, location, size, appearance and progression of palpable masses.

2. Body weight

The body weight of each animal was recorded once before allocation of the animals to groups, on the first day of treatment, once a week during the first 13 weeks of the treatment period and then once every 4 weeks until Week 31. Since a markedly lower body weight gain was noted among the high-dose group, body weight was recorded every 2 weeks from Week 32 until the end of the study.

3. Food intake

The quantity of food consumed by the animals of each cage was recorded once a week, over a 7-day period, during the first 13 weeks of the treatment period and then every 4 weeks until Week 30. Since a markedly lower body weight gain was noted among the high-dose group, food consumption was recorded every 2 weeks from Week 31 until the end of the study. Food consumption was calculated per animal and per day.

4. Laboratory investigations

Oncogenicity animals:

In Week 52 and before the terminal sacrifice (Week 78), blood smears were prepared from samples obtained without anesthesia from a tail vein of each surviving animal. When practicable, this was also done for any animal killed prematurely. The animals were not fasted before sampling.

After discussion with the Sponsor, it was considered that no specific signs of toxicity evidenced the need to determine the differential white cell count.

Chronic phase animals:

In Week 53, before sacrifice, and after an overnight fasting period, blood samples were collected under light isoflurane anesthesia from the orbital sinus of each surviving male and female mouse of all animals.

The following parameters were determined:

Table 3.9.1.2- 3: Laboratory parameters

Alkaline phosphatase (ALP)	IU/L
Aspartate aminotransferase (ASAT)	IU/L
Alanine aminotransferase (ALAT)	IU/L

Bone marrow (oncogenicity and chronic phase animals):

Bone marrow smears were prepared from the femoral bone of all animals killed in Week 53 and at terminal sacrifice. After discussion with the Sponsor, it was considered that no specific signs of toxicity evidenced the need to determine the bone marrow differential cell count.

5. Sacrifice and pathology

On completion of the 52- or 78-week treatment periods, after at least 14 hours fasting, all surviving animals were asphyxiated by carbon dioxide and killed by exsanguination.

Any moribund animals were killed in the same way (except for fasting).

Macroscopic pathology:

A complete macroscopic post-mortem examination was performed on all animals, including any that died during the study or were killed prematurely. This included examination of the external surfaces, all orifices, the cranial cavity, the external surfaces of the brain and spinal cord, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues.

Organ weights:

At study termination, the body weight of all satellite and all principal group animals were recorded before necropsy, and the organs specified in the Tissue Procedures Table were weighed wet as soon as possible after dissection. Paired organs (i.e. epididymides, kidneys, ovaries and testes) were weighed together.

The following organ weights were determined.

Table 3.9.1.2- 4: Organ weights

Adrenals	Liver
Brain (including medulla/pons, cerebellar and cerebral cortex)	Ovaries
Epididymides	Spleen
Heart	Testes
Kidneys	Uterus with cervix

The ratio of organ weight to body weight (recorded immediately before sacrifice) was calculated.

Tissue sampling and fixation:

For all animals including any that died during the study or were killed prematurely, the tissues specified in Table 3.9.1.2- 5 were preserved in 10% buffered formalin (except for the eyes and Harderian glands which were fixed in Davidson's fixative, and the testes and epididymides which were preserved in Bouin's fluid).

Table 3.9.1.2- 5: Sampled organs and tissues

Organs	Preser-vation of tissue	Micro-scopic exami-nation	Organs	Preser-vation of tissue	Micro-scopic exami-nation
Macroscopic lesions	X	X	Ovaries	X	X
Adrenals	X	X	Pancreas	X	X
Aorta	X	X	Pharynx	X	X
Brain (including medulla/ pons cerebellar and cerebral cortex)	X	X	Pituitary gland	X	X
Cecum	X	X	Prostate	X	X
Colon	X	X	Rectum	X	X
Duodenum	X	X	Salivary glands (sublingual, submaxillary)	X	X
Epididymides	X	X	Sciatic nerve	X	X
Esophagus	X	X	Seminal vesicles	X	X
Eyes (with Harderian glands)	X	X	Skeletal muscle	X	X
Femoral bone with articulation	X	X	Skin	X	X
Gall bladder	X	X	Spinal cord (cervical, thoracic, lumbar)	X	X
Heart	X	X	Spleen	X	X
Ileum	X	X	Sternum with bone marrow	X	X
Jejunum	X	X	Stomach with forestomach	X	X
Kidneys	X	X	Testes	X	X
Larynx	X	X	Thymus	X	X
Liver	X	X	Thyroids with parathyroids	X	X
Lungs with bronchi	X	X	Tongue	X	
Lymph nodes (mandibular and mesenteric)	X	X	Trachea	X	X
Mammary glands/area	X	X	Urinary bladder	X	X
Nose	X	X	Uterus (horns and cervix)	X	X
Optic nerve	X	X	Vagina	X	X

6. Histopathology

Tissue processing:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned and stained with hematoxylin-eosin.

Microscopic examination:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned at a thickness of approximately four microns and stained with haematoxylin-eosin.

A microscopic examination was performed on:

- all tissues listed in Table 3.9.1.2- 5 for all oncogenicity animals,
- all macroscopic lesions and liver of all chronic phase animals.

All the slides were sent to a pathologist for peer review of the following slides:

- all organs and tissues of 10% of the control and high dose-group animals,
- all tumors or neoplastic lesions,
- the liver of all animals

The microscopic findings presented in the report reflect the mutually agreed diagnoses.

II. Results and Discussion

A. Results

1. Mortality, clinical results

Mortality:

The number and occurrence of unscheduled deaths was summarized as follows:

Table 3.9.1.2- 6: Mortality

Concentration (ppm)	0	50	400	3,200
Males				
- found dead	5	3	2	3
- prematurely killed	4	3	3	3
Total	9/50	6/50	5/50	6/50
Females				
- found dead	3	3	2	9
- prematurely killed	2	6	2	0
Total	5/50	9/50	4/50	9/50

The number and occurrence of unscheduled deaths was low and comparatively similar in all the control and treated groups, except for the females of the 3,200 ppm group in which mortality was slightly higher than in the control group. This was due to the higher number of animals found dead during the first 52 weeks of treatment. Taking into consideration that a comparable number of unscheduled deaths was noted in the low-dose group, a relationship to treatment with the test substance was considered to be improbable.

At study termination (Week 78), the survival rates were as follows:

Table 3.9.1.2- 7: Survival rate (%)

Concentration (ppm)	0	50	400	3,200
Males (week 1-78)	82	88	90	88
Females (week 1-78)	90	82	92	82

The survival rate was similar in the control and the treated groups. The differences observed between these groups were slight, not statistically significant and not dose-related.

The factors contributing to death or to premature killing were comparatively similar in treated and control groups, so that there was no treatment effect. The mean duration of treatment was similar in the control and treated groups.

Clinical signs:

The nature, incidence and onset of clinical signs were similar in the animals of both sexes from the control and treated groups. None of them suggested a treatment- or dose-relationship. Additionally, they were among those commonly observed in mice. The most frequently recorded signs during the study were non-specific and/or related to the poor clinical condition of aged animals, before death or sacrifice.

No signs of neurotoxic effects were observed during the careful examination performed at weekly intervals.

The number of palpable masses recorded during the in vivo phase of the study did not indicate a dose- or treatment-relationship.

2. Body weights

At 50 and 400 ppm, body weight and body weight gain of treated animals were comparable to control values.

Animals treated at 3,200 ppm lost weight during the first week of the study (week 1-2: -0.2 g in males and -0.1 g in females compared to positive body weight gain of the other dose group animals), leading to statistically significant differences of body weight. After this period, overall body weight gain of animals treated at 3,200 ppm was severely reduced until the end of the dosing period (week 1-78: -45% in males and -35% in females).

Table 3.9.1.2- 8 gives an overview about the body weights and body weight gains.

Table 3.9.1.2- 8: Mean body weight and body weight gain

	Dose level (ppm)						
	0	**50**		**400**		**3,200**	
Males							
Body weight [g] *(% difference to control)*							
Week 1	23.1	23.0	*(-0.4)*	23.2	*(+0.4)*	23.3	*(+0.9)*
Week 2	23.9	23.9	*(±0.0)*	24.0	*(+0.4)*	23.1**	*(-3.3)*
Week 13	29.8	29.9	*(+0.3)*	29.0*	*(-2.7)*	26.9*	*(-9.7)*
Week 26	34.9	34.8	*(-0.3)*	33.8	*(-3.2)*	29.1**	*(-16.6)*
Week 52	40.8	41.3	*(+1.2)*	39.0	*(-4.4)*	31.9**	*(-21.8)*
Week 78	41.4	43.5	*(+5.1)*	42.0	*(+1.4)*	33.3**	*(-19.6)*
Body weight gain [g] *(% difference to control)*							
Week 1-2	0.8	0.9	*(+12.5)*	0.8	*(±0.0)*	-0.2	*(-125.0)*
Week 2-13	5.9	6.0	*(+1.7)*	5.0**	*(-15.3)*	3.9**	*(-33.9)*
Week 13-26	5.1	4.9	*(-3.9)*	4.8	*(-5.9)*	2.2**	*(-56.9)*
Week 26-52	5.8	6.4	*(+10.3)*	5.3	*(-8.6)*	2.8**	*(-51.7)*
Week 52-78	0.6	1.9	*(+216.7)*	3.0**	*(+400.0)*	1.1	*(+83.3)*
Week 1-78	18.3	20.5	*(+12.0)*	18.8	*(+2.7)*	10.0**	*(-45.4)*
Females							
Body weight [g] *(% difference to control)*							
Week 1	19.4	19.3	*(-0.5)*	19.4	*(±0.0)*	19.3	*(-0.5)*
Week 2	20.3	20.4	*(+0.5)*	20.3	*(±0.0)*	19.2**	*(-5.4)*
Week 13	24.5	24.5	*(±0.0)*	24.8	*(+1.2)*	22.8**	*(-6.9)*
Week 26	28.1	28.8	*(+2.5)*	28.7	*(+2.1)*	24.5**	*(-12.8)*
Week 52	33.3	34.5	*(+3.6)*	34.1	*(+2.4)*	26.7**	*(-19.8)*
Week 78	34.7	36.1	*(+4.0)*	36.3	*(+4.6)*	29.2**	*(-15.9)*

	Dose level (ppm)						
	0	**50**		**400**		**3,200**	
Body weight gain [g] *(% difference to control)*							
Week 1-2	0.9	1.1	*(+22.2)*	0.9	*(±0.0)*	-0.1**	*(-111.1)*
Week 2-13	4.2	4.1	*(-2.4)*	4.5	*(+7.1)*	3.6*	*(-14.3)*
Week 13-26	3.6	4.3	*(+19.4)*	3.9	*(+8.3)*	1.7**	*(-52.8)*
Week 26-52	5.2	5.7	*(+9.6)*	5.4	*(+3.8)*	2.2**	*(-57.7)*
Week 52-78	1.4	1.6	*(+14.3)*	2.2	*(+57.2)*	2.5	*(+78.6)*
Week 1-78	15.3	16.8	*(+9.8)*	16.9	*(+10.5)*	9.9**	*(-35.3)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

3. Food intake

Food consumption was similar in the control and the 50 or 400 ppm groups throughout the study. There was a tendency to lower food consumption in animals treated at 3,200 ppm, and mean values of the high-dose group were frequently statistically different from controls. These lower food consumption values correlated with an effect on body weight (see Table 3.9.1.2- 9).

Group mean food consumption values (g/animal/day) are summarized in Table 3.9.1.2- 9.

Table 3.9.1.2- 9: Mean food consumption in g/animal/day *(% difference to control)*

	Dose level (ppm)						
	0	**50**		**400**		**3,200**	
Males							
Week 1-12	5.8	5.7	*(-1.7)*	5.7	*(-1.7)*	5.6**	*(-3.4)*
Week 13-26	5.4	5.6	*(+3.7)*	5.5	*(+1.9)*	4.9**	*(-9.3)*
Week 29-51	5.4	5.5	*(+1.9)*	5.4	*(±0.0)*	4.8**	*(-11.1)*
Week 53-77	5.0	5.1	*(+2.0)*	5.0	*(±0.0)*	4.6**	*(-8.0)*
Week 1-78	5.4	5.5	*(+1.9)*	5.4	*(±0.0)*	5.0**	*(-7.4)*
Females							
Week 1-12	7.8	7.7	*(-1.3)*	7.9	*(+1.3)*	6.7**	*(-14.1)*
Week 13-26	6.7	6.9	*(+3.0)*	6.9	*(+3.0)*	6.1**	*(-9.0)*
Week 29-51	6.0	6.1	*(+1.7)*	6.0	*(±0.0)*	5.9**	*(-1.7)*
Week 53-77	5.6	5.7	*(+1.8)*	5.5	*(-1.8)*	5.1**	*(-8.9)*
Week 1-78	6.4	6.5	*(+1.6)*	6.5	*(+1.6)*	5.9**	*(-7.8)*

** $p \leq 0.01$, statistically different to control

4. Laboratory investigations

When compared with the mean control values, increase in liver enzyme activities was recorded in the females treated with 3,200 ppm, with a high standard variation, however attaining statistical significance for ALP activity.

Taking into account that these differences were due to the contribution of 2/10 females only and in absence of relevant changes in males, it was concluded that there were no differences of biological importance between control and treated animals in the activity of liver enzymes.

At interim sacrifice in Week 53, measurements of the activities of alkaline phosphatase (ALP), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) are summarized in Table 3.9.1.2- 10.

Table 3.9.1.2- 10: Liver enzyme activities (mean, expressed as IU/L), n = 10 *(% difference to control)*

Parameter	Dose level (ppm)					
	0	50		400		3,200	
Males						
Alkaline phosphatase	129	122	*(-5.4)*	114*	*(-11.6)*	135	*(+4.7)*
Aspartate aminotransferase	80	61	*(-23.8)*	62	*(-22.5)*	65	*(-18.8)*
Alanine aminotransferase	28	24	*(-14.3)*	40	*(+42.9)*	64	*(+128.6)*
Females						
Alkaline phosphatase	180	170	*(-5.6)*	183	*(+1.7)*	564**	*(+213.3)*
Aspartate aminotransferase	75	101	*(+34.7)*	115	*(+53.3)*	194	*(+158.7)*
Alanine aminotransferase	45	29	*(-35.6)*	34	*(-24.4)*	145	*(+222.2)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

5. Sacrifice and pathology

Macroscopic post-mortem examination:

After 52 weeks, in the chronic phase groups, liver enlargement was noted in one male given 400 ppm and in two males given 3,200 ppm. Masses and nodules were noted in the liver of two females given 3,200 ppm. These necropsy findings were considered to be treatment-related and correlated with the microscopic changes noted in the liver of these animals (hepatocellular hypertrophy, see microscopic examination).

All the other necropsy findings were found with similar incidences in both control and treated animals and showed no indication of treatment- or dose-relationship.

After 78 weeks, there was a marked increase of liver enlargement at 3,200 ppm, and the number of animals bearing masses and nodules in the liver in treated groups at 400 and 3,200 ppm was higher when compared with controls (see Table 3.9.1.2- 11).

Table 3.9.1.2- 11: Incidence of liver enlargement and masses and nodules in liver (Week 78)

Findings	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	50	50	50	50	50	50	50	50
Liver enlargement	2	7	6	28	8	3	5	29
Liver masses and nodules	3	2	6	10	1	2	3	9

The above-mentioned necropsy findings were considered to be treatment-related and correlated with the hepatic histopathological findings (hepatocellular hypertrophy and hepatocellular adenomas).

Most of the other necropsy findings observed were recognized to be those commonly occurring in aging mice of this strain and age such as enlargement and/or cystic appearance of the lymph nodes, kidneys, ovaries and uterus; enlargement of the spleen and seminal vesicles; paleness of the kidneys and small testes.

All these changes were of similar incidence and severity in both control and treated animals and showed no indication of treatment or dose-relationship.

In addition, the masses and/or nodules were equally distributed among the groups and showed no indication of a treatment-relationship.

Organ weights:

The principal differences (in %) noted between treated and control animals after 52 and 78 weeks were the absolute and relative liver weights (see Table 3.9.1.2- 12).

Table 3.9.1.2- 12: Mean absolute and relative liver weights after 52 & 72 weeks (g) *(% difference to control)*

Parameter	Dose level (ppm)					
	0	50		400		3,200	
Males						
Week 52						
Final body weight (g)	37.66	37.90	*(+0.6)*	42.56	*(+13.0)*	31.25*	*(-17.0)*
Absolute liver weight (g)	1.59	1.71	*(+7.5)*	2.06**	*(+29.6)*	2.15**	*(+35.2)*
Relative liver weight (% of body weight)	4.21	4.53	*(+7.8)*	4.84	*(+15.0)*	6.85	*(+62.7)*
Week 78						
Final body weight (g)	38.58	40.62	*(+5.3)*	39.09	*(+1.3)*	31.15**	*(-19.3)*
Absolute liver weight (g)	1.62	1.85	*(+14.2)*	1.91**	*(+17.9)*	2.37**	*(+46.3)*
Relative liver weight (% of body weight)	4.26	4.65	*(+9.2)*	4.90**	*(+15.0)*	7.62**	*(+78.6)*
Females						
Week 52						
Final body weight (g)	36.23	34.14	*(-5.8)*	34.03	*(-6.1)*	26.58**	*(-26.6)*
Absolute liver weight (g)	1.51	1.44	*(-4.6)*	1.57	*(+4.0)*	2.26**	*(+49.7)*
Relative liver weight (% of body weight)	4.21	4.20	*(+0.2)*	4.61	*(+9.5)*	8.39**	*(+99.3)*
Week 78						
Final body weight (g)	32.61	33.77	*(+3.6)*	33.73	*(+3.4)*	27.17**	*(-16.7)*
Absolute liver weight (g)	1.66	1.64	*(-1.2)*	2.20**	*(+32.5)*	2.59**	*(+56.0)*
Relative liver weight (% of body weight)	5.18	4.94	*(-4.6)*	6.62	*(+27.8)*	9.37**	*(+80.9)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

The higher absolute and relative liver weights noted in the animals given 400 and 3,200 ppm at the end of 52- and 78-week treatment periods, were considered to be treatment-related and correlated with the hepatocellular hypertrophy noted among these animals on all occasions.

At the end of 52 and 78 weeks of treatment, some other differences were noted between treated and control animals in the absolute and relative organ weights. However, as these differences were slight, not dose-related, sometimes without similar trend in both sexes and were without correlative relevant histopathological changes, they were considered to be of no toxicological importance.

6. Histopathology

Non-neoplastic findings (52 weeks of treatment):

Treatment-related microscopic findings were noted in the liver comprising dose-related hepatocellular hypertrophy predominantly centrilobular in males and periportal in females at 400 and 3,200 ppm (see Table 3.9.1.2- 13).

Table 3.9.1.2- 13: Incidence of animals with hepatocellular hypertrophy

Liver findings	Dose level (ppm)							
	Males				Females			
	0	**50**	**400**	**3,200**	**0**	**50**	**400**	**3,200**
Number of animals examined	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy	0	0	5	10	0	0	6	9

The incidence, severity and morphological characteristics of the other microscopic findings encountered were similar in both control and treated animals and did not show an indication of a treatment-relationship.

Neoplastic findings (52 weeks of treatment):

Hepatocellular adenoma was found in one female given 400 ppm and in three female mice given 3,200 ppm. The higher incidence of hepatocellular adenoma at 3,200 ppm which attained statistical significance ($p < 0.0336$) was considered to be treatment-related (see Table 3.9.1.2- 14).

Table 3.9.1.2- 14: Incidence of animals with liver tumors

Liver findings	Dose level (ppm)							
	Males				Females			
	0	**50**	**400**	**3,200**	**0**	**50**	**400**	**3,200**
Number of animals examined	10	10	10	10	10	10	10	10
Hepatocellular adenomas	0	0	0	0	0	0	1	3

Non-neoplastic findings (78 weeks of treatment):

The principal treatment-related non-neoplastic changes were hepatocellular hypertrophy and the higher incidence of altered cell foci as given in Table 3.9.1.2- 15.

Table 3.9.1.2- 15: Incidence of hepatocellular hypertrophy and altered cell foci

Liver findings	Dose level (ppm)							
	Males				Females			
	0	**50**	**400**	**3,200**	**0**	**50**	**400**	**3,200**
Number of animals examined	50	50	50	50	50	50	50	50
Hepatocellular hypertrophy	0	0	20	49	0	0	41	46
Altered cell foci:								
acidophilic	0	3	1	8	0	1	2	20
basophilic	1	2	0	0	1	0	1	1
clear	0	1	0	2	0	1	0	1
vacuolated	0	0	2	4	0	1	0	2
mixed	0	2	2	4	0	0	1	1
Total altered cell foci	**1**	**8**	**5**	**18**	**1**	**3**	**4**	**25**
(%)	***(2)***	***(16)***	***(10)***	***(36)***	***(2)***	***(6)***	***(8)***	***(50)***

No statistical analyses were performed.

High incidence of hepatocellular hypertrophy predominantly centrilobular in males and periportal in females were noted in the males and females given 400 or 3,200 ppm. This was associated with markedly high incidence of altered cell foci (especially acidophilic) in the males and females given 3,200 ppm.

The other non-neoplastic changes in the liver (such as mononuclear cell aggregation, coagulative hepatic cell necrosis, leucocytosis) were of similar incidence and severity in both control and treated animals and were regarded as being of no toxicological importance.

No treatment-related microscopic findings were note in other organs and tissues.

All non-neoplastic changes encountered in the other organs and tissues, like kidneys, heart, lungs, brain, hemolymphoreticular system, reproductive system and endocrine organs, were noted with comparatively similar incidence and severity in the control and treated groups and showed no indication of a treatment-relationship.

Neoplastic findings (78 weeks of treatment):

The number of animals with neoplasms, the number of animals with more than one primary neoplasm and the number of animals with benign and malignant tumors (see Table 3.9.1.2- 16) were comparatively similar in all groups.

Table 3.9.1.2- 16: Incidence of animals with neoplasms

Parameter	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	50	50	50	50	50	50	50	50
Number of animals with neoplasms	17	14	13	16	21	16	17	26
Number of animals with more than one primary neoplasm	4	1	0	3	6	3	1	3
Number of animals with metastases	2	3	0	0	1	2	3	3
Number of animals with benign neoplasms	8	4	7	13	11	7	8	18
Number of animals with unclassified neoplasms	0	0	0	0	0	0	0	0
Number of animals with malignant neoplasms	12	11	6	5	14	10	10	11

No statistical analyses were performed.

At 3,200 ppm, the incidence of hepatocellular adenomas was statistically significantly higher than controls in both sexes. In addition, the incidental tumor analysis as assessed from the evaluation of neoplastic lesions observed at each 100 day-interval in the animals that died or were killed prematurely showed a decrease in the latency of hepatocellular adenoma appearance in the females given 3,200 ppm, when compared with controls (one hepatocellular adenoma already during day 301-400). The incidence and time of onset of the hepatocellular adenomas in the other treated groups (50 and 400 ppm) were comparatively similar to that of the controls (see Table 3.9.1.2- 17). Moreover, no increased incidence of hepatocellular carcinoma were observed in any of the groups after the 78-week treatment period.

The incidence of hepatocellular neoplastic lesions is given in Table 3.9.1.2- 17.

Table 3.9.1.2- 17: Incidence of animals with liver neoplasms

Liver neoplasms	Dose level (ppm)			
	0	**50**	**400**	**3,200**
Males				
Number of animals examined	50	50	50	50
Hepatocellular adenoma *(%)*	5 *(10)*	0 *(0)*	5 *(10)*	11* *(22)*
Incidence in interim death animals / Time period of onset (days) ##	1 / 401-500 1 / 501-558	-	-	1 / 401-500
Hepatocellular carcinoma *(%)*	3 *(6)*	1 *(2)*	0 *(0)*	2 *(4)*
Incidence in interim death animals / Time period of onset (days) ##	1 / 501-558	1 / 501-558	-	1 / 401-500
Total *(%)*	8 *(16)*	1 *(2)*	5 *(10)*	13 *(26)*
Females				
Number of animals examined	50	50	50	50
Hepatocellular adenoma *(%)*	1 *(2)*	2 *(4)*	0 *(0)*	16** *(32)*
Incidence in interim death animals / Time period of onset (days) ##	-	-	-	1 / 301-400 1 / 501-558
Hepatocellular carcinoma *(%)*	0 *(0)*	0 *(0)*	2 *(4)*	0 *(0)*
Incidence in interim death animals / Time period of onset (days) ##	-	-	-	-
Total *(%)*	1 *(2)*	2 *(4)*	2 *(4)*	16** *(32)*

* $p \leq 0.05$, statistically different to control
** $p \leq 0.0005$, statistically different to control
The remaining number of findings of hepatocellular adenoma and carcinoma were observed after terminal sacrifice at study termination.

The neoplastic lesions found in the haemolymphoreticular system (as assessed from the microscopic examination of the liver and lymphoid organs) were diagnosed as lymphocytic malignant lymphoma, malignant lymphoma follicular center cell, mixed type (syn. composite lymphoma) and histiocytic sarcoma.

The incidence of haematopoietic neoplasms is given in Table 3.9.1.2- 18.

Table 3.9.1.2- 18: Incidence of animals with haematopoietic neoplasms

Haematopoietic neoplasms	Dose level (ppm)							
	Males				Females			
	0	**50**	**400**	**3,200**	**0**	**50**	**400**	**3,200**
Number of animals examined	50	50	50	50	50	50	50	50
Lymphocytic malignant lymphoma	0	0	0	0	1	0	0	0
Malignant lymphoma follicular center cell, mixed type	8	5	5	2	11	6	6	9
Histiocytic sarcoma	1	1	0	0	1	1	0	1
Total *(%)*	9 *(18)*	6 *(12)*	5 *(10)*	2 *(4)*	12 *(24)*	7 *(14)*	6 *(12)*	10 *(20)*

From the data cited above, it can be concluded that there was no treatment-related effect on the incidence and distribution of the haematopoietic neoplastic lesions. In addition, the incidence of these lesions is clearly within the range described for C57BL/6 in the literature (up to 20.5% in males and up to 31.3% in females: Frith C.H. (1983)[9]) and within or close to the range of historical control data (males 14-18% and females up to 18-22%; historical control data (HCD) range from two long-term studies (treatment period: 78 weeks) in C57BI/6 mice performed between November 1998 and May 2000 (149 males, 150 females); Citoxlab - Safety and Health Research Laboratories; Anonymous; 2005; M-263591-01-1).

The incidence and the morphological type of the few neoplastic lesions noted in the uterus and vagina were similar to those described for the mice of this strain and age and showed no indication of a treatment-relationship. Few cases of histiocytic sarcoma were noted in the uterus. However, unlike the histiocytic sarcoma originated from the liver, spleen or lymph nodes, in the uterine lesion large areas may be lacking giant cells.

Other few neoplastic lesions in other organs and tissues were recognized as those commonly occurring in mice of this strain and age and showed no indication of treatment-relationship.

In summary the administration of fluopicolide to C57BL/6 for 78 weeks induced after 52 weeks hepatocellular hypertrophy among the males and females given 400 or 3,200 ppm and a higher incidence of hepatocellular adenoma in the females given 3,200 ppm.

After 78 weeks hepatocellular hypertrophy was noted among the males and females given 400 or 3,200 ppm and a markedly higher incidence of hepatocellular adenoma in the males and females given 3,200 ppm.

III. Conclusion

The test substance fluopicolide was administered daily for 78 weeks by the oral route (dietary admixture) to C57BL/6 mice at 50, 400 or 3,200 ppm.

The treatment with the test substance caused a severe reduction of the body weight gain and food consumption at 3,200 ppm, thus indicating that the MTD was reached, higher liver weights, enlarged liver, masses and nodules in the liver at 400 and 3200 ppm, hepatocellular hypertrophy at 400 and 3,200 ppm, and high incidence of altered cell foci and hepatocellular adenoma at 3,200 ppm. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital (see Payraudeau, V.; 2006; M-275342-01-1 and Section 3.9.1.3), which is known to be not relevant for tumour formation in humans.

Consequently, under our experimental conditions, the No Observed Adverse Effect level (NOAEL) was 50 ppm (corresponding to 7.9 mg/kg bw/day for the males and 11.5 mg/kg bw/day for the females) for toxicity and 400 ppm (corresponding to 64.5 mg/kg bw/day for the males and 91.9 mg/kg bw/day for the females) for carcinogenicity.

[9] Frith C.H. (1983) Spontaneous lesions in virgin and retired breeder BALB/C and C57BL/6 mice. Lab. Anim. Sci., 33,273-286.

3.9.1.3 Anonymous; 2006; M-275342-01-1

Study reference:

Anonymous; 2006; AE C638206 (Fluopicolide) - Assessment of hepatocellular proliferation and lack of carcinogenicity potential; M-275342-01-1

Executive Summary:

The dietary administration of fluopicolide produced higher incidence of hepatocellular adenoma (HCA) in high dose male and female mice following a 78-week treatment period (Anonymous.; 2003; M-225595-01-1, see Section 3.9.1.2). Given that these HCA were not observed at lower dose levels in mice, not observed in rats following a 2-year treatment period and taken into account the lack of genotoxicity potential of fluopicolide, the higher incidence of HCA was thus considered to be subsequent to a threshold mechanism with a Phenobarbital-like mechanism of action (hepatocellular hypertrophy and transient cell proliferation) which is a well-known mechanism of action specific to the mouse and of no relevance to humans[10, 11, 12].

In a 28-day explanatory toxicity study (Anonymous.; 2004; M-229594-01-1,see Section 3.9.4.1) fluopicolide was shown to be a strong inducer of total cytochrome P450 and BROD and PROD associated activities. In addition, fluopicolide produced a marked transient liver cell proliferation on Day 7 which returned to control levels on Day 28. These findings were similar to those observed with phenobarbital showing that fluopicolide is a phenobarbital-like compound. Moreover, the PCNA assessment on liver tissue from animals at 3200 ppm (Anonymous.; 2006; M-205579-02-1, see Section 3.12.1.5) showed that fluopicolide did not produce hepatocellular proliferation on Day 90. This is completely consistent with the lack of cell proliferation observed on Day 28 with the BrdU assessment. These findings emphasize that the transient liver cell proliferation followed by a return to control levels (steady state) is necessary for the development of HCA following a long term exposure period to phenobarbital-like product in mice[13, 14, 15].

This mechanism of action is clearly specific to the mouse and of no relevance to humans.

In conclusion, fluopicolide by producing a marked transient liver cell proliferation in high dose mice would allow the development of HCA following a prolonged exposure period. Therefore, in the opinion of Bayer, the higher incidence of HCA observed in high dose male and female mice following a 78-week treatment period with fluopicolide are of no relevance to humans.

Conclusion:

Fluopicolide is thus devoid of any carcinogenicity potential in humans.

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10 Anderson, M. et al. (1992); Oncogenes in mouse liver tumours. In Klein-Szanto, A.J.P., Anderson, M.W., Barrett, J.C. and Slaga, T.J. (Eds), Comparative Molecular Carcinogenesis. Wiley-Liss, New York, PP.187-20

11 Grasso, P. et al. (1991): Evidence for and possible mechanisms of nongenotoxic carcinogenesis in rodent liver. Mutation Res., 248: 271-290

12 Grasso, P. et al. (1991): Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. Ann. Rev. Pharmacol. Toxicol., 31: 253-287

13 Schulte-Hermann, R. (1974). Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit. Rev. Toxicol., 3: 97-158

14 Schulte-Hermann, R. (1979: Adaptive liver growth induced by xenobiotic compounds: its nature and mechanism. Arch.Toxicol., Suppl. 2: 113-124

15 Hildebrand, B. et al. (1991): Validity of considering that early changes may act as indicators for non- genotoxic carcinogenesis. Mutation Res., 248: 217-237

3.9.2 Human data

No human data.

3.9.3 *In vitro* data (e.g. in vitro germ cell and somatic cell mutagenicity studies, cell transformation assays, gap junction intercellular communication tests)

The genotoxic potential of fluopicolide has been investigated in nine in vitro studies, covering the end-points bacterial- and mammalian-cell mutation and clastogenicity, and in five in vivo assays (an unscheduled DNA synthesis assay in rat liver, three mouse micronucleus tests and one Comet assay in mice). Overall, it is concluded that fluopicolide is not genotoxic in vivo, see Section 3.8.

3.9.4 Other data (e.g. studies on mechanism of action)

3.9.4.1 Anonymous; 2004; M-229594-01-1

Study reference:

Anonymous; 2004; AE C638206 - 28-day explanatory toxicity study in the C57BL/6 female mouse; M-229594-01-1

Executive Summary:

In order to find a mode of action for the liver tumors observed in the oncogenicity study in mice, fluopicolide was administered continuously via the diet to a group of 15 female C57BL/6 mice for at least 28 days at concentrations of 0 (control) and 3,200 ppm, equivalent to 575 mg/kg bw/day. Satellite subgroups of 20 female mice were added to each group for interim sacrifice after 7 days of treatment. Clinical signs were recorded daily, body weight and food consumption were measured weekly. A detailed physical examination was performed weekly.

Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. At both interim and final sacrifice times, liver was weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes of the liver. In addition, at interim sacrifice, hepatic cytochrome P-450 isoenzymes were assessed.

At 3,200 ppm, there were no mortalities or clinical signs during the course of the study. There was a body weight loss (-2.1 g) between Days 1-7. The mean body weight was thereafter lower than controls (-6 to -9%). Mean food consumption was lower than controls between Days 1-7 (-25%).

At interim sacrifice, mean terminal body weight was statistically significantly lower (-7%) when compared to controls. Mean absolute and relative liver weights were increased by 27 to 37% compared to controls, 9/20 livers appeared to be dark and 1/20 livers was enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals and a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/20 treated animals when compared to controls. An increased number of mitotic cells and some foci of single cell necrosis/apoptosis were seen in 5/20 treated animals. The mean BrdU labeling index was approx. 6.5-fold higher in treated animals, when compared to controls, indicative of a marked hepatocellular proliferation in the liver. Fluopicolide also induced a marked increase in total cytochrome P450 content (+97%) as well as in BROD (+1785%) and PROD (+1143%) activities.

At final sacrifice, mean terminal body weight was not affected. Mean absolute and relative liver weights were increased by 48 to 56% compared to controls, 11/15 livers appeared to be dark and 3/15 livers were enlarged. There was a diffuse, perilobular to panlobular hepatocellular hypertrophy in all treated animals together with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in 3/15 treated animals, when compared to controls. Minimal single cell necrosis/apoptosis were seen in only 1/15 treated animals and an increased number of mitotic cells in 2/15 treated animals. At terminal sacrifice, there was no increased hepatocellular proliferation (mean BrdU labellimng index) in treated animals when compared to controls.

In conclusion, treatment with fluopicolide at 3200 ppm in female C57BL/6 mice, caused a marked but transient hepatocellular proliferation, which returned to control levels after a total of 28 days treatment. Additionally, fluopicolide induces total cytochrome P450, BROD and PROD. As confirmed by a separate positive control study (see Anonymous.; 2004; M-232813-01-1, see Section 3.9.4.2) fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.3% w/w
Batch no.:	R001737

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Female mice
Strain:	C57BL/6 J@lco mice
Age:	10 weeks of age
Weight at start:	18.0 to 22.2 g
Source:	Charles River Laboratories, L'Arbresle, France
Acclimation period:	Yes
Diet:	Certified rodent powdered and irradiated diet A04C-10 PI from S.A.F.E. (Scientific Animal food and Engineering, Epinay-sur-Orge, France)
Water:	Water ad lib
Housing:	Individually in suspended stainless steel wire mesh cages
Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: October 29, 2003 to January 29, 2004

2. Animal assignment and treatment

The dose levels were set after taking into account the results of a carcinogenicity study (21557 TCS) where hepatocellular adenomas were observed in females at 3,200 ppm. Groups of 35 female mice (20 females designated for interim sacrifice on Day 8 and 15 females designated for final sacrifice on Day 29) were given control diet or the appropriate diet mixture. Table 3.9.4.1- 1 provides an overview of the study design.

Table 3.9.4.1- 1: Study design

Group no.	Dose (ppm)	Number of animals for interim sacrifice	Number of animals for final sacrifice
1	0	20	15
2	3,200	20	15

3. Diet preparation and analysis

Fluopicolide was incorporated into the diet to provide the required concentration. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing.

There was one preparation of the test formulation for the entire study. When not in use the diet formulation was stored at approx. -18 °C.

The stability of the test substance in the diet at 25 and 10,000 ppm has been demonstrated in the 90-day mouse study (Wason, S. M.; 2006; M-205579-02-1, see Section 3.12.1.5) after a freezing period of 7 weeks followed by 1 week at room temperature.

Before the start of the study, homogeneity and concentration were checked on the study mix at 3,200 ppm.

All results for homogeneity and concentration of fluopicolide in the diet were within a range of 97-99% of the nominal concentration. Hence all values were within the in-house target ranges of 85-115% of the nominal concentration. Regarding BrdU, results for concentration on the study solutions at 0.8 g/L were within the range of 104-105% of the nominal concentration.

4. Statistics

Variables analysed:

- Body weight parameters
- Body weight gain/day parameters calculated according to time intervals
- Average food and water consumption/day parameters calculated according to time intervals
- Total cytochrome P-450 content
- Cytochrome P-450 isoenzyme activities
- BrdU labeling indexes
- Organ weight parameters

Statistical analysis:

Means and standard deviations were calculated separately for each group at each time period.

Total cytochrome P-450 content:

The F test was performed to compare the homogeneity of group variances. If the F test was not significant (a=0.05), means were compared using the t-test (2-sided). If the F test was significant, data were transformed using the log transformation. If the F test on log transformed data was not significant (<x=0.05), means were compared using the t-test (2-sided) on log transformed data. If the F test was significant (<x=0.05) even after log transformation, group means were compared using the modified t-test (2-sided).

If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.

Cytochrome P-450 isoenzyme activities:

For each substrate of enzymatic activity (ethoxyresorufin, pentoxyresorufin, benzoxyresorufin, lauric acid), group means were compared using the non-parametric Mann-Whitney exact test (2-sided).

BrdU labeling indices:

Because investigations were interested only in distinguishing between the hypothesis of no difference between means or the hypothesis of an increase of the mean in the treated group compared to the control mean, group means were compared using the non-parametric Mann-Whitney exact test (1-sided).

Statistical analyses were carried out using SAS programs (SAS Software Release 8.2. SAS Institute Inc., Cary, NC, USA).

Body weight gain/day parameters, organ weights:

Mean and standard deviation were calculated for each group and per time period for body weight gain/day parameters.

The F test was performed to compare the homogeneity of group variances. If the F test was not significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

Body weight and average food or water consumption/day parameters:

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters. The F test was performed to compare the homogeneity of group variances. If the F test was not significant (0=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant (a=0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (a=0.05), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data. If the F test was significant (a=0.05) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided). If one or more group variance(s) equaled 0, means were compared using the non-parametric Mann-Whitney test (2-sided). Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals.

Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight and food intake

All animals were weighed twice during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then at approximately weekly intervals throughout the treatment period and before scheduled necropsy. Animals for interim sacrifice were weighed on Days 1 and 7 and before sacrifice only.

The weight of food supplied and of that remaining at the end of the food consumption period was recorded approximately weekly for all animals during the treatment period. From these records the mean daily consumption was calculated. Food spillage was also noted.

3. Cell proliferation assessment (in-life)

Preparation:

Solutions of 5-bromo-2'-deoxyuridine (BrdU) in drinking water were prepared twice at 0.8 g/L. When not in use, the solutions were stored at ambient temperature.

Analysis:

The stability of BrdU in drinking water was demonstrated in a previous study (SA 01416) over a 14-day period under storage and usage conditions similar to those of the current study.

Concentration of BrdU in drinking water was checked for each of the preparations used in the study.

BrdU delivery:

BrdU at 0.8 g/L in drinking water was delivered to selected animals in water bottles for seven days before scheduled sacrifice. Animals selected for interim sacrifice were given BrdU-containing water between Days 1-8 and animals selected for final sacrifice were given BrdU-containing water between Days 22-29.

Water consumption:

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day before scheduled sacrifice. The mean water consumption in g/day was calculated at each scheduled sacrifice.

4. Post mortem examinations

On Days 8 (interim sacrifice) and 29 (final sacrifice), all designated animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of approx. 60 mg/kg body weight). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled, because they were considered to be incidental for this species at this age.

Duodenum and liver were sampled and fixed in 10% neutral buffered formalin for histological and immunohistochemical assessment. Histological sections of the liver (left and median lobes), including a sample of duodenum, were prepared for all animals in all groups for all scheduled sacrifice and stained with hematoxylin and eosin. Histopathological examination was performed on the liver of all animals from all groups.

5. Cell proliferation assessment

An immunohistochemical staining to visualize the incorporation of an analog of a nucleic acid (BrdU) was used to assess hepatocytic cell cycling. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction involved incubation with a monoclonal antibody raised against BrdU, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Feulgen nuclear counterstaining. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals. The labeling index, expressed as the number of BrdU-positive hepatocytes per thousand hepatocytes, was measured separately on random fields comprising approx. 1000 centrilobular and 1000 periportal cells using an automatic image analysis system. The mean labeling index with standard deviation was calculated for each zone, each liver and each group.

6. Hepatotoxicity testing

At interim sacrifice, the remaining portions of the liver pooled by five within each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile to check the hepatotoxic potential of the test substance. Microsomal preparations were not performed from animals sacrificed at the final sacrifice date. Each microsomal sample was identified by the animal number of the first animal from the pool of five.

<u>Total cytochrome P-450 content:</u>

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

<u>Enzyme activities:</u>

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates

- benzoxyresonifin (BROD)
- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate).

Ethoxyresorufin is a highly selective substrate for the isoform IA, the isoform IIB metabolizes preferentially the O-dealkylation of pentoxyresorufin while the benzoxyresonifin O-debenzylation is mainly metabolized by the isoform IIIA. Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Samples were prepared to follow the hydroxylation of lauric acid by the isoform IVA over a period of 10 minutes at 37 °C. Two replicates of each incubation mixture were collected. One replicate was analyzed, the other one was stored frozen. 12-hydroxylauric acid was quantified in the incubation mixtures using the method N° ANL/046-94E.

Rat microsomes induced by well-known reference compounds (3-naphtoflavone, phenobarbital and clofibric acid) were measured at the same time as the study samples to have positive controls for each assay, but results were not reported in the present report.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food and water intake

At 3,200 ppm, there was a reduction of mean body weight gain (-0.35 g/day compared to 0.00 g/day in the control group, $p \leq 0.01$) and a body weight loss (-2.1 g) between days 1-7. The mean body weight gain was thereafter transiently higher and then again lower than controls between days 15-28 resulting in a mean body weight reduction throughout treatment (-6 to -9%).

Table 3.9.4.1- 2: Mean body weight and body weight gain measurements

	Dose level (ppm)		
	0	**3,200**	
Body weight (g) *(% difference to control)*			
Day 1	20.1	20.4	*(+2)*
Day 7	20.1	18.3**	*(-9)*
Day 15	20.5	19.3**	*(-6)*
Day 22	20.5	19.2**	*(-6)*
Day 28	21.0	19.3**	*(-8)*
Body weight gain (g) *(% difference to control)*			
Day 1-7	0.00	-0.35	-
Day 7-15	0.05	0.08	*(+60)*
Day 15-22	0.00	-0.02	-
Day 22-28	0.08	0.03*	*(-62)*

* $p \leq 0.05$; ** $p \leq 0.01$, significantly different from controls

At 3,200 ppm, mean food consumption was lower than controls between days 1-7 (-25 %, $p \leq 0.01$). Thereafter, food consumption was comparable between the treated and the control group. The mean achieved dosage intake of fluopicolide in mg/kg bw/day for Weeks 1 to 4 was 575 mg/kg bw/day.

Table 3.9.4.1- 3: Mean food consumption data (g/day) *(% difference to control)*

	Dose level (ppm)		
	0	**3,200**	
Day 7	3.6	2.7**	*(-25)*
Day 15	3.7	3.5	*(-5)*
Day 22	3.8	3.5	*(-8)*
Day 28	4.0	4.0	*(±0)*

* $p \leq 0.05$; ** $p \leq 0.01$, significantly different from controls

Mean water consumption was unaffected by treatment.

Table 3.9.4.1- 4: Mean water consumption data (g/day) *(% difference to control)*

	Dose level (ppm)		
	0	**3,200**	
Day 7	4.3	4.2	*(-2)*
Day 28	4.6	4.7	*(+2)*

3. Post mortem examinations

Liver weight:

At interim sacrifice, mean terminal body weight was statistically significantly lower (-7%; $p < 0.01$) at 3,200 ppm, when compared to controls.

Mean absolute and relative liver weights were statistically significantly higher at 3,200 ppm, when compared to controls.

Mean brain to body weight ratio was statistically significantly higher at 3,200 ppm (+ 6%, $p < 0.01$) but was considered not toxicologically relevant since it was considered to be related to the lower terminal body weight at 3,200 ppm.

Table 3.9.4.1- 5 gives an overview.

Table 3.9.4.1- 5: Liver weight changes in % of control at interim sacrifice

Parameter	% change
Mean absolute liver weight	+27 ($p < 0.01$)
Mean relative liver weight	+37 ($p < 0.01$)
Mean liver to brain weight ratio	+29 ($p < 0.01$)

At terminal sacrifice, there was no change in mean terminal body weight at 3,200 ppm, when compared to controls. Mean absolute and relative liver weights were statistically significantly higher at 3,200 ppm, when compared to controls.

Table 3.9.4.1- 6: Liver weight changes in % of control at terminal sacrifice

Parameter	% change
Mean absolute liver weight	+48 ($p < 0.01$)
Mean relative liver weight	+56 ($p < 0.01$)
Mean liver to brain weight ratio	+56 ($p < 0.01$)

Mean absolute brain weight was statistically significantly lower at 3,200 ppm (-5%, $p < 0.01$) when compared to controls but the reduction was so slight that it is considered to be within normal biological variations.

Gross pathology:

At interim sacrifice, 9/20 livers appeared to be dark at 3,200 ppm, compared to controls, and 1/20 livers was enlarged. Other macroscopic changes were considered to be incidental or strain-related.

At terminal sacrifice, 11/15 livers appeared to be dark at 3,200 ppm, compared to controls and 3/15 livers were enlarged. Other macroscopic changes were considered to be incidental or strain-related.

Microscopic pathology:

At interim sacrifice, several treatment-related changes were seen in the liver:

- Diffuse, perilobular to panlobular hepatocellular hypertrophy was observed in all treated animals. This hepatocellular hypertrophy was associated with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in treated animals.
- An increased number of mitotic cells was observed in treated animals, when compared to controls.
- Some foci of minimal single cell necrosis/apoptosis was observed in 5 animals at 3200 ppm

Table 3.9.4.1- 7 gives an overview.

Table 3.9.4.1- 7: Incidence and severity of treatment-related changes in the liver at interim sacrifice

	Dose level (ppm)	
	0	**3,200**
Number of animals examined	20	20
Hepatocellular hypertrophy, perilobular to panlobular, diffuse		
Minimal	0	5
Slight	0	13
Moderate	0	2
Total	0	20
Hepatocellular vacuolation, mainly Centrilobular, diffuse		
Minimal	6	3
Slight	12	0
Moderate	2	0
Total	20	3
Increased number of mitoses		
Present	0	5
Single cell necrosis/apoptosis		
Minimal	0	5
Total	0	5

At terminal sacrifice, several treatment-related changes were seen in the liver:

- A diffuse, perilobular to panlobular hepatocellular hypertrophy was observed in all treated animals. This hepatocellular hypertrophy was associated with a marked loss of diffuse, mainly centrilobular hepatocellular vacuolation in treated animals.
- An increased number of mitotic cells was observed in two treated animals, when compared to controls. Minimal single cell necrosis/apoptosis was observed in 1/15 animals only.

Table 3.9.4.1- 8 gives an overview.

Table 3.9.4.1- 8: Incidence and severity of treatment-related changes in the liver at final sacrifice

	Dose level (ppm)	
	0	**3,200**
Number of animals examined	15	15
Hepatocellular hypertrophy, perilobular to panlobular, diffuse		
Slight	0	12
Moderate	0	3
Total	0	15
Hepatocellular vacuolation, mainly Centrilobular, diffuse		
Slight	10	2
Moderate	5	1
Total	**15**	**3**
Increased number of mitoses		
Present	**0**	**2**
Single cell necrosis/apoptosis		
Minimal	0	1
Total	**0**	**1**

The other changes seen were few and considered to be incidental in origin and within the range of expected changes for animals of this strain and age kept under laboratory conditions.

4. Cell cycling assessment

At interim sacrifice, a marked hepatocellular proliferation was noted and considered to be treatment-related:

- Centrilobular, perilobular and total proliferation indexes were significantly and statistically higher in treated animals, when compared to controls.
- Mean BrdU labeling index was approximately 6.5-fold higher in treated animals, when compared to controls.

Table 3.9.4.1- 9: Mean BrdU labeling index (number of BrdU positive cells out of 1000 cells) at interim sacrifice

Interim sacrifice		Centrilobular	Perilobular	Total
Control	animal no.	20	20	20
	Mean	22.95	24.16	23.55
	STD	19.00	20.32	18.97
Fluopicolide	animal no.	20	20	20
	Mean	93.55**	215.88**	152.95**
	STD	35.40	57.91	36.64

**: significantly different from the control group ($p \leq 0.01$)

At terminal sacrifice, there was no increased hepatocellular proliferation in treated animals when compared to controls. The mean BrdU labeling index was even slightly lower in treated animals, when compared to controls (see Table 3.9.4.1- 10).

Table 3.9.4.1- 10: Mean BrdU labeling index (number of BrdU positive cells out of 1000 cells) at terminal sacrifice

Terminal sacrifice		Centrilobular	Perilobular	Total
Control	animal no.	15	15	15
	Mean	25.79	33.29	29.62
	STD	17.94	18.01	16.72
Fluopicolide	animal no.	15	15	15
	Mean	7.23	28.53	17.00
	STD	2.51	10.90	5.27

5. Total cytochrome P450 content

Fluopicolide administration at 3,200 ppm for 7 days induced a marked increase in total cytochrome P450 in female mice (+97% compared to control mean).

Enzymatic activities:

Fluopicolide markedly induced BROD (+1785%) and PROD (+1143%) activities, slightly induced cytochrome P450 (+97%) EROD activity (+79%) and decreased lauric acid hydroxylation compared to control meanafter 7 days of treatment. The magnitude of these changes are presented in Table 3.9.4.1-11.

Table 3.9.4.1- 11: Total cytochrome P-450 content and enzymatic activities at interim sacrifice

Parameter	Fluopicolide at 3,200 ppm % change compared to control mean
P450	+ 97 %
BROD	+ 1785 %
EROD	+ 79 %
PROD	+ 1143 %
Lauric acid	- 67 %

III. Conclusion

In conclusion, treatment with fluopicolide at 3,200 ppm in female C57BL/6 mice, caused a marked but transient hepatocellular proliferation, which returned to control levels after a total of 28 days treatment. Additionally, fluopicolide induces total cytochrome P450, BROD and PROD activities after 7 days of treatment. As confirmed by a separate positive control study (see Langrand-Lerche, C.; 2004; M-232813-01-1, Section 3.9.4.2) fluopicolide induced hepatic changes, both histopathological and in terms of enzyme induction activities with a phenobarbital-like profile.

3.9.4.2 Anonymous; 2004; M-232813-01-1

Study reference:

Anonymous; 2004; Phenobarbital and clofibric acid: Reference 28-day study for hepatotoxicity in the C57BL/6 mouse; M-232813-01-1

Executive Summary:

The potential liver changes caused by two reference compounds: phenobarbital and clofibric acid were assessed after oral administration by gavage at dose levels of 80 or 300 mg/kg bw/day, respectively, for 28 days, to groups of 15 male and 15 female C57BL/6 mice. A similarly constituted group of 15 animals/sex received untreated diet and acted as a control. Satellite subgroups of 20 male and female mice were added to each group for interim sacrifice after 7 days of treatment. Clinical signs were recorded daily, with body weight and food consumption and a detailed physical examination performed weekly. Bromodeoxyuridine (BrdU) was administered in drinking water for 7 days before scheduled sacrifice for cell proliferation assessment. Water consumption was measured during the period of BrdU administration. At both interim and final sacrifice times, livers were weighed and sampled. Hepatic cellular proliferation was assessed as well as morphological changes. In addition, at interim sacrifice, hepatic cytochrome P450 isoenzymes were assessed.

Phenobarbital (80 mg/kg bw/day):

There was no mortality in phenobarbital-treated groups during the study. Reduced motor activity was observed during most of the treatment period for all animals just after administration of the test substance by gavage. In males, a slight reduction mean body weight on Day 7 (-4%) was caused by reductions in body weight gains over the same period. Mean body weight and body weight gain of females were unaffected by treatment. Mean food consumption was decreased by 7% between Days 1-7 in males only. Hepatic effects were recorded as increased liver weights (interim and final sacrifices), dark livers in 5/20 males and 10/20 females at interim sacrifice and in 8/15 males and 9/15 females at final sacrifice, and loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females, corroborated with the persistence of a residual, diffuse periportal hepatocellular vacuolation in some males at bother sacrifices. At the interim sacrifice, marked hepatocellular proliferation was noted in males and females with increases in global proliferation indexes increased compared to controls. At the terminal sacrifice, this measurement was minimal compared to the change at the interim sacrifice and was seen in males only. At interim sacrifice, phenobarbital markedly induced total cytochrome P450 and BROD and PROD activities in both sexes compared to control means, with only a slight increase in males only for EROD activity.

Clofibric acid (300 mg/kg bw/dav):

One male was found dead on Day 6. Two males and two females were killed for humane reasons during the first 12 days of treatment of which one male and one female showed clinical signs indicative of a gavage error that was confirmed at necropsy. Clinical signs observed on the other decedent animals were reduced motor activity, labored respiration, soiled anogenital region, prostration, piloerection and/or coldness to touch. In the surviving animals, one male showed reduced motor activity and labored respiration on Day 6 and one other male showed soiling around the mouth on the first day of treatment. Mean body weight, body weight gain and food consumption were unaffected by treatment. Hepatic effects were recorded as increased liver weights (interim and final sacrifices), dark livers observed in 3/19 males and 8/19 females at interim sacrifice and in 13/13 males and 12/14 females at final sacrifice and a diffuse, centrilobular to panlobular hepatocellular hypertrophy in all males and females at both sacrifices. An increased number of mitotic hepatocytes was noted in 11/19 males and 9/19 females at interim sacrifice only. A loss of centrilobular to diffuse hepatocellular vacuolation was seen in the majority of males and females at both sacrifices, which was corroborated with the persistence of a residual, diffuse periportal hepatocellular vacuolation in some males and females. At the interim sacrifice, marked hepatocellular proliferation was noted in males and females with increases in global proliferation indexes compared to controls. The global proliferation index remained unchanged at the terminal sacrifice. At the interim sacrifice, clofibric acid statistically significantly induced lauric acid hydroxylation in both males and females, but not total cytochrome P450, BROD, PROD or EROD activities.

In conclusion, phenobarbital at 80 mg/kg bw/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which remained statistically significant but slight in males and returned to control levels in females after 28 days of treatment. In addition, phenobarbital was found to be a strong inducer of hepatocellular hypertrophy and of total cytochrome P450, BROD and PROD activities. Clofibric acid at 300 mg/kg bw/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, clofibric acid was found to be a strong inducer of hepatocellular hypertrophy and of lauric acid hydroxylation activities.

I. Materials and Methods

A. Materials

1. Test material

Test substance 1:	Phenobarbital (sodium salt)
Purity:	> 99% w/w
Batch no.:	088H0023

Test substance 2:	Clofibric acid
Purity:	97% w/w
Batch no.:	01220BT-121

2. Vehicle and/or positive control

Vehicle:	0.5% methylcellulose in sterilized water

3. Test animals

Species:	Mice
Strain:	C57BL/6J@lco mice
Age:	10 weeks of age
Weight at start:	21.8 to 25.7 g (males), 17.8 to 21.6 g (females)
Source:	Charles River Laboratories, L'Arbresle, France
Acclimation period:	Yes
Diet:	Certified rodent powdered and irradiated diet A04C-10 PI from S.A.F.E. (Scientific Animal food and Engineering, Epinay-sur-Orge, France)
Water:	Softened tap water ad lib
Housing:	Individually in suspended stainless steel wire mesh cages
Temperature:	22 ± 2 °C
Humidity:	55 ± 15%
Air changes:	15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 05, 2003 to May 26, 2004

2. Animal assignment and treatment

Groups of 35 male and 35 female mice were given the vehicle (0.5% methylcellulose in sterilized water) or the test substance formulations (see Table 3.9.4.2- 1).

Table 3.9.4.2- 1: Study design

Group no.	Group	Dose (mg/kg bw/day)	Interim sacrifice, satellite group 1	Interim sacrifice, satellite group 2	Final sacrifice satellite group 1	Final sacrifice satellite group 2
Males						
1	Control	0	10	10	7	8
2	Phenobarbital	80	10	10	8	7
3	Clofibric acid	300	10	10	7	8
Females						
1	Control	0	10	10	8	7
2	Phenobarbital	80	10	10	7	8
3	Clofibric acid	300	10	10	8	7

3. Diet preparation and analysis

The dosing formulations were prepared by suspending each test substance in 0.5% methylcellulose in sterilized water to produce the required dosing concentrations (w/v). There was one preparation of each test formulation for the study. When not in use, the test formulations were stored at approx. 4 °C.

The stability of phenobarbital at 8 g/L and clofibric acid at 30 g/L in aqueous methylcellulose was studied during the course of the study under conditions of storage and usage similar to those used in the present study. Before the start of the study homogeneity and concentration of phenobarbital and clofibric acid were checked on the study suspensions at 8 and 30 g/L, respectively.

4. Statistics

Means and standard deviations were calculated separately for each group at each time period.

Total cytochrome P450 content:

The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), means were compared using the t-test (2-sided). If the F test was significant, data were transformed using the log transformation. If the F test on log transformed data was not significant ($p > 0.05$), means were compared using the t-test (2-sided) on log transformed data. If the F test was significant ($p < 0.05$) even after log transformation, group means were compared using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using non-parametric procedures.

Cytochrome P450 isoenzyme activities:

For each substrate of enzymatic activity (ethoxyresorufin, pentoxyresorufin, benzoxyresorufin, lauric acid), group means were compared using the non-parametric Mann-Whitney exact test (2-sided).

BrdU labelling indexes:

Because investigations were interested only in distinguishing between the hypothesis of no difference between means or the hypothesis of an increase of the mean in the treated group compared to the control mean, group means were compared using the non-parametric Mann-Whitney exact test (1-sided). Consequently no statistical comparison was performed when the mean of the treated group was lower than the mean of the control group.

Statistical analyses were carried out using SAS programs (SAS Software Release 8.2, SAS Institute Inc., Gary, NC, USA).

Body weight changes, organ weights:

Mean and standard deviation were calculated for each group and per time period for body weight change parameters.

The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p < 0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Body weight and average food or water consumption/day parameters:

Mean and standard deviation were calculated for each group and per time period for average food or water consumption/day parameters. The F test was performed to compare the homogeneity of group variances. If the F test was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p < 0.05$), data were transformed using the Jog transformation. If the F test on log transformed data was not significant ($p > 0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data. If the F test was significant ($p < 0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equalled 0, means were compared using the non-parametric Mann-Whitney test (2-sided).

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). Group means were compared at the 5% and 1% levels of significance.

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight and food intake

All animals were weighed three times during the acclimatization phase. Each animal was weighed on the first day of test substance administration, then at approximately weekly intervals throughout the treatment period. Animals for interim sacrifice were weighed on Days 1 and 7 only. Additionally, diet fasted animals were weighed before scheduled necropsy. The weight of food supplied and of that remaining at the end of the food consumption period was recorded approximately weekly for all animals during the treatment period. From these records the mean daily food consumption was calculated. Food spillage was also noted.

3. Cell proliferation (In-life)

Preparation:

Solutions of 5-bromo-2'-deoxyuridine (BrdU) in drinking water were prepared twice at 0.8 g/L. When not in use, the solutions were stored at ambient temperature.

Analysis:

The stability of BrdU in drinking water was demonstrated in a previous study over a 14-day period under storage and usage conditions similar to those of the current study. Concentration of BrdU in drinking water was checked for each of the preparations used in the study.

BrdU delivery:

BrdU at 0.8 g/L in drinking water was delivered to selected animals in water bottles for seven days before scheduled sacrifice. Animals selected for interim sacrifice were given water containing BrdU between Days 1-8 and animals selected for final sacrifice were given water containing BrdU between Days 22-29.

Water consumption:

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed on the day before scheduled sacrifice. The mean water consumption in g/day was calculated for each scheduled sacrifice.

Postmortem examinations:

On Days 8 (interim sacrifice) and 29 (final sacrifice), all designated surviving animals from all groups were sacrificed. All sacrifices were performed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of approx. 60 mg/kg bw). Animals were diet fasted overnight prior to sacrifice. All animals, surviving, found dead or killed for humane reasons, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Brain and liver were weighed fresh. Duodenum and liver were sampled and fixed in 10% neutral buffered formalin for histological and immunohistochemical assessment. Histological sections of the liver (left and median lobes), including a sample of duodenum, were prepared for all animals in all groups for all scheduled sacrifice and stained with hematoxylin and eosin. Histopathological examination was performed on the livers of all animals from all groups.

Cell proliferation assessment:

An immunohistochemical staining to visualize the incorporation of an analog of a nucleic acid (BrdU) was used to assess hepatocytic cell cycling. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction involved an incubation with a monoclonal antibody raised against BrdU, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Feulgen nuclear counterstaining. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all surviving animals. The labeling index, expressed as the number of BrdU-positive hepatocytes per thousand hepatocytes, was measured separately on random fields comprising approx. 1000 centrilobular and 1000 periportal cells using an automatic image analysis system. The mean labeling index with standard deviation was calculated for each zone, each liver and each group.

Hepatotoxicity testing:

At interim sacrifice, the remaining portions of the liver pooled by five within each group were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile to check the hepatotoxic potential of the test substance. Microsomal preparations were not performed from animals sacrificed at the final sacrifice date. Each microsomal sample was identified by the animal number of the first animal from the pool of five.

Total cytochrome P-450 content:

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. One quantification was performed for each sample.

Enzymatic activities:

Specific cytochrome P450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- benzoxyresorufin (BROD)
- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate). Ethoxyresorufin is a highly selective substrate for the isoform 1A, the isoform IIB metabolizes preferentially the O-dealkylation of pentoxyresorufin, while the benzoxyresorufin O-debenzylation is mainly metabolized by the isoform IIIA. Cytochrome P450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37 °C.

Samples were prepared to follow the hydroxylation of lauric acid by the isoform IVA over a period of 10 minutes at 37 °C. Two replicates of each incubation mixture were collected. One replicate was analyzed, the other one was stored frozen. 12-hydroxylauric acid was quantified in the incubation mixtures using the method N° ANL/046-94E. Rat microsomes induced by well-known reference compounds (3-naphtoflavone, phenobarbital and clofibric acid were measured at the same time as the study samples to have positive controls for each assay. Results are not reported in the present report.

II. Results and Discussion

A. Results

1. Clinical results

Phenobarbital**:**

At 80 mg/kg bw/day, reduced motor activity was observed during most of the treatment period in all treated males and females just after administration of the test substance. No mortality occurred.

Clofibric acid**:**

At 300 mg/kg bw/day, four females showed reduced motor activity on Day 15. Due to the isolated occurrence of this finding, this observation was considered not to be related to the treatment.

At 300 mg/kg bw/day, one male was found dead on Day 6 showing reduced motor activity and laboured respiration on the day of death. Two males were killed for humane reason on Days 2 and 4, respectively, showing piloerection, reduced motor activity, soiled anogenital region and cold to touch or reduced motor activity, hunched posture and focal swelling in the thorax area on the day of sacrifice. Two females were killed for humane reason on Days 7 and 12, respectively. One showed reduced motor activity, prostration and coldness to touching on Day 7 and the other showed reduced motor activity on Days 11 and 12, laboured respiration, soiling around the mouth and focal swelling on the left forelimb on Day 12.

2. Body weight and food intake

Phenobarbital (PB):

Mean body weight of males was 4% and 3% lower than controls on Day 7 ($p < 0.01$) and on Day 28 ($p < 0.05$), respectively. There was a mean cumulative body weight loss of 0.7 g in males between Days 1-7 compared to a mean cumulative body weight gain of 0.2 g in the control group ($p < 0.05$). Cumulative body weight gain between Days 1-28 was also reduced by 46% compared to controls ($p < 0.05$) due to the combined body weight loss observed on Week 1 and a slightly reduced body weight gain observed between Days 22-28 (not statistically significant). In females, mean body weight was 4% lower than controls on Day 7 ($p < 0.01$). There was a mean cumulative body weight loss of 0.5 g in females between Days 1-7 compared to an absence of body weight gain in the control group ($p < 0.01$). Thereafter, mean body weight and body weight gain of females were comparable to controls.

Table 3.9.4.2- 2: Body weight and body weight gains (g)

Day	Treatment	
	Control	Phenobarbital
Males		
1	23.9	23.8
7	24.1	23.1^{+}
15	24.5	24.1
22	24.8	24.4
28	25.3	24.6*
BWG 1-7	0.2	-0.7^{+}
BWG 7-15	0.5	0.2
BWG 15-22	0.8	0.5
BWG 22-28	1.3	0.7*
Females		
1	20.0	19.8
7	20.0	19.3^{+}
15	20.6	20.1
22	21.3	20.7
28	21.6	20.9
BWG 1-7	0.0	-0.5^{+}
BWG 7-15	0.4	0.5
BWG 15-22	1.1	1.1
BWG 22-28	1.4	1.3

* $p < 0.05$; $^{+}$ $p < 0.01$

Mean food consumption was decreased by 7% ($p < 0.01$) between Days 1-7 in males and was thereafter comparable to controls. Mean food consumption of females was unaffected by treatment.

Table 3.9.4.2- 3: Mean food consumption (g/day)

Day	Treatment	
	Control	Phenobarbital
Males		
1-7	4.3	4.0^{+}
7-15	4.5	4.5
15-22	4.4	4.6
22-28	4.4	4.5
Females		
1-7	4.0	3.9
7-15	4.3	4.1
15-22	4.4	4.3
22-28	4.3	4.3

* $p < 0.05$; $^{+}$ $p < 0.01$

Clofibric acid (CA):

Generally mean body weight, body weight gain and food consumption were unaffected by treatment. Mean body weight of males was marginally lower than controls on Day 28 ($p < 0.01$). There was a slight mean body weight loss of 0.03 g/day between Days 15-22 compared to a body weight gain of 0.04 g/day in the control group ($p < 0.05$).

Table 3.9.4.2- 4: Liver weight changes (interim sacrifice)

Day	Treatment	
	Control	Clofibric Acid
Males		
1	23.9	23.6
7	24.1	23.9
15	24.5	24.5
22	24.8	24.2
28	25.3	24.5^{+}
BWG 1-7	0.2	0.3
BWG 7-15	0.5	1.1*
BWG 15-22	0.8	0.9
BWG 22-28	1.3	1.1
Females		
1	20.0	19.9
7	20.0	20.2
15	20.6	21.0
22	21.3	21.0
28	21.6	21.0
BWG 1-7	0.0	0.3
BWG 7-15	0.4	1.0
BWG 15-22	1.1	1.0
BWG 22-28	1.4	1.0

* $p < 0.05$; $^{+}$ $p < 0.01$

Mean food consumption was slightly decreased by 7% ($p < 0.05$) between Days 1-7 in males and was thereafter comparable to controls. Mean food consumption of females was unaffected by treatment, the statistically significant increase in food consumption observed between Days 1-7 being considered not to be related to the treatment, in light of the small magnitude of the increase.

Table 3.9.4.2- 5: Mean food consumption (g/day)

Day	Treatment	
	Control	Clofibric Acid
Males		
1-7	4.3	4.0*
7-15	4.5	4.5
15-22	4.4	4.4
22-28	4.4	4.7
Females		
1-7	4.0	4.1*
7-15	4.3	4.4
15-22	4.4	4.3
22-28	4.3	4.5

* $p < 0.05$; $^{+}$ $p < 0.01$

3. Post mortem examinations

Terminal body weight and organ weights:

Phenobarbital:

Interim sacrifice:

In males, mean terminal body weight was statistically significantly lower (-5%, $p < 0.01$), when compared to controls. In males, mean absolute liver weight and mean liver to brain weight ratio were slightly higher, when compared to controls but these changes were not statistically significant.

Mean liver to body weight ratio was statistically significantly higher, when compared to controls (see Table 3.9.4.2- 6). This statistically significant change was considered to be partially related to the lower terminal body weight. In females, mean absolute and relative liver weights were statistically significantly higher, when compared to controls ($p < 0.01$). These changes were considered to be treatment-related.

Table 3.9.4.2- 6: Liver weight changes (interim sacrifice)

Mean Liver weight changes (% change when compared to controls) Phenobarbital – Interim sacrifice		
	Male	**Female**
Mean absolute liver weight	+ 5%	+ 17%$^{+}$
Mean liver to body weight ratio	+ 10%$^{+}$	+ 19%$^{+}$
Mean liver to brain weight ratio	+ 4%	+ 16%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In males, mean brain to body weight ratio was statistically significantly higher (+7%, $p < 0.01$) when compared to controls: this statistically significant change was considered to be related to the lower terminal body weight in treated animals.

Terminal sacrifice:

There were no statistically significant differences for mean terminal body weights between treated and control males and females.

In both sexes, mean absolute and relative liver weights were statistically significantly higher in treated animals, when compared to controls (see Table 3.9.4.2- 7). These changes were considered to be treatment-related.

Table 3.9.4.2- 7: Liver weight changes (terminal sacrifice)

Mean Liver weight changes (% change when compared to controls) Phenobarbital – Terminal sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 12%$^{+}$	+ 16%$^{+}$
Mean Liver to body weight ratio	+ 16%$^{+}$	+ 14%$^{+}$
Mean Liver to brain weight ratio	+ 11%$^{+}$	+ 20%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In females, mean absolute brain weight was statistically significantly lower (-4%, $p < 0.05$) when compared to controls. With the small magnitude, the fact that this was considered to be within the normal range of biological variation and the potential for it being a secondary effect of reduced bodyweight, this changewas considered to be not treatment-related.

Clofibric acid:

Interim sacrifice:

In males and females, there was no relevant change in mean terminal body weights, when compared to controls. In males and females, mean absolute and relative liver weights were statistically significantly higher, when compared to controls ($p < 0.01$) (see Table 3.9.4.2- 8). These changes were considered to be treatment-related.

Table 3.9.4.2- 8: Liver weight changes after clofibric acid (interim sacrifice)

Mean Liver weight changes (% change when compared to controls) Clofibric acid – Interim sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 19%$^{+}$	+ 22%$^{+}$
Mean Liver to body weight ratio	+ 20%$^{+}$	+ 20%$^{+}$
Mean Liver to brain weight ratio	+ 18%$^{+}$	+ 22%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

Terminal sacrifice:

There were no statistically significant differences for mean terminal body weights between treated and control males and females. In males and females, mean absolute and relative liver weights were statistically significantly higher in treated animals, when compared to controls (see Table 3.9.4.2- 9). These changes were considered to be treatment-related.

Table 3.9.4.2- 9: Liver weight changes after clofibric acid (terminal sacrifice)

Mean Liver weight changes (% change when compared to controls) Clofibric acid – Terminal sacrifice		
	Male	**Female**
Mean absolute Liver weight	+ 26%$^{+}$	+ 29%$^{+}$
Mean Liver to body weight ratio	+ 27%$^{+}$	+ 25%$^{+}$
Mean Liver to brain weight ratio	+ 27%$^{+}$	+ 34%$^{+}$

* $p < 0.05$; $^{+}$ $p < 0.01$

In females, mean absolute brain weight was statistically significantly lower in treated animals (-4%, $p < 0.01$) when compared to controls: this statistically significant change was considered to be within normal ranges of biological variations, and not treatment-related. Mean brain to body weight ratio was statistically significantly lower (-8%, $p < 0.05$) when compared to controls: this statistically significant change was considered to be related to the slightly higher (+3%) terminal body weight at 300 mg/kg bw/day.

Gross pathology:

Unscheduled deaths:

All deaths were from the clofibric acid group. Two males were killed for humane reasons on Days 2 and 4, another male was found dead on Day 6. One animal which was in a moribund state with a focal thoracic swelling) had a mottled, brown lung probably associated with a gavage error (despite the absence of pulmonary lesions at histology). Macroscopically, no clear cause of death was determined for the two other two animals.

Two females were killed for humane reasons on Days 7 and 12. One, with labored respiration) showed an esophageal perforation with a pleura Effusion which was considered to have resulted from a gavage error. No clear cause of death was determined for the other female with prostration, coldness to touching and reduced motor activity): a macroscopic dark liver was noted (correlated with a slight centrilobular to panlobular hepatocellular hypertrophy at histology).

Phenobarbital

Interim sacrifice:

Dark liver was observed in 5/20 males and 10/20 females at 80 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Terminal sacrifice:

Dark liver was noted in 8/15 males and 9/15 females at 80 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Clofibric acid

Interim sacrifice:

Dark liver was observed in 3/19 males and 8/19 females at 300 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Terminal sacrifice:

Dark liver was observed in 13/13 males and 12/14 females at 300 mg/kg bw/day. Other macroscopic lesions were considered to be incidental or strain-related and not treatment-related.

Microscopic pathology:

Unscheduled deaths:

A diffuse congestion in lung and acute inflammation in the subcutis were observed in animal NT3M5636 and a diffuse congestion was found in the liver of animal NT3F5669. These findings had no specific correlation with the macroscopic observations of a gavage error.

A slight centrilobular to panlobular hepatocellular hypertrophy, correlated with a macroscopic dark liver was noted for the female NT3F5660 (as observed in some treated animals at scheduled sacrifice). No microscopic findings indicative of any cause of death were noted for animals NT3M5623, NT3M5629 and NT3F5660.

Phenobarbital:

Interim sacrifice (Table 3.9.4.2- 10):

Several treatment-related changes were seen in the liver in treated males:

- a diffuse centrilobular to midzonal hypertrophy was observed in all males and 18/20 females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males.

Table 3.9.4.2- 10: Phenobarbital liver changes at interim sacrifice

Incidence and severity of treatment-related changes in the liver Interim sacrifice - Phenobarbital				
	Males		**Females**	
Dose (mg/kg/d)	**0**	**80**	**0**	**80**
Animals examined	**20**	**20**	**20**	**20**
Hepatocellular hypertrophy, centrilobular to midzonal diffuse				
Minimal	0	8	0	7
Slight	0	12	0	11
Total	**0**	**20**	**0**	**18**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	13	3	3	5
Slight	6	1	12	7
Moderate	1	0	5	1
Total	**20**	**4**	**20**	**13**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	5	0	0
Slight	0	1	0	0
Total	**0**	**6**	**0**	**0**

Terminal sacrifice (see Table 3.9.4.2- 11):

Several treatment-related changes were seen in the liver in treated males:

- a diffuse centrilobular to midzonal hepatocellular hypertrophy was observed in all males and females.
- A loss of centrilobular to diffuse hepatocellular vacuolation was seen in some males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual,diffuse periportal hepatocellular vacuolation was seen in some males.

Table 3.9.4.2- 11: Phenobarbital liver changes at terminal sacrifice

Incidence and severity of treatment-related changes in the liver Terminal sacrifice - Phenobarbital				
	Males		**Females**	
Dose (mg/kg/d)	**0**	**80**	**0**	**80**
Animals examined	**15**	**15**	**15**	**15**
Hepatocellular hypertrophy, centrilobular to midzonal diffuse				
Minimal	0	8	0	7
Slight	0	7	0	8
Total	**0**	**15**	**0**	**15**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	9	1	6	5
Slight	5	0	8	2
Moderate	1	0	1	0
Total	**15**	**1**	**15**	**7**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	2	0	0
Total	**0**	**2**	**0**	**0**

Clofibric acid

Interim sacrifice (see Table 3.9.4.2- 12):

Several treatment-related changes were seen in the liver in treated males and females:

- a diffuse, centrilobular to panlobular hepatocellular hypertrophy was observed in all males and females.
- an increased number of mitotic hepatocytes was noted in 11/19 males and 9/19 females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in the majority of the males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males and females.

Table 3.9.4.2- 12: Clofibric acid liver changes at interim sacrifice

Incidence and severity of treatment-related changes in the liver Interim sacrifice – Clofibric acid				
	Males		**Females**	
Dose (mg/kg/d)	**0**	**300**	**0**	**300**
Animals examined	**20**	**19**	**20**	**19**
Hepatocellular hypertrophy, centrilobular to panlobular diffuse				
Minimal	0	7	0	11
Slight	0	12	0	8
Total	**0**	**19**	**0**	**19**
Increased number of mitoses				
Present	0	11	0	9
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	13	0	3	3
Slight	6	0	12	0
Moderate	1	0	5	0
Total	**20**	**0**	**20**	**3**
Hepatocellular vacuolation, periportal, diffuse				
Minimal	0	11	0	4
Slight	0	2	0	1
Total	**0**	**13**	**0**	**5**

Terminal sacrifice (see Table 3.9.4.2- 13):

Several treatment-related changes were seen in the liver in treated males and females:

- a diffuse centrilobular to panlobular hepatocellular hypertrophy was observed in all males and females.
- a loss of centrilobular to diffuse hepatocellular vacuolation was seen in all males and females.
- corroborated with the loss of the centrilobular to diffuse vacuolation, persistence of a residual, diffuse periportal hepatocellular vacuolation was seen in some males and females.

Table 3.9.4.2- 13: Clofibric acid liver changes at terminal sacrifice

Incidence and severity of treatment-related changes in the liver Terminal sacrifice – Clofibric acid				
	Males		**Females**	
Dose (mg/kg/d)	**0**	**300**	**0**	**300**
Animals examined	**15**	**13**	**15**	**14**
Hepatocellular hypertrophy, centrilobular to panlobular diffuse				
Minimal	0	0	0	1
Slight	0	5	0	11
Moderate	0	8	0	2
Total	**0**	**13**	**0**	**14**
Hepatocellular vacuolation, centrilobular to diffuse				
Minimal	9	0	6	0
Slight	5	0	8	0
Moderate	1	0	1	0
Total	**15**	**0**	**15**	**0**
Hepatocellular vacuolation, periportal, diffuse				
Slight	0	3	0	3
Total	0	3	0	3

Cell cycle assessment

Interim sacrifice: phenobarbital and clofibric acid (see Table 3.9.4.2- 14):

At interim sacrifice, in males and females, a marked hepatocellular proliferation was noted and was considered to be treatment-related: centrilobular, perilobular and global proliferation indexes were 6.6 to 14.6 fold higher in phenobarbital treated animals and 7.1 to 12.6 fold higher in clofibric acid treated animals, when compared to controls).

Table 3.9.4.2- 14: Cell cycling phenobarbital and clofibric at interim sacrifice

Interim sacrifice Phenobarbital and Clofibric acid				
		Centrilobular	**Perilobular**	**Total**
Males				
Control	N	20	20	20
	Mean	8.0	10.2	9.1
	STD	7.36	7.79	7.00
Phenobarbital	N	20	20	20
	Mean	116.5**	71.9**	92.1**
	STD	47.06	28.61	31.04
Clofibric acid	N	19	19	19
	Mean	100.6**	76.1**	87.5**
	STD	55.76	62.34	57.75
Females				
Control	N	20	20	20
	Mean	12.2	20.7	16.4
	STD	9.65	14.21	9.70
Phenobarbital	N	20	20	20
	Mean	140.7**	137.1**	138.7**
	STD	58.11	37.87	44.16
Clofibric acid	N	19	19	19
	Mean	136.8**	147.8**	142.5**
	STD	76.91	76.58	75.16

** = significantly different from the control group ($p \leq 0.01$)

Terminal sacrifice: phenobarbital and clofibric acid (see Table 3.9.4.2- 15):

Phenobarbital

In males, centrilobular and global proliferation indexes were statistically significantly higher, when compared to controls. There was no relevant change for females, when compared to controls. A centrilobular effect was still present in males but this change was minimal, compared to the effect observed at interim sacrifice.

Clofibric acid

In females, perilobular proliferation index was statistically significantly higher when compared to controls. Global index was similar and centrilobular index was lower, when compared to controls. There was no relevant change for males, when compared to controls. A perilobular effect was still present in females but this change was minimal, compared to the effect observed at interim sacrifice.

Table 3.9.4.2- 15: Cell cycling phenobarbital and clofibric at terminal sacrifice

Terminal sacrifice Phenobarbital and Clofibric acid				
		Centrilobular	**Perilobular**	**Total**
Males				
Control	N	15	15	15
	Mean	13.4	20.1	16.9
	STD	5.34	7.39	5.77
Phenobarbital	N	15	15	15
	Mean	34.4**	15.1	23.7*
	STD	17.50	7.05	10.29
Clofibric acid	N	13	13	13
	Mean	12.9	21.3	17.4
	STD	6.62	15.39	20.21
Females				
Control	N	15	15	15
	Mean	29.8	29.1	29.4
	STD	28.29	18.67	22.96
Phenobarbital	N	15	15	15
	Mean	25.8	31.2	28.7
	STD	23.69	20.50	21.31
Clofibric acid	N	14	14	14
	Mean	16.4	42.5*	30.0
	STD	10.21	19.16	13.83

* = significantly different from the control group ($p \leq 0.05$); ** = significantly different from the control group ($p \leq 0.01$)

Special testing

Total cytochrome P450 content

Phenobarbital

Phenobarbital administration for 7 days induced a marked statistically significant increase in total cytochrome P-450 in both sexes compared to control means (+95% and +77% for the males and the females, respectively).

Clofibric acid

Clofibric acid administration for 7 days induced a slight increase in total cytochrome P-450, but without reaching statistical significance (+28% and +24% for the males and the females, respectively).

Enzymatic activities

Phenobarbital (see Table 3.9.4.2- 16):

Phenobarbital markedly induced BROD and PROD activities in both sexes compared to control means. The magnitude of the changes are indicated in the table below. A slight increase was also observed in EROD activity for males only. No significant changes were observed in lauric acid hydroxylation.

Table 3.9.4.2- 16: Enzymatic activities of phenobarbital

Phenobarbital		
% change compared to control means		
	Males	Females
P450	+95%	+77%
BROD	+6326%	+1494%
EROD	+ 83%	NC
PROD	+1920%	+819%
Lauric acid	NC	NC

NC = no significant change

Clofibric acid (see Table 3.9.4.2- 17):

Clofibric acid significantly induced lauric acid hydroxylation in both males and females. The magnitude of the changes is indicated in the table below. The slight decreases observed in males for EROD activity and in females for BROD and PROD activities were considered not to be toxicologically meaningful.

Table 3.9.4.2- 17: Enzymatic activities of clofibric acid

Clofibric acid		
% change compared to control means		
	Males	Females
P450	+28%	+24%
BROD	NC	-34%
EROD	-27%	NC
PROD	NC	-44%
Lauric acid	+173%	+112%

NC = no significant change

III. Conclusion

Phenobarbital at 80 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which remained statistically significant but slight in males and returned to control levels in females after 28 days of treatment. In addition, phenobarbital was found to be a strong inducer of hepatocellular hypertrophy and of total cytochrome P-450 and BROD and PROD activities. Clofibric acid at 300 mg/kg/day induced a marked hepatocellular proliferation in male and female C57BL/6 mice after 7 days of treatment, which returned to control levels after 28 days of treatment. In addition, clofibric acid was found to be a strong inducer of hepatocellular hypertrophy and of lauric acid hydroxylation activities.

3.9.4.3 Anonymous; 2017; M-600904-01-1 + Anonymous; 2017; M-603455-01-1

Study references:

Anonymous; 2017; Fluopicolide - Preliminary concentration range finding study in cultured male and female C57BL/6 mouse hepatocytes; M-600904-01-1

+

Anonymous; 2017; Fluopicolide - Enzyme and DNA-synthesis induction in cultured male and female C57BL/6 mouse hepatocytes; M-603455-01-1

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female C57BL/6 mouse hepatocyte cultures. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM, was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of Cyp2b and Cyp3a- activities and increased cell proliferation). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

In a preliminary range finding study, fluopicolide administration to C57BL/6 male and female mouse hepatocytes in culture resulted in cytotoxicity at concentrations > 3 µM and > 10 µM respectively (Anonymous.; 2017; M-600904-01-1).

Therefore, in this study, male and female hepatocytes were treated with fluopicolide up to and including 3 µM or 10 µM respectively, resulting in a 48% decrease in ATP levels in male hepatocytes at 3 µM and a 55% decrease in ATP levels in female hepatocytes at 10 µM.

Fluopicolide induced replicative DNA synthesis in a dose-dependent manner with maximal induction at 0.3 µM (1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes). PB induced replicative DNA synthesis to a maximum of 1.8-fold and 2.2-fold in the male and female hepatocytes, respectively; the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin O-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of Cyp2b and Cyp3a induction. In male C57BL/6 mouse hepatocytes, Fluopicolide caused a dose dependant increase in PROD and BROD. BQ was also increased in these cells following administration of fluopicolide (1 and 2 µM only).

In female C57BL/6 mouse hepatocytes, fluopicolide induced a dose dependent increase in PROD, but not BROD or BQ activities.

PB (1000 µM) administration to both male and female mouse hepatocytes induced PROD, BROD and BQ activities. Treatment with the positive control items PB and EGF gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide induced both hepatocellular S-phase replicative DNA synthesis and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures.

Taken together, these data suggest that fluopicolide activated the nuclear hormone receptor constitutive androstane receptor (Car) in male and female C57BL/6 mouse hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178 Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Test animals

Species:	Mice (male and female)
Strain:	C57BL/6 mice
Age:	10 weeks old at study start
Source:	Taconic Biosciences, 273 Hover Avenue, Germantown, NY 12526
Acclimation period:	yes
Diet:	RM1 pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) (the specification of the diet is held by the Medical School Resource Unit (MSRU))
Water:	Not mentioned
Housing:	Housing in groups on saw-dust in solid-bottom, polypropylene cages.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	14-15/hour
Photoperiod:	12 hours

B. Study conduct

1. Study Dates

Study initiation date:	05 May 2017
Experimental start date:	15 May 2017
Experimental finish date:	14 July 2017

2. Experimental procedures

2.1 Hepatocyte isolation

Mice were terminally anaesthetised using Euthatal™ and hepatocytes isolated by in situ perfusion according to Mitchell A.M. et al., (1984). Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion (Laboratory Method Sheet (LMS) Tic-002), were in excess of 81% for the males and 78% for the females. Hepatocytes used in this study were pooled from multiple independent perfusions of each sex.

2.2 Hepatocyte culture

Primary monolayers of hepatocytes were cultured in plastic tissue culture flasks/plates (25 cm^2 flasks at 2 x 10^6 cells/flask; 6-well plates at 0.8 x 10^6 cells/well; and 96-well plates at 0.02 x 10^6 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Following isolation, hepatocytes were cultured in Leibowitz CL15 medium (LMS Tic-001) for 4 hours to allow adherence. The medium was then changed and the hepatocytes were exposed to PB (10, 100 and 1000 µM) or to fluopicolide (male mouse hepatocytes: 0.03, 0.1, 0.3, 1, 2 and 3 µM; females mouse hepatocytes: 0.03, 0.1, 0.3, 1, 3 and 10 µM), to epidermal growth factor (EGF) (25 ng/mL) or a vehicle control [0.1% v/v DMSO]. All test substances were formulated in DMSO. The final concentration of DMSO in all hepatocyte cultures was 0.1% (v/v).

Fluopicolide concentrations were defined in a preliminary range finding study (Chatham, L.; 2017; M-600904-01-1). Fluopicolide caused marked cytotoxicity in male and female C57BL/6 mouse primary hepatocytes at concentrations greater than 1 µM, with more pronounced cytotoxicity in male, than female C57BL/6 mouse primary hepatocytes. Therefore, the concentrations used in the main study were 0.03, 0.1, 0.3, 1, 2 and 3 mM for the male and 0.03, 0.1, 0.3, 1, 3 and 10 µM for the female mouse primary hepatocytes, respectively.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test substance concentration) were cultured in 6-well plates.

To determine cytotoxicity hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

2.3 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer and subjected to sonication and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al*. [16].

2.4 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions. Results were expressed relative to control cells.

[16] Lowry, O.R., Rosebrough, N.J., Fair, A.L. and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

2.5 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analog of the nucleoside thymidine that is incorporated into newly synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of culture. At the end of the culture period (96 hours), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

2.6 Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*[17].

2.7 Benzyloxyresorufin-0-debenzylation (BROD)

The activity of Cyp2b and Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al.*[17].

2.8 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline.

2.9 Statistics

Statistical comparisons between fluopicolide-treated mouse hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose dependant decreases in ATP levels in the male and female mouse hepatocytes, falling to 48% and 55% of control values at 3 and 10 µM, respectively. These data are in agreement with the dose range finding study (Chatham, L.; 2017; M-600904-01-1), where ATP levels of 53% and 62% relative to control were measured at 3 and 10 µM in the male and female C57BL/6 mouse hepatocytes, respectively.

Although some cytotoxicity was observed in male mouse hepatocytes after treatment with 100 µM PB this was not deemed to be biologically relevant as the ATP values remained above 85%. Neither 10 µM nor 1000 µM PB depleted ATP in male or female hepatocytes, as can be seen in Table 3.9.4.3- 1.

[17] Burke *et al.* (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. Biochem. Pharmacol. 34: 3337-3345.

Table 3.9.4.3- 1: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence)	
	Male Mouse hepatocytes	Female Mouse hepatocytes
Vehicle control (0.1% [v/v] DMSO)	482328 ± 12663 (100 ± 2.6)	467685 ± 25761 (100 ± 5.5)
PB 10 µM	457427 ± 30294 (94.8 ± 6.3)	485305 ± 8335 (103.8 ± 17.8)
PB 100 µM	418519 ± 8489*** (86.8 ± 1.8)	476751 ± 36014 (101.9 ± 7.7)
PB 1000 µM	452015 ± 28540* (93.7 ± 5.9)	506639 ± 61550 (108.3 ± 13.2)
Fluopicolide 0.03 µM	465107 ± 28825 (96.4 ± 6.00)	452015 ± 9038 (96.6 ± 1.9)
Fluopicolide 0.1 µM	42884 ± 9398*** (88.1 ± 1.9)	470627 ± 39236 (100.6 ± 8.4)
Fluopicolide 0.3 µM	441382 ± 14490*** (91.5 ± 3.0)	419130 ± 16967** (89.6 ± 3.6)
Fluopicolide 1 µM	375500 ± 21877*** (77.9 ± 4.5)	427745 ± 5186** (91.5 1.1)
Fluopicolide 2 µM	300665 ± 21692*** (62.3 ± 4.5)	-------
Fluopicolide 3 µM	231918 ± 13432*** (48.1 ± 2.8)	380615 ± 13882*** (81.4 ± 3.0)
Fluopicolide 10 µM	--------	256467 ± 7142*** (54.8 ± 1.5)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

2. Biochemical assay results

In the male mouse hepatocytes, fluopicolide caused dose dependant statistically significant increases in PROD and BROD activities (indicative of Cyp2b and Cyp2b/3a, respectively). Fluopicolide only slightly induced BQ activities (indicative of Cyp3a) at 1 and 2 µM (1.4- and 1.5-fold respectively). PROD, BROD and BQ activities were reduced at the highest concentration of fluopicolide (3 µM); this was due to cytotoxicity observed at this concentration (see Table 3.9.4.3- 1).

PB (1 mM) caused significant increases in PROD (6.5-fold), BROD (4.9-fold) and BQ (7.7-fold) activities in the male mouse hepatocytes.

The pattern of PROD activity in female mouse hepatocytes exposed to fluopicolide was similar to that of males. Here, the test substance caused a dose dependant increase up to and including 1 µM fluopicolide, where PROD activity at 1 µM was induced 1.7-fold relative to control. PROD activity at 3 µM fluopicolide was 1.6-fold relative to control but was not statistically significant in this instance. At 10 µM fluopicolide, PROD activity was reduced to 13% of control values coinciding with the cytotoxicity observed at this concentration of fluopicolide. Although there was a slight increase in BROD activity (maximum of 1.2- fold at 3 µM) this was not statistically significant. BQ activity was slightly reduced after treatment with fluopicolide, however, this was only statistically significant at 10 µM. Again, all three enzyme activity measurements were decreased at the top concentration assessed.

As in the male hepatocytes, PB (1 mM) caused significant increases in PROD, BROD and BQ activities in the female mouse hepatocytes, increasing activities by 2.6-, 1.6- and 4.4-fold, respectively.

All results are summarized in Table 3.9.4.3- 2 and Table 3.9.4.3- 3.

Table 3.9.4.3- 2: Biochemical measurements following PB or fluopicolide administration (males)

Test substance and concentration	Males		
	Mouse hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	5.89 ± 0.249 (100 ± 4.2)	24.74 ±5.15 (100 ± 20.8)	0.241 ± 0.022 (100 ± 9.3
PB 10 µM	5.00 ± 0.469* (84.9 ±8.0)	21.66 ± 1.71 (87.6 ± 6.9)	0.228 ± 0.026 (94.3 ± 10.8)
PB 100 µM	5.45 ± 0.078* (92.5 ± 1.3)	19.54 ± 0.60 (79.0 ± 2.4)	0.241 ± 0.037 (100.0 ± 15.2)
PB 1000 µM	38.07 ± 2.757*** (646.5 ± 46.8)	121.37 ± 16.67*** (490.6 ± 67.4)	1.858 ± 0.186*** (770.2 ± 77.1)
Fluopicolide 0.03 µM	6.34 ± 0.356 (107.6 ± 6.1)	22.53 ± 0.57 (91.1 ± 2.3)	0.255 ± 0.024 (93.1 ± 9.8)
Fluopicolide 0.1 µM	7.91 ± 0.393*** (134.3 ± 6.7)	29.84 ± 6.05 (120.6 ± 24.4)	0.266 ± 0.007 (110.2 ± 2.9)
Fluopicolide 0.3 µM	10.74 ± 0.583*** (182.4 ± 9.9)	34.01 ± 2.37* (137.5 ± 9.6)	0.247 ± 0.008 (102.2 ± 3.5)
Fluopicolide 1 µM	14.40 ± 0.272*** (244.6 ± 4.6)	51.51 ± 4.64** (208.3 ± 18.7)	0.340 ± 0.021** (140.9 ± 8.7)
Fluopicolide 2 µM	17.6 ± 1.955*** (298.9 ± 33.2)	62.96 ± 9.56** (254.5 ±38.6)	0.354 ± 0.023** (146.8 ± 9.5)
Fluopicolide 3 µM	11.58 ± 3.411* (196.6 ± 57.9)	34.52 ± 6.14 (139.5 ± 24.8)	0.220 ± 0.28 (91.4 ± 11.8)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Table 3.9.4.3- 3: Biochemical measurements following PB or fluopicolide administration (females)

Test substance and concentration	Females		
	Mouse hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	13.51 ± 2.19 (100 ± 16.2)	44.90 ± 11.17 (100 ± 24.9)	0.396 ± 19.4 (100 ± 19.4)
PB 10 µM	15.43 ± 0.24 (114.2 ± 1.7)	43.97 ± 12.50 (97.9 ± 27.8)	0.357 ± 0.041 (90.3 ±10.3)
PB 100 µM	22.58 ± 5.51 (167.1 ± 40.8)	54.55 ± 12.95 (121.5 ± 28.8)	0.358 ± 0.069 (90.4 ± 17.4)
PB 1000 µM	34.73 ± 2.53*** (257.0 ± 18.7)	69.99 ± 8.71* (155.9 ± 19.4)	0.938 ± 0.081*** (236.9 ± 20.3)
Fluopicolide 0.03 µM	13.44 ± 0.42 (99.5 ± 3.1)	34.51 ± 6.83 (76.9 ± 15.2)	0.303 ± 0.032 (76.5 ± 8.0)
Fluopicolide 0.1 µM	13.25 ± 3.02 (98.0 ± 22.3)	30.38 ± 8.69 (67.7 ± 19.4)	0.278 ± 0.044 (70.2 ± 11.0)
Fluopicolide 0.3 µM	20.49 ± 0.89** (151.6 ± 6.6)	51.04 ±10.90 (113.7 ± 24.3)	0.371 ± 0.027 (93.7 ± 6.9)
Fluopicolide 1 µM	23.05 ± 0.6** (170.6 ± 4.4)	50.86 ± 1.79 (113.6 ± 4.0)	0.327 ± 0.017 (82.6 ± 4.3)
Fluopicolide 3 µM	21.33 ± 5.04 (157.8 ± 37.3)	53.10 ± 11.20 (118.3 ± 24.9)	0.269 ± 0.047 (67.9 ± 11.9)
Fluopicolide 10 µM	1.76 ± 0.28*** (13.0 ± 2.1)	7.15 ± 0.80** (15.9 ± 1.8)	0.042 ± 0.002*** (10.6 ± 0.5)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p<0.05$; ** $p<0.01$; ***$p<0.001$.

3. Replicative DNA synthesis (S-phase)

In the male mouse hepatocytes, fluopicolide caused dose-dependent increases in replicative DNA synthesis; maximal induction was 1.7-fold at 0.3 µM. At 1 µM fluopicolide, replicative DNA synthesis was 1.6-fold relative to control. Replicative DNA synthesis (S-phase) was not analysed at 2 and 3 µM due to reduced cell numbers at these concentrations. PB (100 µM) and EGF (25 mg/mL) also caused significant increases in replicative DNA synthesis of 1.8-fold and 8.3-fold, respectively.

Similarly, in the female mouse hepatocytes, fluopicolide caused dose-dependent increases in replicative DNA synthesis, with the maximal response being seen at 0.3 µM (2.3-fold). The increases in replicative DNA synthesis at 1 and 3 µM were lower, at 1.5- and 1.4-fold, respectively, relative to control. Due to reduced cell numbers, S-phase was not analysed at 10 µM fluopicolide. PB caused significant increases in replicative DNA synthesis at all concentrations, the maximal response (2.2-fold) was observed at 100 µM. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (6.0-fold) in the female hepatocytes.

The results are summarized in Table 3.9.4.3- 4.

Table 3.9.4.3- 4: Replicative DNA synthesis (S-phase) assessment following PB, EGF or Fluopicolide administration

Test substance and concentration	S-Phase labelling index	
	Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	0.28 ± 0.06 (100 ± 21.2)	1.07 ± 0.16 (100 ± 14.5)
PB 10 µM	0.24 ± 0.04 (87 ± 14.0)	2.22 ± 0.19*** (206.5 ±18.1)
PB 100 µM	0.52 ±0.08*** (184.2 ± 28.6)	2.41 ± 0.24*** (224.5 ± 22.6)
PB 1000 µM	0.24 ± 0.04 (84.9 ± 13.7)	2.06 ± 0.22*** (191.9 ± 20.6)
Fluopicolide 0.03 µM	0.31 ± 0.03 (111.6 ± 11.6)	1.83 ± 0.11*** (170.7 ± 10.6)
Fluopicolide 0.1 µM	0.38 ± 0.10 (135.0 ± 35.6)	2.45 ± 0.40*** (227.8 ± 37.6)
Fluopicolide 0.3 µM	0.48 ± 0.05*** (171.9 ± 18.8)	2.50 ± 0.18*** (171.9 ± 18.8)
Fluopicolide 1 µM	0.46 ± 0.07** (163.5 ± 24.0)	1.61 ± 0.45* (149.6 ± 41.7)
Fluopicolide 2 µM	#	------
Fluopicolide 3 µM	#	1.52 ± 0.26* (141.3 ± 24.3)
Fluopicolide 10 µM	-------	#
EGF 25 ng/mL	2.32 ± 0.68*** (830.4 ± 242.7)	6.45 ± 0.90*** (600.1 ± 83.6)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control p<0.05; ** p<0.01; ***p<0.001. # not counted due to abnormal morphology and cell density following treatment with Fluopicolide.

III. Conclusion

Fluopicolide induced replicative DNA synthesis in a dose-dependent manner. In both sets of hepatocytes maximal induction occurred at 0.3 µM: 1.7-fold in male hepatocytes and 2.3-fold in female hepatocytes. Similar increases in replicative DNA synthesis were observed in cells exposed to PB, where fold increases of 1.8 and 2.2 were seen in male and female hepatocytes, respectively. As expected, the positive control EGF caused a robust proliferative response in both male and female mouse hepatocytes (8.3-fold in males and 6.0- fold in females).

Fluopicolide caused dose dependant increases in the activity of Cyp2b as determined by PROD and BROD activity in both sexes. However, BQ activities were slightly increased in the male mouse hepatocytes but not in the female hepatocytes, suggesting that Cyp3a was less affected by fluopicolide administration than was Cyp2b. In line with expectations, PB caused induction of both Cyp2b and Cyp3a enzyme activities in both sexes of mouse hepatocytes.

Treatment with the positive control items PB and EGF gave the expected set of responses, indicating the suitability of the test system.

In conclusion, fluopicolide induced hepatocellular S-phase (replicative DNA synthesis) and Cyp2b enzyme activity in both male and female C57BL/6 mouse primary hepatocyte cultures. Taken together, these data suggest that Fluopicolide activated the nuclear hormone receptor constitutive androstane receptor (Car) in male and female C57BL/6 mouse hepatocytes.

3.9.4.4 Anonymous; 2017; M-600908-01-1 + Anonymous; 2017; M-604080-01-1

Study references:

Anonymous; 2017; Fluopicolide - Preliminary concentration range finding study in cultured male and female CarKO/PxrKO mouse hepatocytes; M-600908-01-1

+

Anonymous; 2017; Fluopicolide - Enzyme and DNA-synthesis induction in cultured male and female CarKO/PxrKO mouse hepatocytes; M-604080-01-1

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (Cyp) enzyme activities in isolated male and female constitutive androstane receptor knockout/pregnane x receptor knockout (CarKO/PxrKO) mouse hepatocyte cultures. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM, was tested in parallel as an assay control to confirm CarKO/PxrKO hepatocytes responded to the reference compound in the expected manner (i.e. poor induction of Cyp2b- and Cyp3a- activities and no cell proliferation). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

Fluopicolide administration to CarKO/PxrKO male and female mouse hepatocytes in culture resulted in severe cytotoxicity at concentrations > 3 and > 10 µM respectively (Chatham, L.; 2017; M-600908-01-1). Therefore, in this study, male and female hepatocytes were treated with fluopicolide up to and including 3 or 10 µM respectively. This resulted in a decrease of 65% in ATP levels in male hepatocytes at 3 µM and a 61% decrease in ATP levels in female hepatocytes at 10 µM.

Treatment with fluopicolide or PB did not induce replicative DNA synthesis in male or female CarKO/PxrKO hepatocytes at any concentration but the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin-O-depentylation (PROD), benzyloxyresorufin 0-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of Cyp2b and Cyp3a induction. Fluopicolide did not cause any increases in PROD, BROD or BQ in male or female CarKO/PxrKO mouse hepatocytes.

PB administration (1000 µM only) to male mouse hepatocytes only slightly induced PROD, BROD and BQ to 3.5-, 1.5- and 1.7-fold respectively. 1000 µM PB also caused only slight induction in female mouse hepatocytes in PROD and BROD of 1.6- and 1.8-fold respectively, with no induction observed in BQ.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, Cyp2b or Cyp3a enzyme activity in male or female CarKO/PxrKO mouse primary hepatocyte cultures. Taken together, these data suggest that fluopicolide requires the presence of the nuclear hormone receptors Car and/or Pxr to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178 Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Test animals

Species:	Mice (male and female)
Strain:	Male and female CarKO/PxrKO mice (C57BL/6-Nrli2tm3Arle/Nrli3tmUArte, product number, 8222-M or 8222-F, respectively)
Age:	11-12 weeks old at study start
Source:	Taconic Biosciences, 273 Hover Avenue, Germantown, NY 12526
Acclimation period:	yes
Diet:	RM1 pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) (the specification of the diet is held by the Medical School Resource Unit (MSRU))
Water:	Not mentioned
Housing:	Housing in groups on saw-dust in solid-bottom, polypropylene cages.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	14-15/hour
Photoperiod:	12 hours

B. Study conduct

1. Study Dates

Study initiation date: 02 June 2017

Experimental start date: 05 June 2017

Experimental finish date: 12 June 2017

2. Experimental procedures

2.1 Hepatocyte isolation

Mice were terminally anaesthetised using Euthatal™ and hepatocytes isolated by in situ perfusion according to Mitchell A.M, Bridges, J.W. & CR. Elcombe (1984)[18]. Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion, were in excess of 81% for the males and 78% for the females. Hepatocytes used in this study were pooled from multiple independent perfusions of each sex.

2.2 Hepatocyte culture

Primary monolayers of hepatocytes were cultured in plastic tissue culture flasks/plates (25 cm^2 flasks at 2 x 10^6 cells/flask; 6-well plates at 0.8 x 10^6 cells/well; and 96-well plates at 0.02 x 10^6 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Following isolation, hepatocytes were cultured in Leibowitz CL15 medium for 4 hours to allow adherence. The medium was then changed and the hepatocytes were exposed to PB (10, 100 and 1000 µM) or to fluopicolide (male mouse hepatocytes: 0.03, 0.1, 0.3, 1, 2 and 3 µM; females mouse hepatocytes: 0.03, 0.1, 0.3, 1, 3 and 10 µM), to epidermal growth factor (EGF) (25 ng/mL) or a vehicle control [0.1% v/v DMSO]. All test substances were formulated in DMSO. The final concentration of DMSO in all hepatocyte cultures was 0.1% (v/v).

Fluopicolide concentrations were defined in a preliminary range finding study (Chatham, L.; 2017; M-600908-01-1). In this study, fluopicolide caused pronounced cytotoxicity in CarKO/PxrKO mouse primary hepatocytes at concentrations greater than 1 and 3 µM in male and female, respectively. Therefore, the concentrations used in the main study were 0.03, 0.1, 0.3, 1, 2 and 3 µM for the male and 0.03, 0.1, 0.3, 1, 3 and 10 µM for the female CarKO/PxrKO mouse primary hepatocytes, respectively.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test item concentration) were cultured in 6-well plates.

To determine cytotoxicity, hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

[18] Mitchell A.M, Bridges, J.W. & CR. Elcombe (1984) Factors influencing peroxisome proliferation in cultured rat hepatocytes. Arch. Toxicol. 55: 239-246

2.3 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer and subjected to sonication and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al.*[16].

2.4 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions.

Results were expressed relative to control cells.

2.5 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analog of the nucleoside thymidine that is incorporated into newly synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of culture. At the end of the culture period (96 hours), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

2.6 Pentoxyresorufin-O-depentylation (PROD)

The activity of Cyp2b in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al*.[17] according to LMS Fluor-002.

2.7 Benzyloxyresorufin-0-debenzylation (BROD)

The activity of Cyp2b and Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin.

2.8 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of Cyp3a in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline.

2.9 Statistics

Statistical comparisons between fluopicolide-treated mouse hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose dependant decreases in ATP levels in the male and female mouse hepatocytes, falling to 35% and 39% of control values at 3 and 10 µM, respectively. These data are in agreement with the dose range finding study (Chatham, L.; 2017; M-600908-01-1), where ATP levels of 36% and 46% relative to control were measured at 3 and 10 µM in the male and female CarKO/PxrKO mouse hepatocytes, respectively.

Although some cytotoxicity was observed in male mouse hepatocytes after treatment with PB (10, 100 and 1000 µM) and female hepatocytes (100 µM only) this was not deemed to be biologically relevant as the ATP values remained above 85%.

An overview is given in Table 3.9.4.4- 1.

Table 3.9.4.4- 1: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence units)	
	Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	441352 ± 17781 (100 ± 4.0)	412962 ± 29443 (100.0 ± 7.1)
PB 10 µM	392636 ± 18325*** (89.0 ± 4.2)	415156 ± 19045 (100.5 ± 4.6)
PB 100 µM	406457 ± 16496** (92.1 ± 3.7)	373077 ± 13602* (90.3 ± 3.3)
PB 1000 µM	415126 ± 17249* (94.1 ± 3.9)	398332 ± 24209 (96.5 ± 5.9)
Fluopicolide 0.03 µM	413286 ± 16455* (93.6 ± 3.7)	407496 ± 17572 (98.7 ± 4.3)
Fluopicolide 0.1 µM	399551 ± 10509*** (90.5 ± 2.4)	387127 ± 21231 (93.7 ± 5.1)
Fluopicolide 0.3 µM	384198 ± 22427*** (87.1 ± 5.1)	363244 ± 44708* (88.0 ± 10.8)
Fluopicolide 1 µM	336433 ± 8650*** (77.9 ± 4.5)	369433 ± 27823* (89.5 ± 6.7)
Fluopicolide 2 µM	225484 ± 11574*** (51.1 ± 2.6)	Not analyzed
Fluopicolide 3 µM	153540 ± 10158*** (34.8 ± 2.3)	309758 ± 25430*** (75.0 ± 6.2)
Fluopicolide 10 µM	Not analyzed	162451 ± 5137*** (39.3 ± 1.2)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

2. Biochemical assay results

In the male CarKO/PxrKO mouse hepatocytes, fluopicolide caused decreases in PROD activity (indicative of Cyp2b), with no activity at all observed after treatment with 3 µM fluopicolide.

This lack of activity was due to cytotoxicity observed at this concentration. Fluopicolide reduced BROD and BQ activities (indicative Cyp2b/3a and Cyp3a respectively) in a dose dependant manner.

PB administration (1000 µM only) to male mouse hepatocytes caused small but significant increases in PROD, BROD and BQ by 3.5-, 1.5- and 1.7-fold respectively.

In female CarKO/PxrKO mouse hepatocytes, treatment with fluopicolide caused decreases in PROD, BROD and BQ activities. Enzyme assays for 3 and 10 µM fluopicolide were n=l and n=2 respectively, therefore, statistics have not been carried out on these concentrations.

1000 µM PB also caused small, but significant inductions in PROD and BROD by 1.6- and 1.8-fold respectively. BQ activity was decreased compared to control after treatment with 1000 µM PB.

All results are summarized in Table 3.9.4.4- 2 and Table 3.9.4.4- 3.

Table 3.9.4.4- 2: Biochemical measurements following PB or fluopicolide administration (males)

Test substance and concentration	Males Mouse KO hepatocytes		
	PROD (pmol resorufin/min/mg)	**BROD (pmol resorufin/min/mg)**	**BQ (nmol 7-OH quinoline/min/mg)**
Vehicle control (0.1% [v/v] DMSO)	0.14 ± 0.103 (100 ± 73.7)	4.18 ± 0.73 (100 ± 17.3	0.081 ± 0.007 (100 ± 8.7)
PB 10 µM	0.16 ± 0.064 (116.5 ± 45.6)	3.09 ± 0.47 (73.8 ± 11.3)	0.074 ± 0.004 (91.1 ± 4.7)
PB 100 µM	0.15 ± 0.070 (104.2 ± 50.4)	2.64 ± 0.74 (63.1 ± 17.8)	0.082 ± 0.007 (100.9 ± 8.0)
PB 1000 µM	0.49 ± 0.084** (354.7 ± 60.3)	6.07 ± 0.27* (145.1 ± 6.6)	0.142 ± 0.011** (174.1 ± 13.9)
Fluopicolide 0.03 µM	0.17 ± 0.038 (121.6 ± 27.2)	3.23 ± 0.36 (77.1 ± 8.7)	0.084 ± 0.005 (103.0 ± 6.3)
Fluopicolide 0.1 µM	0.16 ± 0.032 (118.2 ± 23.0)	2.69 ± 0.04* (64.2 ± 1.0)	0.082 ± 0.001 (100.4 ± 1.7)
Fluopicolide 0.3 µM	0.18 ± 0.059 (182.4 ± 9.9)	3.24 ± 0.23 (77.5 ± 5.5)	0.076 ± 0.006 (93.1 ± 7.4)
Fluopicolide 1 µM	0.08 ± 0.039 (60.6 ± 28.3)	2.26 ± 0.04* (54.0 ± 1.1)	0.072 ± 0.006 (89.0 ± 8.0)
Fluopicolide 2 µM	0.02 ± 0.012 (11.0 ± 8.5)	1.27 ± 0.08** (30.4 ± 1.8)	0.043 ± 0.0002*** (52.8 ± 0.2)
Fluopicolide 3 µM	#	0.51 ± 0.13*** (12.1 ± 3.0)	0.027 ± 0.003*** (33.0 ± 3.7)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. # no activity was determined in that sample set.

Table 3.9.4.4- 3: Biochemical measurements following PB or fluopicolide administration (females)

Test substance and concentration	Females		
	Mouse KO hepatocytes		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	2.08 ± 0.103 (100 ± 73.7)	11.74 ± 1.53 (100 ± 13.0)	0.509 ± 0.049 (100 ± 9.7)
PB 10 µM	1.83 ± 0.17 (88.1 ± 8.1)	12.56 ± 0.09 (107.0 ± 0.8)	0.502 ± 0.046 (98.6 ± 8.9)
PB 100 µM	1.61 ± 0.45 (77.7 ± 21.9)	12.9 ± 2.01 (103 ± 17.1)	0.432 ± 0.090 (84.8 ± 17.7)
PB 1000 µM	3.30 ± 0.14*** (257.0 ± 18.7)	21.69 ± 0.45*** (184.7 ± 3.8)	0.384 ± 0.025* (75.5 ± 4.9)
Fluopicolide 0.03 µM	1.70 ± 0.08* (81.8 ± 4.0)	12.33 ± 2.30 (105.0 ± 19.6)	0.591 ± 0.069 (116.2 ± 13.5)
Fluopicolide 0.1 µM	1.75 ± 0.25 (84.3 ± 11.8)	10.51 ± 0.41 (89.5 ± 19.4)	0.463 ± 0.056 (91.0 ± 11.1)
Fluopicolide 0.3 µM	2.01 ± 0.19 (97.0 ± 7.7)	12.41 ± 0.69 (105.7 ± 5.9)	0.480 ± 0.030 (94.4 ± 5.8)
Fluopicolide 1 µM	1.41 ± 0.21** (68.0 ± 10.2)	8.81 ± 0.72* (75.0 ± 6.2)	0.417 ± 0.062 (81.9 ± 12.2)
Fluopicolide 3 µM	0.57[a] (27.3)	4.74[a] (40.3)	165[a] (32.5)
Fluopicolide 10 µM	#[b]	0.36 ± 0.07[b] (3.1 ± 0.6)	0.005 ± 0.000[b] (1.1 ± 0.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. # no activity was determined in that sample set.
[a] one samples analyzed at the dose group; [b] two samples analyzed at the dose group.

3. Replicative DNA synthesis (S-phase)

In the male mouse hepatocytes, fluopicolide did not cause any significant increases in replicative DNA synthesis at any concentration analysed. S-phase labelling index was not analysed at 2 and 3 µM due to reduced cell numbers at these concentrations. EGF (25 ng/mL) caused a significant increase in replicative DNA synthesis of 5.8-fold, whereas PB did not cause any induction in replicative DNA synthesis.

Similarly, in the female mouse hepatocytes, fluopicolide did not cause any increases in replicative DNA synthesis at any concentration analysed. Due to reduced cell numbers, S-phase labelling index was not analysed at 3 or 10 µM fluopicolide. PB did not cause any significant increases in replicative DNA synthesis at any concentration analysed. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (3.2-fold) in the female hepatocytes.

The results are summarized in the following table.

Table 3.9.4.4- 4: Replicative DNA synthesis (S-phase) assessment following PB, EGF or fluopicolide administration

Test substance and concentration	S-Phase labelling index	
	Mouse hepatocytes	
	Males	Females
Vehicle control (0.1% [v/v] DMSO)	0.52 ± 0.13 (100 ± 24.9)	1.76 ± 0.35 (100 ± 20.1)
PB 10 µM	0.60 ± 0.09 (116.3 ± 16.8)	1.53 ± 0.18 (86.7 ± 10.4)
PB 100 µM	0.43 ± 0.11 (83.1 ± 21.8)	1.85 ± 0.36 (104.7 ± 20.4)
PB 1000 µM	0.42 ± 0.09 (81.0 ± 17.2)	1.68 ± 0.49 (95.3 ± 28.0)
Fluopicolide 0.03 µM	0.43 ± 0.07 (83.9 ± 14.4)	1.55 ± 0.24 (87.6 ± 13.8)
Fluopicolide 0.1 µM	0.48 ± 0.05 (92.1 ± 9.1)	1.63 ± 0.15 (92.6 ± 8.6)
Fluopicolide 0.3 µM	0.48 ± 0.18 (93.8 ± 33.5)	1.35 ± 0.21 (76.7 ± 12.2)
Fluopicolide 1 µM	0.40 ± 0.06 (78.1 ± 12.0)	1.30 ± 0.14 (73.9 ± 7.9)
Fluopicolide 2 µM	#	------
Fluopicolide 3 µM	#	#
Fluopicolide 10 µM	-------	#
EGF 25 ng/mL	3.01 ± 0.93*** (582.4 ± 180.7)	5.65 ± 1.05*** (320.2 ± 59.4)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 5 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
not counted due to abnormal morphology and cell density following treatment fluopicolide.

III. Conclusion

Based on the toxicity observed in the preliminary ATP assay (Chatham, L.; 2017; M-600908-01-1) the highest concentration of fluopicolide used to treat male and female mouse hepatocytes was 3 and 10 µM respectively. At these concentrations, ATP levels were depleted to 36% (male) and 46% (female) of control values.

Neither fluopicolide nor PB induced replicative DNA synthesis at any concentrations evaluated. As expected, the positive control, EGF, caused a robust proliferative response in both male and female mouse hepatocytes (5.8-fold in males and 3.2- fold in females).

Fluopicolide did not cause any increases in the activity of Cyp2b or Cyp3a as determined by PROD, BROD and BQ activity in both sexes. In line with expectations, PB did not cause robust induction of Cyp2b or Cyp3a enzyme activities in either sex of CarKO/PxrKO mouse hepatocytes.

Treatment with the positive control for replicative DNA synthesis, EGF, gave the expected set of responses, indicating the suitability ofthe test system.

In conclusion, fluopicolide did not induce either hepatocellular S-phase replicative DNA synthesis, Cyp2b or Cyp3a enzyme activity in male or female CarKO/PxrKO mouse primary hepatocyte cultures. Taken together, these data suggest that Fluopicolide requires the presence of the nuclear hormone receptors Car and/or Pxr to induce replicative DNA synthesis and enzyme activity in male and female mouse hepatocytes.

3.9.4.5 Anonymous; 2017; M-600911-01-1 + Anonymous; 2017; M-604094-01-1

Study references:

Anonymous; 2017; Fluopicolide - Preliminary concentration range finding study in cultured human hepatocytes from three individual donors; M-600911-01-1

+

Anonymous; 2017; Fluopicolide - Enzyme and DNA-synthesis induction in cultured human hepatocytes from three individual donors; M-604094-01-1

Executive Summary:

The aim of this study was to investigate the potential for fluopicolide to stimulate cell proliferation (measured as the change in replicative DNA synthesis during S-phase of the cell cycle) and modulate cytochrome P450 (CYP) enzyme activities in cryopreserved male and female human hepatocyte cultures from three independent donors. Cytotoxicity, as evaluated by adenosine 5'-triphosphate (ATP) depletion, was assessed in parallel.

Phenobarbital (PB) at 10, 100 and 1000 µM, was tested in parallel as an assay control to confirm hepatocytes responded to the reference compound in the expected manner (induction of CYP2B- and CYP3A-activities). In addition, Epidermal Growth Factor (EGF, 25 ng/mL) was included as a positive control for hepatocyte proliferation.

In a preliminary study, fluopicolide administration to male and female human hepatocytes in culture resulted in cytotoxicity at concentrations > 3 and > 10 µM respectively (Chatham, L.; 2017; M-600911-01-1). In this study, male and female human hepatocytes were treated with fluopicolide up to and including 10 or 100 µM, respectively, resulting in an estimated 38% decrease in ATP levels in male hepatocytes at 10 µM and estimated 69% or 57% decreases in ATP levels in female hepatocytes at 100 µM (donor 8239 and 1765, respectively).

Neither administration with fluopicolide nor PB induced replicative DNA synthesis in cultured male or female human hepatocytes. However, the proliferative capability of these cells in culture was confirmed using EGF (25 ng/mL).

Hepatic pentoxyresorufin O-dependentylation (PROD), benzyloxyresorufin O-debenzylase (BROD) and benzoxyquinoline-O-debenzylation (BQ) rates are indicative of CYP2B and CYP3A induction. In male human hepatocytes, Fluopicolide caused a dose-dependent increase in BQ. BROD was also slightly increased in these cells following administration of fluopicolide. PB administration to male human hepatocytes induced BROD and BQ in a dose-dependent manner. PROD activity could not be analysed as levels were below the level of quantification.

Treatment with fluopicolide resulted in dose-dependent increases in BQ activity in female hepatocytes from both donors. Fluopicolide caused no increased PROD or BROD activity in female hepatocytes, however, slight, but significant, decreases in BROD activity were observed at the top concentrations. In female human hepatocytes, PB induced a dose-dependent increase in BROD and BQ in both donors, however, only donor 1765 responded in a dose-dependent manner after treatment with PB. Treatment with the positive control item EGF gave the expected set of responses, indicating the suitability of the test system.

In summary, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and females)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

In conclusion, these data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels) with no effect on DNA-synthesis in male or female human hepatocytes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Fluopicolide
Purity:	98.2% w/w
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	0.1% v/v DMSO
Positive controls:	Phenobarbital sodium salt (PB), catalogue no. P-5178 Epidermal growth factor (EGF), catalogue no. E-9644)
Supplier:	Sigma-Aldrich Company Ltd, Poole, Dorset, UK

3. Human Hepatocytes

Primary male and female human hepatocytes (cryopreserved and plateable) were sourced from Life Technologies, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR. Hepatocytes were sourced from a single male donor with reference number 8210 (Caucasian, 51 yrs.) and two female donors with reference numbers 8239 (Caucasian, 52 yrs.) and 1765 (Caucasian, 37 yrs.). Data sheets are provided in the report.

B. Study conduct

1. Study Dates

Study initiation date:	27 June 2017
Experimental start date:	03 July 2017
Experimental finish date:	21 July 2017

2. Experimental procedures

2.1 Hepatocyte culture

Viabilities of the hepatocyte preparations, as determined by Trypan Blue exclusion (Laboratory Method Sheet (LMS) Tic-002), were in excess of 80%.

Primary monolayers of hepatocytes were cultured in collagen-coated plastic tissue culture flasks/plates (25 cm^2 flasks at 3.2 x 10^6 cells/flask; 6-well plates at 1.6 x 10^6 cells/well; and 96-well plates at 4 x 10^4 cells/well). In all 96-well plate cultures, the outside wells of the culture plates were filled with sterile phosphate buffered saline to reduce culture media evaporation.

Hepatocytes were resuscitated in Cryopreserved Hepatocyte Thaw Medium, then cultured in Cryopreserved Hepatocyte Plating Medium at 37 °C in a humidified incubator under an atmosphere of 95% air/5% CO_2 for 6 hours to allow adherence. The medium was then changed to Leibowitz human complete L15 (HCL15) medium and the hepatocytes exposed to PB at 3 concentrations (10, 100 and 1000 µM) as an assay control, the test substance at 6 concentrations (the male human donor was treated with 0.03, 0.1, 0.3, 1, 3 and 10 µM, the female human donors were treated with 0.3, 1, 3, 10, 30 and 100 µM fluopicolide) and a vehicle control [0.1% v/v DMSO].

The concentrations of fluopicolide used were selected with reference to the preliminary range finding study (Chatham, L.; 2017; M-600911-01-1). In this range finder, fluopicolide caused pronounced cytotoxicity in primary cultures of hepatocytes from one male donor at concentrations greater than 3 µM and two female donors at concentrations greater than 10 or 100 µM, with more pronounced cytotoxicity being observed in the male primary hepatocytes. Therefore, following discussions with the sponsor, the concentrations to be taken to the main study were 0.03, 0.1, 0.3, 1, 3 and 10 µM for the male donor, and 0.3, 1, 3, 10, 30 and 100 µM for the two female donors.

Hepatocytes were cultured for 96 hours and the culture medium, including test and control substances, was replaced at 24 h intervals. Hepatocytes used for enzyme assays (n=3 per test substance concentration) were cultured in 25 cm^2 flasks. To determine replicative DNA synthesis, hepatocytes (n=5 per test substance concentration) were cultured in 6-well plates. To determine cytotoxicity, hepatocytes (n=6 per test substance concentration) were cultured in 96-well plates.

2.2 Hepatocyte culture harvest

After 96 hours in culture, hepatocytes were either fixed in methanol for assessment of the cell cycle S-phase labelling index or harvested into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH 7.4) for subsequent enzyme activity analysis. Hepatocytes harvested into SET were scraped into the buffer, subjected to sonication, and stored at -70 °C until analysis. Protein was determined by the method of Lowry *et al.*[16].

2.3 Hepatocyte cytotoxicity

Hepatocyte toxicity, following 96 hours of culture, was assessed by measuring ATP depletion (LMS-Spec-009) using the CellTitre-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions.

Results were expressed relative to control cells.

2.4 Replicative DNA synthesis (cell cycle S-phase labelling index)

The number of cells undergoing replicative DNA synthesis (cell cycle S-phase labelling index) in any given cell population can be determined by measuring the incorporation of 5'-bromo-2'-deoxyuridine (BrdU) by immunocytochemistry (ICC). BrdU is a synthetic analog of the nucleoside thymidine that is incorporated into newly-synthesised DNA; the incorporated BrdU is detected by ICC. Cell cycle S-phase labelling index was determined over the last 3 days of the culture. At the end of the culture period (96 hrs), ICC was performed on fixed cells; the number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

2.5 Pentoxyresorufin-O-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, as described by Burke *et al.*[17] according to LMS Fluor-002.

2.6 Benzyloxyresorufin-0-debenzylation (BROD)

The activity of CYP2B and CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin, as described by Burke *et al.*[17], according to LMS Fluor-002.

2.7 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, as described by the Corning Gentest HTS technical bulletin and according to LMS Fluor-005.

2.8 Statistics

Statistical comparisons between fluopicolide-treated human hepatocytes and their respective control groups were undertaken for all numerical data sets using a 2-tailed Student's t-test.

II. Results and Discussion

A. Results

1. Cytotoxicity determination by ATP depletion

Fluopicolide caused dose-dependent decreases in ATP levels in male and female human hepatocytes, falling to 62%, 31% and 43% of control values at 10 µM (donor 8210) and 100 µM (donors 8239 and 1765), respectively. These data are in broad agreement with the dose range finding study (Chatham, L.; 2017; M-600911-01-1) where declines in ATP levels of 58% and 37%, relative to control, were measured at 10 µM (donor 8210) and 100 µM (donor 8239), respectively. However, Donor 1765 did not suffer as great a decline in ATP levels at 100 µM, compared to control, in this study compared with the previous dose range finding study (43% vs. 70%, respectively).

No cytotoxicity was observed in either male or female human hepatocytes after treatment with PB.

An overview is given in Table 3.9.4.5- 1.

Table 3.9.4.5- 1: ATP assay following PB or fluopicolide administration

Test substance and concentration	ATP content (luminescence units)		
	Male human hepatocytes (Donor 8210)	**Female human hepatocytes (Donor (8239)**	**Female human hepatocytes (Donor 1765)**
Vehicle control (0.1% [v/v] DMSO)	301240 ± 15625 (100 ± 5.2)	82474 ± 6272 (100.0 ± 7.6)	72652 ± 4952 (100.0 ± 6.8)
PB 10 µM	375108 ± 26769*** (124.5 ± 8.9)	103164 ± 9620** (125.1 ± 11.7)	74811 ± 4250 (103.0 ± 5.8)
PB 100 µM	334775 ± 17404** (111.1 ± 5.8)	102030 ± 10472** (123.7 ± 12.7)	81209 ± 6504* (111.8 ± 9.0)
PB 1000 µM	308482 ± 19913 (102.4 ± 6.6)	85064 ± 7689 (103.1 ± 9.3)	76352 ± 6198 (105.1 ± 8.5)
Fluopicolide 0.03 µM	319470 ± 16891 (106.1 ± 5.6)	-------	-------
Fluopicolide 0.1 µM	335622 ± 17799** (111.4 ± 5.9)	-------	-------
Fluopicolide 0.3 µM	334441 ± 29050* (111.0 ± 9.6)	87427 ± 10547 (106.0 ± 12.8)	74283 ± 4862 (102.2 ± 6.7)
Fluopicolide 1 µM	315283 ± 27099 (104.7 ± 9.0)	95004 ± 8775* (115.2 ± 10.6)	67440 ± 6446 (92.8 ± 8.9)
Fluopicolide 3 µM	283059 ± 8089* (94.0 ± 2.7)	73538 ± 6146* (89.2 ± 7.5)	62939 ± 6116* (86.6 ± 8.4)
Fluopicolide 10 µM	186593 ± 18036*** (61.9 ± 6.0)	56856 ± 5868*** (68.9 ± 7.1)	63557 ± 5527* (87.5 ± 7.6)

Test substance and concentration	ATP content (luminescence units)		
	Male human hepatocytes (Donor 8210)	Female human hepatocytes (Donor (8239)	Female human hepatocytes (Donor 1765)
Fluopicolide 30 µM	-------	41382 ± 3699*** (50.2 ± 4.5)	50481 ± 3742*** (69.5 ± 5.2)
Fluopicolide 100 µM	-------	25465 ± 2523*** (30.9 ± 1.2)	31253 ± 23131 (43.0 ± 5.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 6 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control. ; * statistically different from control
p < 0.05; ** p < 0.01; ***p < 0.001.

2. Biochemical assay results

In the male human hepatocytes, PROD activities could not be determined as the values were below the limit of quantification, therefore, no results are presented for this assay in the male Donor 8210. Fluopicolide caused slight increases in BROD and BQ activities to a maximum of 1.5- and 2.6-fold respectively. All levels of PB resulted in statistically significant increases in BROD and BQ activities in the male human hepatocytes, with maximum increases observed at 1 mM (2.0- and 5.3-fold respectively).

PROD activity was not altered after exposure to fluopicolide in hepatocytes from female human Donor 8239. However, BROD activity was significantly reduced at 30 and 100 µM fluopicolide. BQ activity increased in a dose-dependent manner, to a maximum of 1.7- fold induction compared to control, this increase was observed at 3 µM.

Fluopicolide induced small, but statistically significant, increases in PROD and BQ activities in female human Donor 1765, to a maximum of 1.5- and 2.8-fold respectively, with a dose-dependent increase observed in BQ activities. No increases were observed in BROD activity after administration with fluopicolide and a small but statistically significant decrease was observed after treatment with 100 µM fluopicolide.

Treatment with PB resulted in increased PROD, BROD and BQ activities in the female human hepatocytes from Donor 8239 to a maximum of 1.1-, 1.7- and 3.6-fold, respectively. PB also caused significant increases in PROD, BROD and BQ activities in the female human hepatocytes from Donor 1765, to a maximum of 2.3-, 3.9- and 5.4-fold, respectively.

The results are summarized in Table 3.9.4.5- 2 to Table 3.9.4.5- 4.

Table 3.9.4.5- 2: Biochemical measurements following PB fluopicolide administration in Donor 8210 (male)

Test substance and concentration	Human hepatocytes (Donor 8210)	
	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
	Male	
Vehicle control (0.1% [v/v] DMSO)	0.53 ± 0.006 (100 ± 11.1)	0.110 ± 0.004 (100 ± 3.5)
PB 10 µM	0.71 ± 0.04** (135.5 ± 7.2)	0.141 ± 0.007** (127.6 ± 6.)3
PB 100 µM	1.06 ± 0.10** (200.8 ± 19.6)	0.243 ± 0.0016*** (220.6 ± 14.7)
PB 1000 µM	2.88 ± 0.34*** (547 ± 65.2)	0.579 ± 0.023*** (525.6 ± 20.52)

Test substance and concentration	Human hepatocytes (Donor 8210)	
	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
	Male	
Fluopicolide 0.03 µM	0.71 ± 0.05* (135.6 ± 9.4)	0.146 ± 0.008** (132.3 ± 7.1)
Fluopicolide 0.1 µM	0.59 ± 0.09 (112.9 ± 16.6)	0.122 ± 0.006* (110.7 ± 5.4)
Fluopicolide 0.3 µM	0.65 ± 0.05* (124.3 ± 9.2)	0.164 ± 0.007*** (149.0 ± 5.9)
Fluopicolide 1 µM	0.77 ± 0.07** (145.7 ± 12.9)	0.193 ± 0.009*** (174.8 ± 8.1)
Fluopicolide 3 µM	0.78 ± 0.03** (147.6 ± 4.8)	0.285 ± 0.013*** (258.4 ± 11.9)
Fluopicolide 10 µM	0.40 ± 0.04 (76.6 ± 6.8)	0.227 ± 0.024 (206.1 ± 22.1)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$

Table 3.9.4.5- 3: Biochemical measurements following PB or fluopicolide administration in Donor 8239 (female)

Test substance and concentration	Female		
	Donor 8239		
	PROD (pmol resorufin/min/mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control 0.1% [v/v] DMSO)	0.21 ± 0.08 (100 ± 38.8)	0.81 ± 0.14 (100 ± 17.7)	0.17 ± 0.08 (100 ± 44.98)
PB 10 µM	0.20 ± 0.04 (97.3 ± 18.7)	0.78 ± 0.07 (97.1 ± 8.9)	0.19 ± 0.08 (108.63 ± 48.71)
PB 100 µM	0.23 ± 0.08 (111.1 ± 38.8)	108 ± 0.02* (134.4 ± 2.4)	0.28 ± 0.11 (161.3 ± 62.4)
PB 1000 µM	0.20 ± 0.07 (96.2 ± 32.0)	1.39 ± 0.14** (171.9 ± 17.9)	0.57 ± 0.27 (333.84 ± 156.54)
Fluopicolide 0.3 µM	0.20 ± 0.01 (97.1 ± 6.1)	0.78 ± 0.03 (96.4 ± 3.4)	0.19 ± 0.08 (109.09 ± 45.68)
Fluopicolide 1 µM	0.21 ± 0.09 (102.1 ± 43.6)	0.88 ± 0.05 (108.4 ± 6.6)	0.22 ± 0.09 (130.49 ± 50.03)
Fluopicolide 3 µM	0.14 ± 0.01 (69.4 ± 3.2)	0.64 ± 0.08 (78.8 ± 9.9)	0.29 ± 0.12 (169.49 ± 68.44)
Fluopicolide 10 µM	0.21 ± 0.03 (101.4 ± 15.4)	0.55 ± 0.13 (68.4 ± 16.4)	0.16 ± 0.08 (95.05 ± 45.44)
Fluopicolide 30 µM	0.19 ± 0.02 (92.2 ± 10.2)	0.54 ± 0.03 (66.6 ± 3.3)	0.07 ± 0.03 (38.90 ± 15.82)
Fluopicolide 100 µM	#	0.34 ± 0.14 (42.0 ± 17.2)	#

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
no activity observed at this concentration.

Table 3.9.4.5- 4: Biochemical measurements following PB or fluopicolide administration in Donor 1765 (female)

Test substance and concentration	Female		
	Donor 1765		
	PROD (pmol resorufin/min/ mg)	BROD (pmol resorufin/min/mg)	BQ (nmol 7-OH quinoline/min/mg)
Vehicle control (0.1% [v/v] DMSO)	0.12 ± 0.01 (100 ± 12.31)	0.87 ± 0.01 (100 ± 79)	0.08 ± 0.01 (100 ± 15.04
PB 10 µM	0.12 ± 0.07 (101.0 ± 6.4)	0.96 ± 0.12 (110.29 ± 13.87)	0.09 ± 0.01 (114.14 ± 14.45)
PB 100 µM	0.15 ± 0.018 (128.7 ± 15.1)	1.63 ± 0.25** (184.67 ± 28.93)	0.18 ± 0.01 (224.72 ± 18.66)
PB 1000 µM	0.28 ± 0.04 (230.3 ± 35.1)	3.42 ± 0.024*** 392.82 ± 28)	0.42 ± 0.03 (542.27 ± 39.73)
Fluopicolide 0.3 µM	0.11 ± 0.019 (95.6 ± 16.1)	0.86 ± 0.11 (98.91 ± 12.26)	0.08 ± 0.02 (100.67 ± 21.77)
Fluopicolide 1 µM	0.09 ± 0.01 (75.0 ± 10.6)	0.95 ± 0.06 (109.36 ± 7.39)	0.12 ± 0.01 (152.30 ± 17.4)
Fluopicolide 3 µM	0.10 ± 0.008 (80.2 ± 6.5)	1.01 ± 0.12 (116.5 ± 13.22)	0.20 ± 0.03 (254.20 ± 32.06)
Fluopicolide 10 µM	0.103 ± 0.008 (85.3 ± 6.3)	0.92 ± 0.07 (105.39 ± 8.43)	0.22 ± 0.01 (286 ± 16.39)
Fluopicolide 30 µM	0.175 ± 0.036* (145.6 ± 30.2)	0.71 ± 0.11 (81.3 ± 12.83)	0.06 ± 001 (72.93 ± 1.87)
Fluopicolide 100 µM	0.132 ± 26.5 (109.8 ± 26.5)	0.72 ± 0.086* (82.52 ± 9.90)	0.03 ± 0.001 (34.26 ± 0.09)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 3 per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.
no activity observed at this concentration.

3. Replicative DNA synthesis (S-phase)

In the male human hepatocytes, Donor 8210, treatment with either fluopicolide or PB did not result in any increases in replicative DNA synthesis. S-phase was not analysed at 10 µM fluopicolide due to reduced cell numbers at these concentrations. EGF (25 ng/mL) caused significant increases in replicative DNA synthesis of 7.4-fold.

Similarly, in the female human hepatocytes, treatment with either fluopicolide or PB did not result in any increases in replicative DNA synthesis. Due to reduced cell numbers, S-phase was not analysed at 30 µM (Donor 8239) or 100 µM (Donors 8239 and 1765) fluopicolide. As expected, EGF (25 ng/mL) significantly induced replicative DNA synthesis (9.7-fold for Donor 8239 and 5.9-fold for Donor 1765) in the female human hepatocytes.

The results are summarized in Table 3.9.4.5- 5.

Table 3.9.4.5- 5: Replicative DNA synthesis (S-phase) assessment following PB, EGF or fluopicolide administration

Test substance and concentration	S-phase labelling index		
	Male human hepatocytes (Donor 8210)	Female human hepatocytes (Donor (8239)	Female human hepatocytes (Donor 1765)
Vehicle control (0.1% [v/v] DMSO)	0.28 ± 0.04 (100 ± 15.8)	0.22 ± 0.07 (100 ± 30.0)	0.16 ± 0.06 (100 ± 38.9)
PB 10 µM	0.30 ± 0.06 (107.5 ± 22.8)	0.25 ± 0.07 (115.1 ± 29.9)	0.13 ± 0.03 (82.1 ± 19.7)
PB 100 µM	0.32 ± 0.08 (113.0 ± 28.7)	0.27 ± 0.04 (121.8 ± 18.8)	0.17 ± 0.08) (110.2 ± 52.2)

Test substance and concentration	S-phase labelling index		
	Male human hepatocytes (Donor 8210)	**Female human hepatocytes (Donor (8239)**	**Female human hepatocytes (Donor 1765)**
PB 1000 µM	0.30 ± 0.07 (105.8 ± 23.4)	0.30 ± 0.07 (137.5 ± 29.8)	0.19 ± 0.04 (121.2 ± 24.7
Fluopicolide 0.03 µM	0.33 ± 0.07 (118.7 ± 24.2)	-------	-------
Fluopicolide 0.1 µM	0.34 ± 0.07 (120.3 ± 23.6)	-------	-------
Fluopicolide 0.3 µM	0.30 ± 0.07 (107.7 ± 24.0)	0.24 ± 0.06 (108.4 ± 25.8)	0.17 ± 0.04 (111.5 ± 26.1)
Fluopicolide 1 µM	0.29 ± 0.07 (103.85 ± 26.10	0.22 ± 0.10 (101.2 ± 46.8)	0.17 ± 0.08 (112.7 ± 53.1)
Fluopicolide 3 µM	0.24 ± 0.06 (86.9 ± 21.0)	0.29 ± 0.09 (131.9 ± 42.0)	0.19 ± 0.06 (120.3 ± 39.6)
Fluopicolide 10 µM	#	0.16 ± 0.04 (73.82 ± 42.0)	0.12 ± 0.04 (77.3 ± 26.74)
Fluopicolide 30 µM	-------	#	0.09 ± 0.03 (58.63 ± 21.19)
Fluopicolide 100 µM	-------	#	#
EGF 25 ng/mL	2.07 ± 0.2*** (736.01 ± 57.3)	2.12 ± 0.16*** (968.16 ± 73.89)	0.91 ± 0.11 (589.42 ± 71.19)

Values are mean ± SD. Values in parenthesis are mean % control ± SD; n= 5per group.
A student's t-test (2-sided) was performed on the results; * statistically different from control
$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$. # Concentrations not counted due to abnormal morphology and cell density following treatment with fluopicolide.

III. Conclusion

Neither fluopicolide nor PB induced replicative DNA synthesis in male or female human hepatocytes. As expected, the positive control EGF caused a robust proliferative response in both male and female human hepatocytes (7.4 -fold for male Donor 8210, 9.7-fold for female Donor 8239 and 5.9-fold for female Donor 1765).

Fluopicolide caused dose-dependent increases in the activity of CYP3A as determined by BQ activity in both sexes. PROD activities were not increased in the female human hepatocytes and levels could not be detected in male human hepatocytes, suggesting that CYP2B was not affected by fluopicolide administration.

In conclusion, treatment of cultured male or female human hepatocytes with fluopicolide resulted in weak induction of CYP3A enzyme activity (BROD (male only) and BQ activities (male and female)). There was no evidence of fluopicolide or PB-stimulated proliferation in cultured male or female human hepatocytes.

Treatment with the positive control item EGF gave the expected set of responses, indicating the suitability of the test system.

These data suggest that fluopicolide is a weak activator of human PXR (as shown by the effects on CYP3A enzyme activity levels) with no effect on DNA-synthesis in male or female human hepatocytes.

3.10 Reproductive toxicity

Table 3.10- 1: Summary of reproductive studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Pilot reproductive study in rats (diet) 0, 50, 200, 750 and 2,500 ppm [equivalent to 0, 4/4, 17/18, 65/67 and 197/204 mg/kg bw/day for M/F]	Parental toxicity: 200 ppm [17/18 mg/kg bw/day in M/F] Offspring: 750 ppm [65/67 mg/kg bw/day in M/F] Reproductive effects: 2,500 ppm [197/204 mg/kg bw/day in M/F]	Parental toxicity: 750 ppm [65/67 mg/kg bw/day in M/F] Offspring: 2500 ppm [197/204 mg/kg bw/day in M/F] Reproductive effects: >2,500 ppm [197/204 mg/kg bw/day in M/F]	↓ bodyweight gain and food consumption (F) ↓ bodyweight gain	Anonymous; 2002; M-215068-01-1
2-generation study in rats (diet) 0, 100, 500 and 2,000 ppm [equivalent to 0, 5.2/6.4, 25.5/32.9 and 103.4/127.3 mg/kg bw/day for M/F premating]	Parental toxicity: 500 ppm [25.5/32.9 mg/kg bw/day in M/F] Offspring: 500 ppm [35.8 mg/kg bw/day] Reproductive effects: 2,000 ppm [103.4/127.3 mg/kg bw/day]	Parental toxicity: 2,000 ppm [103.4/127.3 mg/kg bw/day] Offspring: 2000 ppm [103.4/127.3 mg/kg bw/day] Reproductive effects: >2,000 ppm [103.4/127.3 mg/kg bw/day]	↓ bodyweight gain and food consumption (M/F) ↑ liver and kidney weights (M/F) ↓ spleen weights (F) ↑ incidence of centrilobular hepatocyte hypertrophy ↑ incidence of degenerative and regenerative changes in kidneys (M/F) ↓ bodyweight gain	Anonymous; 2003; M-232532-01-1 Anonymous; 2004; M-247289-01-1
Developmental toxicity range finding study in rats (gavage) 500 and 1,000 mg/kg bw/day	Maternal toxicity: <500 mg/kg bw/day Developmental toxicity: <500 mg/kg bw/day	Maternal toxicity: 500 mg/kg bw/day Developmental toxicity: 500 mg/kg bw/day	↓ food consumption ↓ mean foetal weight and crown-rump length	Anonymous; 2000; M-198488-01-1
Developmental toxicity study in rats (gavage) 0, 5, 60 and 700 mg/kg bw/day	Maternal toxicity: 60 mg/kg bw/day Developmental toxicity: 60 mg/kg bw/day	Maternal toxicity: 700 mg/kg bw/day Developmental toxicity: 700 mg/kg bw/day	↓ bodyweight gain and food consumption ↓ mean foetal weight and crown-rump length ↓ ossification ↑ incidence of minor defects	Anonymous; 2004; M-202155-02-1
Developmental toxicity range finding study in rabbits (gavage) 25, 50, 100, 250, 500 and 1,000 mg/kg bw/day	Maternal toxicity: 25 mg/kg bw/day Developmental toxicity: 50 mg/kg bw/day	Maternal toxicity: 50 mg/kg bw/day Developmental toxicity: >50 mg/kg bw/day	↓ bodyweight gain and food consumption ↑ incidence of premature deliveries	Anonymous; 2000; M-211192-01-1

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Developmental toxicity study in rabbits (gavage) 0, 5, 20 and 60 mg/kg bw/day	Maternal toxicity: 20 mg/kg bw/day	Maternal toxicity: 60 mg/kg bw/day	↑ mortality and ↓ in body weight gain due to drastically reduced feed consumption ↑ incidence of premature deliveries	Anonymous; 2004; M-202513-02-1
	Developmental toxicity: 20 mg/kg bw/day	Developmental toxicity: 60 mg/kg bw/day	↓ mean foetal weight and crown-rump length	
Supplementary information: External expert statement regarding fluopicolide's reproductive and developmental toxicity potentials	-	-	This document contains a summary and review of the two-generation and the developmental toxicity studies with rats and rabbits by an external expert for reproductive toxicity concluding that on the basis of the available studies, fluopicolide should not be classified as a reproductive toxicant.	Anonymous; 2018; M-638869-01-1

M = male, F = female

In a **preliminary** study treatment levels for a two-generation study in **rats** were examined. Fluopicolide was administered orally, via the diet, at concentration levels of 0, 50, 200, 750 or 2,500 ppm to groups of 8 males and 8 females, 15 days prior to pairing until termination after weaning of the resulting litters. Selected offspring (constituting an F1 generation) continued to receive the diets from about the time of weaning until termination following attainment of sexual maturation.

This preliminary study showed that dietary concentrations of 2,500 ppm of fluopicolide induced general toxicity observed as bodyweight gain reductions in F0 males during the premating period, and in F0 females during the gestation and lactation period. A transient effect in F0 females was also observed during the gestation period at 750 ppm. F1 offspring body weight gains were also reduced at 2,500 ppm. Reproductive parameters were considered to be unaffected by treatment with fluopicolide up to and including the highest tested dose of 2,500 ppm. Therefore, a dose level of maximum up to 2,500 ppm was regarded as suitable as the high concentration in a main study of reproductive performance.

In the main **2-generation study in rats** the influence of fluopicolide on the fertility and reproductive performance of two successive generations was assessed in male and female rats of the Crl: CD® (SD) IGS BR strain. Fluopicolide was administered continuously in the diet at concentrations of 0, 100, 500 or 2,000 ppm to groups of rats throughout the two generations. The F0 generation, which comprised 28 males and 28 females in each group, received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation.

Body weight gain and food consumption were low for adult animals treated at 2,000 ppm throughout the study, with the exception of the low body weight gain which was not apparent in the females following parturition. Oestrous cycles, mating performance, fertility and fecundity were similar in all groups. Gestation length, parturition process and sperm parameters were unaffected by treatment. The return of females to oestrous cycling following lactation was not influenced by treatment in either generation. Sexual maturation, as assessed by the age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation, was also not affected by treatment with fluopicolide at doses up to and including 2,000 ppm.

Litter parameters at birth of the F1 and F2 progeny and their survival to weaning showed no detrimental effects of treatment. Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet.

At 2,000 ppm, kidney and liver weights were high for parental males and females in both generations, when compared with the controls and a retrospective histopathological examination showed treatment-related findings in both organs (centrilobular hepatocyte hypertrophy and degenerative and regenerative changes in kidneys) at this dose level. Group mean body weight-relative liver weights were also slightly higher for females treated at 500 ppm, when compared with the controls and centrilobular hepatocyte hypertrophy was also present in in males at 500 ppm from both generations. Since these findings are common in the livers of rodents which have been administered xenobiotics, they are considered to be an adaptive change and not a toxic effect of treatment at this dose level.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL). The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

A **rat range finding study** was conducted to select suitable doses of fluopicolide for a subsequent developmental toxicity (teratogenicity) study in Sprague Dawley rats. Groups of 4 mated female Sprague Dawley rats received technical fluopicolide in aqueous methylcellulose (1% w/v) by oral gavage once daily at the dose levels of 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy and were sacrificed on Day 21 of pregnancy.

The key maternal findings in this study included a reduced gain in body weight throughout the period of treatment at 1,000 mg/kg bw/day (-34% compared to 500 mg/kg bw/day and -27% when corrected for gravid uterine weight). Food consumption showed a marked initial (Days 7-10) decrease at this dose level and a slight reduction at 500 mg/kg bw/day. No compound-related effects were observed at necropsy of the animals. Post-implantation loss was elevated at 1,000 mg/kg bw/day and included one total resorption. Mean fetal weight and crown-rump length were reduced at 1000 and 500 mg/kg bw/day. Therefore, the highest dose selected for the definitive study of developmental toxicity was 700 mg/kg bw/day.

In the **main developmental toxicity study in rats**, groups of 23 mated female Sprague Dawley rats received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 60 or 700 mg/kg bw/day from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection). They were sacrificed on Day 21 of pregnancy.

Body weights and weight gains were decreased in the animals from the high dose group, especially at the beginning of the treatment period during gestational Days 7-10 (-24% when compared with the control value), a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. These animals showed also a slight initial decrease in food consumption after beginning of treatment. Overall body weight gain (Days 1-21) was 9% lower than the concurrent control value and 12% lower when corrected for gravid uterine weight.

Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group. However, litter size, number of live and dead foetuses as well as sex ratios were unaffected by the administration of the test substance. Incidences of early and late conceptuses undergoing resorption were also not affected by the administration of the test compound up to and including the highest tested dose level of 700 mg/kg bw/day.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor skeletal defects at the thoracic vertebrae, sternebrae and ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses. In addition a delayed ossification was detected at 700 mg/kg bw/day which indicated together with the decreased foetalweight and length a generally retarded foetal development at this maternally toxic dose level.

In conclusion, oral administration of fluopicolide to the pregnant rat at the dose of 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gains and slightly decreased food consumption. Mean foetal body weights and crown-rump lengths were also slightly decreased at700 mg/kg bw/day. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification were observed more frequently at this dose level and are considered secondary to the above described maternal toxicity. Fluopicolide was not teratogenic in this developmental toxicity study in rats.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 60 mg/kg bw/day or below. Therefore, with regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

A **rabbit range finding study** was conducted in rabbits in order to select a suitable high dose level of fluopicolide for a subsequent developmental toxicity study in Himalayan rabbits. Groups of 4 mated female Himalayan rabbits received fluopicolide suspended in 1% (w/v) aqueous methyl cellulose by oral gavage once daily at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw/day from Day 6-28 of pregnancy (Day 0: day of mating) and were sacrificed on Day 29 of pregnancy.

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study. At the dose of 50 mg/kg bw/day one animal aborted on Day 29. No clinical signs of toxicity were observed at 25 mg/kg bw/day. Body weight gains and food consumption were decreased during the treatment period at 50, 100, 250, 500 or 1,000 mg/kg bw/day. Body weight gains were not impaired in the animals from the 25 mg/kg bw/day group.

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

One animal from the 25 and 50 mg/kg bw/day group each was not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section the remaining animals from these groups. Gravid uterus and foetal weights were normal and embryofoetal development was unaffected.

Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.

In the **main rabbit developmental toxicity study**, groups of 23 mated female Himalayan rabbits received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 20 or 60 mg/kg bw/day from Day 6-28 of gestation and were sacrificed on Day 29 of gestation.

Three animals of the high dose group were found dead and 15 animals of this group were killed after abortion or premature delivery from Day 22-29 of gestation (12 dams aborted whole litters (with no live pups), whilst 2 dams delivered prematurely with partial live litters (dam 178 delivered 1 dead and 4 live pups and dam 180 delivered 7 dead and 3 live pups). In addition a further dam prematurely delivered 7/7 live pups. These dams showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous feces, and discoloured urine. One animal from the intermediate dose group was killed after premature delivery on Day 28 of gestation. This animal showed decreased defecation

and reduced hay consumption. The dossier submitter considers this to be a spuorious finding and not related to treatment with fluopicolide, owing to its isolated occurrence in this dose group, supported by the the dose-range finding study, in which no abortions were observed at a similar dose (25 mg/kg bw/day) and only one dam aborted at a much higher dose (50 mg/kg bw/day). Furthermore, according to published historical data, up to 20% abortions have been reported for this strain of rabbit (Viertel & Trieb 2002[19]).

Body weight gains and food consumption were markedly decreased in the animals from the high dose group.Gravid uterus weights were slightly lower in the animals from at the same dose level; however body-weight gains were markedly decreased even when the gravid uterine weight was taken into account.

At necropsy, tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discoloration of the liver were observed in single animals from the high dose group. No compound-related effects were observed in the low and intermediate dose group.

The study report author stated that three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation; however, further examination of the raw data has revealed that twelve dams aborted their litters whilst two dams prematurely delivered litters that contained 1/5 and 7/10 dead foetuses respectively; a further dam prematurely delivered a litter conaining only live foetuses (7/7 live foetuses). It is more likely that the observed abortions are secondary to the maternal toxicity observed at this dose, and not a consequence of malformations in the aborted foetuses; no malformations were detected in these foetuses or in foetuses at the lower doses. Furthermore, variations which could potentially progress into malformations (with an increase in dose) were not detected at 50 or 20 mg/kg bw/day. Mean fetal body weights, crown-rump lengths and placental weights were decreased in the animals from the high dose group. Of the remaining surviving litters, litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound (litters from dams found dead or killed after abortion/premature delivery were excluded from subsequent calculations). Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test substance. Morphological examination of the fetuses did not reveal any compound-related effects up to and including the highest tested dose level of 60 mg/kg bw/day

Oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused severe maternal toxicity as evidenced by mortality, high incidence of premature delivery and decreases in body weight gain and food consumption. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the hightest tested dose. No teratogenic effects were observed in the fetuses at any dose level.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 20 mg/kg bw/day. Therefore, the NOAEL is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

In addition, an **external expert for reproductive toxicology** (Mary Moxon has more than 40 years' experience of conducting and supervising developmental and reproduction studies for regulatory purposes, within the industrial sector and contract research organisations. Mary is also experienced in fetal pathology and a former fetal pathology facility manager) was asked for review of the experimental study results in relation to the criteria for classification. Her conclusion supports Bayer's assessment that on the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

[19] Viertel B, Trieb G. The Himalayan rabbit (Oryctolagus cunuiculus L.): Spontaneous incidences of endpoints from prenatal developmental toxicity studies, Laboratory animals. 2003; 27, 19-36.

3.10.1 Animal data

3.10.1.1 Anonymous; 2002; M-215068-01-1

Study reference:

Anonymous; 2002; AE C638206 - Preliminary study of effects on reproductive performance in CD rats by dietary administration; M-215068-01-1

Deviations:	As a dose-range finding study the study was not intended to comply with OECD guidelines.

Executive Summary:

This preliminary study was performed to enable selection of treatment levels for a two-generation study.

For this purpose fluopicolide was administered orally, via the diet, at inclusion levels of 0, 50, 200, 750 or 2,500 ppm to groups of 8 males and 8 females. Treatment started 15 days prior to pairing and continued uninterrupted until termination after weaning of the resulting litters. Selected offspring (constituting an F1 generation) continued to receive the diets from about the time of weaning until termination following attainment of sexual maturation. Exposure to fluopicolide was achieved for the F0 adults and the F1 generation throughout the study.

The general condition of the F0 animals remained similar in all groups. One female receiving 2,500 ppm was killed due to parturition problems (dystocia) on Day 22 of gestation. The isolated nature of this finding suggested that it was more likely to be coincidental than treatment-related.

Body weight gains were lower (-22%) for males treated at 2,500 ppm at the end of the premating period. For females treated at 2,500 ppm, body weight gains were also lower before pairing and during gestation days 0-13 (approx. -20%). A transient effect in F0 females was also observed during the gestation period at 750 ppm. Food consumption was reduced for females treated with 2,500 ppm for most periods during gestation, for gestation periods days 3-5 and 6-9 also at 750 ppm, and during lactation at 2,500 ppm (Days 7-13). The achieved intake during the first few days of treatment was slightly lower for the F0 animals treated at 2,500 ppm than expected suggesting a transient palatability effect. The mean compound intakes during the pre-mating period were approx. 4, 17, 65 and 197 mg/kg bw/day for males and 4, 18, 67 and 204 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm, respectively.

Oestrous cycles, mating performance, fertility and fecundity were considered to be unaffected by treatment with fluopicolide. Gestation length appeared to be slightly longer for the females treated at 2,500 ppm than the controls, but the group size was too low to determine whether this was a true treatment-related finding. Gestation index and the parturition process were considered to be unaffected. Litter parameters incorporating litter size, offspring survival and sex ratio were considered not to be affected by treatment with fluopicolide.

The general condition of the F1 generation was similar in all groups and there were no deaths. Body weight gains for the F1 offspring in the group treated at 2,500 ppm were 87% and 85% of control groups for males and females respectively from approx. Day 14 of age, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet. Food consumption and body weight gains continued to be lower for animals treated at 2,500 ppm following the pattern established before weaning. The timing of balano-preputial separation for the F1 animals was unaffected by treatment. There appeared to be a slight delay in vaginal opening for females treated at 2,500 ppm when compared to the concurrent control. However, a treatment-relation is questionable.

No treatment-related findings were seen at macroscopic examination of the F0 or any of the F1 animals.

This preliminary study showed that dietary concentrations of 2,500 ppm of fluopicolide induced toxicity observed as body weight gain reductions in F0 in both sexes during the premating period, and in F0 females during the gestation period. A transient effect in F0 females was also observed during the gestation period at 750 ppm. F1 offspring bodyweight gains were also reduced at 2,500 ppm from approx. Day 14 of age. Therefore, a dose level up to 2,500 ppm would be suitable as the high concentration in a main study of reproductive performance. This concentration would be expected to elicit a measurable degree of parental toxicity without affecting fertility or reproductive performance.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Basal diet, no positive control

3. Test animals

Species:	Rat
Strain:	Crl:CD®(SD)lGS BR strain (Sprague-Dawley origin)
Age:	Males: 9-10 weeks, females: 8-9 weeks
Weight at start:	Males: 280 to 317 g, females: 191 to 219 g.
Source:	Charles River (UK) Limited, Margate, Kent, England
Acclimation phase:	Yes
Diet:	Powdered laboratory animal diet (UAR VFR1 Certified, manufactured by Usine d'Alimentation Rationnelle in France) supplied by Charles River UK, Margate, Kent, England
Water:	Polythene or polycarbonate bottles with sipper tubes
Housing:	The animal room had its own supply of filtered air, which was passed to the atmosphere without recirculation. Housing in TR18 cages from Arrowmight Biosciences, Hereford, England or RB3 modified and RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England. The cages consisted of stainless steel (TR18) or high-density polypropylene (RB3 and RB3 modified) bodies with lids of stainless steel grid. TR18 and RB3 modified cages had stainless steel grid floors.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	Not given (see above)
Photoperiod:	12 hours

B. Study design

1. In life dates: February 21 to May 25, 2001

2. Animal assignment and treatment:

On receipt, animals were non-selectively allocated to cages, up to four of one sex being placed in each cage. Before commencement of treatment, the animals within each sex were ranked by body weight into blocks (5 g range). Surplus animals were discarded from amongst those with outlying body weights and 40 animals of each sex were allocated to the five groups, and re-housed four of one sex/cage, by selecting animals from each body weight range in rotation. The groups contained populations of rats with comparable initial mean body weight and body weight range.

Individuals were then assigned a number and identified by tail tattoo.

The study design is given in Table 3.10.1.1- 1.

Table 3.10.1.1- 1: Study design – F0 generation

Test group	Dose level (ppm)	Number of animals	
		Male	Female
1	0	8	8
2	50	8	8
3	200	8	8
4	750	8	8
5	2,500	8	8

Cage labels, identifying the occupants by experiment, animal number, sex and treatment group, were color-coded.

3. Diet preparation and analysis:

Formulations were prepared on a weekly basis. A pre-mix, of a suitable dietary concentration, was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equaled this mixture was then added and stirred with a spoon. This doubling-up procedure was followed until a visibly homogenous premix of the required weight was achieved, and the premix blended in a Turbula Mixer. A second premix was formulated from this first premix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,500 and 750 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 200 and 50 ppm formulations were prepared by direct dilution of the second premix with plain diet. Blending was achieved by mixing in a Turbula Mixer.

Dosages and concentrations were expressed in terms of the test material as supplied. F0 males and females were fed the diets for 15 days before pairing for mating. Treatment was continued throughout mating and until termination after weaning of the litters. Animals selected to form the F1 generation received the diets from the time of weaning until termination following sexual maturation.

Information on the homogeneity of mixing, stability and concentration of the test substance in the diet were determined by Huntingdon Life Sciences. The homogeneity and the stability, during ambient temperature storage for 22 days, were confirmed for fluopicolide in VRF 1 formulation at nominal concentrations of 50 and 2,500 ppm. The storage period represented the maximum time from preparation to completion of use.

Additionally, samples of the formulations were taken for the first and last study preparations and analysed by Huntingdon Life Sciences for test substance content. The mean concentrations of fluopicolide in test diet formulations analysed during the study were between 90.8% and 101% of nominal concentrations and were considered satisfactory.

4. Statistics

Statistical evaluation of data was only performed where considered appropriate. The following parameters were analysed, and results are presented in relevant tables of this report:

Bodyweights and bodyweight change, food consumption of females during gestation and lactation, and litter data including offspring body weights.

For data recorded and/or processed by the Xybion computer system (adult organ weights and weekly body weight change) for the parental animals, homogeneity of variance was assessed using Bartlett's test (Bartlett, 1937). Whenever this was found to be statistically significant a Behrens-Fisher test (Cochran and Cox, 1957) was used to perform pairwise comparison, otherwise a Dunnett's test (Dunnett, 1955/64) was used.

For the remaining data statistical analysis was performed using an in-house programme developed by Huntingdon Life Sciences which used the following criteria:

If 75% of the data (across all groups) shared the same value, then a frequency analysis was applied.

Treatment groups were compared using a Mantel test for a trend in proportions (Mantel 1963) and also pairwise Fisher's Exact tests (Fisher 1973) for each dose group against the control.

If Bartlett's test for variance homogeneity (Bartlett 1937) was not significant at the 1% level, or if it was not significant after first a logarithmic or second a square-root transformation, then parametric analysis was applied. If the F1 test for monotonicity of dose-response (Healey 1999) was not significant at the 1% level, then Williams' test was performed for a monotonic trend (Williams 1971, 1972). If the F1 test was significant, showing that the dose-response was not monotonic, then Dunnett's test (Dunnett 1955, 1964) was applied instead. Both the Williams' and Dunnett's test use the error mean square from a one-way analysis of variance.

If Bartlett's test was significant at the 1% level, even after transformation, then non-parametric analysis was applied to the untransformed data. If the H1 test for monotonicity of dose-response (Healey 1999) was not significant at the 1% level, then Shirley's test was applied for a monotonic trend (Shirley 1977). If the H1 test was significant, showing that the dose-response was not monotonic, then Dunn's test (Dunn 1964) was applied instead.

Significant (i.e. $p<0.05$) inter-group differences from the control are reported.

C. Methods

1. Observations

All animals were inspected regarding clinical signs at least twice daily throughout the study and any visible signs of reaction to treatment were recorded, with details of type, severity, time of onset and duration.

Animals killed for reasons animal welfare were subjected to a thorough macroscopic examination of the visceral organs and specimens of abnormal tissues were retained.

2. Body weight and food intake

Males were weighed on the first day of treatment and twice weekly until termination. Females were weighed on the first day of treatment and then twice weekly until mating was detected. Subsequently the females were weighed on Days 0, 6, 13 and 20 after mating and on Days 1, 4, 7, 14 and 21 of lactation. F1 selected animals were weighed weekly from nominal 4 weeks of age until termination following sexual maturation (approx. 8 weeks of age).

Food consumption was recorded twice weekly for the F0 animals until they were paired for mating.

Food consumption for the females was recorded Days 0-2, 3-5, 6-9, 10-12, 13-16 and 17-19 after mating and Days 1-3, 4-6 and 7-13 during lactation.

Food consumption for the F0 selected animals was recorded weekly for nominal Weeks 4 to 5 and 5 to 6 of age - described as Weeks 4 or 5 in the results.

3. Oestrous cycles

For 10 days before pairing of the F0 animals, vaginal smears were taken daily from all females to establish the normality or otherwise of the oestrous cycle. This was continued after pairing with the male until evidence of mating was observed.

4. Mating

After the scheduled period of treatment, the F0 females were paired on a one-to-one basis with males from the same treatment group.

Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa. The day on which a sperm positive vaginal smear or at least three copulation plugs were found was designated Day 0 of gestation. Once mating had occurred, the males and females were separated and vaginal smearing discontinued.

The pre-coital interval (females only), i.e. the time elapsing between initial pairing and detection of mating was recorded.

5. Parturition and duration of gestation

From Day 20 after mating, the females were inspected three times daily for the onset, progress and completion of parturition.

All females were permitted to deliver their young naturally and rear their own offspring until Day 21 of lactation (litter standardisation was performed at Day 4 of lactation).

6. Observations of litters

All offspring were examined at approx. 24 hours after birth (Day 1), and each live offspring was identified within the litter by a toe tattoo. The following was recorded for each litter:

a) Number born (live and dead).
b) Individual bodyweights of live offspring.
c) Individual sexes.
d) Observations on individual offspring.

Clinical signs, mortality:

Litters were observed daily on clinical signs for evidence of ill health or a reaction to treatment.

Mortality and litter size was checked, daily records were maintained of mortality and consequent changes in litter size. Wherever possible, any offspring found dead were examined externally and internally.

On Day 4 of age, litters containing more than ten offspring were reduced to ten by random culling, leaving, wherever possible, five male and five female offspring in each litter.

Sex ratio:

The offspring were sexed on Days 1, 4 (before and after culling) and 21 of age.

Body weight:

The offspring were weighed individually on Days 1, 4 (before culling), 7, 14 and 21 of age.

F1 selection:

Following weaning, 12 male and 12 female offspring from the F1 litters in each of the groups were selected to form the F1 generation.

Where possible, at least one male and one female were selected from each litter using random numbers within litters after grossly atypical animals had been excluded. Additional male/female offspring were then selected from litters chosen at random within each group to achieve twelve male and twelve female offspring per group. The animals were assigned a number and identified by tail tattoo.

The design conditions and serial observations were as described for the first generation.

Table 3.10.1.1- 2: Study design – F1 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	12	12
2	50	12	12
3	200	12	12
4	750	12	12
5	2,500	12	12

Sexual maturation:

Sexual maturation of the selected animals was assessed as follows:

Males: Examined daily from Day 38 of age until balano-preputial separation occurred. Body weight was recorded on the day of completion.

Females: Examined daily from Day 28 of age until vaginal opening occurred. Body weight was recorded on the day of vaginal opening.

7. Necropsy, pathology:

All adult animals, weaned animals and any late neonates (> 10 days of age) that were killed for reasons of animal welfare were killed by carbon dioxide inhalation.

Offspring culled on Day 4 of age, and any that were killed for reasons of animal welfare before Day 10 of age, were killed by an intraperitoneal injection of sodium pentobarbitone.

Any parental animals or offspring that were found dead or killed for reasons of animal welfare were subjected to external and internal necropsy examination as soon as possible. Those found dead outside the normal working day were stored in a refrigerator, designated for this purpose, and necropsied the following day.

F0 males were killed after the females had successfully littered. Females that littered and reared offspring to weaning were killed after their respective litters were weaned.

Macroscopic pathology F0 animals:

All animals were subjected to a detailed macroscopic examination for evidence of disease or adverse reaction to treatment. Samples of abnormal tissues were weighed and retained in appropriate fixative. For all females, the number of implantation sites was recorded.

Offspring (sporadic deaths and unselected F1 offspring):

For early neonates that were found dead, an assessment of the stomach for milk content was made.

Offspring culled on Day 4 of age that were grossly normal were discarded without macroscopic examination.

Any deaths in late neonates or weaned offspring not selected for continuation of the study (killed at approx. 4 weeks of age) were examined externally and internally for macroscopic abnormalities. Specimens of abnormal tissues were retained in Industrial methylated spirit.

F1 selected animals:

Males and females were killed after sexual maturation was complete (at approx. 7 to 8 weeks of age) and were subjected to gross necropsy. The animals were examined externally and internally for macroscopic abnormalities. Specimens of abnormal tissues were retained in industrial methylated spirit pending possible future examination.

II. Results and Discussion

A. Observations

F0 generation

1. Clinical signs, mortality - F0 generation

Dosages of up to 2,500 ppm, administered by the dietary route, had no effect upon the general condition of the animals and there were no signs that were considered to be treatment-related.

One female (Animal Number 1078) receiving 2,500 ppm was killed due to parturition problems (dystocia) on day 22 of gestation. Findings at necropsy included red staining around the uro-genital region and lower ventral abdomen, reduced and dehydrated contents in the caecum, few and very firm faecal pellets in the colon and rectum and a small pale spleen. The uterus contained twenty implantations, one empty site and one site containing only a placenta. The isolated nature of the parturition problems observed for this animal suggested that it was more likely to be coincidental than treatment-related. Apart from this female there were no other mortalities among adult animals.

2. Body weight and food intake - F0 generation

Body weight:

In males, body weight gains were lower at 2,500 ppm compared to controls; the divergence was apparent from the first week of treatment (Days 0-7: -25%, $p < 0.01$) and persisted for the 7 weeks of treatment (Days 0-49: -22%, $p < 0.01$). Body weight gains in the other male groups were considered to be largely unaffected with no clear dosage related pattern emerging.

In females, bodyweight gains were slightly lower before pairing (Days 0-14: -20%, not statistically significant) and during gestation (approx. 82-92% of Control values, $p < 0.05$) for females at 2,500 ppm when compared with the controls. A transient effect in F0 females was also observed during the gestation period at 750 ppm. No clear differences were apparent during lactation.

An overview is given in Table 3.10.1.1- 3.

Table 3.10.1.1- 3: Body weight and body weight gains (g) of parental animals (F0 generation)

	Generation	Dose level [ppm] 0	50		200		750		2,500	
Males										
Body weights [g] (% difference to control)										
Day 0	Pre- and postmating	301	302	*(±0)*	302	*(±0)*	294	*(-2)*	292	*(-3)*
Day 7		361	354	*(-2)*	361	*(±0)*	351	*(-3)*	337	*(-7)*
Day 14		420	404	*(-4)*	408	*(-3)*	399	*(-5)*	379	*(-10)*
Day 21		455	437	*(-4)*	443	*(-3)*	427	*(-6)*	411	*(-10)*
Day 28		485	468	*(-3)*	471	*(-3)*	453	*(-7)*	433	*(-11)*
Day 35		509	497	*(-2)*	504	*(-1)*	488	*(-4)*	458	*(-10)*
Day 42		546	534	*(-2)*	530	*(-3)*	519	*(-5)*	489	*(-10)*
Day 49		572	560	*(-2)*	550	*(-4)*	540	*(-6)*	504	*(-12)*
Body weight gains [g] (*% difference to control*)										
Day 0-7		60	52	*(-13)*	59	*(-2)*	57	*(-5)*	45**	*(-25)*
Day 0-14		119	102	*(-14)*	106	*(-11)*	105	*(-12)*	87**	*(-27)*
Day 0-49		271	258	*(-5)*	248	*(-9)*	246	*(-9)*	212**	*(-22)*
Females										
Body weights [g] (*% difference to control*)										
Day 0	Premating	205	203	*(-1)*	205	*(±0)*	204	*(±0)*	208	*(+2)*
Day 7		230	228	*(-1)*	231	*(±0)*	223	*(-3)*	223	*(-3)*
Day 14		246	245	*(±0)*	251	*(+2)*	243	*(-1)*	240	*(-2)*
Body weight gains [g] (*% difference to control*)										
Day 0-7		25	25	*(±0)*	25	*(±0)*	18	*(-28)*	15	*(-40)*
Day 0-14		40	42	*(+5)*	46	*(+15)*	39	*(-2)*	32	*(-20)*
Body weights [g] (*% difference to control*)										
GD 0	Gestation	255	257	*(+1)*	258	*(+1)*	254	*(±0)*	249	*(-2)*
GD 6		292	296	*(+1)*	300	*(+3)*	285	*(-2)*	280	*(-4)*
GD 13		334	335	*(±0)*	342	*(+2)*	327	*(-2)*	313	*(-6)*
GD 20		418	426	*(+2)*	436	*(+4)*	415	*(-1)*	399	*(-4)*
Body weight gains [g] (*% difference to control*)										
GD 0-6		38	40	*(+5)*	43	*(+13)*	31	*(-18)*	31	*(-18)*
GD 0-13		80	79	*(-1)*	85	*(+6)*	73	*(-9)*	65*	*(-19)*
GD 0-20		163	169	*(+4)*	178	*(+9)*	161	*(-1)*	150	*(-8)*
Body weights [g] (*% difference to control*)										
LD 1	Lactation	313	321	*(+3)*	330	*(+5)*	310	*(-1)*	291	*(-7)*
LD 4		328	331	*(+1)*	348	*(+6)*	329	*(±0)*	314	*(-4)*
LD 7		347	345	*(-1)*	363	*(+5)*	336	*(-3)*	325	*(-6)*
LD 14		360	367	*(+2)*	370	*(+3)*	358	*(-1)*	337	*(-6)*
LD 21		350	360	*(+3)*	363	*(+4)*	349	*(±0)*	334	*(-5)*

	Generation	Dose level [ppm]							
		0	50		200		750		2,500	
Body weight gains [g] (*% difference to control*)										
LD 1-4		15	11	*(-27)*	18	*(+20)*	19	*(+27)*	22	*(+47)*
LD 1-7		34	24	*(-29)*	33	*(-3)*	26	*(-23)*	34	*(±0)*
LD 1-14		47	46	*(-2)*	40	*(-15)*	48	*(+2)*	46	*(-2)*
LD 1-21		38	39	*(+3)*	33	*(-13)*	39	*(+3)*	43	*(+13)*

GD: gestation day; LD: lactation day

* / ** statistically significant difference from control, $p \leq 0.05$ / $p \leq 0.01$

Food intake:

In males, food consumption recorded during the two-week pre-pairing phase, was slightly low (approx. 74-90% of control values) for males receiving 2,500 ppm when compared with the controls.

In females, during the first week of treatment, food consumption was slightly low (approx. 75-90% of control value) for females receiving 2,500 ppm when compared with the controls. This trend did not continue into the second week, but re-appeared during gestation (approx. 82-93% of control values, $p < 0.05$ for 4/6 periods). Food consumption was also low (approx. 90-93% of control values, $p < 0.05$ for 2/6 periods) during gestation for females receiving 750 ppm. No clear effects of treatment were apparent for food consumption during lactation apart from a possible reduction at 2,500 ppm which developed during the second week (approx. 83% of control values, $p < 0.01$).

The other dose groups were considered to be unaffected. The following tables give an overview.

Table 3.10.1.1- 4: Food consumption (g/rat/week) prior to pairing – F0 generation

Time period	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Day 1-14	106	120	118	119	95	81	79	83	78	74
(% control)		*(113)*	*(111)*	*(112)*	*(90)*		*(97)*	*(102)*	*(96)*	*(91)*

Table 3.10.1.1- 5: Food consumption (g/rat/day) of dams during gestation (g) - F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 0-2	27	27	28	25	24
Days 3-5	28	29	29	26*	26**
(% control)				(93)	(93)
Days 6-9	30	29	30	27**	26**
(% control)				(90)	(87)
Days 10-12	29	29	30	27**	27**
(% control)				(93)	(93)
Days 13-16	29	28	31	27	27
Days 17-19	33	31	33	30	27*
(% control)					(82)

* $p \leq 0.05$; ** $p \leq 0.01$ statistically significantly different to controls
(% of controls if statistically significant difference)

Table 3.10.1.1- 6: Food consumption (g/rat/day) of dams during lactation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 1-3	42	40	43	39	45
Days 4-6	54	52	52	52	49
Days 7-13	75	73	77	70	62**

** $p \leq 0.01$ statistically significantly different to controls

The only consistent effect on food conversion efficiency was that it appeared slightly low for both sexes receiving 2,500 ppm when compared with the controls which was statistically not significant.

Achieved dosages:

The achieved dosage at all dietary concentrations for both sexes was considered to be satisfactory and sufficient exposure to fluopicolide was achieved. During the first few days of treatment the achieved intake at 2,500 ppm was slightly lower than might have been expected (counter to the usual pattern of higher values initially, declining as the animals grow). This could suggest a transient palatability effect. Fluctuations during gestation and lactation were in line with expectation and considered to be related to changes in the physiological demands during these periods that affected the homeostasis of the females. The mean compound intakes during the pre-mating period were approx. 4, 17, 65 and 197 mg/kg bw/day for males and 4, 18, 67 and 204 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm, respectively.

Table 3.10.1.1- 7: Achieved dosage (mg/kg bw/day) before pairing – F0 generation

Time period	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Days 1-3	-	5	18	68	194	-	5	18	65	175
Days 4-7	-	4	17	64	208	-	4	18	67	209
Days 8-10	-	4	16	67	201	-	4	18	70	225
Days 11-14	-	4	15	59	185	-	4	18	64	208

Table 3.10.1.1- 8: Achieved dosages (mg/kg bw/day) during gestation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 0-6	-	5	21	72	236
Days 6-13	-	5	19	67	220
Days 13-20	-	4	16	58	192

Table 3.10.1.1- 9: Achieved dosages (mg/kg bw/day) during lactation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 1-4	-	6	26	92	370
Days 4-7	-	8	29	116	384
Days 7-14	-	10	42	151	471

3. Reproductive results – F0 generation

Oestrous cycles:

The regularity and duration of the oestrous cycles were unaffected at dosages of up to 2,500 ppm.

Mating performance and fertility:

Mating performance and fertility were unaffected by treatment as can be seen in Table 3.10.1.1- 10.

Table 3.10.1.1- 10: Mating performance and fertility – F0 generation

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Number mating	8	8	8	8	8
Number achieving pregnancy	8	8	8	8	8
Conception rate (%)	100	100	100	100	100

Gestation length, gestation index and parturition:

Gestation length was within the usual range of 22 to 23.5 days for all females. The gestation lengths for females receiving 2,500 ppm appeared to be slightly longer than the controls, but all remained within 23 days. This slight shift was possibly attributable to the slightly lower body weights of these females but the group size was too low to be certain whether this was a true treatment related finding.

Gestation index was slightly low for animals in group 5 (2,500 ppm), but this was attributable to the one female with parturition problems on Day 22 of gestation and not related to treatment with fluopicolide.

Table 3.10.1.1- 11: Gestation length and gestation index – F0 generation

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Number of pregnant animals	8	8	8	8	8
Number of litters born	8	8	8	8	7
Gestation index (%)	100	100	100	100	88
No. of animals with defined gestation length (% of animals)					
Gestation length: 22 days	5 (63)	5 (63)	4 (50)	5 (63)	1 (13)
Gestation length: 22.5 days	1 (13)	2 (25)	4 (50)	2 (25)	3 (38)
Gestation length: 23 days	2 (25)	1 (13)	0 (0)	1 (13)	4 (50)

Offspring results:

Litter responses

The general condition of the offspring was unaffected at any dosage.

Litter size and offspring survival were not affected. The numbers of implantation sites, total litter size and live litter size on day 1 of age and offspring survival assessed by post-implantation survival, live birth, viability and lactation indices were considered to be similar in all groups.

Regarding sex ratio, the ratio of male to female offspring showed some inter-group variation in particular with a slightly lower proportion of males to females at 2,500 ppm. This difference was considered incidental and no effect of treatment on sex ratio was indicated.

An overview is given in Table 3.10.1.1- 12.

Table 3.10.1.1- 12: Uterine implantations, litter data

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Litter number	8	8	8	8	8
Implantations (mean)	15.5	16.6	16.0	17.1	15.7
Total litter size on day 1	14.6	15.8	15.0	16.1	14.6
Post implantation survival index (%)	94.3	94.6	94.0	93.7	92.5
Live birth index (%)	100.0	100.0	99.1	98.5	100.0
Viability index (%)	99.3	98.3	99.3	99.4	97.4
Lactation index (%) – day 7	98.8	100.0	100.0	100.0	92.9
Lactation index (%) – day 21	98.8	100.0	100.0	100.0	92.9
Sex ratio (day 1):					
Males	7.5	8.6	7.6	8.4	6.6
Females	7.1	7.1	7.4	7.8	8.0
Sex ratio (day 21):					
Males	4.8	5.0	5.4	5.3	4.1
Females	5.1	5.0	4.6	4.8	5.1

Offspring body weights (F1 pups)

Overall group mean body weight gains from Day 1 to 21 of age were slightly low for both male (approx. 87% of control values, $p < 0.05$) and female (approx. 85% of control values, $p < 0.05$) offspring in Group 5 (2,500 ppm) when compared with the controls. These lower body weights first appeared on Day 14 of age, coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect. Body weight gains in the other groups were considered to be unaffected.

An overview is given in Table 3.10.1.1- 13.

Table 3.10.1.1- 13: Body weight and body weight gains (g) of F1 pups

	Dose level (ppm)								
	0	50		200		750		2,500	
Males									
Body weights [g] (*% difference to control*)									
Day 1 (before cull)	6.8	6.8	*(±0.0)*	6.7	*(-1.5)*	6.8	*(±0.0)*	7.1	*(+4.4)*
Day 4 (before cull)	9.5	9.4	*(-1.1)*	9.2	*(-3.2)*	8.9	*(-6.3)*	9.7	*(+2.1)*
Day 4 (after cull)	9.6	9.6	*(±0.0)*	9.2	*(-4.2)*	8.9	*(-7.3)*	9.7	*(+1.0)*
Day 7 (after cull)	14.7	15.3	*(+4.1)*	14.8	*(+0.7)*	14.2	*(-3.4)*	15.3	*(+4.1)*
Day 14 (after cull)	32.2	32.1	*(-0.3)*	31.9	*(-0.9)*	30.4	*(-5.6)*	29.9	*(-7.1)*
Day 21 (after cull)	51.5	49.8	*(-3.3)*	50.5	*(-1.9)*	47.6	*(-7.6)*	46.0	*(-10.7)*
Body weight gain (g) Day 1-21	44.7	43.0	*(-3.8)*	43.8	*(-2.0)*	40.8	*(-8.7)*	38.8*	*(-13.2)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Females									
Body weights [g] (*% difference to control*)									
Day 1 (before cull)	6.3	6.4	*(-1.6)*	6.4	*(-1.6)*	6.5	*(+3.2)*	6.7	*(+6.3)*
Day 4 (before cull)	9.0	8.8	*(-2.2)*	8.8	*(-2.2)*	8.5	*(-5.6)*	8.9	*(-1.1)*
Day 4 (after cull)	9.0	8.9	*(-1.1)*	8.9	*(-1.1)*	8.7	*(-3.3)*	9.0	*(±0.0)*
Day 7 (after cull)	14.1	14.4	*(+2.1)*	14.4	*(+2.1)*	14.1	*(±0.0)*	13.7	*(-2.8)*
Day 14 (after cull)	31.4	30.2	*(-3.8)*	31.1	*(-1.0)*	30.1	*(-4.1)*	25.1*	*(-20.1)*
Day 21 (after cull)	48.8	46.3	*(-5.1)*	48.5	*(-0.6)*	46.3	*(-5.1)*	42.9*	*(-12.1)*
Body weight gain (g) Day 1-21	42.5	39.9	*(-6.1)*	42.1	*(-0.9)*	39.9	*(-6.1)*	36.2*	*(-14.8)*

* $p \leq 0.05$ statistically significantly different to controls

Examination results of unselected offspring and F0 parents

Necropsy of unselected offspring and F0 adults

There were no macroscopic observations at necropsy that suggested any test article-related effects of treatment upon the offspring.

Macroscopic examination of the F0 animals at necropsy revealed no test article-related findings that were considered to be related to the dietary administration of fluopicolide.

F1 generation

1. Clinical signs, mortality - F1 generation

Dosages of up to 2,500 ppm, administered by the dietary route, had no effect upon the general condition of the animals and there were no signs that were considered to be treatment-related. There were no deaths in the F1 generation.

2. Body weight and food intake - F1 generation

Body weight gain was statistically significantly decreased in males at 2,500 ppm (Week 1-3: -11% compared to control) and also slightly reduced in females.

Body weight gains in the other groups were considered to be unaffected.

Food consumption was slightly low (not statistically significant) for animals receiving 2,500 ppm when compared with the controls (approx. 87-90% of control values in males and 91-93% in females). The other groups were unaffected.

Overall food conversion efficiency for the F1 generation was similar in all groups.

Table 3.10.1.1- 14: Body weight and body weight gains (g) of F1 animals

Timepoint #	Dose level (ppm)							
	0	50		200		750		2,500	
Males								
Body weights [g] (*% difference to control*)								
Week 1	148	147	*(+0.7)*	156	*(+5.4)*	148	*(±0.0)*	131	*(-11.5)*
Week 2	210	210	*(±0.0)*	218	*(+3.8)*	207	*(-1.4)*	188	*(-10.5)*
Week 3	277	277	*(±0.0)*	283	*(+2.2)*	271	*(-2.2)*	244	*(-11.9)*
Body weight change (g) Week 1-3	186	187	*(+0.5)*	186	*(±0.0)*	180	*(-3.2)*	165*	*(-11.3)*
Females								
Body weights [g] (*% difference to control*)								
Week 1	126	124	*(-1.6)*	136	*(+7.9)*	128	*(+1.6)*	116	*(-7.9)*
Week 2	161	160	*(-0.6)*	171	*(+6.2)*	165	*(+2.5)*	151	*(-6.2)*
Week 3	190	186	*(-2.1)*	200	*(+5.3)*	196	*(+3.2)*	178	*(-6.3)*
Body weight change (g) Week 1-3	106	106	*(±0.0)*	110	*(+3.8)*	113	*(+6.6)*	103	*(-2.8)*

* $p \leq 0.05$ statistically significantly different to controls

\# F1 selected animals were weighed weekly from nominal 4 weeks of age until termination following sexual maturation (approximately 8 weeks of age).

Achieved dosage:

The achieved dosages for the selected offspring were considered to be satisfactory. As expected, intake of the test substance was higher in these younger animals when compared with their parents but did not suggest that these levels could not be tolerated by the young animals. The mean compound intakes for the F1 animals were approx. 6, 24, 93 and 316 mg/kg bw/day for males and 6, 25, 95 and 313 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm respectively.

Sexual maturation of the F1 generation:

The timing of balano-preputial separation for the F1 animals was unaffected by treatment. There appeared to be a delay of 3 days in vaginal opening (VO) for females treated at 2,500 ppm when compared to control value ($p < 0.01$); however, this is considered more an incidental finding than a treatment-related effect, particularly when compared to the control value of the definitive two-generation study in rats of 35.0±3.0 days (see Blee, M. A. B.; 2003; M-232532-01-1, Section 3.10.1.2) and considering the low animal number. Furthermore, no effect on sexual development was observed at 2,000 ppm in the main study. Even if considered treatment-related the slight delay in VO would be assumed secondary to the lower body weights seen in this group rather than a selective effect of treatment.

Table 3.10.1.1- 15: Sexual development – F1 generation (group mean)

Parameter	Dose level (ppm)				
	0	**50**	**200**	**750**	**2,500**
Males					
Preputial separation (days)	44±1.9	45±1.8	43±1.7	45±1.0	44±2.2
Body weight at preputial separation (g)	227	228	222	227	210
Females					
Vaginal opening (days)	33±2.0	34±2.1	33±2.1	34±1.4	36±2.9**
Body weight at vaginal opening (g)	111	115	112	118	118

** $p \leq 0.01$ statistically significantly different to controls

Necropsy of F1 animals:

Macroscopic examination at necropsy of the F1 animals at around 7 to 8 weeks of age did not reveal test substance-related findings that were considered to be related to the dietary administration of fluopicolide.

III. Conclusion

This preliminary study showed that dietary concentrations up to 2,500 ppm of fluopicolide induced toxicity observed as body weight gain reductions in F0 in both sexes during the premating period, and in F0 females during the gestation period. A transient effect on bodyweight gain in F0 females was also observed during the gestation period at 750 ppm. F1 offspring body weight gains were also reduced at 2,500 ppm from approx. Day 14 of age. A dose level up to 2,500 ppm would be suitable as the high concentration in a main study of reproductive performance. This concentration would be expected to elicit a measurable degree of parental toxicity without affecting fertility or reproductive performance.

3.10.1.2 Anonymous; 2003; M-232532-01-1

Study reference:

Anonymous; 2003; Study of reproductive performance in CD rats treated continuously through two successive generations by dietary administration (Volume 1 of 3) Code: AE C638206; M-232532-01-1

Deviations:	Deviations from the current OECD guideline (416, 2001): None.

Executive Summary:

The influence of fluopicolide on the fertility and reproductive performance of two successive generations was assessed in male and female rats of the Crl: CD® (SD) IGS BR strain. Fluopicolide was administered continuously in the diet at inclusion levels of 0, 100, 500 or 2,000 ppm (parts per million) to groups of rats throughout the two generations.

The F0 generation, which comprised 28 males and 28 females in each group, received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation. Both sexes received similar treated diets as their parents for a minimum of 10 weeks from selection, throughout pairing, gestation, littering and lactation. Sexual maturation, fertility and reproductive capacity of the F1 generation were assessed and the resulting F2 offspring were monitored for survival and development until weaning.

All F0 and F1 adult animals were subjected to a detailed necropsy, the reproductive organs and other selected organs were weighed and retained. Sperm motility, morphology and counts were assessed for all F0 and F1 males in the control and high treatment groups. Histopathological examinations were performed on designated tissues from ten parent males and ten parent females in the control and high dosage groups, and abnormal tissues from all other parental animals. Unselected F1 and F2 offspring were killed on day 34 of age. Where possible, one male and one female from each litter were subjected to a necropsy examination, the reproductive organs retained, and the brain, spleen, and thymus weighed and retained.

The general condition of the F0 and F1 adults was satisfactory throughout the study. There were no treatment-related deaths in either generation for the adult animals. Body weight gain and food consumption were low for adult animals treated at 2,000 ppm throughout the study, with the exception of the low body weight gain which was not apparent in the females following parturition. Food conversion efficiency was considered unaffected. The achieved dosage at all dietary concentrations for both sexes was considered satisfactory and exposure levels in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the treatment period. The intake generally fell to approx. 40 to 50% of the initial values during the course of each pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation.

Oestrous cycles, mating performance, fertility and fecundity were similar in all groups. Gestation length and the parturition process were unaffected by treatment. The return of females to oestrous cycling following lactation was not influenced by treatment in either generation.

Sexual maturation, as assessed by the age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation, was not affected by treatment with fluopicolide at doses up to and including 2,000 ppm.

Litter parameters at birth of the F1 and F2 progeny and their survival to weaning showed no detrimental effects of treatment. Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning, coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet.

Macroscopic examination at necropsy of F0 parents, the unselected F1 offspring or the F2 offspring did not indicate any adverse effect of treatment. At 2,000 ppm, kidney and liver weights were high for parental males and females in both generations, when compared with the controls. Group mean body weight-relative liver weights were also slightly higher for females treated at 500 ppm, when compared with the controls. Differences in spleen and/or thymus weights treated at 2,000 ppm were generally attributed to the lower body weights in these groups, although body weight-relative spleen weights were also low for females at this level for all generations.

Sperm analysis in males (control and high treatment groups only) of both generations did not reveal any treatment-related findings.

A retrospective histopathological examination of the liver and kidneys (see Anonymous.; 2004; M-247289-01-1, Section 3.10.1.3) showed treatment-related findings in both organs. In the kidneys treatment-related changes were noted in both sexes from both generations at 2,000 ppm and consisted of cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females. In the liver treatment-related changes were present in both sexes at 2,000 ppm and in males at 500 ppm from both generations and consisted of centrilobular hepatocyte hypertrophy.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL), based on the likelihood of the increased liver weights and the slighty increased centrilobular hepatocyte hypertrophy at this dose level being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Basal diet, no positive control

3. Test animals

Species:	Rat
Strain:	Crl:CD®(SD)lGS BR strain (Sprague-Dawley origin)
Age:	Approximately 6 weeks
Weight at start:	37 to 223 g for the males and 120 to 189 g for the females
Source:	Charles River (UK) Limited, Margate, Kent, England
Acclimation phase:	Yes
Diet:	Powdered laboratory animal diet (UAR VFR1 Certified, manufactured by Usine d'Alimentation Rationnelle in France) supplied by Charles River UK, Margate, Kent, England
Water:	Polythene or polycarbonate bottles with sipper tubes
Housing:	The animal room had its own supply of filtered air, which was passed to the atmosphere without recirculation. Depending on the phase of the study, rats were housed in TR18 cages from Arrowmight Biosciences, Hereford, England or RB3 modified and RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England. The cages consisted of stainless steel (TR18) or high-density polypropylene (RB3 and RB3 modified) bodies with lids of stainless steel grid
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	Not given (see above)
Photoperiod:	12 hours

B. Study design

1. In life dates: July 11, 2001 to June 19, 2002

2. Animal assignment and treatment:

Upon receipt of the animals, each litter was identified and allocated to cages, a maximum of four littermates of one sex being placed in each cage. Before commencement of treatment, the animals within each sex were weighed and the three litters per sex showing the greatest within-litter variation in body weight were discarded to leave 28 litters of males and 28 litters of females. One animal from each of these litters was randomly allocated to each study group, assigned a number and identified by tail tattoo. This procedure ensured that for each sex, all groups contained populations of rats with a similar genetic background and comparable initial mean body weight and body weight range. No group contained more than one male and one female from the same litter.

The four treatment groups comprising the F0 generation were as follows:

Table 3.10.1.2- 1: Study design – F0 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	28	28
2	100	28	28
3	500	28	28
4	2,000	28	28

24 males and 24 females per group were selected to form the F1 generation when the F1 offspring were approx. four weeks of age. A minimum of one male and one female per litter were selected from as many litters as possible by using a random number procedure within each available litter.

Where appropriate, additional offspring were selected from randomly selected litters until the required number of animals were selected for the F1 generation. Each selected animal was assigned a number and identified by tail tattoo.

The four treatment groups comprising the F1 generation were as follows:

Table 3.10.1.2- 2: Study design – F1 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	24	24
2	100	24	24
3	500	24	24
4	2,000	24	24

3. Diet preparation and analysis:

Fresh diet was prepared at Huntingdon Life Sciences, Huntingdon Research Centre at various intervals during the study in batches covering up to two weeks of treatment and prepared up to one week in advance of the first day of use. A pre-mix of a suitable dietary concentration was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equalled this mixture was then added and stirred in with a spoon. This doubling-up procedure was followed until a visibly homogenous pre-mix of the required weight was achieved, and the pre-mix blended in a Turbula Mixer. A second pre-mix was formulated from this first pre-mix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,000 and 500 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 100 ppm formulation was prepared by direct dilution of the second pre-mix with plain diet. Blending was achieved by mixing in a Turbula Mixer.

Blending in the Turbula Mixer was set at 100 cycles (approx. six minutes duration). Dosages and concentrations were expressed in terms of the test material as supplied.

Information on the homogeneity of mixing, stability and concentration of the test material in the diet was determined by Huntingdon Life Sciences as part of the preliminary study (see Anonymous.; 2002; M-215068-01-1), Section 3.10.1.1). In that study the homogeneity and stability, during ambient temperature storage for 22 days, were confirmed for fluopicolide in VRF 1 formulation at nominal concentrations of 50 and 2,500 ppm. The storage period represented the maximum time from preparation to completion of use.

In this main study, samples (nominally 200 g) of treated diets were taken at approx. 10-week intervals equivalent to:

a) Start of treatment (week 1 of F0 generation)
b) First week of pairing (week 11 of F0 generation)
c) Week of selection of second generation (week 18 of F0 generation)
d) During pre-pairing phase of second generation (week 8 of F1 generation)
e) Third week of lactation for second generation (week 17 of F1 generation)

These samples were analysed by Huntingdon Life Sciences for test material content using the High Performance Liquid Chromatography assay.

The mean concentrations of fluopicolide in formulations, prepared for use during Weeks 1, 11 and 18 of treatment of the F0 generation and Weeks 8 and 17 of treatment of the F1 generation, ranged from 87.8 to 101% of nominal concentrations and were considered satisfactory.

The test substance was administered at constant concentration (ppm) to the treated animals in their diet, which was available on an ad libitum basis. Males and females of the F0 generation were treated for 10 weeks before pairing and throughout the study until termination. Animals of the F1 generation had access to the same diet as their parents throughout, but the F1 generation was deemed to formally start at nominal Week 4 (approx. 4 weeks of age). They were treated from nominal week 4 for approx. 10 weeks before pairing, and until termination when litters were weaned. The F0 and F1 Control animals received untreated diet from the same batch over the same period.

4. Statistics

Statistical analyses were performed on the majority of data presented and results of these tests, whether significant or non-significant, are presented on the relevant tables. For some parameters, such as mating performance, the similarity of the data was such that analyses were not considered to be necessary.

For data recorded and/or processed by the Xybion computer system for the parental animals, homogeneity of variance was assessed using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparison, otherwise a Dunnett's test was used. Intergroup differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

The following sequence of statistical tests was used for body weight and body weight change during gestation and lactation, body weight and body weight change for offspring, food consumption pre-pairing, during gestation and lactation and offspring organ weights:

> If Bartlett’s test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response (F1 test) was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.

> If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response (H1 test) was not significant at the 1 % level, Shirley's test for a monotonic trend was applied.

Significant differences between Control and treated groups were expressed at the 5% ($p<0.05$) or 1% ($p<0.01$) level.

C. Methods

1. Observations

All animals were observed at least twice daily throughout the study and any visible signs of reaction to treatment were recorded, with details of type, severity, time of onset and duration. In addition a more detailed weekly examination was performed throughout the treatment period.

2. Body weight and food intake

F0 males were weighed on the day that treatment commenced then weekly thereafter. F1 males were weighed at the formal start of the generation, twice weekly until sexual maturation and then weekly thereafter. F0 and F1 females were weighed on the same schedule until pairing and then on Days 0, 6, 13, and 20 after mating, and on Days 1, 4, 7, 14, and 21 of lactation.

Food consumption was recorded on a cage basis (four animals per cage) for F0 and F1 males and females weekly until pairing. Food consumption for females after mating was recorded on an individual basis on Days 0-5, 6-12 and 13-19 after mating and on Days 1-3, 4-6 and 7-13 of lactation.

After Day 14 of lactation, food intake is increasingly influenced by the offspring as they start to eat solid food and is no longer an accurate reflection of maternal intake.

3. Oestrous cycles - pre-pairing, through mating and post-weaning

For 22 days before pairing of the F0 and F1 generations, daily vaginal smears were taken, using cotton swabs, from all females and these were examined to establish the duration and regularity of the oestrous cycle. After pairing with the male, smearing (vaginal lavage) was continued until evidence of mating was observed.

Following weaning, daily vaginal smears were taken from all females on Days 25 to 28 after birth prior to necropsy and used to determine the stage of the oestrous cycle at termination.

Females that failed to litter were killed on Day 25 after mating and females experiencing total litter loss were retained until sufficient litters had been reared to eliminate the requirement for a second pairing. In both instances, females were despatched to necropsy without being smeared.

4. Mating

Following the scheduled period of treatment (10 weeks of treatment for the F0 generation; 10 weeks after selection for the F1 generation), males and females from within the same treatment groups were paired on a one-to-one basis for a period of up 3 weeks. If there was no positive indication of mating after 14 days, the male partner was replaced by a proven male from within the same group provided the female had shown evidence of oestrus. Care was taken to avoid pairing siblings.

Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa and the stage of the oestrous cycle. The day on which evidence of mating was found was designated Day 0 of gestation.

Once mating occurred, the males and females were generally separated and smearing was discontinued. However after inconclusive mating, smearing was continued for up to 5 days to confirm positive mating.

The time elapsing between initial pairing and detection of mating was recorded.

5. Parturition and duration of gestation

From Day 20 after positive evidence of mating, females were inspected three times daily for evidence of onset, progress and completion of parturition. Females from each group were permitted to deliver their young naturally and rear their own offspring until Day 21 of lactation.

The time elapsing between the detection of mating and commencement of parturition was reported as the duration of gestation.

Females were observed daily for evidence of abnormal maternal behaviour.

6. Observations of F1 and F2 litters

All offspring were examined at approx. 24 hours after birth (Day 1 of age) and the following parameters were recorded for each litter:

a) Number of offspring (live and dead)
b) Individual bodyweights of live offspring
c) Sex ratio
d) Observations on individual offspring

Litters were observed daily for evidence of ill health or a reaction to treatment. Daily records were maintained of mortality and consequent changes in litter size. Wherever possible, any offspring found dead were examined externally and internally.

On Day 4 of age, litters containing more than ten offspring were reduced to ten by random culling, leaving, whenever possible, five male and five female offspring in each litter.

The offspring were sexed at weighing and sexes reported for Days 1, 4 (before and after culling) and 21 of age.

Individual F1 offspring were weighed on Days 1, 4 (before culling), 7, 14, 21, 25 and 28 of age.

Some F1 offspring which were selected to form the F1 generation were weighed on Day 27 of age (nominal week 4 - formal commencement of the F1 generation) instead of Day 28. This deviation from the protocol is considered not to have affected the integrity of the study.

Individual F2 offspring were weighed on the same occasions as the F1 offspring however, they were also weighed on Day 31 of age; these data are retained within the raw data but are not presented within the report. Some F2 litters were weighed on Day 26 of age instead of Day 25 in error. This deviation from the protocol is considered to have no impact on the scientific integrity of the study.

On Day 1 of age, the ano-genital distance was measured for all F1 offspring derived from control dams in order to provide background control data. These data have not been presented within the report however, records are held with the raw data.

Sexual maturation of the F1 selected animals was assessed as follows:

Males: Examined daily from Day 38 of age until balano-preputial separation occurred. Body weight was recorded on the day of completion.

Females: Examined daily from Day 28 of age until vaginal opening occurred. Body weight was recorded on the day of vaginal opening.

7. Necropsy, organ weights, histopathology

Euthanasia:

All adult and weaned animals killed at scheduled termination, and offspring (> 14 days of age) that were killed for reasons of animal welfare, were killed by carbon dioxide inhalation.

Offspring culled on Day 4 of age, and any killed in extremis or for humane reasons before Day 14 of age, were killed by an intraperitoneal injection of pentobarbitone sodium. Grossly normal culled offspring were discarded without formal necropsy. Grossly abnormal culled offspring were examined externally and internally and any abnormal tissues retained.

Any parental animals or offspring that were found dead or killed for reasons of animal welfare were subjected to external and internal necropsy examination as soon as possible. Those found dead outside the normal working day were stored in a refrigerator, designated for this purpose, and necropsied the following day.

In the parental F0 and F1 generation, males were killed once the majority of litters had weaned and it had been established that further litters were not required.

Females that littered and reared offspring were killed at Day 28 of lactation after completion of post-weaning vaginal smears. Females that failed to litter were killed on Day 25 after mating and females experiencing total litter loss were retained until sufficient litters had been reared to eliminate the requirement for a second pairing (Day 17/18 of theoretical lactation).

Regarding the offspring (sporadic deaths, unselected F1 offspring and F2 offspring), for early neonates that were found dead, an assessment of the stomach for milk content was made.

Any early neonates that were found dead were retained, where possible, in industrial methylated spirit prior to an external and internal examination. Other offspring that died before scheduled termination were subjected to external and internal examination.

F1 weaned offspring, not selected for continuation of the study, were killed on Day 34 of age following completion of the selection process for the next generation. For practical reasons, the majority of offspring from five unselected F1 litters (from females 2F 1147, 3F 1175, 3F 1186, 3F 1187 and 4F 1202), born later than the other litters, were killed before Day 34 of age, however, one male and one female in each of these litters was retained until Day 34 in order to collect comparable organ weights. F2 offspring were all killed on Day 34 of age.

Macroscopic pathology:

All parental animals were subjected to a detailed macroscopic examination for evidence of disease or an adverse reaction to treatment.

The necropsy procedure included a review of the history of each animal, and a detailed examination of the cranial, thoracic, abdominal and pelvic cavities, and their viscera. The external and cut surfaces of the organs and tissues were examined, either before or after weighing, as appropriate. The number of uterine implantation sites was recorded for the adult females. Abnormalities, interactions and changes were noted, the requisite organs weighed and the required tissue samples preserved in fixative.

Unselected F1 offspring and all F2 offspring were examined macroscopically for evidence of disease, or adverse reaction to treatment, and for one offspring of each sex per litter (where possible) appropriate organs were weighed and retained. Specimens of any abnormal tissues were also retained.

Sperm analysis (F0 and F1 adult males):

Immediately after sacrifice the left vas deferens, epididymis and testis of each male was removed and the epididymis and testis were weighed and the following sperm parameters determined.

Sperm motility:

A sample of sperm was expressed from the vas deferens into pre-warmed (37 °C) medium M 199, which contained 0.5% w/v bovine serum albumin (BSA Fraction V). A sample for assessment was taken into a 100 µm depth cannula by capillary action and where possible at least 200 sperm per animal analysed using the Hamilton Thorne IVOS Computer Assisted Sperm Analyser (CASA) version 12.00. The percentages of motile and progressively motile sperm were reported.

Sperm morphology:

A 200 µL aliquot of the sperm/medium mixture (described above) was diluted with 800 µL of 4% neutral buffered formaldehyde. After staining with nigrosine and eosin, an air-dried smear was prepared and examined by light microscopy for the assessment of sperm morphology. Where possible at least 200 sperm were assessed for each male. The percentages of normal sperm and major categories of abnormal sperm were reported.

Sperm count:

The left cauda epididymis of each male was weighed; those for the low and intermediate groups (groups 2 and 3) were frozen. For the control and high treatment groups (groups 1 and 4), 10 mL of a mixture of 0.9% saline, 0.01% merthiolate and 0.05% Triton X-100 (SMT) was added and the cauda epididymis was homogenised for at least one minute. An aliquot of this mixture was added to a preprepared IDENT stain tube (obtained from Microm UK) before being assessed for sperm count using CASA. The concentration (Million/g) and total number of sperm were reported.

Homogenisation-resistant spermatids count:

The left testis of each male of the Control and high treatment groups (groups 1 and 4) was homogenised for at least two minutes in 25 mL of SMT. An aliquot of this mixture was added to a pre-prepared IDENT stain tube before being assessed for homogenisation-resistant spermatid count using CASA. The concentration (Million/g) and total number of spermatids were reported. Testes of the low and intermediate treatment groups (groups 2 and 3) were frozen.

Organ weights:

Parental animals:

The following organs, taken from each F0 and F1 parental animal, were dissected free of adjacent fat and other tissue, and the weights were recorded:

Adrenal glands	Prostate (ventral lobe)
Brain	Seminal vesicles and coagulating gland
Epididymides (L+R)	Spleen
Kidneys	Testes (L+R)
Liver	Thyroid
Ovaries (L+R)	Thymus
Pituitary^{+}	Uterus with cervix and oviducts

$^{+}$: weighed post-fixation
L+R: paired organs weighed separately

The weight of each organ was expressed as a percentage of the bodyweight recorded immediately prior to necropsy for all adults surviving to scheduled terminal kill.

Offspring (unselected F1 offspring and F2 offspring):

Where possible the following organs were taken from one male and one female randomly selected from each litter on day 34 of age, dissected free from adjacent fat and other tissue, and the weight recorded:

Brain
Spleen
Thymus

The weight of each organ was expressed as a percentage of the body weight recorded immediately prior to necropsy for each offspring.

Samples of the following tissues were preserved in 10% neutral buffered formalin (NBF), except for the testes and epididymides, which were preserved in Bouin's fluid for at least 24 hours before transfer to 70% industrial methylated spirit:

Parental animals:

Abnormalities*	Pituitary
Adrenal glands	Prostate (ventral lobe)
Brain	Seminal vesicles and coagulating gland
Right epididymis	Spleen
Kidneys	Right testis
Liver	Thyroid
Mammary glands - caudal	Uterus with cervix and oviducts
Ovaries	Vagina

* Preserved for all animals including F1 and F2 offspring

Offspring (F1 offspring and F2 offspring for organ weight determinations):

The offspring randomly selected for organ weights also had the following tissues retained:

Abnormalities*	Spleen
Brain	Testes
Epididymides	Thymus
Ovaries	Uterus with cervix and oviduct
Prostate (ventral lobe)	Vagina
Seminal vesicles and coagulating gland	

* Preserved for all animals including F1 and F2 offspring

Histology

Tissue samples from animals specified in the following table were dehydrated, embedded in paraffin wax, sectioned at four to five micron thickness and stained with haematoxylin and eosin. Testes were stained using a standard PAS method.

Table 3.10.1.2- 3: Histology

Tissue	Regions to be examined	Special notes
Abnormalities		Examined for all animals including F1/F2 offspring
Adrenal glands		
Epididymis - right	Longitudinal section to allow examination of caput, corpus and cauda	
Mammary glands - caudal		
Ovaries with oviduct	Midline section	5 Sections cut at approximately l00µm (left and right) intervals from the inner third of each ovary to reach the mid-line
Pituitary		
Prostate (ventral lobe) Seminal vesicles and coagulating gland		
Testis (right)		Qualitative evaluation with awareness of stages of spermatogenesis
Uterus with cervix		
Vagina	Transverse section cut at approximately 5 mm from vulva	

Microscopical examination

Parental animals:

Microscopic examination was performed as follows. The tissues specified in the previous table were examined for ten parent males and ten parent females of the control and high treatment groups (groups 1 and 4) sacrificed on completion of the scheduled treatment period and for all adult animals killed or dying before scheduled termination. Mammary glands were retained and examined.

Examination of the ovary of F0 females was limited to a qualitative assessment of the presence of primordial follicles, growing follicles and corpora lutea. For F1 females, in addition to the general qualitative examination of ovarian tissue, a quantitative assessment was made of the primordial follicle population. For this, five sections were cut at about 100 µm intervals from the inner third of each ovary and the primordial follicles manually counted. Primordial follicle counts were limited to 10 females in each of the control and high treatment groups (groups 1 and 4) and females (all groups) that failed to mate or conceive, or that suffered a total litter loss.

A separate, retrospective, histopathological examination of the liver and kidneys (see Anonymous.; 2004; M-247289-01-1, Section 3.10.1.3) was additionally performed to determine if there were treatment-related effects in the identified target organs.

II. Results and Discussion

A. Observations:

F0 generation

1. Clinical signs, mortality - F0 generation

The general condition of F0 males and females was satisfactory throughout the generation; no clinical signs were seen that were considered associated with treatment. There were no unscheduled deaths amongst the males, but one female (Animal Number 1176) at 500 ppm was killed in extremis shortly after parturition. This female was found with pale skin and eyes, hunched posture and piloerection having delivered 10 offspring, three of which were dead; of the seven live offspring five still had the placenta attached. All surviving offspring were killed at the same time as the dam. Findings noted at macroscopic necropsy examination were unremarkable. In view of the isolated nature of this death, and in the absence of similar in-life signs being observed for any other animals, it was considered to be a coincidental event unrelated to treatment.

Green staining was observed on the cage tray paper of seven pairs of animals after mating (one pair at 100 ppm, four pairs at 500 ppm and two pairs at 2,000 ppm). In addition, green staining was also observed on the cage tray paper of one cage of male animals at 2,000 ppm following their return to the home cage after separation from the females. In view of the sporadic nature of this finding and the absence of similar findings during/after pairing of the F1 generation, this green staining was considered to be of no toxicological significance.

2. Body weight and food intake - F0 generation

Body weight:

In males, at 2,000 ppm group mean body weight and cumulative body weight change values were lower than that of control from the commencement of treatment (Week 1 of treatment, 6% lower than control value, $p < 0.05$) and remained lower throughout the treatment period to termination (Weeks 1-16 of treatment, 7% lower than control value, not statistically significant) with differences frequently attaining statistical significance.

In 500 ppm males initial group mean body weight and cumulative body weight change values were similar to control. From Week 5 of treatment until termination, group mean body weight gain values were occasionally slightly lower or higher than those of the controls. Conversely, therefore due to the lack of a consistent pattern or other supporting data the slight differences in this parameter at this level were considered to reflect normal biological variation. There was also no evidence of an adverse effect of treatment with fluopicolide on group mean body weight or cumulative bodyweight change values in 100 ppm males.

In 2,000 ppm females mean body weight and cumulative body weight change values prior to pairing displayed a similar pattern of lower gains (Weeks 1-10 of treatment, 14% lower than control value, $p < 0.01$) like that observed in the males at this dosage level. Low mean body weight gain (up to 17% lower than control values, $p < 0.01$) continued during the first two weeks of gestation, however, by the end of the gestation period overall body weight change was similar to control, although absolute body weight remained lower (approx. 5% lower than control value). Following parturition, there was no evidence to suggest that treatment with fluopicolide had any noticeable effect on the normal pattern of body weight change associated with the demands of lactation.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment with fluopicolide on body weight or body weight change either prior to pairing, during gestation or during lactation.

An overview is given in Table 3.10.1.2- 4.

Table 3.10.1.2- 4: Body weight and body weight gains (g) of parental animals – F0 generation

	Generation	Dose level [ppm]						
		0	100		500		2,000	
Males								
Body weights [g] (% difference to control)								
Week 0	Pre- and postmating	191	189	*(-1.0)*	188	*(-1.6)*	190	*(-0.5)*
Week 4		414	410	*(-1.0)*	408	*(-1.4)*	398	*(-3.9)*
Week 8		532	521	*(-2.1)*	517	*(-2.8)*	498*	*(-6.4)*
Week 12		573	561	*(-2.1)*	556	*(-3.0)*	539	*(-5.9)*
Week 16		619	608	*(-1.8)*	603	*(-2.6)*	588	*(-5.0)*
Body weight gains [g] (*% difference to control*)								
Week 0-4		223	221	*(-0.9)*	220	*(-1.3)*	208	*(-6.7)*
Week 0-8		341	333	*(-2.3)*	328	*(-3.8)*	308*	*(-9.7)*
Week 0-12		382	372	*(-2.6)*	367	*(-3.9)*	348*	*(-8.9)*
Week 0-16		428	419	*(-2.1)*	415	*(-3.0)*	398	*(-7.0)*
Females								
Body weights [g] (% difference to control)								
Week 0	Premating	151	155	*(+2.6)*	151	*(±0.0)*	152	*(+0.7)*
Week 4		241	254	*(+5.4)*	247	*(+2.5)*	232	*(+3.7)*
Week 8		286	294	*(+2.8)*	283	*(-1.0)*	268	*(-6.3)*
Week 10		297	309	*(+4.0)*	298	*(+0.3)*	277	*(-6.7)*
Body weight gains [g] (*% difference to control*)								
Week 0-4		90	99	*(+10.0)*	96	*(+6.7)*	80*	*(-11.1)*
Week 0-8		134	139	*(+3.7)*	133	*(-0.7)*	116**	*(-13.4)*
Week 0-10		146	154	*(+5.5)*	148	*(+1.4)*	126**	*(-13.7)*
Body weights [g] (% difference to control)								
GD 0	Gestation	300	312	*(+4.0)*	309	*(+3.0)*	283	*(-2.3)*
GD 6		331	340	*(+2.7)*	335	*(+1.2)*	308*	*(-6.9)*
GD 13		362	371	*(+2.5)*	366	*(+1.1)*	335**	*(-7.5)*
GD 20		439	453	*(+3.2)*	449	*(+2.3)*	419	*(-4.6)*
Body weight gains [g] (% difference to control)								
GD 0-6		30	28	*(-6.7)*	26*	*(-13.3)*	25**	*(-16.7)*
GD 0-13		62	59	*(-4.8)*	57	*(-8.1)*	52**	*(-16.1)*
GD 0-20		138	141	*(+2.2)*	141	*(+2.2)*	136	*(-1.4)*
Body weights [g] (% difference to control)								
LD 1	Lactation	344	357	*(+3.8)*	341	*(-0.9)*	316**	*(-8.1)*
LD 4		356	373	*(+4.8)*	362	*(+1.7)*	335*	*(-5.9)*
LD 7		368	382	*(+3.8)*	372	*(+1.1)*	346*	*(-6.0)*
LD 14		377	394	*(+4.5)*	379	*(+0.5)*	346**	*(-8.2)*
LD 21		366	370	*(+1.1)*	370	*(+1.1)*	347*	*(-5.2)*
Body weight gains [g] (*% difference to control*)								
LD 1-4		12	16	*(+33.3)*	22**	*(+83.3)*	19**	*(+58.3)*
LD 1-7		24	25	*(+4.2)*	31	*(+29.2)*	30	*(+25.0)*
LD 1-14		33	37	*(+12.1)*	39	*(+18.2)*	31	*(-6.1)*
LD 1-21		22	14	*(-36.4)*	29	*(+31.8)*	31	*(+40.9)*

GD: gestation day; LD: lactation day

* / ** statistically significantly different from control, $p \leq 0.05$ / $p \leq 0.01$

Food intake:

In males, group mean food consumption values at 2,000 ppm were slightly lower than control from Week 1 to 8 prior to pairing (up to 7% lower than the control values, $p < 0.05$) of the F1 generation, after which parity with the control group was attained.

At 500 and 100 ppm, there was no evidence of an effect of treatment on food consumption during the pre-pairing period. It was noted that group mean values at 100 ppm were slightly lower than control. However, this is considered to be a reflection of the slightly lower body weight and body weight gain observed at this dosage level and not a treatment-relationship.

In 2,000 ppm females mean food consumption values during the pre-pairing phase (up to 8% lower than control values, $p < 0.05$) were consistently slightly lower than control values and this pattern continued throughout the gestation (up to 16% lower than control values, $p < 0.01$) and lactation phases (up to 10% lower than control value, $p<0.05$).

There was no evidence of an adverse effect of treatment with fluopicolide on food consumption at 500 or 100 ppm during the pre-pairing, gestation or lactation phases.

An overview is given in Table 3.10.1.2- 5 to Table 3.10.1.2- 7.

Table 3.10.1.2- 5: Food consumption (g/rat/week) prior to pairing – F0 generation

Time period	Dose level (ppm)						
	0	100		500		2,000	
Males							
Week 1	172	171	*(-0.6)*	169	*(-1.7)*	158**	*(-8.1)*
Week 10	193	197	*(+2.1)*	190	*(-1.6)*	187	*(-3.1)*
Females							
Week 1	127	127	*(±0.0)*	125	*(-1.6)*	115**	*(-9.4)*
Week 10	128	136	*(+6.3)*	136	*(+6.3)*	123	*(-3.9)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 3.10.1.2- 6: Food consumption (g/rat/day) of dams during gestation (g) - F0 generation

Time period	Dose level (ppm)						
	0	100		500		2,000	
Days 0-5	24	24	*(±0.0)*	25	*(+4.2)*	22**	*(-8.3)*
Days 6-12	27	28	*(+3.7)*	28	*(+3.7)*	25**	*(-7.4)*
Days 13-19	28	29	*(+3.6)*	29	*(+3.6)*	27	*(-3.6)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 3.10.1.2- 7: Food consumption (g/rat/day) of dams during lactation – F0 generation

Time period	Dose level (ppm)						
	0	100		500		2,000	
Days 1-3	34	37	*(+8.8)*	36	*(+5.9)*	35	*(+2.9)*
Days 4-6	50	49	*(-2.0)*	51	*(+2.0)*	48	*(-4.0)*
Days 7-13	68	68	*(±0.0)*	68	*(±0.0)*	60**	*(-13.3)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Food conversion efficiency of males and females before pairing, assessed during the 10 week pre-pairing treatment period, did not indicate any adverse effect of treatment on food utilisation for either sex, with values for each inclusion level being comparable to Control, indicating that food intake and bodyweight/bodyweight gain were in balance.

Achieved test substance doses:

The achieved dosage at all dosage levels for both sexes generally reflected the intended five and four-fold intervals between dietary levels and exposure to fluopicolide was considered to be satisfactory. Exposure levels in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the 10 week pre-mating period and throughout gestation and lactation. The intake fell to approx. 50% of the initial values during the course of the pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation (see Table 3.10.1.2- 8 to Table 3.10.1.2- 10).

Table 3.10.1.2- 8: Achieved dosage (mg/kg bw/day) prior to pairing – F0 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Week 1	-	11.0	55.0	206.4	-	10.7	54.0	199.6
Week 10	-	5.2	25.5	103.4	-	6.4	32.9	127.3

Table 3.10.1.2- 9: Achieved dosages (mg/kg bw/day) during gestation – F0 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-6	-	7.4	38.9	150.3
Days 6-13	-	7.8	39.5	156.6
Days 13-20	-	7.1	35.8	145.4

Table 3.10.1.2- 10: Achieved dosages (mg/kg bw/day) during lactation – F0 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-4	-	7.4	38.9	150.3
Days 4-7	-	7.8	39.5	156.6
Days 7-14	-	7.1	35.8	145.4

3. Reproductive results – F0 generation

Oestrous cycles:

There was no evidence of an adverse effect of treatment with fluopicolide on oestrous cycle regularity at any dosage level investigated.

Pre-coital interval and mating performance:

There was no evidence of an adverse effect of treatment with fluopicolide on mating performance and fertility, as assessed by pre-coital interval, percentage mating, conception rate and fertility index at any dosage level investigated.

Table 3.10.1.2- 11 gives an overview.

Table 3.10.1.2- 11: Mating performance and fertility – F0 generation

Parameter	Dose level (ppm)			
	0	100	500	2000
Number mating	28	28	28	28
Number achieving pregnancy	27	26	27	28
Conception rate (%)	96	93	96	100
Fertility index (%)	96	93	96	100

Gestation length, gestation index and parturition:

With the exception of one control female, two females at 100 ppm and one female at 500 ppm, all females were pregnant and gave birth to live offspring.

There was no adverse effect of treatment with fluopicolide on gestation length or gestation index, with the length of the gestation phase being between 22 and 23 days for females in all groups. There were no difficulties evident during the parturition process that were considered to be related to treatment (see Table 3.10.1.2- 12).

Table 3.10.1.2- 12: Gestation length and gestation index – F0 generation

Parameter	Dose level (ppm)			
	0	100	500	2000
Number of pregnant animals	27	26	27	28
Number of litters born	27	26	27	28
Gestation index (%)	100	100	100	100
No. of animals with defined gestation length (% of animals)				
Gestation length: 22 days	10 (37)	11 (42)	13 (48)	8 (29)
Gestation length: 22.5 days	11 (41)	7 (27)	9 (33)	11 (39)
Gestation length: 23 days	6 (22)	8 (31)	5 (19)	9 (32)

Offspring results

Litter responses

There were no instances of total litter loss and with the exception of the female at 500 ppm, which was killed in extremis prior to day 1 of lactation, all females reared their litters to maturity. The following assessment is based on 27, 26, 26 and 28 litters surviving to weaning at 0, 100, 500 and 2,000 ppm, respectively.

The general condition of the offspring was similar in all groups and offspring from the test groups showed no adverse clinical findings either while they were being suckled or later as they started to eat the treated diet.

There was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated on the number of uterine implantation sites (recorded at termination), litter size at birth, survival of offspring to litter standardisation on Day 4 and subsequent survival to weaning.

Assessment of the sex ratio, from Day 1 to 21 after birth, did not indicate any adverse effects of treatment with fluopicolide upon the survival of either sex throughout this period.

An overview is given in Table 3.10.1.2- 13.

Table 3.10.1.2- 13: Uterine implantations, litter data

Parameter	Dose level (ppm)			
	0	**100**	**500**	**2,000**
Litter number	27	26	26	28
Implantations (mean)	14.4	14.7	15.7	14.9
Total litter size on day 1	13.5	14.1	14.6	13.9
Live birth index (%)	98.3	97.8	99.3	98.1
Viability index (%)	98.9	95.9	98.3	99.3
Lactation index (%) – day 7	100.0	99.6	100.0	100.0
Lactation index (%) – day 21	100.0	98.8	99.6	99.6
Sex ratio (day 1):				
Males	6.3	7.0	7.4	7.5
Females	7.1	7.0	7.2	6.3
Sex ratio (day 21):				
Males	4.7	4.7	4.9	5.0
Females	4.9	4.4	5.0	4.8

Offspring body weights (F1 pups)

Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning (approx. 8% lower than control values, $p < 0.01$), coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect (see also external expert statement Moxon, M.; 2018; M-638869-01-1 in Section 3.10.1.8). This is supported by the fact that reduced food consumption and body weight development was observed in all repeated dose rat studies during the initial treatment phase and also in the parental animals of the present reproductive study (see Table 3.10.1.2- 4 and Table 3.10.1.2- 5).

At 500 and 100 ppm there was no evidence of an adverse effect of treatment with fluopicolide on offspring body weight or body weight change from birth and through to weaning.

An overview is given in Table 3.10.1.2- 14.

Table 3.10.1.2- 14: Body weight and body weight gains (g) of F1 pups

Day of Age	Dose level (ppm)					
	0	100		500		2,000	
Males						
Body weights [g] (*% difference to control*)						
Day 1 (before cull)	6.8	6.8	*(0.0)*	6.7	*(-1.5)*	7.0	*(+2.9)*
Day 4 (before cull)	9.5	9.6	*(+1.1)*	9.4	*(-1.1)*	9.4	*(-1.1)*
Day 4 (after cull)	9.6	9.5	*(-1.0)*	9.4	*(-2.1)*	9.4	*(-2.1)*
Day 7 (after cull)	15.7	15.1	*(-3.8)*	15.2	*(-3.2)*	14.8	*(-5.7)*
Day 14 (after cull)	33.0	32.9	*(-0.3)*	32.6	*(-1.2)*	30.4**	*(-7.9)*
Day 21 (after cull)	52.3	52.0	*(-0.6)*	51.5	*(-1.5)*	48.0**	*(-8.2)*
Day 25 (after cull)	70.5	69.8	*(-1.0)*	69.3	*(-1.7)*	64.4**	*(-8.7)*
Day 28 (after cull)	86.7	85.5	*(-1.4)*	85.1	*(-1.8)*	79.3**	*(-8.5)*
Body weight change (g) Day 1-28	79.9	78.7	*(-1.5)*	78.4	*(-1.9)*	72.2**	*(-9.6)*
Females						
Body weights [g] (*% difference to control*)						
Day 1 (before cull)	6.4	6.4	*(0.0)*	6.3	*(-1.6)*	6.6	*(+3.0)*
Day 4 (before cull)	9.1	9.2	*(+1.1)*	8.9	*(-2.2)*	9.0	*(-1.1)*
Day 4 (after cull)	9.1	9.3	*(+2.2)*	8.9	*(-2.2)*	9.0	*(-1.1)*
Day 7 (after cull)	14.9	14.6	*(-2.0)*	14.4	*(-3.4)*	14.2	*(-4.7)*
Day 14 (after cull)	31.9	32.3	*(+1.3)*	31.1	*(-2.5)*	29.4**	*(-7.8)*
Day 21 (after cull)	50.1	50.5	*(+0.8)*	48.8	*(-2.6)*	46.3**	*(-7.6)*
Day 25 (after cull)	66.2	67.2	*(+1.5)*	64.9	*(-2.0)*	61.1**	*(-7.7)*
Day 28 (after cull)	79.4	80.4	*(+1.3)*	77.9	*(-1.9)*	73.8**	*(-7.1)*
Body weight change (g) Day 1-28	73.0	74.0	*(+1.4)*	71.6	*(-1.9)*	67.2**	*(-7.9)*

** $p \leq 0.01$, statistically significantly different from control

Examination results of unselected offspring and F0 parents

Necropsy of offspring

The nature and incidence of findings observed at macroscopic examination of offspring dying before weaning and the unselected offspring killed at weaning, after selection of the F1 generation, did not suggest any adverse effect of treatment with fluopicolide. The majority of the offspring dying before weaning presented with no milk in the stomach. This is a common finding in such offspring and is considered to reflect a possible lack of maternal care. The incidence of pups dying after litter standardisation was negligible and there was no evidence of an increase in mortality associated with transition to treated diet.

Organ weights of unselected F1 offspring:

Both male and female offspring treated at 2,000 ppm displayed slightly lower absolute spleen (Males: 11% lower than control values, $p < 0.05$; Females: 17% lower than control value, $p < 0.01$) and thymus weights (Males: 11% lower than control value, $p < 0.01$; Females: 9% lower than control value, $p < 0.05$) than the concurrent control, largely related to the lower group mean terminal body weight observed at this dosage which is supported by the fact that the body weight-relative organ weight values for male offspring were similar to those of the controls. Similarly, body weight-relative thymus weights for female offspring were considered unaffected, however, body weight-relative spleen values were slightly low (8% lower than control value, $p < 0.05$). Absolute and body weight-relative brain weights were considered unaffected by treatment.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment on absolute or body weight-relative organ weights. Absolute values at 500 ppm tended to be slightly lower than counterpart controls, but they were considered to be within the expected range of biological variation.

An overview is given in Table 3.10.1.2- 15.

Table 3.10.1.2- 15: Organ weights of unselected offspring – F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	**100**	**500**	**2,000**	**0**	**100**	**500**	**2,000**
Litter number	27	25	26	28	27	24	26	28
Terminal body weight (g)	128.0	128.1	127.4	118.9**	113.7	114.3	109.1	102.6**
Spleen weight								
Absolute (g)	0.493	0.490	0.454	0.441*	0.402	0.400	0.369	0.334**
Relative (%)	0.3846	0.3839	0.3577	0.3708	0.3523	0.3482	0.3376	0.3255*
Thymus weight								
Absolute (g)	0.544	0.521	0.512	0.486**	0.468	0.477	0.457	0.424*
Relative (%)	0.4265	0.4091	0.4021	0.4101	0.4129	0.4177	0.4215	0.4154

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Necropsy of F0 parents

Macroscopic necropsy findings for males and females at termination were unremarkable and there was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated.

Organ weights F0 parents

In both males and females treated at 2000 ppm, group mean absolute and bodyweight-relative kidney and liver weights were high and spleen weights were low, when compared with the controls; these instances often attained statistical significance, with the most noticeable and consistent difference being recorded for the liver.

An overview is given in Table 3.10.1.2- 16.

Table 3.10.1.2- 16: Organ weights of F0 parents

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Terminal body weight (g)	618.8	605.7	600.0	587.5	325.0	339.2	327.2	305.7*
Liver weight								
Absolute (g)	22.49	23.44	22.54	26.94**	15.58	16.49	16.12	17.84**
Relative (%)	3.628	3.871*	3.756	4.594**	4.800	4.858	4.926	5.828**
Spleen weight								
Absolute (g)	0.908	0.854	0.854	0.800**	0.613	0.634	0.591	0.524**
Relative (%)	0.1473	0.1424	0.1429	0.1369	0.1878	0.1874	0.1804	0.1719*
Kidney weight								
Absolute (g)	4.03	4.01	4.05	4.44*	2.65	2.81	2.63	2.67
Relative (%)	0.654	0.663	0.678	0.757**	0.817	0.829	0.805	0.873**

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

In addition, other inter-group differences in body weight-relative organ weights were observed, however, these were in organs not influenced to the same extent by overall body weight (e.g. the brain, prostate, seminal vesicles, testes, epididymides and thyroids with parathyroids). As the absolute weights of these organs were unaffected, the apparent increases in body weight-relative weights were considered to be an artefact of the adjustment to terminal body weight rather than an effect of treatment.

Absolute and body weight-relative organ weights for animals receiving 500 or 100 ppm were considered unaffected by treatment.

Sperm analysis and morphology of F0 males

Quantitative (Computer Assisted Sperm Analyser) assessment of the sperm parameters (motility, progressive motility, sperm count, homogenisation resistant spermatids) and visual assessment of sperm morphology did not reveal an effect by treatment with fluopicolide.

Marginal reductions in % motile sperm and % normal morphology and increases in the number of decapitate sperm were apparent at 2,000 ppm; however these were accompanied by much larger standard deviations and were attributable to an individual (Animal Number 1104) and these changes did not attain statistical significance. Sperm concentrations and total sperm counts from the cauda epididymis and testis were similar in the control and treated groups (see Table 3.10.1.2- 17).

Table 3.10.1.2- 17: Sperm results of F0 males

Parameter	Dose level (ppm)	
	0	2,000
Motile sperm (%)	95±9	90±22
Sperm count (mill/g) (cauda epididymis)	586±159	600±181
Sperm count (mill/g) (testis)	54±12	54±17
Sperm morphology		
Normal (%)	96.9±8.7	94.6±16.6
Decapitate (%)	2.2±8.4	4.3±16.4
Abnormal (%)	0.9±0.7	1.1±0.9

4. Pathology

Histopathology F0 parents:

Microscopic examination of the organs and tissues taken from the F0 males and females did not reveal any obvious findings that were considered to be related to treatment.

A retrospective histopathological examination of the liver and kidneys (see Anonymous.; 2004; M-247289-01-1, Section 3.10.1.3) showed treatment-related findings in both organs. In kidneys an increased incidence of a number of degenerative and regenerative changes (cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females) were seen at 2,000 ppm. An increased incidence of cortical tubular basophilia and interstitial inflammation was also seen in males and females treated at 500 ppm, respectively. In the liver an increased incidence of centrilobular hepatocyte hypertrophy was seen in males and females at 2,000 ppm and males given 500 ppm. The latter finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

A mammary gland tumour was recorded in a control female, one female receiving 100 ppm and one female treated at 2,000 ppm. In view of the incidence and distribution of these tumours they were considered to have arisen by chance and be unrelated to treatment with fluopicolide.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Qualitative examination of the primordial follicle population of the F0 females exposed to fluopicolide suggested that they were similar to the controls.

Oestrous cycles at termination F0 females:

Vaginal smears taken post-weaning (days 25 to 28 of lactation) showed that most females had returned to normal oestrous cycles and attained oestrus before termination and, therefore, fluopicolide had no effect on this parameter.

F1 generation

1. Clinical signs, mortality – F1 generation

The general condition of F1 animals was satisfactory throughout the generation; no clinical signs were seen that were considered associated with treatment and there were no unscheduled deaths.

2. Body weight and food intake – F1 generation

Body weight

In males, at the commencement of the F1 generation (at nominal Week 4 of age) group mean body weights at 2,000 ppm were lower (11% lower than control value, $p < 0.01$) than control reflecting the pattern established prior to weaning; values remained consistently lower than those of the controls throughout the generation, frequently attaining statistical significance up to Week 4 (approx. eight weeks of age). However, differences in cumulative body weight gain when compared with control were small indicating that, despite lower absolute body weights, a similar pattern of growth occured, with overall body weight gain values (Day 0-112) being only 4% lower than control.

In 500 and 100 ppm males there was no evidence of an adverse effect on group mean body weight or body weight gain following treatment with fluopicolide. It was noted that at 100 ppm, body weight and body weight gain were slightly lower than control. In the absence of any effect of treatment in 500 ppm males, these values at 100 ppm were considered to be coincidental and reflect the slightly lower group mean body weight of these animals at the start of the male F1 generation.

In 2,000 ppm females mean body weight values were lower than control from the commencement of the female F1 generation throughout the pre-pairing, gestation and lactation phases (up to 13% lower than control values, $p < 0.01$). Group mean body weight gain values were similar or slightly lower than control up to week 5 of the F1 generation after which some divergence was noted (8% lower than control at the end of the pre-pairing phase; Day 0-70). This pattern of reduced body weight gain (up to 15% lower than control values, $p < 0.05$ or $p < 0.01$) in females continued throughout the gestation phase, however, following parturition, there was no evidence to suggest that treatment with fluopicolide affected the normal pattern of body weight gain during the lactation period with overall gain by weaning being superior to the concurrent control.

In 500 and 100 ppm females there was no evidence of an adverse effect of treatment with fluopicolide on body weight or body weight gain either prior to pairing, during gestation or during lactation.

An overview of the body weight development in males and females in the different periods is given in Table 3.10.1.2- 18.

Table 3.10.1.2- 18: Body weight and body weight gains (g) - F1 generation

	Generation	Dose level [ppm]							
		0	100		500		2,000		
Males									
Body weights [g] (*% difference to control*)									
Day 0[#]	**Pre- and postmating**	91	87	*(-4)*	90	*(-1)*	81**	*(-11)*	
Day 7		146	139	*(-5)*	144	*(-1)*	132*	*(-10)*	
Day 14		205	197	*(-4)*	204	*(±0)*	191	*(-7)*	
Day 21		267	259	*(-3)*	265	*(-1)*	250*	*(-6)*	
Day 28		327	316	*(-3)*	324	*(-1)*	308	*(-6)*	
Day 35		372	361	*(-3)*	369	*(-1)*	353	*(-5)*	
Day 42		409	398	*(-3)*	407	*(±0)*	387	*(-5)*	
Day 49		441	428	*(-3)*	439	*(±0)*	418	*(-5)*	
Day 56		468	453	*(-3)*	465	*(-1)*	442	*(-6)*	
Day 63		486	472	*(-3)*	486	*(±0)*	462	*(-5)*	
Day 70		503	490	*(-3)*	506	*(+1)*	478	*(-5)*	
Day 77		514	500	*(-3)*	516	*(+1)*	486	*(-5)*	
Day 84		531	518	*(-2)*	538	*(+1)*	508	*(-4)*	
Day 91		546	533	*(-2)*	552	*(+1)*	521	*(-5)*	
Day 98		553	539	*(-2)*	557	*(+1)*	525	*(-5)*	
Day 105		568	553	*(-3)*	569	*(±0)*	540	*(-5)*	
Day 112		580	560	*(-3)*	583	*(+1)*	548	*(-5)*	
Body weight gains [g] (*% difference to control*)									
Day 0-7		55	52	*(-5)*	54	*(-2)*	51	*(-7)*	
Day 0-14		114	111	*(-3)*	114	*(±0)*	110	*(-3)*	
Day 0-49		350	341	*(-3)*	348	*(-1)*	337	*(-4)*	
Day 0-84		441	432	*(-2)*	448	*(+2)*	427	*(-3)*	
Day 0-112		489	474	*(-3)*	492	*(+1)*	467	*(-4)*	

	Generation	Dose level [ppm]							
		0	100		500		2,000		
Females									
Body weights [g] (*% difference to control*)									
Day 0[#]	**Premating**	82	81	*(-1)*	82	*(±0)*	74*	*(-10)*	
Day 7		121	123	*(+2)*	122	*(+1)*	112*	*(-7)*	
Day 14		159	162	*(+2)*	156	*(-2)*	147*	*(-7)*	
Day 21		185	192	*(+4)*	185	*(±0)*	174	*(-6)*	
Day 28		211	219	*(+4)*	209	*(-1)*	196*	*(-7)*	
Day 35		229	239	*(+4)*	228	*(±0)*	215*	*(-6)*	
Day 42		247	258	*(+5)*	243	*(-2)*	227**	*(-8)*	
Day 49		259	271	*(+5)*	256	*(-1)*	241*	*(-7)*	
Day 56		272	284	*(+4)*	273	*(±0)*	254*	*(-7)*	
Day 63		285	292	*(+3)*	285	*(±0)*	265*	*(-7)*	
Day 70		297	300	*(+1)*	291	*(+2)*	271**	*(-9)*	
Body weight gains [g] (*% difference to control*)									
Day 0-7		40	42	*(+5)*	40	*(±0)*	38	*(-5)*	
Day 0-14		77	81	*(+5)*	74	*(-4)*	73	*(-5)*	
Day 0-49		177	190	*(+7)*	174	*(-2)*	167	*(-6)*	
Day 0-70		215	219	*(+2)*	209	*(-3)*	197*	*(-8)*	
Body weights [g] (*% difference to control*)									
GD 0	**Gestation**	298	302	*(+1)*	295	*(-1)*	267**	*(-10)*	
GD 6		325	329	*(+1)*	321	*(-1)*	289**	*(-11)*	
GD 13		356	358	*(+1)*	352	*(-1)*	317**	*(-11)*	
GD 20		440	448	*(+2)*	440	*(±0)*	398**	*(-9)*	
Body weight gains [g] (*% difference to control*)									
GD 0-6		27	26	*(-4)*	26	*(-4)*	23*	*(-15)*	
GD 0-13		58	56	*(-3)*	57	*(-2)*	50**	*(-14)*	
GD 0-20		142	146	*(+3)*	144	*(+1)*	132*	*(-7)*	
Body weights [g] (*% difference to control*)									
LD 1	**Lactation**	344	342	*(-1)*	339	*(-1)*	299**	*(-13)*	
LD 4		351	357	*(+2)*	347	*(-1)*	309**	*(-12)*	
LD 7		362	367	*(+1)*	357	*(-1)*	318**	*(-12)*	
LD 14		367	373	*(+2)*	361	*(-2)*	321**	*(-12)*	
LD 21		359	364	*(+1)*	355	*(-1)*	320**	*(-11)*	
Body weight gains [g] (*% difference to control*)									
LD 1-4		7	15	*(+114)*	8	*(+14)*	9	*(+29)*	
LD 1-7		18	25	*(+39)*	18	*(±0)*	18	*(±0)*	
LD 1-14		23	31	*(+35)*	22	*(-4)*	22	*(-4)*	
LD 1-21		15	22	*(+47)*	16	*(+7)*	21	*(+40)*	

GD: gestation day; LD: lactation day
* / ** statistically significantly different from control, $p \leq 0.05$ / $p \leq 0.01$
[#] formal commencement of F1 generation; animals nominal week 4 of age

Food intake

Group mean food consumption values for males at 2,000 ppm were slightly lower than control from Week 1 to 8 prior to pairing (up to 7% lower than the control values, $p < 0.05$) of the F1 generation, after which it was comparable to the control group.

In 500 and 100 ppm males, there was no evidence of an effect of treatment on food consumption during the pre-pairing period. It was noted that group mean values at 100 ppm were slightly lower than control, however this is considered to be a reflection of the slightly lower body weight and body weight gain observed at this dosage level and not a treatment-relationship.

In 2,000 ppm females group mean food consumption values during the pre-pairing phase were consistently slightly lower than control values (up to 8% lower than control values, $p < 0.05$), and this pattern continued throughout the gestation (up to 16% lower than control values, $p < 0.01$) and lactation phases (up to 10% lower than control value, $p < 0.05$).

There was no evidence of an adverse effect of treatment with fluopicolide on food consumption in 500 or 100 ppm females during the pre-pairing, gestation or lactation phases.

An overview of the food uptakes in males and females in the different periods is given in Table 3.10.1.2-19 to Table 3.10.1.2- 21.

Table 3.10.1.2- 19: Food consumption (g/rat/week) prior to pairing – F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2000	0	100	500	2,000
Week 1 (% control)	118	111	115	110* (93)	100	102	102	99 (99)
Week 10 (% control)	194	191	197	193 (99)	141	142	140	133 (94)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 3.10.1.2- 20: Food consumption (g/rat/day) of dams during gestation – F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-5 (% control)	25	25	25	22** (88)
Days 6-12 (% control)	28	27	27	25** (89)
Days 13-19 (% control)	31	30*	29*	26** (84)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 3.10.1.2- 21: Food consumption (g/rat/day) of dams during lactation – F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-3	42	45	40	38
Days 4-6 (% control)	51	54	49	46* (90)
Days 7-13 (% control)	72	73	69	66* (92)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Food conversion efficiency, assessed during the 10 week pre-pairing treatment period, did not indicate any adverse effect of treatment on food utilisation for either sex, with values for each inclusion level being comparable to control, indicating that food intake and body weight/body weight gain were in balance.

Formal treatment of the F1 generation begins at approx. 4 weeks of age compared to 6 weeks of age in the F0 generation. This automatically results in a higher exposure level in the F1 generation provided food intake and body weight gains are not adversely affected.

Achieved test article doses:

The achieved dosage at all dosage levels for both sexes continued to reflect the intended five and fourfold intervals between dietary levels, and exposure to fluopicolide was considered to be satisfactory.

Exposure levels well in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the 10-week pre-mating period and during gestation and lactation. The intake fell to approximately 40 to 50% of the initial values during the course of the pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation.

An overview of the achieved dosages in males and females in the different periods is given in Table 3.10.1.2- 22 toTable 3.10.1.2- 24.

Table 3.10.1.2- 22: Achieved dosage (mg/kg bw/day) prior to pairing - F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Week 1	-	14.1	70.3	296.5	-	14.2	71.6	304.2
Week 10	-	5.7	28.3	117.1	-	6.8	34.6	141.6

Table 3.10.1.2- 23: Achieved dosages (mg/kg bw/day) during gestation - F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-6	-	8.0	40.3	158.9
Days 6-13	-	7.9	40.2	162.6
Days 13-20	-	7.3	37.2	147.0

Table 3.10.1.2- 24: Achieved dosages (mg/kg bw/day) during lactation - F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-4	-	12.8	58.6	252.2
Days 4-7	-	14.8	69.9	294.1
Days 7-14	-	19.7	96.0	414.9

Sexual maturation:

There was no evidence of an adverse effect of treatment with fluopicolide on the time of completion of vaginal opening or balanopreputial separation at any dosage level investigated (see Table 3.10.1.2- 25).

Table 3.10.1.2- 25: Sexual development – F1 generation (group mean ± SD)

Parameter	Dose level (ppm)			
	0	100	500	2,000
Males				
Preputial separation (days)	44.4±1.7	44.6±3.5	45.0±2.1	45.9±2.4
Body weight at preputial separation (g)	219.8	217.4	223.5	220.9
Females				
Vaginal opening (day)	35.0±3.0	34.4±2.1	34.9±1.9	35.2±2.2
Body weight at vaginal opening (g)	116.7	118.5	115.8	111.3

SD: Standard deviation

3. Reproductive results - F1 generation

Oestrous cycles:

There was no evidence of an adverse effect of treatment with fluopicolide on establishment or regularity of oestrous cycles at any dosage level investigated (see Table 3.10.1.2- 26).

Table 3.10.1.2- 26: Oestrus cycle - F1 generation

Incidences	Dose level (ppm)			
	0	100	500	2,000
Number of animals	24	24	24	24
Regular 4- or 5-day cycles	17	23	20	17
Irregular cycles	0	0	0	0
Acyclic	7	1	4	7

Pre-coital interval and mating performance:

There was no evidence of an adverse effect of treatment with fluopicolide on mating performance and fertility, as assessed by pre-coital interval, percentage mating, conception rate and fertility index at any dosage level investigated (see Table 3.10.1.2- 27).

Table 3.10.1.2- 27: Mating performance and fertility - F1 generation

Parameter	Dose level (ppm)			
	0	100	500	2,000
Number mating	24	24	24	24
Number achieving pregnancy	24	24	23	23
Conception rate (%)	100	100	100	96
Fertility index (%)	100	100	96	96

Gestation length, gestation index and parturition:

With the exception of one female at 2,000 ppm, all females were pregnant and gave birth to live offspring.

There was no adverse effect of treatment with fluopicolide on gestation length or gestation index with the length of the gestation phase being between 22 and 23 days for females in all groups. There were no difficulties evident during the parturition process that were considered to be related to treatment (see Table 3.10.1.2- 28).

Table 3.10.1.2- 28: Gestation length and gestation index – F1 generation

Parameter	Dose level (ppm)			
	0	**100**	**500**	**2,000**
Number of pregnant animals	24	24	24	23
Number of litters born	24	24	24	23
Gestation index (%)	100	100	100	100
No. of animals with defined gestation length (% of animals)				
Gestation length: 22 days	13 (54)	14 (58)	17 (71)	9 (39)
Gestation length: 22.5 days	5 (21)	7 (29)	2 (8)	8 (35)
Gestation length: 23 days	6 (25)	3 (13)	5 (21)	6 (26)

Offspring results

Litter responses

Two females experienced total litter loss, one at 500 ppm on Day 4 of lactation and one at 2,000 ppm on Day 3 of lactation. Most coincidental instances of total litter loss in the CD rat occur during this period and the isolated incidences in this study were considered not to be related to treatment with fluopicolide. The following assessment is based on 24, 24, 23 and 22 litters surviving to weaning at 0, 100, 500 and 2,000 ppm, respectively.

The general condition of the offspring was similar in all groups and showed no adverse responses to treatment of the F1 parents.

There was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated on the number of uterine implantation sites (recorded at termination), litter size at birth, survival of offspring to litter standardisation on day 4 and subsequent survival to weaning.

Assessment of the sex ratio, from Day 1 to 21 after birth, did not indicate any adverse effects of treatment with fluopicolide upon the survival of either sex throughout this period.

An overview is given in Table 3.10.1.2- 29.

Table 3.10.1.2- 29: Uterine implantations, litter data

Parameter	Dose level (ppm)			
	0	**100**	**500**	**2,000**
Litter number	24	24	23	22
Implantations (mean)	15.6	16.1	15.4	15.3
Total litter size on day 1	14.8	15.4	15.0	14.4
Live birth index (%)	98.3	96.5	99.3	99.4
Viability index (%)	98.8	97.1	97.7	97.2
Lactation index (%) – day 7	100.0	98.8	99.6	100.0
Lactation index (%) – day 21	99.2	96.2	98.3	99.1

Parameter	Dose level (ppm)			
	0	100	500	2,000
Sex ratio (day 1):				
Males	6.8	7.4	8.0	7.0
Females	7.9	8.0	6.9	7.4
Sex ratio (day 21):				
Males	5.0	5.0	5.0	5.2
Females	4.9	4.6	4.6	4.7

Offspring body weights (F2 pups)

At 2,000 ppm, F2 offspring of both sexes showed statistically significantly reduced body weight compared to control from postnatal Day 14 onwards. Consequently, cumulative body weight gain (Day 1-28) was reduced by 14% and 11% for males and females, respectively.

Body weight and body weight gains of the F2 offspring treated at 500 or 100 ppm were considered unaffected by treatment with fluopicolide.

An overview is given in Table 3.10.1.2- 30.

Table 3.10.1.2- 30: Body weight and body weight gains (g) of F2 pups

	Dose level (ppm)					
	0	100		500		2,000	
Males						
Body weights [g] (*% difference to control*)						
Day 1 (before cull)	6.5	6.5	*(±0)*	6.5	*(±0)*	6.6	*(+2)*
Day 4 (before cull)	9.2	9.1	*(-1)*	9.0	*(-2)*	9.0	*(-2)*
Day 4 (after cull)	9.3	9.2	*(-1)*	9.0	*(-3)*	9.0	*(-3)*
Day 7 (after cull)	14.7	14.7	*(±0)*	14.2	*(-3)*	14.0	*(-5)*
Day 14 (after cull)	31.4	31.3	*(±0)*	30.7	*(-2)*	28.4**	*(-9)*
Day 21 (after cull)	44.9	44.7	*(±0)*	43.8	*(-2)*	38.9**	*(-13)*
Day 25 (after cull)	63.2	61.9	*(-2)*	60.6	*(-4)*	54.9**	*(-13)*
Day 28 (after cull)	78.6	76.2	*(-3)*	75.1	*(-4)*	68.9**	*(-12)*
Body weight gain (g) Day 1-28	72.1	69.7	*(-3)*	68.5	*(-5)*	62.3**	*(-14)*
Females						
Body weights [g] (*% difference to control*)						
Day 1 (before cull)	6.1	6.1	*(±0)*	6.1	*(±0)*	6.2	*(+2)*
Day 4 (before cull)	8.6	8.6	*(±0)*	8.5	*(-1)*	8.5	*(-1)*
Day 4 (after cull)	8.7	8.7	*(±0)*	8.5	*(-2)*	8.6	*(-1)*
Day 7 (after cull)	13.9	14.0	*(+1)*	13.7	*(-1)*	13.4	*(-4)*
Day 14 (after cull)	29.9	30.2	*(+1)*	29.6	*(-1)*	27.3**	*(-9)*
Day 21 (after cull)	42.8	43.7	*(+2)*	42.3	*(-1)*	37.7**	*(-12)*
Day 25 (after cull)	59.5	59.4	*(±0)*	57.4	*(-3)*	53.1**	*(-11)*
Day 28 (after cull)	72.5	72.0	*(-1)*	69.8	*(-4)*	65.3**	*(-10)*
Body weight gain (g) Day 1-28	66.5	65.9	*(-1)*	63.7	*(-4)*	59.1**	*(-11)*

** $p \leq 0.01$, statistically significantly different from control

Examination results of the F2 offspring and F1 parents

Necropsy of offspring

The nature and incidence of findings observed at macroscopic examination of F2 offspring dying before weaning or at scheduled termination (Day 34 of age), did not suggest any adverse effect of treatment with fluopicolide. The majority of the offspring dying before weaning presented with no milk in the stomach. This is a common finding in such offspring and is considered to reflect a possible lack of maternal care. As in the previous generation, there were virtually no deaths after standardisation of litter size on Day 4 of age.

Organ weights of offspring on Day 34 of age

Both male and female offspring treated at 2,000 ppm displayed lower absolute spleen (Males: 19% lower than control value, $p < 0.01$; Females: 19% lower than control value, $p < 0.01$) and thymus weights (Males: 16% lower than control value, $p < 0.01$; Females: 11% lower than control, $p < 0.05$) than the concurrent control at termination, largely related to the lower group mean terminal body weight observed at this dosage. In females, body weight-relative spleen values were slightly low (9% lower than control value, $p < 0.05$), when compare with the controls.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment on absolute or body weight-relative organ weights of male offspring. Female offspring at these dietary concentrations displayed slightly low absolute spleen weights although this was considered to be consistent with the slightly lower group mean terminal body weight observed and not treatment-related.

An overview is given in Table 3.10.1.2- 31.

Table 3.10.1.2- 31: Organ weights of unselected offspring – F2 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Litter number	24	24	23	22	24	24	23	22
Terminal body weight (g)	116.4	111.5	110.9	100.4**	103.2	97.1	97.7	92.3**
Spleen weight								
Absolute (g)	0.439	0.423	0.428	0.356**	0.375	0.331*	0.335*	0.305**
Relative (%)	0.3772	0.3805	0.3842	0.3543	0.3643	0.3423	0.3424	0.3309*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Necropsy of F1 parents

Macroscopic necropsy findings for males and females at termination were unremarkable and there was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated.

Organ weights F1 parents

In both males and females treated at 2,000 ppm, group mean absolute and body weight-relative liver and kidney weights were higher than those of the controls, the liver being the organ most noticeably and consistently affected. The females in this group also displayed lower absolute spleen weights, but the body weight-relative spleen weights were not significantly different from controls.

Table 3.10.1.2- 32: Organ weights of F1 parents

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Terminal body weight (g)	585.5	567.2	587.5	553.6	320.2	327.7	312.7	290.1**
Liver weight								
Absolute (g)	21.88	21.03	22.88	26.73**	15.31	15.78	16.18	17.95**
Relative (%)	3.732	3.704	3.895	4.833**	4.781	4.813	5.184**	6.189**
Spleen weight								
Absolute (g)	0.843	0.789	0.821	0.777	0.665	0.686	0.624	0.567*
Relative (%)	0.1435	0.1392	0.1398	0.1408	0.2071	0.2105	0.1995	0.1954
Kidney weight								
Absolute (g)	3.82	3.81	3.84	4.49**	2.52	2.66	2.52	2.58
Relative (%)	0.653	0.672	0.656	0.811**	0.790	0.812	0.807	0.890**

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Group mean body weight-relative liver weights were also slightly high (8% higher than control values, $p < 0.01$) for females treated at 500 ppm, when compared with the controls.

In addition, other inter-group differences in body weight-relative organ weights were observed, however, these were in organs that were not influenced to the same extent by overall body weight, such as the brain, seminal vesicles and thyroids with parathyroids. In these cases, the absolute values were considered unaffected, therefore, the apparent increase in body weight-relative weight were considered to be attributable to the adjustment to terminal body weight rather than an effect of treatment.

Sperm analysis and morphology of F1 males

The numbers of motile and progressively motile sperm (from the vas deferens) and the numbers of caudal epididymal sperm and testicular spermatids were similar in all groups. In addition, assessment of sperm morphology suggested that fluopicolide had no adverse effects upon spermatogenesis or upon sperm maturation.

Table 3.10.1.2- 33: Sperm results of F1 males

Parameter	Dose level (ppm)	
	0	2,000
Motile sperm (%)	90±19	90±7
Sperm count (mill/g) (cauda epididymis)	577±148	587±122
Sperm count (mill/g) (testis)	111±35	127±25
Sperm morphology		
Normal (%)	94.1±20.1	97.6±3.9
Decapitate (%)	5.0±20.2	1.3±2.9
Abnormal (%)	0.9±0.9	1.1±1.2

4. Pathology

Histopathology F1 parents:

Microscopic examination of the organs and tissues taken from the F1 males and females did not reveal any obvious findings that were considered to be related to treatment.

A retrospective histopathological examination of the liver and kidneys (see Anonymous.; 2004; M-247289-01-1, Section 3.10.1.3) showed treatment-related findings in both organs. In kidneys an increased incidence of a number of degenerative and regenerative changes (cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females) were seen at 2,000 ppm. In the liver an increased incidence of centrilobular hepatocyte hypertrophy was seen in males and females at 2,000 ppm and males given 500 ppm. The latter finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Oestrous cycles at termination F1 females:

Vaginal smears taken post-weaning (Days 25 to 28 of lactation) showed that most females had returned to oestrous cyclicity and attained oestrus before termination and, therefore, fluopicolide had no effect on this parameter.

Fluopicolide had no apparent effect on the primordial follicle count in any group tested when compared to the control values.

III. Conclusion

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL), based on the likelihood of the increased liver weights and the slighty increased centrilobular hepatocyte hypertrophy at this dose level being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

An additional histopathological examination to the 2-generation rat study is summarized in Section 3.10.1.3.

3.10.1.3 Anonymous; 2004; M-247289-01-1

Study reference:

Anonymous; 2004; AE C638206 Additional Microscopic Examination to a Study of Reproductive Performance in CD Rats Treated Continuously Through Two Successive Generations by Dietary Administration - Volumes 1 and 2; M-247289-01-1

Executive Summary:

The objective of this evaluation was to extend the histopathological examination to target organs (based on other toxicity studies) from all F0 and F1 animals of the 2-generation rat study (see Anonymous.; 2003; M-232532-01-1, Section 3.10.1.2). In this study the influence of fluopicolide on the fertility and reproductive capacity of two successive generations was assessed in male and female rats of the CD strain by continuous administered via the diet at concentrations of 0, 100, 500 or 2,000 ppm throughout two generations.

In the kidneys treatment-related changes were noted in both sexes from both generations at 2,000 ppm and consisted of cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females.

In the liver treatment-related changes were present in both sexes at 2,000 ppm and in males at 500 ppm from both generations and consisted of centrilobular hepatocyte hypertrophy. This finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

The additional histopathology on this study confirmed treatment-related findings in the kidneys of animals in the F0 and Fl generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2,000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

Results:

This histopathological examination revealed an increased incidence of centrilobular hepatocyte hypertrophy in F0 males and females given fluopicolide at 2,000 ppm and males given 500 ppm.

In the kidneys, an increased incidence of a number of degenerative and regenerative changes was seen in animals given fluopicolide at 2,000 ppm. An increased incidence of cortical tubular basophilia and interstitial inflammation was seen in males and females treated at 500 ppm, respectively.

An overview is given in Table 3.10.1.3- 1.

Table 3.10.1.3- 1: Histopathological findings in the liver and kidney - F0 generation

Findings	Dose level (ppm)							
	Males				Females			
	0	**100**	**500**	**2,000**	**0**	**100**	**500**	**2,000**
Number of organs examined	28	28	28	28	28	28	27	28
Liver								
Hepatocyte hypertrophy, centrilobular	0	0	9**	28**	0	0	0	12**
Kidney								
Cortical tubular basophilia total	12	16	19	28**	13	10	19	2
Cortical tubules with hyaline droplets total	1	1	0	28**	0	0	0	0
Granular casts, medulla	0	0	1	20**	0	0	0	0
Interstitial inflammation	6	5	9	15*	0	3	5*	2
Cortical scarring	2	2	2	10*	1	2	3	3
Hyaline tubular casts	2	2	4	10*	3	1	3	0
Cortical tubular dilatation	1	0	0	0	0	0	3	12**
Corticomedullary mineralization	0	0	0	1	3	4	3	10

* $p \leq 0.05$; ** $p \leq 0.01$

Also in the F1 generation an increased incidence of centrilobular hepatocyte hypertrophy was seen in animals given fluopicolide at 2,000 ppm and males given 500 ppm.

In the kidneys an increased incidence of a number of degenerative and regenerative changes were seen in animals given fluopicolide at 2,000 ppm.

An overview is given in the following table.

Table 3.10.1.3- 2: Histopathological findings in the liver and kidney – F1 generation

Findings	Dose level (ppm)							
	Males				Females			
	0	**100**	**500**	**2,000**	**0**	**100**	**500**	**2,000**
Number of organs examined	24	24	24	24	24	24	24	24
Liver								
Hepatocyte hypertrophy, centrilobular	0	0	8**	24**	0	0	0	20**
Kidney								
Cortical tubular basophilia total	10	11	11	23**	10	7	9	22**
Cortical tubules with hyaline droplets total	0	0	0	22**	0	0	0	0
Granular casts, medulla	0	0	0	14**	0	0	0	0
Cortical scarring	0	2	1	6*	1	1	0	1
Hyaline tubular casts	2	4	5	13**	1	1	1	0
Cortical tubular dilatation	1	0	0	5	1	1	2	11**
Corticomedullary mineralization	0	0	0	0	3	0	5	8

* $p \leq 0.05$; ** $p \leq 0.01$

The additional histopathology of this study confirmed treatment-related findings in the kidneys of animals in the F0 and F1 generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The hyaline droplets in the cortical tubules of male rats are considered likely to represent accumulation of α2μ-globulin within the lysosomes. It is generally regarded that α2μ-globulin nephropathy is a male rat specific toxic response to the administration of certain types of chemicals (hydrocarbon nephropathy). However, there is a clear effect of administration of fluopicolide on the kidney of female animals as well. This indicates that the kidney is a target organ for this compound.

The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2,000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

III. Conclusion

The additional histopathology of this study confirmed treatment-related findings in the kidneys of animals in the F0 and F1 generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

3.10.1.4 Anonymous; 2000; M-198488-01-1

Study reference:

Anonymous; 2000; AE C638206 - Code: AE C638206 00 1C99 0005 - Rat oral developmental toxicity (teratogenicity) range finding study; M-198488-01-1

Deviations:	As a dose-range finding study the study was not intended to comply with OECD guidelines.

Executive Summary:

This range finding study was conducted in order to select suitable doses of fluopicolide for a subsequent developmental toxicity (teratogenicity) study in Sprague Dawley rats.

Groups of 4 mated female Sprague Dawley rats received technical fluopicolide in aqueous methylcellulose (1% w/v) by oral gavage once daily at the dose levels of 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy.

Animals were observed daily for mortality and clinical signs of toxicity. Body weight and food consumption were determined regularly throughout the study. At necropsy the dams were examined for macroscopically visible changes. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths and placental weights were determined.

No deaths occurred throughout the study. Two animals from the 1,000 mg/kg bw/day group showed pultaceous loose faeces from Day 10 up to Day 13 of pregnancy. At 1,000 mg/kg bw/day body weight gains were lower than normal and also compared to the 500 mg/kg bw/day group throughout the treatment period. In addition, food consumption showed a marked initial decrease in this group between Days 7 and 10 and was slightly reduced during this period also at 500 mg/kg bw/day. No compound-related effects were observed at necropsy of the animals.

Incidence of post-implantation loss was increased at 1,000 mg/kg bw/day even after one animal from this group with total litter loss was excluded from the calculations. In addition, mean foetal weight and crown-rump length were reduced at this dose. Slight decreases in foetal weights and foetal crown-rump lengths were also observed at 500 mg/kg bw/day.

Based on the results of this study, the high dose in the main study should be between 500 and 1,000 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206 (fluopicolide)
Batch:	PP/241024/2 & PP241067/1
Purity:	97.6% w/w (T/041/99, dated 18 or 23 August, 1999)

2. Vehicle and/or positive control:

1% (w/v) methyl cellulose in deionised water

3. Test animals

Species:	Rat
Strain:	Hsd: Sprague Dawley SD
Age:	approximately 8-10 weeks
Weight at start:	191 – 215 g
Source:	Harlan Winkelmann GmbH, Gartenstraße 27, 33178 Borchen, GERMANY
Acclimation phase:	yes
Diet:	commercial diet for laboratory rats Ssniff R-Z (V1324)2, *ad libitum*
Water:	tap water in plastic bottles, *ad libitum*
Housing:	in fully air-conditioned rooms in Macrolon cages (Type 3) on soft wood granulate
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 21 to October 12, 1999

2. Animal assignment and treatment:

The test animals were assigned to the following groups (see Table 3.10.1.4- 1).

Table 3.10.1.4- 1: Study design

Test group	Dose level [mg/kg bw/day]	Number of females
1	500	4
2	1,000	4

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 500 or 1,000 mg/kg bw/day from Day 7-20 of pregnancy.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 500 or 1,000 mg/kg bw as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 7-20 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals in the pre-oestrus or oestrus phase were mated overnight with sexually mature males in the ratio 1 male : 1 female and were caged individually after detection of sperm in vaginal smears. The day of sperm detection was defined as Day 1 of pregnancy, and the day of mating was defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

Due to the low animal number and the lack of an untreated control group no statistical analyses were performed.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 1, 4, 7, 10, 14, 17, 19 and 21 of pregnancy, and food consumption between Days 1-4, 4-7, 7-10, 10-14, 14-17, 17-19 and 19-21.

3. Caesarean section and foetal evaluation:

The animals were killed on Day 21 of pregnancy and the foetuses removed by Caesarean section. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was recorded. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities and then the foetuses were killed by CO_2 asphyxia.

II. Results and Discussion

A. Observations:

1. Maternal data:

No deaths occurred throughout the study. Two animals from the 1,000 mg/kg bw/day group showed pultaceous faeces from Day 10 up to day 13 of pregnancy. Females of the 500 mg/kg bw/day group showed no clinical signs.

2. Body weight and food consumption:

At 1,000 mg/kg bw/day body weight gain was reduced throughout the treatment period (Day 7-21: -34% compared to 500 mg/kg bw/day dose group).

Table 3.10.1.4- 2: Maternal body weight and body weight gain *(% of 500 mg/kg bw/day group*)

	Dose level [mg/kg bw/day]	
	500	**1,000**
Body weight [g]		
Day 1	199.5	204.7
Day 4	209.0	211.7
Day 7	221.5	224.3
Day 10	228.5	227.3
Day 14	245.5	242.7
Day 17	269.0	262.0
Day 19	300.8	274.0
Day 21	326.3	293.7
Cumulative body weight gain [g] *(% difference to 500 mg/kg bw/day dose group)*		
Day 1-7	22	19.6 *(-11)*
Day 7-21 [a]	104.8	69.4 *(-34)*
Day 1-21 [b]	126.8	89.0 *(-30)*
Gravid uterus weight [g]		
Day 21	63.1	42.5

[a] During treatment
[b] During pregnancy

Food consumption showed an initial decrease at 1,000 mg/kg bw/day after start of treatment between Days 7 and 10 (-16% compared to Day 4-7 value) and was slightly reduced during the same period at 500 mg/kg bw/day (-7% compared to Day 4-7 value). Food consumption was generally lower at 1,000 mg/kg bw/day compared to the 500 mg/kg bw/day dose group.

Table 3.10.1.4- 3: Food consumption during gestation

	Dose level [mg/kg bw/day]	
	500	**1,000**
Mean Food Consumption [g/animal/day]		
Day 1-4	15.0	13.3
Day 4-7	18.2	19.7
Day 7-10	17.0	16.5
Day 10-14	21.4	21.3
Day 14-17	21.7	21.0
Day 17-19	25.2	19.8
Day 19-21	22.9	20.2
Total [g/animal]	397.4	376.3

3. Necropsy findings:

No compound-related effects were observed at necropsy of the animals.

4. Caesarean section data:

Incidence of post-implantation loss was increased at 1,000 mg/kg bw/day. One animal from this group showed total litter loss and in addition another female had a high incidence of resorptions (75% of implantations). Mean foetal weight and crown-rump length were also reduced at this dose level compared to control values of the main study (foetal weight 3.7 g, crown-rump length 36 mm). Slight decreases in foetal weights and foetal crown-rump lengths were also observed at 500 mg/kg bw/day. Placental weights were comparable in both groups.

Table 3.10.1.4- 4: Summary of caesarean section parameters

Parameter	Dose level [mg/kg bw/day]	
	500	1,000
No. pregnant / no. mated	4 / 4	4 / 4
No. of dams with resorptions only	0	1[#]
No. dams with live foetuses	4	3
Mean no. corpora lutea/dam	14.0	17.3
Mean no. implantation sites/dam	13.0	15.0
Pre-implantation loss (% of corpora lutea)	9.1	14.6
Post-implantation loss (% implants)	0.0	31.7
Mean no. of resorptions/dam	0.0	5.7
No of dead foetuses	0.0	0.0
Total no. of live fetuses	52	28
Mean no. live fetuses/dam	13.0	9.3
Sex ratio (% males)	Not determined	
Mean foetal weight (g)	3.1	2.8
Crown-rump length (mm)	34.1	32.6
Placental weight (g)	0.496	0.468

[#] Excluded from all calculations

III. Conclusion

Based on the results of this study the high dose in the main study should be between 500 and 1,000 mg/kg bw/day.

3.10.1.5 Anonymous; 2004; M-202155-02-1

Study reference:

Anonymous; 2004; AE C638206 Code: AE C638206 00 1C99 0005 Rat oral developmental toxicity (teratogenicity) study; M-202155-02-1

Deviations: The following deviations from the OECD-Guideline 414 (2018) occurred:

- dose levels were not set in two- to four-fold intervals
- thyroid-related parameters (thyroid gland weight, histopathological assessment of the thyroid gland) were not assessed as required in the newest guideline version
- anogenital distance of all live rodent foetuses was not measured as required in the newest guideline version
- No blood samples were taken from dams at study termination for assessment of thyroid hormones T4, T3 and thyroid stimulating hormone (TSH) as required in the newest guideline version

At the time of the study conduct, the test guideline required only that the intermediate dose should be located geometrically between the low and high dose levels.Therefore, the deviations are considered not to compromise the outcome of the study and the measured parameters are considered sufficient to assess the developmental toxicity of fluopicolide.

Executive Summary:

Groups of 23 mated female Sprague Dawley rats received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 60 or 700 mg/kg bw from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy. The dosing volume was 5 mL/kg bw.

Behaviour and state of health were observed daily in all groups. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. Gravid uterus weight was recorded. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths, sex ratios of the foetuses and placental weights were determined. The foetuses were examined for external, visceral and skeletal anomalies.

There were no deaths during the study. No clinical signs were observed in any of the animals. Body weights and weight gains were decreased in the animals from the high dose group, especially at the beginning of the treatment period during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. These animals showed also a slight initial decrease in food consumption after beginning of treatment. Body weight gains and food consumption remained unaffected by the administration of the test compound in the other dose groups.

Gravid uterus weights were comparable in all groups. No compound-related effects were observed at necropsy.

Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group. However, litter size, number of live and dead foetuses as well as sex ratios were unaffected by the administration of the test substance. Incidences of early and late conceptuses undergoing resorption were also not affected by the administration of the test compound up to and including the highest tested dose level of 700 mg/kg bw/day.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor defects comprising aplastic, dysplastic or fused thoracic vertebral arches, aplastic, dysplastic, fragmented, fused or dislocated vertebral centres, fragmented and displaced sternebrae as well as aplastic, dysplastic, shortened, fused, wavy and/or thickened ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the fetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses

Signs of retarded development were reflected by increased incidences of ossification of less than two caudal vertebral centres, weakly or non-ossified sternebrae and non-ossified metacarpale 5, metatarsale 5 and phalanx III of 1st to 5th toe at 700 mg/kg bw/day.

In conclusion, oral administration of fluopicolide to the pregnant rat at the dose of 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gains and slightly decreased food consumption. Mean foetal body weights and crown-rump lengths were also slightly decreased at the highest dose level. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification were observed more frequently at this dose level and are considered secondary to the above described maternal toxicity. Fluopicolide was not teratogenic in this developmental toxicity study in rats.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 60 mg/kg bw/day or below. Therefore, with regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206 (fluopicolide)
Batch:	PP/241024/2 & PP241067/1
Purity:	1) 97.6% w/w (T/041/99, dated 23 August 1999)
	2) 97.8% w/w (T/060/99, dated 01 December 1999)

2. Vehicle and/or positive control:

1% w/v methyl cellulose in deionised water (tylose slime)

3. Test animals

Species:	Rat
Strain:	Hsd: Sprague Dawley SD
Age:	approximately 8-10 weeks
Weight at start:	mean group weights: 226.6 – 227.7 g
Source:	Harlan Winkelmann GmbH, Gartenstraße 27, 33178 Borchen, GERMANY
Acclimation phase:	yes
Diet:	commercial diet for laboratory rats Ssniff R-Z (V1324)2, *ad libitum*
Water:	tap water in plastic bottles, *ad libitum*
Housing:	in fully air-conditioned rooms in Macrolon cages (Type 3) on soft wood granulate
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 2, 1999 to June 15, 2000

2. Animal assignment and treatment

The test animals were assigned randomly (computer-generated algorithm) to the following groups (see Table 3.10.1.5- 1).

Table 3.10.1.5- 1: Study design

Test group	Dose level [mg/kg bw/day]	Number of females
1	0	23
2	5	23
3	60	23
4	700	23

3. Duration of dosing

Mated female Sprague Dawley rats received fluopicolide from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy.

4. Dose preparation and administration:

The rats received fluopicolide at the dose levels of 0, 5, 60 or 700 mg/kg bw/day as a suspension in 1% w/v methyl cellulose (in deionised water) orally by gavage once daily from Day 7-20 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Test substance analysis

For each concentration, samples were taken towards the start, middle and end of the dosing period (Day 7 to 20). All samples were stored deep frozen prior to analysis. The samples from start and end of the dosing period were analysed. The achieved concentrations of fluopicolide in aqueous methyl cellulose (1%) directly after preparation and after 4 hour storage at room temperature from different container locations were between 80 and 107% of the nominal concentrations confirming a sufficient test substance content, homogeneity and stability in the dosing solution.

6. Mating

Virgin female animals in the pre-oestrus or oestrus phase were mated overnight with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of sperm detection was defined as Day 1 of gestation, and the day of mating was defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

7. Statistics

The statistical evaluation is based on the assumption of a monotone dose-response relationship. Statistical comparisons of the low dose groups with the simultaneous control group were only carried out if significant effects were detectable in the high dose group. In the univariate analysis, two-sided questions (body weight of dams, relative food consumption, crown-rump length, foetal weight and placenta weight) were generally tested as follows: a two-sided comparison with the high dose group was followed by a one-sided test for the low-dose group. In case of the caesarean section data of the foetuses (crown-rump length, foetal weight and placental weight), multivariate statistics were first of all calculated and used in selecting relevant dose groups. For the individual parameters, sequential comparisons with the high dose group and sequential tests at the 5 % level for the low dose were then conducted.

The t-tests and the test statistics of Wilks are based on common variance estimations for all study groups. For the Wilcoxon test the exact distribution of the meaned ranks was calculated.

In the case of the daily food consumption of the dams, the mean consumption per 100 g body weight was always calculated between two successive measurement times and evaluated by the rank sum test after Wilcoxon. In examining the body weights of the dams, the change in weight was determined in comparison to the initial weight. The univariate evaluation was carried out using t-tests.

The caesarean section data of the foetuses were used to calculate litter mean values.

Multivariate evaluation was carried out using the test statistics of Wilks. In the univariate analysis, t-tests were used.

The number of corpora lutea, implantation sites and live foetuses, and quotas of dead embryonic primordia undergoing resorption in the animals were likewise analysed using one-sided Wilcoxon tests.

The findings obtained at autopsy and at body cross-section and skeletal examination of the foetuses were evaluated separately for the foetuses and for the litters by Jackknife t-test at significance levels of 5%. It was examined whether the relative frequencies of findings in the dose groups deviated from those findings in the control group.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily (on weekends and public holidays once daily).

2. Body weights and food consumption:

Body weights were determined on Days 1, 4, 7, 10, 14, 17, 19 and 21 of pregnancy, and food consumption was recorded between Days 1-4, 4-7, 7-10, 10-14, 14-17, 17-19 and 19-21 of pregnancy.

3. Caesarean section:

The animals were killed on Day 21 of pregnancy and the foetuses removed by Caesarean section. All animals were examined externally and internally (thoracic and abdominal contents) for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was determined. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The implantation sites in the uterus were counted after staining with ammonium sulphide.

The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities. Then the foetuses were killed by CO_2 asphyxia.

4. Foetal evaluation:

Approx. 50% of the foetuses of each litter were fixed in alcohol, necropsied, sexed and checked for anomalies of the internal organs. The carcasses were placed in a solution of potassium hydroxide for clearing and stained with Alizarin red S (bones) and Alcian blue (cartilage). The skeletons were examined and checked for stage of development and abnormalities with the aid of a stereo-microscope.

Foetuses found dead in the uterus at caesarean section were fixed in alcohol and examined for external and skeletal anomalies. The remaining foetuses were transferred in Bouin's solution, examined for organ anomalies referring to Wilson's slicing technique[20] and sexed.

Visceral and skeletal changes were subdivided into four categories (major defects, minor defects, variations and retardations) based on the severity of the finding and/or the spontaneous incidence of the finding.

[20] Wilson, J.G.: Embryological considerations in teratology. In Teratology: Principles and Techniques (J.G. Wilson, J. Warkany, Ed.), page 251-277. University of Chicago Press, Chicago, IL (1965)

II. Results and Discussion

A. Observations:

1. Maternal data:

No deaths or clinical signs were observed during the whole study.

2. Body weight and food consumption:

Body weights were slightly but statistically significantly decreased in the animals from the high dose group during treatment on Days 10-21. The statistically significant decrease in body weight at Day 4 in the high dose group is considered to be fortuitous, since treatment started on day 7. Body weight gain was also decreased at 700 mg/kg bw/day (-8% during treatment period and -12% during gestation if corrected for gravid uterus weight). Especially at the beginning of the treatment period the body weight gain was markedly reduced by 24% during Day 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development.

Table 3.10.1.5- 2: Maternal body weight and body weight gain

	Dose level [mg/kg bw/day]						
	0	**5**		**60**		**700**	
Body weight [g] *(% difference to control)*							
Day 1	227.3	227.7	*(±0)*	226.6	*(±0)*	226.7	*(±0)*
Day 4	235.7	234.8	*(±0)*	234.0	*(-1)*	231.4*	*(-2)*
Day 7	248.4	249.1	*(±0)*	245.9	*(-1)*	245.2	*(-1)*
Day 10	259.2	260.1	*(±0)*	257.1	*(-1)*	253.5*	*(-2)*
Day 14	275.1	275.4	*(±0)*	272.2	*(-1)*	267.5*	*(-3)*
Day 17	301.2	300.6	*(±0)*	295.9	*(-2)*	291.3*	*(-3)*
Day 19	330.1	329.4	*(±0)*	326.1	*(-1)*	319.3*	*(-3)*
Day 21	363.0	366.6	*(+1)*	360.1	*(-1)*	350.5*	*(-3)*
Day 21 [a, #]	292.54	291.46	*(±0)*	287.99	*(-2)*	284.10	*(-3)*
Cumulative body weight gain [g] *(% difference to control)* [#]							
Day 1-7	21.1	21.4	*(+1)*	19.3	*(-9)*	18.5	*(-14)*
Day 7-10	10.8	11.0	*(+2)*	11.2	*(+4)*	8.2	*(-24)*
Day 10-14	16.0	15.2	*(-5)*	15.1	*(-6)*	14.0	*(-12)*
Day 14-17	26.0	25.2	*(-3)*	23.6	*(-9)*	23.8	*(-8)*
Day 17-19	29.0	28.8	*(-1)*	30.3	*(+4)*	28.0	*(-3)*
Day 19-21	32.9	37.2	*(+13)*	34.0	*(+3)*	31.2	*(-5)*
Day 7-21 [b]	114.6	117.5	*(+3)*	114.2	*(±0)*	105.3	*(-8)*
Day 1-21	135.7	138.9	*(+2)*	133.5	*(-2)*	123.8	*(-9)*
Day 1-21 [a]	65.2	63.8	*(-2)*	61.4	*(-6)*	57.4	*(-12)*
Gravid uterus weight [g] (% difference to control)							
Day 21	70.46	75.14	*(+7)*	72.11	*(+2)*	66.40	*(-6)*

* Significantly different from control
\# No statistical analyses were performed.
a Corrected for gravid uterus weight
b Treatment period only

The animals from the high dose group showed a slight initial decrease in food consumption (not statistically significant) after begin of the treatment period (see Table 3.10.1.5- 3).

Table 3.10.1.5- 3: Food consumption during gestation

	Dose level [mg/kg bw/day]					
	0	**5**		**60**		**700**	
Mean Food Consumption [g/100 g body weight] *(% difference to control)*						
Day 1-4	6.6	6.3	*(-5)*	6.6	*(±0)*	6.3	*(-5)*
Day 4-7	8.1	8.5	*(+5)*	8.3	*(+2)*	8.5	*(+5)*
Day 7-10	8.5	8.9	*(+5)*	8.9	*(+5)*	8.3	*(-2)*
Day 10-14	8.2	8.3	*(+1)*	8.5	*(+4)*	8.5	*(+4)*
Day 14-17	8.1	7.9	*(-2)*	7.7	*(-5)*	8.0	*(-1)*
Day 17-19	8.4	8.4	*(±0)*	8.5	*(+1)*	8.3	*(-1)*
Day 19-21	7.4	7.5	*(+1)*	7.5	*(+1)*	7.5	*(+1)*
Total [g/animal]	426.8	427.9	*(±0)*	429.2	*(+1)*	416.1	*(-3)*

Body weight gains and food consumption remained unaffected by the administration of the test substance in the other dose groups.

3. Necropsy findings:

Gravid uterus weights were comparable in all groups (see Table 3.10.1.5- 2). No compound-related effects were seen at necropsy of the animals.

4. Caesarean section data:

One female from the control and intermediate dose group and two females from the low and high dose group each did not become pregnant.

There was no increase in the incidence of the numbers of early and late conceptuses undergoing resorption and dead foetuses at any dose level up to and including 700 mg/kg bw/day. Most of the resorptions had diameters between 2 and 8 mm, indicating that they were early conceptuses undergoing resorption. One dead foetus was observed in the low dose group.

An overview of relevant parameters is given in Table 3.10.1.5- 4.

Table 3.10.1.5- 4: Results of gestation and Caesarean section

Parameter	Dose level [mg/kg bw/day]			
	0	**5**	**60**	**700**
No. pregnant / no. mated	22 / 23	21 / 23	22 / 23	21 / 23
No. dams with live foetuses	22	21	22	21
Mean no. corpora lutea/dam	15.5	16.0	14.9	15.1
Mean no. implantation sites/dam	13.8	14.8	14.1	13.3
Pre-implantation loss (% of corpora lutea)	10.77	7.04	5.34	12.50
Post-implantation loss (% implants)	8.18	6.75	4.53	1.55
Mean no. of resorptions/dam	0.86	0.95	0.64	0.24
Early resorptions/dam	0.86	0.90	0.64	0.24
Dead foetuses/dam	0.00	0.05	0.00	0.00

***** Statistically significantly different from control

5. Foetal Data:

Litter size as well as number of live and dead foetuses remained unaffected by the administration of the test substance. Sex ratio of the foetuses was also not altered by the administration of the test substance. Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group only.

Table 3.10.1.5- 5: Foetal data *(% difference to control)*

Parameter	Dose level [mg/kg bw/day]			
	0	**5**	**60**	**700**
Total no. of live foetuses	284	291	297	274
Mean no. live foetuses/dam	12.9	13.9	13.5	13.0
Sex ratio (% males)	53.9	47.4	41.2	48.2
Mean foetal weight (g)	3.7	3.7 *(±0)*	3.6 *(-3)*	3.4* *(-8)*
Crown-rump length (mm)	36.2	36.0 *(-1)*	36.1 *(±0)*	34.8* *(-4)*
Mean placental weight (g)	0.57	0.53 *(-7)*	0.53 *(-7)*	0.52* *(-9)*

***** Statistically significantly different from control

External, skeletal and visceral examination:

No compound-related effects were observed at external examination of the foetuses scheduled for skeletal examination. In all cases there was no dose-dependency, and statistical examination did not reveal differences between the groups.

Foetal findings including increased incidences of a small number of minor skeletal defects and evidence of retarded development that could be ascribed to treatment were generally only observed in the highest dose group of 700 mg/kg bw/day. Increased incidences of skeletal findings above the historical control data range in the intermediate dose group are attributed to one single foetus (60 L05) with multiple malformations at the vertebral column and the pelvis. Although increased incidences of thoracic and rib findings were also found in the highest dose group, the findings in the one foetus in the intermediate group are considered incidental since only one foetus was affected and the defects were different to the ones observed at 700 mg/kg bw/day (see also Table 3.10.1.5- 8).

An overview of relevant findings is given in Table 3.10.1.5- 6.

Table 3.10.1.5- 6: Selected external and skeletal foetal findings (foetal (litter) incidence in %)

Parameter	Classification	Dose level [mg/kg bw/day]				HCD[#]
		0	5	60	700	
External examination						
No.of foetuses (litters) examined		136 (22)	141 (21)	144 (22)	132 (21)	3031 (435)
Eye						
- microphthalmia	major defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.8 (4.8)	0.0-0.0
Skeletal examination						
No.of foetuses (litters) examined		148 (22)	150 (21)	153 (22)	142 (21)	3031 (435)
Tail/lumbar-, sacral-, caudal vertebra / pelvic girdle						
- tail and vertebra aplasia, ilium & ischium dysplasia bilateral	major defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	0.0 (0.0)	0.0-0.5
Thoracic vertebral arches						
- Aplasia, dysplasia, fused, fused with attached rib	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	2.8 (14.3)	0.0-0.5
Thoracic vertebral centra						
- aplasia, dysplasia, fused, fragmented, dislocated	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	7.0* (28.6)	0.0-1.2
Caudal vert. centra						
- ossification of less than 2	retardation	6.8 (31.8)	34.0* (81.0)	24.2* (72.7)	82.4* (100.0)	11.0-35.3
Sternebrae						
- fragmented, longitudinally displaced	minor defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.1 (14.3)	0.0-1.4
- non- or weakly ossified	retardation	6.1 (36.4)	20.7* (47.6)	20.9* (63.6)	71.8* (100.0)	12.8-36.3

Parameter	Classification	Dose level [mg/kg bw/day]				HCD[#]
		0	5	60	700	
Rib						
- aplasia, dysplasia, shortened, fused, primordium of only 9	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	4.2 (14.3)	0.0-0.3
- wavy and/or thickened	minor defect	0.7 (4.5)	0.7 (4.8)	0.0 (0.0)	3.5 (9.5)	0.0-1.5
- extra rib at 7th cervical vertebra - short or normally long – uni- or bilateral	variation	2.0 (9.1)	0.0 (0.0)	0.7 (4.5)	3.5 (14.3)	0.0-0.3
Pectoral girdle						
- scapula shortened and bent costad right	minor defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.7 (4.8)	0.0-0.3
Forepaw						
- metacarpal 5 non-ossified bilateral	retardation	4.7 (22.7)	27.3* (66.7)	19.0* (63.6)	71.8* (100.0)	7.1-33.6
Hindpaw						
- metatarsal 5 non-ossified bilateral	retardation	0.7 (4.5)	0.7 (4.8)	1.3 (9.1)	7.0 (28.6)	0.0-5.7
- 1st to 5th toe non-ossified bilateral	retardation	1.4 (9.1)	0.0 (0.0)	0.0 (0.0)	5.6 (23.8)	0.0-2.3

***** Statistically different from control, $p \leq 0.05$
[#] As presented in study report (two-sided 95% tolerance interval range of HCD (per study) of foetal incidences in
% based on 21 studies (3031 foetuses, 435 litters) in Sprague Dawley rats
[a] Attributed to one small foetus (weight 2.7 g and C/R 26 mm) with multiple malformations

Major defects:

There was one foetus (60 LO5, 2.7 g) with multiple malformations at the vertebral column and the pelvis in the intermediate dose group and one foetus (90 R04, 2.0 g) with microphthalmia in the high dose group. The latter one might be secondary to a general developmental retardation (foetal weight -46% compared to control) and both findings are considered to be incidental due to their isolated occurrence.

In addition, the microphthalmia incidence was within historical control ranges identified in a publication (Lang, P. L.; 1993; M-259312-01-1)[21]. The data of this document cover studies which were conducted in the years before 1993 which is not too far from the experimental duration of present study which was from November 2, 1999 to June 15, 2000. This overview provided historical control range data for the finding microphthalmia on gestation Day 21 of up to 1.09% for foetal and of up to 8.70% for litter incidences. An overview is given in Table 3.10.1.5- 7.

[21] Charles River (1993): Historical Control Data for Development and Reproductive Toxicity Studies using the CrI:CD®BR Rat, Compiled by MARTA (Middle Atlantic Reproduction and Teratology Association); Edited by Patricia L. Lang, Ph.D., Consultant in Toxicology, September 1993 (M-259312-01-1).

Table 3.10.1.5- 7: Historical control data range for microphthalmia on gestation day 20 and 21 according to public literature

Eye: microphthalmia	Foetal incidence (%)			Litter incidence (%)		
	Average	SD	Max.	Average	SD	Max.
Gestation day 20[a]	0.027	0.17	1.68	0.195	1.19	12.00
Gestation day 21[b]	0.028	0.15	1.09	0.310	1.51	8.70

[a] Gestation day 20: total studies: 154, total litters: 3240, total foetuses: 22892
[b] Gestation day 21: total studies: 69, total litters: 1458, total foetuses: 14976

Therefore, the foetal and litter incidences of microphthalmia in the present study are clearly within the historical ranges of the data provided by Charles River[21] for Sprague-Dawley rats.

Minor defects:

The incidence of foetuses and litters with minor defects was clearly elevated in the 700 mg/kg bw/day group. However, by definition, minor defects are non-lethal, generally not detrimental to postnatal development and therefore, are considered not to have adverse consequences for the fetuses in postnatal life in contrast to major defects/malformations. In this case, most minor defects represent a perturbation of ossification which is transient in nature and will be resolved as ossification progresses (see also external expert statement Moxon, M.; 2018; M-638869-01-1 in Section 3.10.1.8).

Minor defects observed more frequently in the foetuses from the high dose group consisted of aplastic, dysplastic or fused thoracic vertebral arches (0/148, 0/150, 1/153, 4/142), aplastic, dysplastic, fragmented, fused or dislocated thoracic vertebral centres (0/148, 0/150, 1/153, 10/142*), fragmented or longitudinally displaced sternebrae (0/148, 0/150, 0/153, 3/142), aplastic, dysplastic, shortened, fused or primordium of only 9 ribs (0/148, 0/150, 1/153, 6/142) as well as wavy and/or thickened ribs (1/148, 1/150, 0/153, 5/142).

Table 3.10.1.5- 8: Detailed foetal and litter data for minor skeletal defects

Parameter	Group dose level (mg/kg bw/day)			
	0	5	60	700
Skeletal				
No.of foetuses examined	148	150	153	142
No. of litters examined	22	21	22	21
Cervical vertebra				
- primordium of only 6	-	-	-	77 R01
Thoracic vertebral arches				
- aplasia 10, 11, 12, 13th bilateral	-	-	60 L05	-
- aplasia 2nd or 7th unilateral or 9th bilateral	-	-	-	77 R01, 77 R05 79 R07
- dysplasia 9th bilateral	-	-	60 L05	-
- fused 6th & 7th unilateral	-	-	-	84 R06
- fused 9th with attached rib	-	-	60 L05	-

Parameter	Group dose level (mg/kg bw/day)			
	0	5	60	700
Thoracic vertebral centra				
- aplasia of 9, 10, 11, 12 and 13th bilateral	-	-	60 L05	-
- aplasia 2nd or 9th	-	-	-	77 R05 79 R07
- dysplasia 8th	-	-	60 L05	-
- fragmented 6,7,11, 12, 13th (and/or)	-	-	-	71 R07 74 R03 77 R01 84 R02, 84 R04 88 L03
- fused 6 & 7th	-	-	-	77 R01
- dislocated 8th	-	-	60 L05	-
- dislocated 6th or 11th	-	-	-	84 L05, 84 R06
Sternebrae				
- fragmented and/or longitudinally displaced 4th	-	-	-	77 R05 79 R07 88 R06
Rib				
- anlage of only 9 bilateral	-	-	60 L05	-
- aplasia 2nd, 7th or 9th unilateral	-	-	-	77 R01, 77 R05 79 R07
- dysplasia 9th unilateral	-	-	60 L05	-
- fused 8 & 9th proximal part, unilateral	-	-	-	79 R07
- 13th shortened unilateral	-	-	-	75 L05, 75 R02, 75 R04
- wavy 9th, 10th ,11th (and/or)	14 R07	43 L06	-	79 L01, 79 R03
- thickened 3,4,5,6, 7, 8, 9, 10, 11th (and/or)	-	43 L06	-	79 L01, 79 L03, 79 R03, 79 R07 89 R07
Pectoral girdle				
- scapula shortened and bent costad right unilateral	-	-	-	79 R03

Because individual foetuses sometimes had more than one minor skeletal defect the overall incidence of affected animals was low (foetal incidence: 18/142; litter incidence 8/21). Of the 34 minor skeletal defects listed in Table 3.10.1.5- 8, 62% can be ascribed to only two litters (77 and 79). Therefore, the level of concern regarding the increased incidences of minor defects is considered low (ECETOC Monograph 31, 2002)[22].

Moreover, the elevation in minor skeletal defects was correlated with the lower mean foetal body weight and the lower dam body weight gain during gestation and is thus considered mainly caused by general retardation secondary to the maternal toxicity induced at 700 mg/kg bw/day (see Figure 3.10.1.5- 1 and Figure 3.10.1.5- 2).

[22] ECETOC. 2002. Guidance on Evaluation of Reproductive Toxicity Data. Monograph No.31

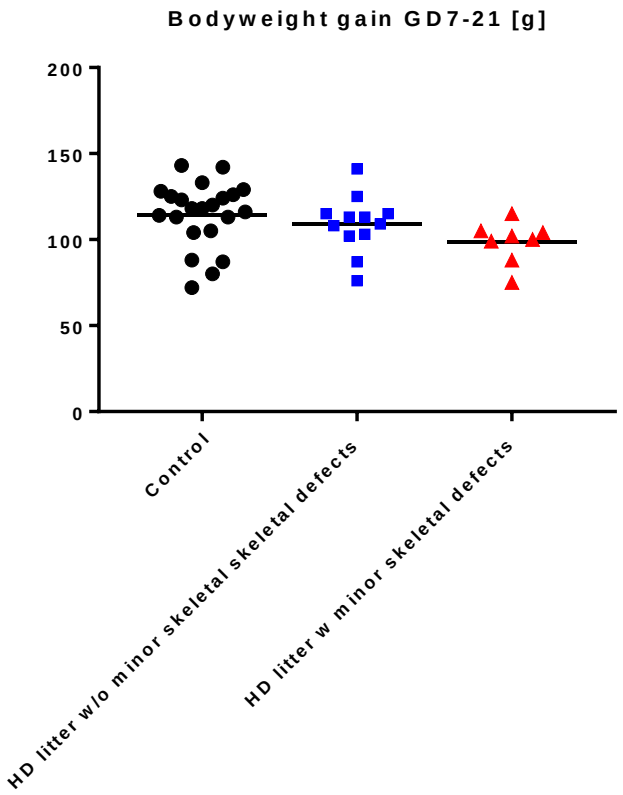


Figure 3.10.1.5- 1: Maternal body weight gain (GD7-21) of the control and the high dose group (HD) split into dams that had litters without minor skeletal defects and dams with litters with minor skeletal defects (individual values and mean)

If the dams of the high dose group are grouped into dams with litters showing minor skeletal defects and dams that had litters without minor skeletal defects it is clearly visible that there is a correlation between dam body weight gain and the occurrence of minor skeletal defects in the litter (Figure 3.10.1.5-1). The body weight gain of the high dose dams with litters without minor skeletal defects was 109 g compared to 115 g of the control dams (95% of control) whereas the body weight gain of the dams with litters with minor skeletal defects was only 99 g (86% of control).

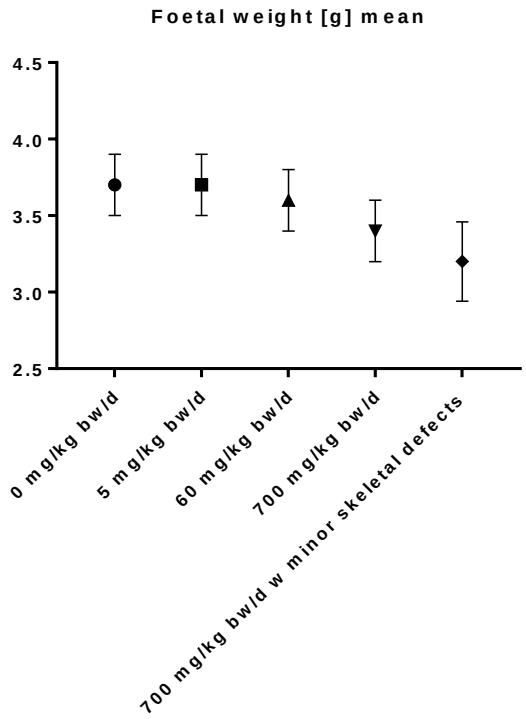


Figure 3.10.1.5- 2: Mean foetal weight of control and treatment groups plus foetal weight of foetuses with minor skeletal defects of the highest dose group (mean ± SD).

Secondary to maternal toxicity the mean foetal body weight at the highest dose level was statistically significantly decreased by 8% compared to control. If only taken into account the foetuses with minor skeletal defects the weight difference was even higher (-14%; see Figure 3.10.1.5- 2).

Both figures support the hypothesis that the above described skeletal findings are correlated with the lower mean foetal body weight and the lower dam body weight gain during gestation observed at the highest dose level.

Statistical evaluation did not reveal any other skeletal or visceral differences between the groups, and all other incidences were within or only slightly above the historical tolerance limit. Therefore, a compound-related effect is not evident. No abnormalities were detected by examination of cartilage.

Variations:

The incidence of an extra rib at the 7th cervical vertebra (3/148, 0/150, 1/153, 5/142) was above the upper limit of the historical confidence interval in the high dose group. However, the value of the control group was also above the HCD range, the difference between control and high dose group was low, and statistical evaluation did not reveal differences between the groups. Therefore, a compound-related effect is questionable. In all other cases the incidences were within or slightly above the historical tolerance limit. Additionally, statistical evaluation did not reveal differences.

Retardations:

Statistical evaluation revealed significant increases in the incidence of ossification of less than 2 caudal vertebral centres (10/148, 51/150*, 37/153*, 117/142*), non- or weakly ossified sternebrae (9/148, 31/150*, 32/153*, 102/142*) and non-ossified metacarpale 5 of the forepaw (7/148, 41/150*. 29/153*, 102/142*) in the foetuses from the high dose group. The incidence of non-ossified metatarsale 5 of the hindpaw (1/148, 1/150, 2/153, 10/142) and of non-ossified phalanx III of 1st to 5th toe of the hindpaw (2/148, 0/150, 0/153, 8/142) was also increased in this group. Since the incidences in the high dose group were distinctly higher than the historical tolerance limit, these changes are considered to be related to treatment and indicate a general developmental retardation of foetal development at this dose level.

Concerning the statistical significant changes in the low and intermediate dose group, the incidences were well within the historical tolerance interval, whereas the control incidences were below the historical control data range (see Table 3.10.1.5- 6). Additionally, there was no dose-dependency. Therefore, the increased number of retardations observed in the low and intermediate dose groups is considered not to be treatment-related and only statistically different to control because of unusual high ossification grade of control foetuses.

All other findings were within the historical tolerance interval, and statistical evaluation did not reveal differences between the groups

Findings in dead foetuses:

The dead foetus in the low dose group was stunted and showed weak or absent ossification of several bones. No major defects were observed.

III. Conclusion

Oral administration of fluopicolide to the pregnant rat at 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gain and slightly decreased food consumption especially at the beginning of treatment during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. Mean foetal body weights and crown-rump lengths were also slightly but statistically significantly decreased at this dose level. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification considered secondary to the above described maternal toxicity were observed more frequently in the foetuses at this high dose level. Fluopicolide was not teratogenic in this prenatal developmental toxicity study in rats and did not cause any maternal toxicity or embryotoxicity at 60 mg/kg bw/day or below.

With regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal and developmental toxicity.

3.10.1.6 Anonymous; 2000; M-211192-01-1

Study reference:

Anonymous; 2000; Rabbit oral developmental toxicity (teratogenicity range finding study) – AE C638206 - Code: AE C638206 00 1C99 0005; M-211192-01-1

Deviations:	As a dose-range finding study the study was not intended to comply with OECD guidelines.

Executive Summary:

The present study was conducted in order to select suitable dose levels of fluopicolide for a subsequent developmental toxicity study in Himalayan rabbits.

Groups of 4 mated female Himalayan rabbits received technical fluopicolide suspended in 1% (w/v) aqueous methyl cellulose by oral gavage once daily at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw from Day 6-28 of pregnancy (Day 0: day of mating) and were sacrificed on Day 29 of pregnancy.

Animals were observed daily for mortality and clinical signs of toxicity. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Gravid uterus weight was recorded. Foetal body weights, crown-rump lengths and placental weights were determined.

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study. These animals showed non-specific symptoms including impairments of motility and consciousness, decreased defaecation and hay consumption, hyperactivity and discoloured urine.

At the dose of 50 mg/kg bw/day one animal showed decreased defaecation and discoloured tray. This animal aborted on Day 29. The other animals of the 50 mg/kg bw/day dose group did not show any clinical signs of toxicity. No clinical signs of toxicity were observed at 25 mg/kg bw/day.

Body weight loss and markedly reduced food consumption was observed in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals. At 50 mg/kg bw/day, body weight gain and food consumption was reduced throughout the treatment period compared to the 25 mg/kg bw/day dose group animals. Slightly reduced food consumption was also noted in the low dose (25 mg/kg bw/day) animals during the treatment period compared to pre-treatment values.

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

One animal at 25, 50 and 250 mg/kg bw/day were not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section in the remaining animals from these groups. Gravid uterus and foetal weights were normal and embryofoetal development was unaffected.

Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.

I. Materials and Methods

A. Materials

1. Test material:

Identification: AE C638206

Batch: PP/241024/2 & PP/241067/1

Purity: 97.6 - 97.8% w/w (T/041/99, dated 23 August, 1999, T/060/99, dated 01 December, 1999)

2. Vehicle and/or positive control:

1% (w/v) methyl cellulose in deionised water

3. Test animals

Species:	Rabbit
Strain:	Chbb:HM(SPF) Kleinrusse
Age:	approximately 5-10 months
Weight at start:	2314-2949 g
Source:	Chemisch pharmazeutische Fabrik Dr. Karl Thomae GmbH, D-88387 Biberach, Germany
Acclimation phase:	yes
Diet:	commercial diet for laboratory rabbits Ssniff K-H (V2333) ad libitum in food racks, additionaly 40-50 g hay daily
Water:	Ad libitum from automatic dispensers
Housing:	in fully air-conditioned rooms in V2A-steel-cage typ: HD3 Fa. Hulskamp;
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 23, 1999 to February 16, 2000

2. Animal assignment and treatment:

The test animals were assigned to the following groups (see Table 3.10.1.6- 1).

Table 3.10.1.6- 1: Study design

Test group	Dose level (mg/kg bw)	Number of females
1	25	4
2	50	4
3	100	4
4	250	4
5	500	4
6	1,000	4

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide orally by gavage once daily from Day 6-28 of pregnancy.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw/day as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 6-28 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals were mated with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of mating is defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

Due to the low animal number and the lack of an untreated control group no statistical analyses were performed.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 0, 3, 6, 8, 10, 13, 16, 19, 23, 26 and 29 of pregnancy, and food consumption between Days 0-3, 3-6, 6-8, 8-10, 10-13, 13-16, 16-19, 19-23, 23-26 and 26-29.

3. Caesarean section and fetal evaluation:

The animals are killed by intravenous injection of T61® (Hoechst) on Day 29 of pregnancy and the foetuses removed by Caesarean section. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was determined. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities. The fetuses were killed by CO_2 asphyxia and the crown-rump length was recorded.

II. Results and Discussion

A. Observations:

1. Maternal data:

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were either found dead, killed moribund or killed after abortion up to Day 23 of the study.

In detail, at 1,000 mg/kg bw/day, animals were found dead on Day 13 (2 of 4 animals), 14 (1 of 4 animals) and 15 (1 of 4 animals). At 500 mg/kg bw/day, one animal was found dead on Day 15 and the remaining three animals were found dead on Day 16. Two animals of the 250 mg/kg bw/day dose group were found dead on Day 18 and 21, whereas two additional animals were killed moribund on Day 17 and 23. At 100 mg/kg bw/day, two animals were found dead (Day 16 and 20), one animal was killed moribund on Day 22 and another animal was killed after abortion on Day 22.

Animals found dead, killed moribund or killed after abortion showed non-specific symptoms including impairment of motility and consciousness, respiratory sounds, coat bristling, decreased defaecation and hay consumption, tray beeding discolored, hyperactivity, hypoactivity and discoloured urine.

At the dose of 50 mg/kg bw/day one animal showed decreased defaecation and discoloured tray. This animal aborted on Day 29. The other animals in the 50 mg/kg bw/day dose group did not show any clinical signs of toxicity. No clinical signs of toxicity were observed at 25 mg/kg bw/day.

Table 3.10.1.6- 2: Maternal mortality data until study termination

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Total animal number per group	4	4	4	4	4	4
Maternal mortality						
No. of animals **found dead**	0	0	2	2	4	4
Day(s) of death/sacrifice	-	-	16, 20	18, 21	15-16	13-15
No. of animals **killed moribund**	0	0	1	2	0	0
Day(s) of death/sacrifice	-	-	22	17, 23	-	-
No. of animals **killed after abortion**	0	1	1	0	0	0
Day(s) of death/sacrifice	-	29	22	-	-	-
Total number animals dead/sacrificed before study termination on day 29	0	0	4	4	4	4

2. Body weight and food consumption:

Mean body weights were impaired in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals (Day 22, Day 23, Day 16 and Day 15, respectively).
At 50 mg/kg bw/day, body weight gain was reduced (-57%) throughout the treatment period especially in the one animal that aborted on day 29 compared to the mean body weight gain of the 25 mg/kg bw/day dose group animals.

Table 3.10.1.6- 3: Mean maternal body weight and body weight gain[#]

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Body weight [g]						
Day 0	2750.3	2471.3	2676.0	2599.0	2763.3	2613.5
Day 3	2807.7	2524.3	2746.3	2554.3	2813.8	2681.8
Day 6	2781.3	2517.3	2725.5	2618.7	2794.0	2695.3
Day 8	2778.0	2543.3	2725.0	2640.0	2724.0	2640.3
Day 10	2788.3	2539.3	2709.8	2630.7	2723.8	2604.3
Day 13	2790.3	2567.7	2629.0	2574.0	2669.5	2496.5
Day 16	2837.0	2603.7	2522.8	2507.0	2578.0	-
Day 19	2831.0	2612.7	2381.3	2351.0	-	-
Day 23	2868.3	2599.3	-	-	-	-
Day 26	2934.0	2641.7	-	-	-	-
Day 29	3002.0	2612.3	-	-	-	-
Cumulative body weight gain [g]						
Day 0-6	31.0	46.0	49.5	19.7	30.7	81.8
Day 6-29	220.7	95.0	-	-	-	-
Day 0-29	251.7	141.0	-	-	-	-

[#] Non-pregant dams were excluded from the means

\- No data available due to mortality rate of 100 % of pregnant dams in the respective groups

Reduced mean food consumption was observed in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals (Day 22, Day 23, Day 16 and Day 15, respectively).

At 50 mg/kg bw/day, mean total food consumption was decreased at the end of the study compared to the food consumption of the 25 mg/kg bw/day dose group animals. Slightly reduced food consumption was also noted in the low dose (25 mg/kg bw/day) animals during the treatment period compared to pre-treatment values.

Table 3.10.1.6- 4: Mean food consumption during gestation[#]

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Mean Food Consumption [g/animal/day]						
Day 0-3	111.8	81.3	98.8	38.4	81.3	74.8
Day 3-6	107.3	89.8	100.9	78.5	62.7	86.4
Day 6-8	97.5	93.8	80.0	90.3	34.4	32.1
Day 8-10	85.7	88.5	72.6	71.8	25.8	17.3
Day 10-13	93.1	89.3	33.6	25.9	7.5	2.2
Day 13-16	70.2	72.1	1.8	2.6	1.3	-
Day 16-19	96.7	68.3	1.9	3.0	-	-

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Day 19-23	84.9	55.9	-	-	-	-
Day 23-26	81.4	63.0	-	-	-	-
Day 26-29	84.0	23.5	-	-	-	-
Total Day 0-29	2569.0	2050.3	-	-	-	-

[#] Non-pregant dams were excluded from the means

- No data available due to mortality rate of 100 % of pregnant dams in the respective groups

3. Necropsy findings:

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

4. Caesarean section data:

One animal at 25 mg/kg bw/day, 50 mg/kg bw/day and 250 mg/kg bw/d were not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section in the remaining animals from these groups.

Table 3.10.1.6- 5: Results of gestation and Caesarean section

Parameter	**Dose level [mg/kg bw/day]**	
	25	**50**
No. pregnant / no. mated	3 / 4	3 / 4
No of abortions	0	1
No. dams with live foetuses	3	2
Mean no. corpora lutea/dam	8.7	8.5
Mean no. implantation sites/dam	8.3	8.0
Pre-implantation loss (% of corpora lutea)	4.2	6.3
Post-implantation loss (% implants)	0.0	5.6
Mean no. of resorptions/dam	0.0	0.0
Early resorptions/dam	0.0	0.0
Dead foetuses/dam	0.0	0.0

5. Foetal data

Foetal parameters were not affected by treatment with fluopicolide neither at 25 mg/kg bw/day nor at 50 mg/kg bw/day.

Table 3.10.1.6- 6: Foetal data

Parameter	Dose level [mg/kg bw/day]	
	25	**50**
Total no. of live foetuses	25	15
Mean no. live foetuses/dam	8.3	7.5
Sex ratio (% males)	Not determined	
Mean foetal weight (g)	37.8	35.3
Crown-rump length (mm)	89.6	89.6
Mean placental weight (g)	4.6	4.4

III. Conclusions

Based on the results of this study, a dose level around 50 mg/kg bw/day was considered to be an appropriate high dose for the main study.

3.10.1.7 Anonymous; 2004; M-202513-02-1

Study reference:

Anonymous; 2004; AE C638206 Code: AE C638206 00 1C99 0005 Rabbit Oral Developmental Toxicity (Teratogenicity) Study; M-202513-02-1

Deviations: The following deviations from the OECD-Guideline 414 (2018) occurred:

- according to the guideline, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death (exceeding approx. 10%) or severe suffering. In the present study, the high dose resulted in a mortality rate > 10%. Therefore, the number of females with implantation sites that were available at necropsy (fewer than 16 animals) limits the validity of the assessment of the high dose results. Although this outcome might suggest that a new study is required to allow examination of foetuses from dams experiencing tolerable maternal toxicity, the small dose space between 20 mg/kg bw/day (NOEL) and 60 mg/kg bw/day makes the selection of a dose that might be tolerated but induce some toxicity difficult.

Therefore, the study is considered to be sufficient to assess the developmental toxicity of fluopicolide.

Executive Summary:

The present study was conducted in order to determine the effects of fluopicolide on maternal health and embryonic and foetal development. Groups of 23 mated female Himalayan rabbits received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 20 or 60 mg/kg bw from Day 6 to day 28 of gestation (Day 0: day of mating) and were sacrificed on Day 29 of gestation. The dosing volume was 5 mL/kg bw.

Behaviour and state of health were observed daily in all groups. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. Gravid uterus weight was recorded. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths, sex ratios of the foetuses and placental weights were determined. The foetuses were examined for external, visceral and skeletal anomalies.

Three animals of the 60 mg/kg bw/day dose group were found dead and 15 animals of this group were killed after premature delivery during Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces, and discoloured urine. One animal of the 20 mg/kg bw/day dose group was killed after premature delivery on Day 28 of gestation. This animal also showed decreased defecation and reduced hay consumption. Since up to 20% abortions are covered by historical control data this isolated single premature delivery is considered incidental and not treatment-related.

Body weight gains and food consumption were markedly decreased in animals of the 60 mg/kg bw/day dose group throughout the treatment period (-86%). In addition, gravid uterus weights were slightly lower at the same dose level.

At necropsy, tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discoloration of the liver were observed in single animals of the high dose group. No compound-related effects were observed in the low and intermediate dose group.

Dead foetuses were present in most premature deliveries. Mean foetal body weights and crown-rump lengths were decreased in foetuses at 60 mg/kg bw/day. Litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test substance. External, skeletal and visceral examination of the foetuses did not reveal any compound-related effects.

In conclusion, oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused increased incidences of premature deliveries, reduced foetal crown-rump lengths and foetal weights. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the highest tested dose.

At doses up to and including 20 mg/kg bw/day, fluopicolide did not cause any maternal toxicity or embryotoxicity. There was no evidence of treatment-related teratogenic effects at any dose level.

Therefore, the No Observed Adverse Effect Level (NOAEL) of the present study is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206
Batch:	PP/241024/2 & PP241067/1
Purity:	97.8% w/w (PP/241024/2 & PP241067/1)

2. Vehicle and/or positive control:

1 % w/v methyl cellulose in deionised water

3. Test animals

Species:	Rabbit
Strain:	Chbb:HM(SPF) Himalayan rabbit
Age:	approximately 5-10 months
Weight at start:	2145-3095 g
Source:	Chemisch pharmazeutische Fabrik, Dr. Karl Thomae GmbH, D-88387 Biberach Germany
Acclimation phase:	yes
Diet:	commercial diet for laboratory rabbits Ssniff K-H (V2333)1 ad libitum in food racks, additionally 40-50 g hay daily
Water:	tape water, ad libitum from automatic dispensers
Housing:	in fully air-conditioned rooms in V2A-steel-cage type: HD3 Fa. Hulskamp
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 28 to November 22, 2000

2. Animal assignment and treatment:

The test animals were assigned randomly (computer-generated algorithm) to the following groups (see Table 3.10.1.7- 1).

Table 3.10.1.7- 1: Study design

Test group	Dose level (ppm)	Number of females
1	0	23
2	5	23
3	20	23
4	60	23

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide orally by gavage once daily from Day 6-28 of gestation.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 0, 5, 20 or 60 mg/kg bw/day as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 6-28 of gestation. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test suspension was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals were mated with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of mating is defined as Day 0 of gestation. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

The statistical evaluation is based on the assumption of a monotone dose-response relationship. Statistical comparisons of the low dose groups with the simultaneous control group were only carried out if significant effects were detectable in the high dose group. In the univariate analysis, two-sided questions (body weight of dams, relative food consumption, crown-rump length, fetal weight and placental weight) were generally tested as follows: a two-sided comparison with the high dose group was followed by a one-sided test for the low-dose group.

In case of the caesarean section data of the fetuses (crown-rump length, fetal weight and placental weight), multivariate statistics were first of all calculated and used in selecting relevant dose groups. For the individual parameters, sequential comparisons with the high dose group and sequential tests at the 5% level for the low dose were then conducted.

The t-tests and the test statistics of Wilks are based on common variance estimations for all study groups. For the Wilcoxon test the exact distribution of the meaned ranks was calculated.

In the case of the daily food consumption of the dams, the mean consumption per 100 g body weight was always calculated between two successive measurement times and evaluated by the rank sum test after Wilcoxon. In examining the body weights of the dams, the change in weight was determined in comparison to the initial weight. The univariate evaluation was carried out using t-tests.

The caesarean section data of the fetuses were used to calculate litter mean values. Multivariate evaluation was carried out using the test statistics of Wilks. In the univariate analysis, t-tests were used.

The number of corpora lutea, implantation sites and live fetuses, and dead embryonic primordia undergoing resorption in the animals were likewise analysed using one sided Wilcoxon tests.

The findings obtained at autopsy and at organ cross-section and skeletal examination of the fetuses were evaluated separately for the fetuses and for the litters by Jackknife t-test at a significance levels of 5%. It was examined whether the relative frequencies of findings in the dose groups deviated from those findings in the control group.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 0, 3, 6, 8, 10, 13, 16, 19, 23, 26 and 29 of gestation, and food consumption was determined between Days 0-3, 3-6, 6-8, 8-10, 10-13, 13-16, 16-19, 19-23, 23-26 and 26-29.

3. Caesarean section:

The animals were sacrificed on Day 29 of gestation by intravenous injection of T61 HOECHST® and the fetuses removed by Caesarean section. Gravid uterus weight was determined. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. The live and dead fetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically.

4. Foetal evaluation:

The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities. Afterwards the fetuses were killed by CO_2-asphyxia and the crown-rump length recorded. All foetuses were fixed in alcohol, necropsied, sexed and checked for anomalies of the internal organs. Eyes, brain, heart and kidneys were cross-sectioned and examined for anomalies. All carcasses were placed in a solution of potassium hydroxide for clearing and stained with alizarin red S and Alcian blue. The skeletons (bone and cartilage) were examined and checked for stage of development and abnormalities with the aid of a magnifier. The foetuses found dead at caesarean section were examined for external anomalies.

Visceral and skeletal changes were subdivided into four categories (major defects, minor defects, variations and retardations) based on the severity and/or the spontaneous incidence of the finding.

II. Results and Discussion

A. Observations:

1. Maternal data:

The study authors reported that three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation; however, further examination of the raw data has revealed that 12 dams aborted whole litters (with no live pups), whilst 2 dams delivered prematurely with partial live litters (dam 178 delivered 1 dead and 4 live pups and dam 180 delivered 7 dead and 3 live pups). In addition a further dam prematurely delivered 7/7 live pups. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces and discolored urine. One animal of this dose group showed increased salivation. One animal from the intermediate dose group (20 mg/kg bw/day) was killed after aborting on Day 28 of gestation. This animal showed decreased defecation and reduced hay consumption. The dossier submitter considers this to be a spuorious finding and not related to treatment with fluopicolide, owing to its isolated occurrence in this dose group, supported by the the dose-range finding study, in which no abortions were observed at a similar dose (25 mg/kg bw/day) and only one dam aborted at a much higher dose (50 mg/kg bw/day). Furthermore, according to published historical data, up to 20% abortions have been reported for this strain of rabbit (Viertel & Trieb 2002[16]). Therefore, this isolated single abortion at this dose is considered incidental and not treatment-related.

Table 3.10.1.7- 2: Maternal mortality data until study termination

	Dose level [mg/kg bw/day]			
	0	**5**	**20**	**60**
Total animal number per group	23	23	23	23
Maternal mortality				
No. of animals **found dead**	0	0	0	3
Day(s) of death/sacrifice	-	-	-	24-25, 29
No. of animals **killed moribund**	0	0	0	0
Day(s) of death/sacrifice	-	-	-	-
No. of animals **killed after premature delivery**	0	0	1	15
Day(s) of death/sacrifice	-	-	28	22-28
Total number animals dead/sacrificed before study termination	0	0	1	18

2. Body weight and food consumption:

Body weight gains were markedly decreased in the animals from the high dose group throughout the treatment period (Day 6-29: -86%). Body weights were statistically significant lower than control on Days 26 and 29 of gestation.

In addition, gravid uterus weights were slightly lower at 60 mg/kg bw/day compared to control; nevertheless, body weights were still markedly reduced compared with controls even when the gravid uterine was taken into account.

An overview is given in Table 3.10.1.7- 3.

Table 3.10.1.7- 3: Mean maternal body weight and body weight gain

	Dose level [mg/kg bw/day]					
	0	**5**		**20**		**60**	
Body weight [g] *(% difference to control)*						
Day 0	2491.9	2424.5	*(-3)*	2503.6	*(±0)*	2496.6	*(±0)*
Day 3	2556.5	2528.0	*(-1)*	2590.8	*(+1)*	2598.0	*(+2)*
Day 6	2580.9	2531.7	*(-2)*	2599.6	*(+1)*	2614.2	*(+1)*
Day 8	2571.8	2512.9	*(-2)*	2587.6	*(+1)*	2607.8	*(+1)*
Day 10	2570.1	2519.4	*(-2)*	2603.0	*(+1)*	2595.6	*(+1)*
Day 13	2596.9	2537.5	*(-2)*	2616.6	*(+1)*	2614.2	*(+1)*
Day 16	2653.6	2601.8	*(-2)*	2676.6	*(+1)*	2640.8	*(±0)*
Day 19	2677.8	2624.0	*(-1)*	2683.8	*(+1)*	2660.2	*(±0)*
Day 23	2729.0	2656.0	*(-3)*	2728.3	*(±0)*	2693.8	*(-1)*
Day 26	2788.9	2725.5	*(-2)*	2798.4	*(±0)*	2668.6*	*(-4)*
Day 29	2852.6	2785.2	*(-2)*	2859.8	*(±0)*	2652.4*	*(-7)*
Day 29 [a]	2476.9	2424.4	*(-2)*	2480.0	*(±0)*	2348.5	*(-5)*
Cumulative body weight gain [g] *(% difference to control)* [#]						
Day 0-6	89.0	107.2	*(+20)*	96.0	*(+8)*	117.6	*(+32)*
Day 6-29 [b]	271.7	253.5	*(-7)*	260.2	*(-4)*	38.2	*(-86)*
Day 0-29	360.7	360.7	*(±0)*	356.2	*(-1)*	155.8	*(-57)*
Gravid uterus weight [g] *(% difference to control)*						
Day 29	375.7	360.8	*(-4)*	379.8	*(+1)*	303.9	*(-19)*

* Statistically different from control, $p \leq 0.05$
[#] No statistical analyses were performed.
[a] Corrected for gravid uterus weight
[b] Treatment period only
Animals not surviving to day 29 after mating, with total litter loss or non pregnant animals are excluded from the means

Likewise, food consumption was markedly decreased in the animals from the high dose group throughout the treatment period (especially in second half of gestation).

Table 3.10.1.7- 4: Mean food consumption during gestation

	Dose level [mg/kg bw/day]					
	0	**5**		**20**		**60**	
Mean Food Consumption [g/100 g body weight] *(% difference to control)*						
Day 0-3	3.5	3.7	*(+6)*	3.4	*(-3)*	4.3	*(+23)*
Day 3-6	4.1	4.1	*(±0)*	4.1	*(±0)*	4.7	*(+15)*
Day 6-8	3.5	3.5	*(±0)*	3.4	*(-3)*	3.5	*(±0)*
Day 8-10	3.6	3.4	*(-6)*	3.4	*(-6)*	3.2	*(-11)*
Day 10-13	3.6	3.4	*(-6)*	3.4	*(-6)*	3.2	*(-11)*
Day 13-16	3.4	3.3	*(-3)*	3.1	*(-9)*	2.8	*(-18)*
Day 16-19	3.7	3.6	*(-3)*	3.4	*(-8)*	2.7	*(-27)*
Day 19-23	3.7	3.6	*(-3)*	3.5	*(-5)*	3.0	*(-19)*
Day 23-26	3.5	3.7	*(+6)*	3.5	*(±0)*	2.0*	*(-43)*
Day 26-29	3.5	3.6	*(+3)*	3.4	*(-3)*	1.6*	*(-54)*

* Statistically different from control, $p \leq 0.05$
Animals not surviving to day 29 after mating or resorptions or non pregnant animals are excluded from the means

Body weight, body weight gains and food consumption remained generally unaffected by the administration of fluopicolide at 20 mg/kg bw/day and below. However, the one dam at the mid dose level that had a premature delivery on Day 28 (No. 142) also showed markedly reduced feed consumption during the three days prior death (19% of control; Figure 3.10.1.7- 1).

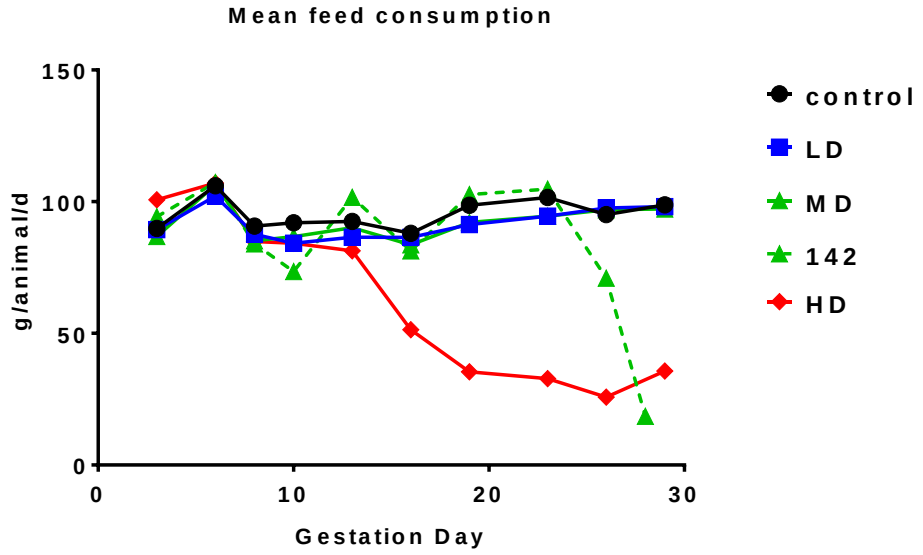


Figure 3.10.1.7- 1: Mean food consumption during treatment period in g/animal/day. The food consumption of the one dam at the mid dose level that had a premature delivery on Day 28 (No. 142) is shown separately as green dashed line. LD = low dose (5 mg/kg bw/day) MD = mid dose (20 mg/kg bw/day) HD = high dose (60 mg/kg bw/day)

3. Necropsy findings:

Gross examination of dams at necropsy showed tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discoloration of the liver in single animals of the high dose group (see Table 3.10.1.7- 5).

No treatment-related effects were observed in the animals from the low and intermediate dose group.

Table 3.10.1.7- 5: Gross findings at necropsy (Number of animals affected)

Parameter	Dose level [mg/kg bw/day]			
	0	5	20	60
Number of animals examined	23	23	23	23
Stomach				
Tautly filled (full of mash)	0	0	0	6
Urinary bladder				
Filled with red fluid	0	0	0	2
Uterus				
Filled with red fluid	0	0	0	1
Liver				
Lobus dexter access. yellow discolored	0	0	0	1

4. Caesarean section data:

With the exception of three females at 5 mg/kg bw/day and one female at 20 mg/kg bw/day, all animals became pregnant. In addition, one female of the control group had total pre-implantation loss.

In most cases in which premature delivery occurred, dead foetuses were observed. At study termination, one dead foetus occurred in the control, low and high dose group each, and three dead foetuses were observed in the intermediate dose group. These incidences of dead foetuses are not unusual in the rabbit strain used and therefore, a compound-related effect is not evident.

An overview including all other Caesarean section parameters is given in the following tables.

Table 3.10.1.7- 6: Results of gestation and Caesarean section

Parameter	Dose level [mg/kg bw/day]				HCD[#]
	0	5	20	60	
No. pregnant / no. mated	23 / 23	20 / 23	22 / 23	23 / 23	
No. of intercurrent death	0	0	0	3	
No. dams with premature delivery	0 (0.0%)	0 (0.0%)	1 (4.5%)	15 (65.2%)	0.0%-20.0%
No. dams with live foetuses	22[a]	20	21	5	
Mean no. corpora lutea/dam	8.3	8.3	8.4	8.2	6.4-10.0
Mean no. implantation sites/dam	7.5	7.6	7.8	7.0	5.1-8.5
Pre-implantation loss (% of corpora lutea)	10.8	9.2	7.7	16.1	
Post-implantation loss (% implants)	4.1	12.2	10.9	10.3	
Total intrauterine deaths/dam	0.32	0.95	0.76	0.60	0.0-1.25
Early resorptions/dam	0.27	0.90	0.62	0.40	
Dead foetuses/dam	0.05	0.05	0.14	0.20	

[a] One pregnant female had total pre-implantation loss

[#] Ranges of studies performed with animals of the same strain and breeder between 1968 and 1999 including 1144 litters (Viertel B, Trieb G. The Himalayan rabbit (Oryctolagus cuniculus L.): spontaneous incidences of endpoints from prenatal developmental toxicity studies. Lab Anim. 2003 Jan;37(1):19-36)

5. Foetal Data:

Mean foetal body weights and crown-rump lengths were statistically significantly decreased in foetuses at 60 mg/kg bw/day. Mean number of live foetuses (per dam) remained unaffected by the administration of the test substance. Likewise, sex ratio of the foetuses was not altered by the administration of the test substance.

Table 3.10.1.7- 7: Foetal data

Parameter	Dose level [mg/kg bw/day]			
	0	**5**	**20**	**60**
Total no. of live foetuses	157	132	147	32
Mean no. live foetuses/dam	7.1	6.6	7.0	6.4
Sex ratio (% males)	53.5	58.3	51.7	43.8
Mean foetal weight (g)	37.8	38.8	39.4	32.4*
Crown-rump length (mm)	93.3	93.9	95.1	88.1*
Mean placental weight (g)	4.91	5.13	5.00	4.53

* Statistically different from control, $p \leq 0.05$

The increased incidence of abortions/premature deliveries with mainly dead foetuses and the reduced foetal weights and crown-rump lengths in the highest dose group in the present study are considered as secondary consequences of severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to markedly reduced feed consumption.
As shown in Table 3.10.1.7- 8, mean food consumption was drastically reduced to 9% of control in the high dose dams that died or had premature deliveries from gestation Day 19 until death or sacrifice whereas the high dose dams that survived until termination showed only a food consumption reduction to 60% of control which is in good agreement with published results in rabbits (Matsuzawa *et al*. 1981[23]; Petrere *et al*. 1993[24]; Cappon *et al*. 2005[25]; Menchetti *et al*. 2015[26]).

Table 3.10.1.7- 8: Mean maternal food consumption in g/animal/day during gestation *(% of control)*

Parameter	Dose level [mg/kg bw/day]				
	0	**5**	**20**	**60**	
				Died or aborted during gestation	**Survived until termination**
Number of dams	21	20	21	18	5
Day 6-19	92.4	87.2 *(94%)*	87.5 *(95%)*	64.0 *(69%)*	79.9 *(86%)*
Day 19- sacrifice[a]	98.5	96.7 *(98%)*	70.0 *(71%)*	9.1 *(9%)*	58.9 *(60%)*

[a] death in case of 3 animals in the high dose group

[23] Matsuzawa T, Nakata M, Goto I, Tsushima M. Dietary deprivation induces fetal loss and abortion in rabbits. Toxicology. 1981;22(3):255-9.

[24] Petrere JA, Rohn WR, Grantham LE 2nd, Anderson JA. Food restriction during organogenesis in rabbits: effects on reproduction and the offspring. Fundam Appl Toxicol. 1993 Nov;21(4):517-22.

[25] Cappon GD, Fleeman TL, Chapin RE, Hurtt ME. Effects of feed restriction during organogenesis on embryo-fetal development in rabbit. Birth Defects Res B Dev Reprod Toxicol. 2005 Oct;74(5):424-30.

[26] Menchetti L, Brecchia G, Canali C, Cardinali R, Polisca A, Zerani M, Boiti C. Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming. Res Vet Sci. 2015 Feb;98:7-12

External, skeletal and visceral examination:

External, skeletal and visceral examination of live foetuses did not reveal any treatment-related abnormalities. Minor defects and variations observed were within the historical range of the rabbit strain used, without any dose-dependency and/or statistical evaluation did not reveal differences between the groups.

Furthermore, dead foetuses, foetuses of dams which died or which had premature deliveries did not show any major defects.

III. Conclusions

In conclusion, oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused increased incidences of premature deliveries, reduced foetal crown-rump lengths and reduced foetal and placental weights. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the hightest tested dose. External, skeletal and visceral examination of live foetuses did not reveal any treatment-related abnormalities.

At doses up to and including 20 mg/kg bw/day, fluopicolide did not cause any maternal toxicity or embryotoxicity. There was no evidence of treatment-related teratogenic effects at any dose level.

Therefore, the No Observed Adverse Effect Level (NOAEL) of the present study is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

3.10.1.8 Anonymous; 2018; M-638869-01-1

Study reference:

Anonymous; 2018; Fluopicolide: Review of potential for classification for reproductive and developmental toxicity; M-638869-01-1

Executive Summary

Fluopicolide is due for review with respect to the potential for classification and labelling (Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures). This review has been undertaken to assess the potential for classification of fluopicolide as a reproductive toxicant.

On the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

I. Reproductive Toxicity

A two-generation study has been conducted in the rat (see Anonymous.; 2003; M-232532-01-1, Section 3.10.1.2) which is consistent with current OECD test guideline 416. The F0 generation, which comprised 28 males and 28 females in each group, received diet with inclusion levels of 0, 100, 500 or 2,000 ppm for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point, 24 male and 24 female offspring per group were selected to form the F1 generation. Both sexes received diets as did their parents for a minimum of 10 weeks from selection, throughout pairing, gestation, littering and lactation. Sexual maturation, fertility and reproductive capacity of the F1 generation were assessed and resulting F2 offspring were monitored for survival and development until weaning.

Parental toxicity was achieved at the highest dose tested but fertility and reproductive performance were unaffected in both generations. The only effect in pups was a lower body weight at the highest dose which attained statistical significance at Day 14, for male and female pups from both generations, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity and not a developmental effect. Furthermore, no adverse effect on lactation was indicated.

The study design included endocrine sensitive endpoints including oestrus cyclicity, parturition, sexual maturation and these were not affected by treatment with fluopicolide.
Macroscopic examination at necropsy of the unselected F1 offspring or the F2 offspring did not indicate any adverse effect of treatment. Differences in spleen and thymus weights of the offspring treated at 2,000 ppm were generally attributed to the lower body weights in this group, although body weight-relative spleen weights were low for female offspring at this dose for both generations.

Macroscopic examination at necropsy of the F0 and F1 adults revealed no treatment-related findings. At 2,000 ppm, kidney and liver weights were high for males and females in both generations, when compared with the controls. Body weight-relative liver weights were also slightly high for the F1 females treated at 500 ppm (considered likely to be an adaptive liver change). Treatment-related histopathological change was observed in the liver and kidneys of males and females at 2,000 ppm and in the liver of males at 500 ppm.

Absolute and body weight-relative spleen weights were slightly low for the F0 parental animals treated at 2,000 ppm and absolute spleen weights were also low for the F1 females in this group. Histopathological examination of the spleen was not conducted and the significance of the weight changes cannot be determined. Sperm analysis in males (control and high dose groups only) of both generations did not reveal any treatment-related findings.

The dietary concentration of 500 ppm should be considered as the NOAEL based on the likelihood of the increased liver weights in F1 females being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The NOEL for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

With reference to the ECHA Guidance on the application of the CLP criteria[1], there are no results from this study of reproduction and fertility in the rat that determine a need for classification, i.e. there are no adverse effects sexual function and fertility and no adverse effect on prenatal or postnatal development of the offspring, in the presence of parental toxicity. Lower body weight from postnatal Day 14, in the F1 offspring at 2,000 ppm, was not a developmental effect but a palatability effect and/or systemic toxicity resulting from direct consumption of the diet. Furthermore, no adverse effect on lactation was indicated.

II. Developmental Toxicity

1. Rat

A study was conducted in order to determine the effects of fluopicolide on the maternal state of health, embryonic and fetal development when administered orally during pregnancy (see Anonymous; 2004; M-202155-02-1, Section 3.10.1.5). For the definitive study, groups of 23 mated female Sprague Dawley rats received fluopicolide by gavage in 1% methylcellulose at dose levels of 5, 60 or 700 mg/kg bw/day on Days 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were terminated on Day 21 of pregnancy. The study design was based on international test guidelines (including OECD TG 414 adopted 1981). Although the study predates the current version of the guideline (adopted in 2018), there are no significant deviations other than the omission of the recently included thyroid hormone measurements, thyroid weight and histopathology and fetal anogenital distance; the treatment period is consistent with the current guideline requirement. However, with respect to dose selection, the current test guideline describes two- to four-fold intervals between doses as being frequently optimal for setting the descending dose levels and that the addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. At the time of the study conduct, the test guideline required only that the intermediate dose be located geometrically between the low and high dose levels and the doses were selected based on a factor of 12 and on the results of a preliminary study (see Anonymous; 2000; M-198488-01-1, Section 3.10.1.4).

The NOAEL for maternal toxicity and fetotoxicity was 60 mg/kg bw/day, based on decreased body weight gain in dams (8% of controls for Days 7-21) and a reduction in mean fetal body weight (8%) and crown–rump length (4%) in fetuses at 700 mg/kg bw/day. Litter size, number of live and dead fetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test compound.

Visceral and skeletal findings were subdivided into four categories (major defects, minor defects, variations and retardations) based on the severity of the finding and/or the spontaneous incidence of the finding. OECD TG 414 (2018) defines malformation/major abnormality as structural change considered detrimental to the animal (may also be lethal) and usually rare and, variation/minor abnormality as structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population. Fluopicolide was found not to be teratogenic in the rat; the incidence of major malformations was low and clearly incidental to the administration of fluopicolide.

The incidence of fetuses/litters with specific minor external/visceral defects or variations was low and not statistically significant (see Table 3.10.1.8- 1); there was no evidence of any treatment-related increase in incidence or dose-related trend. The findings relating to the presence of blood or haematoma are due to handling at necropsy. All findings were considered incidental to treatment.

Table 3.10.1.8- 1: Incidence of external/visceral variations and minor defects including those detected at body cross-section# (no. (%) of fetuses and litters)

Variation / Minor Defect	Dose Level (mg/kg bw/day)			
	0	**5**	**60**	**700**
Number of fetuses examined Number of litters examined	148/136# (284) 22	150/141# (291) 21	153/144# (297) 22	142/132# (274) 21
Thoracic cavity - blood	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.8)
Thoracic cavity – blood#	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Heart pericardium - blood	1 (0.7) 1 (4.5)	1 (0.7) 1 (4.8)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Abdominal cavity – blood#	2 (1.5) 2 (9.1)	5 (3.5) 5 (23.8)	4 (2.8) 3 (13.6)	3 (2.3) 2 (9.5)
Lung lobus inferior medialis – reduced in size	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.8)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Liver lobes -haematoma#	1 (0.7) 1 (4.5)	2 (1.4) 2 (9.5)	1 (0.7) 1 (4.5)	4 (3.0) 4 (19.0)
Kidney displaced mediad - right	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)
Kidney displaced caudad – right#	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.8) 1 (4.8)
Kidney pelvis distended - unilateral	5 (3.4) 3 (13.6)	1 (0.7) 1 (4.8)	2 (1.3) 2 (9.1)	5 (3.5) 3 (14.3)
Kidney pelvis distended – unilateral or bilateral#	1 (0.7) 1 (4.5)	1 (0.7) 1 (4.8)	3 (2.1) 3 (13.6)	5 (3.8) 4 (19.0)
Kidney pelvis distended – overall incidence	6 (2.1) 4 (18.2)	2 (0.7) 2 (9.5)	5 (1.7) 4 (18.2)	10 (3.6) 4 (19.0)
Ureter distended – unilateral or bilateral#	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	4 (3.0) 3 (14.3)
Ureter distended – overall incidence	1 (0.4) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	4 (1.5) 3 (14.3)

Overall incidence includes all fetuses examined irrespective of examination technique

Fetuses from the high dose group showed increased incidences of a small number of minor skeletal defects and evidence of retarded development. To better understand the nature of the observed minor defects, it is necessary to consider the individual fetal data. This enables collation of the findings for each fetus and aids visualisation of the anomaly/ies. The selected minor defects are common to several fetuses and several litters in the high dose group, as shown below:

0 mg/kg bw/day: **Litter 14 Fetus R07** RIB, 10th, Right, Wavy

5 mg/kg bw/day: **Litter 43 Fetus L06** RIB, 6th-8th, Right, Thickened, RIB, 10th-11th, Right, Wavy

60 mg/kg bw/day: **Litter 60 Fetus L05** (2.7 g body weight - litter mean 3.785g)

THORACIC VERT.ARCH, 9th, Right, Fused with attached rib, THORACIC VERT.ARCH, 9th, Bilateral, Dysplasia, THORACIC VERT.ARCH, 10th-13th, Bilateral, Aplasia, THORACIC VERT.CENTRA, 8th, Dysplasia,

THORACIC VERT.CENTRA, 8th, Dislocated, THORACIC VERT.CENTRA, 9th-13th, Aplasia,

LUMBAR, SACRAL, CAUDAL VERTEBRA, All vertebrae, Aplasia, Major defect

RIB, Bilateral, Anlage of only 9, RIB, 9th, Left, Dysplasia

PELVIC GIRDLE, Ilium & Ischium, Bilateral, Dysplasia Major defect

700 mg/kg bw/day:

Litter 71 Fetus R07 THORACIC VERT.CENTRA, 11th, Fragmented

Litter 74 Fetus R03 THORACIC VERT.CENTRA, 11th & 12th, Fragmented

Litter 75 Fetus L05 RIB, 13th, Left, Shortened

Litter 75 Fetus R02 RIB, 13th, Left, Shortened

Litter 75 Fetus R04 RIB, 13th, Left, Shortened

Litter 77 Fetus R01 THORACIC VERT.ARCH, 7th, Left, Aplasia, THORACIC VERT.CENTRA, 6th /7th, Fragmented,

THORACIC VERT.CENTRA, 6th and 7th, Left, Fused, RIB, 7th, Left, Aplasia

Litter 77 Fetus R05 THORACIC VERT.ARCH, 2nd, Right, Aplasia, THORACIC VERT.CENTRA, 2nd, Aplasia

STERNEBRA, 4th, Fragmented & Longitudinally displaced, RIB, 2nd, Right, Aplasia

Litter 79 Fetus R03 RIB, 4th, Bilateral, Thickened, RIB, 5th, Left, Thickened, RIB, 6th-7th, Bilateral, Thickened,

RIB, 8th-9th, Right, Thickened, RIB, 11th, Bilateral, Wavy

Litter 79 Fetus R07 THORACIC VERT.ARCH, 9th, Bilateral, Aplasia, THORACIC VERT.CENTRA, 9th, Aplasia

STERNEBRA, 4th, Longitudinally displaced, RIB, 5th-7th, Right, Thickened, RIB, 9th, Right, Aplasia,

RIB, 8th and 9th, Left, Fused, proximal part

Litter 79 Fetus L01 RIB, 3rd-8th, Bilateral, Thickened, RIB, 9th-11th, Bilateral, Wavy

Litter 79 Fetus L03 RIB, 4th-7th, Bilateral, Thickened

Litter 84 Fetus R02 THORACIC VERT.CENTRA, 11th, Fragmented

Litter 84 Fetus R04 THORACIC VERT.CENTRA, 13th, Fragmented

Litter 84 Fetus R06 THORACIC VERT.ARCH, 6th-7th, Right, Fused, THORACIC VERT.CENTRA, 6th, Dislocated

Litter 84 Fetus L05 THORACIC VERT.CENTRA, 11th, Dislocated

Litter 88 Fetus L03 THORACIC VERT.CENTRA, 12th, Fragmented

Litter 88 Fetus R06 STERNEBRA, 4th, Longitudinally displaced

Litter 89 Fetus R07 RIB, 9th-11th, Right, Thickened

The data show that 18 (12.7%) fetuses from 8 (38.1%) high dose litters showed minor defect/s of the thoracic region and an effect due to treatment is indicated. However, the nature of the minor defects is not indicative of permanent structural change detrimental to the animal i.e. malformation (according to OECD TG 414) and therefore, are considered not to have adverse consequences for the fetuses in postnatal life. The observations represent a perturbation of ossification, transient in nature, being resolved as ossification progresses.

Fluopicolide tested at 700 mg/kg bw/day, induced maternal toxicity in terms of reduced body weight gain particularly following the onset of dosing (↓ 24% of controls Days 7-10). It is considered likely that the early development of the rat fetuses was impeded by the magnitude of the maternal effect between gestation days 7-10. However, as the minor skeletal anomalies were not indicative of permanent structural change or abnormality, it is considered that they should be considered non-adverse such that classification of fluopicolide as a developmental toxicant is not warranted. This conclusion is supported by reference to the CLP guidance[1] which states that 'classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in fetal body weight or retardation of ossification when seen in association with maternal toxicity'.

It is also important to note that there is no reason to dispute the minor nature of the findings on the basis of the terminology used. Where the aplasia or dysplasia is considered to be a malformation, the term major defect is clearly ascribed (e.g. fetus L05 from litter 60). The performing laboratory has a clear structure for categorising fetal findings and the fetal data indicate that the criteria have been applied correctly.

Skeletal observations considered indicative of retarded development were also of increased incidence at the highest dose only. Their occurrence was consistent with the lower fetal body weight in this dose group. This is because the extent of fetal ossification depends to some extent on fetal size and it is a common observation that smaller fetuses (from larger litters) show an increased incidence of delayed ossification when compared with larger fetuses (from smaller litters) of the same dose group (OECD GD 43). Although statistically significant differences were attained at lower doses, the incidence of affected fetuses was not dose-related and no association with treatment was indicated.

The NOAEL for maternal toxicity and fetotoxicity was 60 mg/kg bw/day, based on slightly decreased body weight in dams and a reduction in mean fetal body weight and crown–rump length in fetuses at 700 mg/kg bw/day. Further evidence of fetotoxicity at this dose was increased incidences of minor defects of the thoracic vertebrae, sternebrae and ribs, as well as delayed ossification. Fluopicolide was not teratogenic in the prenatal developmental toxicity study in rats.

2. Rabbit

A study was conducted in order to determine the effects of fluopicolide on the maternal state of health, embryonic and fetal development when administered orally during pregnancy (see Anonymous; 2004; M-202513-02-1, Section 3.10.1.7). The study design was based on international test guidelines (including OECD TG 414 adopted 1981). Although the study predates the current version of the guideline (adopted in 2018), there are no significant deviations; the treatment period is consistent with the current guideline requirement. However, the highest dose tested did induce more than 10% maternal deaths and maternal toxicity leading to abortion such that only 5 litters were available at term for examination which is inadequate for meaningful assessment.

Groups of 23 mated female Himalayan rabbits received fluopicolide by gavage in 1% methylcellulose at dose levels of 5, 20 or 60 mg/kg bw/day on Days 6-28 of pregnancy (Day 0: day of mating) and were terminated on Day 29 of pregnancy.

There was no clear indication from the preliminary study (see Anonymous; 2000; M-211192-01-1, Section 3.10.1.6) that the highest dose of 60 mg/kg bw/day would not be tolerated. However, in the definitive study, three animals given 60 mg/kg bw/day were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces and discoloured urine. Body weight gains and food consumption were markedly decreased in the animals from the 60 mg/kg bw/day group.

With respect to the litters, dead fetuses were present in most premature deliveries. Mean fetal body weights, crown-rump lengths and placental weights were decreased in the 5 high dose group litters at term. Litter size, number of live and dead fetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were unaffected. Morphological examination of the fetuses did not reveal any compound-related effects but the number of litters in the high dose group precluded meaningful evaluation.

The intermediate dose of 20 mg/kg bw/day is a no observed effect level (NOEL) for both maternal and developmental toxicity indicating the steepness of the dose-response. Whilst a dose level higher than 20 mg/kg bw/day has not been fully evaluated for developmental effects, the steepness of the dose-response for maternal effects makes selection of a dose that might be tolerated and induce some toxicity difficult to predict. Given that the generally accepted analytical tolerance for achieved concentration of test substance in dosing formulations is ± 15%, the lower tolerance value for 60 mg/kg bw/day is 51 mg/kg bw/day and the upper tolerance value for 20 mg/kg bw/day is 23 mg/kg bw/day. Should a dose level higher than 20 mg/kg bw/day be required for evaluation, it would need to be in the range of 24-50 mg/kg bw/day (mid-range value 37 mg/kg bw/day). Whether or not the mid-range value would induce maternal toxicity is unknown. Given the lack of evidence of fluopicolide to induce adverse developmental effects in the absence of maternal toxicity, the justification for further animal experimentation is questionable.

There are no grounds for classification for developmental toxicity on the basis of this study which should be considered a valid assessment of the potential of fluopicolide to induce developmental toxicity in the rabbit.

III. Conclusion

On the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

3.10.2 Human data

No human data.

3.10.3 Other data (e.g. studies on mechanism of action)

No other data.

3.11 Specific target organ toxicity – single exposure

The acute studies that are relevant for the assessment of the specific target organ toxicity of fluopicolide after single exposure are reported in chapters 3.1 to 3.3. An acute neurotoxicity study is also available and is summarised below.

According to the CLP criteria a classification for STOT-SE needs to be considered if the substance causes non-lethal target organ toxicity after a single exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitisation, genotoxicity, carcinogenicity and reproductive toxicity should be taken into consideration).

Based on the results after acute exposure to fluopicolide in toxicological studies with single and repeated dosing, no significant toxic effects on specific target organs were observed at non-lethal dose levels. Thus, classification of fluopicolide for STOT-SE Category 1 or 2 is not warranted.

There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to fluopicolide. Classification of fluopicolide for STOT-SE Category 3 is therefore also not warranted.

No classification / labelling is warranted regarding Specific Target Organ Toxicity – Single Exposure (STOT-SE) for fluopicolide when comparing the study results with CLP criteria according to the Regulation (EC) No 1272/2008[1].

3.11.1 Animal data

3.11.1.1 Anonymous; 2002; M-208046-01-1

Study reference:

Anonymous; 2002; Neurotoxicity study by a single gavage administration to CD rats followed by a 14-day observation period AE C638206; M-208046-01-1

Deviations:	Deviations from the current OECD guideline (424, 1997): None.

Executive Summary:

Groups of ten males and ten females received fluopicolide via oral gavage administration, at dosages of 0 (control group 1), 10, 100 or 2,000 mg/kg bw. The control group received the vehicle, 1% methylcellulose, at the same volume-dosage as treated rats and served to generate contemporaneous control data.

Functional observational battery (FOB) observations were performed before commencement and on Day 1 (6 hours after dosing), 8, and 15. Body weights were recorded pre-treatment, immediately before dosing, on Days 8 and 15 and before necropsy. A body weight recording was also made each time an FOB was performed. Food consumption was recorded weekly. On completion of the study period the animals were sacrificed and examined macroscopically; the brain was measured for length and width and weighed. Selected tissues were retained for all animals and examined for five males and five females in the 0 and 2,000 mg/kg bw groups.

No animals died, no signs were seen at routine observations and bodyweights, food consumption, food conversion efficiency and brain weights and dimensions were unaffected by treatment.

Macropathological and histopathological examination of the tissues did not reveal any findings related to the administration of fluopicolide.

At the neurobehavioural screening, low body temperatures were recorded in animals receiving 2,000 mg/kg bw and a higher incidence of excessive grooming was seen in females receiving this dosage as compared with controls.

It is concluded that fluopicolide had a transient effect on body temperature in CD rats when given as a single oral gavage administration at a dosage of 2,000 mg/kg bw. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9% (w/w)
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	1% methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats
Age:	42-48 days (males), 44-50 days (females) at dosing
Weight at start:	Males: 135 to 152 g, females: 118 to 138 g.
Source:	Charles River (UK) Limited, Margate, Kent, England.
Acclimation period:	Yes
Diet:	Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England except overnight before dosing and continuing until the last FOB of the day had been performed.
Water:	Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), was freely available, via polycarbonate bottles fitted with sipper tubes.
Housing:	Modified RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England, which were made of a plastic body with a stainless steel mesh lid and floor
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 21 to August 2, 2001

2. Animal assignment and treatment

During the pre-treatment period animals were weighed and ranked in order of body weight. Animals were then distributed amongst the treatment groups by body weight blocking to ensure that all groups contained populations of animals with similar initial mean and range of body weights. During the pretreatment functional observational battery examinations it was noted that female No. 56 had severe ocular lesions. This animal was replaced with a spare female taken from the same batch of animals with a similar body weight.

As far as was practicable the distribution of animals in the room was designed to minimise the effect of any spatially variable component of the environment.

In addition the cage batteries were rotated around the arrival room twice weekly. This ensured that each battery occupied different positions in the room during the course of the study.

Animals were assigned to the groups as follows.

Table 3.11.1.1- 1: Study design

Group	Treatment	Dose (mg/kg bw)	Number of males	Number of females
1	Vehicle control	0	10	10
2	Fluopicolide	10	10	10
3	Fluopicolide	100	10	10
4	Fluopicolide	2,000	10	10

The required amount of fluopicolide was weighed out and transferred to a suitably sized mortar and ground using a pestle. A small amount of 1% methylcellulose was added and mixed with the fluopicolide using the pestle to form a smooth paste. Further small amounts of 1% methylcellulose were added to form a smooth, pourable suspension. The suspension was added to a measuring cylinder that had been pre-rinsed with 1% methylcellulose and also contained a small amount of the vehicle in the bottom. Small amounts of 1% methylcellulose were added to the mortar and pestle to remove all traces of fluopicolide with the rinsing being added to the cylinder. The required volume was then made up in the cylinder using 1% methylcellulose and the suspension was transferred to a beaker, rinsing out the cylinder with the suspension to remove any concentrate and finally scraping out the cylinder. The suspension was then mixed using a high-shear homogeniser to further break down any agglomerates of fluopicolide and to produce an homogenous suspension.

Each dose was prepared and divided into four aliquots, one for each day of dosing. Doses were stored in a refrigerator (approx. 4 °C) and issued to the animal unit as required.

Before treatment the suitability of the proposed mixing procedure and the homogeneity and stability of the test substance in the vehicle at concentrations of 1 and 200 mg/mL was assessed.

The mean concentration of fluopicolide in the formulations prepared for dosing ranged from 89.8% to 94.0% of nominal concentrations and was considered satisfactory.

Animals received the test substance or control formulations by single oral gavage administration at a volume-dosage of 10 mL/kg bw. Animals were dosed in four batches over four days, in the order in which the FOB observations were performed. The volume administered to each animal was calculated from the body weight measured immediately before the administration. Formulations were inverted approx. 20 times before dosing. They were also stirred with a magnetic stirrer before and throughout the dosing procedure.

All animals were starved overnight before dosing and remained without food until after the last FOB observation of the day was performed.

All animals received a single dose followed by fourteen days of observation.

C. Methods

1. Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

After each single dose, detailed observations were recorded before and after dosing; these observations were recorded at the following times in relation to dosing:

- Immediately before dosing.
- Immediately after dosing on return of the animal to its cage.
- 1-2 hours after dosing.
- Throughout the day observations were made as part of the functional observation battery screening examinations.
- As late as possible in the working day.

Cages and cage-trays were inspected daily for evidence of ill health, such as blood or loose faeces. During the acclimatisation period, observations of the animals and their cages were made at least once per day.

2. Body weights and food consumption

Each animal was weighed on the day of allocation, on the day of dosing and on Days 8 and 15 of the study. Body weights were also recorded before necropsy. Further weightings occurred as part of the neurobehavioural screening.

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded for the two weeks of the study. From these records the weekly consumption per animal was calculated. Food conversion efficiencies were calculated for each sex/group for the two weeks of treatment.

3. Neurobehavioural screening - functional observational battery

The functional observational battery (FOB) and motor activity examinations were performed on all animals before the commencement of treatment, on Day 1 at six hours after dosing and on Days 8 and 15 of the study. Any deviation from normal was recorded with respect to nature and, where appropriate, degree of severity.

The observer was unaware of the treatment of the individual animal because the animal numbering system was such that it was difficult to identify a treatment group by just the animal number. Prior to the observations the animal unit staff ensured that the labels on the cages gave the animal/cage number and contained no information on the group number, test material or dosages. This was recorded in the daybook as was the reversion to full cage labels after the observations were completed. In this study all animals were observed. In order to reduce bias during the observations, the animals were also examined in a random cage order.

Observations in the home cage:

The following parameters were assessed in the home cage, without disturbing the animal:

Convulsions, tremors, twitches
Palpebral closure
Posture
Spontaneous vocalisations

Observations in the hand:

During and after removal from the home cage, the following parameters were assessed:

Ease of removal from the cage
Exophthalmos
Fur condition
Piloerection
Reactivity to handling
Salivation/lacrimation
Vocalisation on handling

Observations in the arena:

The animal was placed in a standard arena (approx. 653 x 500 mm) with the floor marked into six sections of equal size for scoring activity counts (see below). The animal was observed for the following parameters during a 3-minute recording period:

Activity counts
Arousal
Tremor, Twitches and Convulsions
Defecation count
Gait
Grooming
Palpebral closure
Posture
Rearing count
Respiration
Urination

Manipulations:

After completion of arena observations, the following measurements, reflexes and responses were recorded:

Approach response
Body temperature
Body weight
Grip strength - fore and hindlimbs
Landing footsplay
Pupil reflex
Righting reflex
Startle response
Tail pinch response
Touch response

Motor activity:

Motor activity was measured by automated infra-red equipment, recording individual animal activity at regular intervals over a one-hour period. The infra-red beams were arranged at two levels to record rearing and cage floor activity.

During the testing of the females, one of the stations on the activity monitoring equipment had a beam malfunction and could not be used. This resulted in two females, Nos. 43 and 79, not being tested. These females were from different treatment groups and the failure to record motor activity data for these animals does not prevent meaningful inter-group comparisons for this parameter. The overall validity of the study was not, therefore, compromised.

4. Necropsy

Animals sacrificed at the end of the scheduled treatment period were killed by an overdose of barbiturate by intra-peritoneal injection and exposure of the heart to permit perfusion with glutaraldehyde (1.5%):paraformaldehyde (4%) fixative via the aorta.

Macroscopic pathology:

All animals were subjected to a detailed necropsy. The necropsy procedures included a review of the history of each animal and a detailed examination of the external features and orifices, the neck and associated tissues and the cranial, thoracic, abdominal and pelvic cavities and their viscera. The requisite organs were measured and weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Abnormalities and interactions were noted and the required tissue samples preserved in fixative.

Anatomical measurements:

The brain from each animal was transected from the spinal cord above the first cervical spinal nerve and the olfactory lobes removed. Measurements were taken of the length between the rostral part of the cerebral hemispheres to the most caudal part of the cerebellum and also the width between the widest parts of the cerebral hemispheres.

Brain weights:

The brain, taken from each animal, was dissected free of adjacent fat and other contiguous tissue and the weight recorded.

5. Pathology

Tissues preserved for histopathology:

Samples of the following tissues were preserved in glutaraldehyde (1.5%):paraformaldehyde (4%) fixative by *in situ* perfusion followed by immersion:

Brain
Dorsal root fibres - cervical and lumbar
Dorsal root ganglia - cervical and lumbar
Eyes
Optic nerves
Sciatic nerve (right)
Skeletal muscle - gastrocnemius, right
Spinal cord
Tibial nerves - at knee (right)
Tibial nerve - calf muscle branch (es), (right)
Ventral root fibres - cervical and lumbar

Tissues preserved but not examined:

Samples of the tissues listed below were not processed histologically, but are held in fixative against any future requirement for microscopic examination:

Sciatic nerve, left *(in situ*)*
Skeletal muscle - gastrocnemius, left
Tibial nerve - at knee, left *(in situ*)*
Tibial nerve - calf muscle branch(es), left *(in situ*)*
* The carcass was retained.

Histology:

Tissue samples were taken from the five male and five female rats with the lowest animal numbers from the control and high dosage groups (Groups 1 and 4) sacrificed on completion of the treatment period and were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Brain: Six sections including all major regions (three cross sections of the forebrain, one cross section of each of the mid-brain, cerebellum and pons and medulla oblongata).
Spinal cord: Transverse and longitudinal sections at cervical and lumbar swellings.
Dorsal root ganglia: One cervical and one lumbar.
Dorsal root fibres: One longitudinal section at one cervical level, and at one lumbar level.
Ventral root fibres: One longitudinal section at one cervical level, and at one lumbar level.
Eyes (retina): One longitudinal section of each.
Optic nerves: One longitudinal section of each.
Skeletal muscle (gastrocnemius): One transverse section.
For eyes and optic nerves, sections of both the left and right organs were examined.

In addition, tissue samples from all animals sacrificed on completion of the scheduled treatment period were processed: Using resin embedding procedures, sectioned at approx. 2 micron thickness and stained with toluidine blue:

Sciatic nerve: Longitudinal and transverse sections at the sciatic notch and the mid-thigh.
Tibial nerve: Longitudinal and transverse sections at the knee and of calf muscle branch(es).

Microscopy:

Microscopic examination was performed as follows:

The tissues specified above were examined for the five male and five female rats with the lowest animal numbers from Groups 1 and 4 sacrificed on completion of the scheduled treatment period.

Findings were either reported as "Present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe.

The initial examination was undertaken by the Study Pathologist. The results of this examination were then subjected to a routine peer review by a second pathologist. The diagnosis reported here represent consensus opinions of both pathologists.

6. Statistics and evaluation

For body weight change, if 75% of the data (across all groups) were the same value, c, say, then a frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the control both for i) values <c versus values >=c, and for ii) values <=c versus values >c, as applicable.

If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.

If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Steel's test was performed instead.

For brain weights changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

The FOB numerical data were subjected to statistical analysis: activity and rearing in the standard arena, body temperature, body weight, landing footsplay, grip strength and motor activity. The following statistical analyses were performed:

> If the data consisted predominantly of one particular value (relative frequency of the mode exceeds 75%), the proportion of animals with different values from the mode was analysed using Fisher's Exact test. Otherwise, Bartlett's test was performed to test for variance heterogeneity between groups. Where significant (1% level) heterogeneity was found, the data were transformed, first logarimically and if appropriate by square root, and re-tested for heterogeneity. If no statistically significant heterogeneity of variance was detected (with or without transformation), a one way analysis of variance was carried out. If the analysis of variance showed evidence (at the 5% level) of differences between the groups, Student's Mest was used to test for differences between treatment groups and the control group. If heterogeneity was significant and could not be stabilised by transformation, the Kruskal-Wallis test on ranks was performed on the untransformed data. If the Kruskal-Wallis test showed evidence (5% level) of differences between the groups, the Wilcoxon Rank-Sum test was used to test for differences between the treatment groups and the control group.

Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the controls ($p > 0.05$).

II. Results and Discussion

A. Results

1. Clinical signs and mortality

No animals died as a result of the single administration of fluopicolide. There were no treatment-related signs seen in the animals immediately after dosing and in the routine observations.

2. Body weights and food consumption

The body weight gains of the animals were very variable both within and among the groups. There was no dosage-relationship in this variability and the bodyweight gains of the animals are considered to be unaffected by treatment.

Food consumption in the treated animals was similar to the controls. Food conversion efficiency was unaffected by treatment.

3. Neurobehavioural screening - functional observational battery

Observations in the home cage:

Home cage observations were unaffected by treatment.

Observations in the hand:

There were no differences considered to be associated with treatment.

Observations in the arena:

There were no observations noted in the rats during the time spent in the arena that were considered to be related to the administration of fluopicolide.

Increased grooming in females at 2,000 mg/kg on Day 1 was isolated in occurrence and thus considered to be of doubtful significance. Furthermore, the frequency of grooming was similar in all groups at Days 8 and 15 of the study, demonstrating that no permanent change in behaviour had been effected.

Females receiving 2,000 mg/kg showed a high incidence of elevated gait on the day of dosing, compared with controls, but the variable incidence in all treatment groups, including controls, on subsequent study occasions indicates that this was unlikely to represent an effect of treatment.

Males receiving 2,000 mg/kg showed an increased incidence of head shaking on the day of dosing, compared with controls (four versus none), but this behaviour was also observed in three control females and was not considered to be treatment-related.

FOB parameters were unaffected at Days 8 and 15.

Table 3.11.1.1- 2: Selected results following arena observations on Day 1

Parameter	Grade	Dose level (mg/kg bw)							
		Males				Females			
		0	10	100	2,000	0	10	100	2,000
Number of animals	-	10	10	10	10	10	10	10	10
Grooming	0	8	8	8	4	4	2	2	0
	1	2	2	2	6	6	8	7	6
	2	0	0	0	0	0	0	1	4
Elevated gait	-	3	0	2	3	3	2	2	7
Head shaking	-	0	0	1	4	3	1	0	1

Manipulations:

Compared with controls, a statistically significant reduction in body temperature was recorded in both males and females that had received 2,000 mg/kg bw, at the expected time of peak effect on the day of dosing. The temperature of most males in this group was between 36 and 37 °C but for some females it fell below 35 °C. Body temperature showed full recovery, with all groups showing similar group mean values on Days 8 and 15 of the study.

On Day 8 of the study, females that had received 2,000 mg/kg bw showed a statistically significant reduction in forelimb grip strength, compared with controls. Grip strength values for males and females were, however, unaffected on the day of dosing, and in the absence of reduced hindlimb values for females in these groups, or similar changes in males at Days 8 and 15, this effect was not considered to be associated with treatment.

Table 3.11.1.1- 3: Selected results following manipulation observations on Days 1, 8 and 15 (mean)

Parameter	Day	Dose level (mg/kg bw)							
		Males				Females			
		0	10	100	2,000	0	10	100	2,000
Number of animals	-	10	10	10	10	10	10	10	10
Body temperature (°C)	1	37.3	37.2	37.0	36.5***	36.9	36.8	36.8	36.0*
	8	37.5	37.4	37.4	37.4	37.4	37.3	37.3	37.5
	15	37.3	37.3	37.3	37.3	37.3	37.4	37.5	37.3
Fore limb grip strength (g)	1	561	557	586	555	559	534	523	523
	8	570	582	580	573	612	567	577	510**
	15	594	598	636	588	616	619	554	550
Hind limb grip strength (g)	1	203	189	208	194	204	196	185	190
	8	227	192	207	202	216	223	222	196
	15	225	198	212	209	212	220	220	204

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

Motor activity:

Motor activity scores for males in all treated groups were statistically significantly lower than those of the controls on the day of dosing (Day 1), at the expected time of peak effect. Both high beam and low beam scores (rearing and cage floor activity, respectively) were similarly reduced. There was, however, not always a dosage-relationship and pre-treatment high and low beam scores for males in the treated groups were also lower than the controls (up to -25% and -18% for high and low beam scores, respectively) before commencement of the study. Taking both of these factors into account, the reduced activity scores shown by males in all treated groups on the day of dosing were not considered to be treatment-related.

Scores for females on the day of dosing (Day 1) showed considerable inter-group variation but there were no consistent difference considered to be associated with treatment. This further supports the absence of treatment-related findings in the male groups. Scores for both males and females on Days 8 and 15 of the study were considered unaffected.

Table 3.11.1.1- 4: Selected results following motor activity measurements on days 1, 8 and 15 ***(% difference to control)***

	Beam	**Dose level (mg/kg bw)**							
		0	**10**		**100**		**2,000**	
Males								
Number of animals	-	10	10		10		10	
Pre-treatment	High	80.0	71.2	*(-11)*	65.9	*(-18)*	59.9	*(-25)*
	Low	471.4	420.8	*(-11)*	388.0	*(-18)*	416.8	*(-12)*
Day 1	High	111.9	71.6*	*(-36)*	78.4	*(-30)*	68.8*	*(-38)*
	Low	637.4	415.2*	*(-35)*	403.4**	*(-37)*	368.5**	*(-42)*
Day 8	High	133.7	118.2	*(-12)*	115.6	*(-13)*	109.7	*(-18)*
	Low	697.5	696.1	*(±0)*	693.3	*(-1)*	631.3	*(-9)*
Day 15	High	134.0	131.5	*(-2)*	118.0	*(-12)*	119.9	*(-10)*
	Low	714.9	680.4	*(-5)*	633.4	*(-11)*	604.8	*(-15)*
Females								
Number of animals	-	10	10		10		10	
Pre-treatment	High	65.3	68.6	*(+5)*	100.7	*(+54)*	77.2	*(+18)*
	Low	422.9	416.9	*(-1)*	500.3	*(+18)*	441.6	*(+4)*
Day 1	High	108.3	111.8	*(+3)*	91.6	*(-15)*	142.3	*(+31)*
	Low	626.1	626.6	*(±0)*	466.1	*(-25)*	520.9	*(-17)*
Day 8	High	117.7	132.7	*(+13)*	146.8	*(+25)*	151.4	*(+29)*
	Low	650.1	772.6	*(+19)*	757.6	*(+17)*	716.4	*(+10)*
Day 15	High	152.4	197.9	*(+30)*	156.9	*(+3)*	173.6	*(+14)*
	Low	762.2	996.2	*(+31)*	894.4	*(+17)*	872.5	*(+15)*

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

4. Necropsy and pathology

Brain weights:

Brain weights were similar for control and treated animals.

Anatomical (cerebral hemisphere) measurements:

Measurements of the cerebral hemisphere did not reveal any differences in brain length or width between the control and treated animals.

Macropathology:

Macropathological examination did not reveal any findings related to the administration of fluopicolide.

Histopathology:

There were no findings which were considered to be related to treatment.

III. Conclusion

The single oral gavage administration of fluopicolide to CD rats at dosages of 10, 100 or 2,000 mg/kg bw had no effects on body weights, food consumption, food conversion efficiency, brain weights and brain measurements. Macropathological and histopathologcial examination of the tissues did not reveal any findings related to the administration of the test substance.

The only notable observation related to treatment was seen in males and females at 2,000 mg/kg bw at the expected time of peak effect and consisted of low body temperatures.

It is concluded that fluopicolide had a transient effect on body temperature in CD rats when given as a single oral gavage administration at a dosage of 2,000 mg/kg bw. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

3.11.2 Human data

No human data.

3.11.3 Other data

No other data.

3.12 Specific target organ toxicity – repeated exposure

3.12.1 Animal data

Numerous short term toxicity studies with fluopicolide have been conducted in mice, rats and dogs at lengths of 4, 13 and 52 weeks. With the exception of a 28-day study by dermal application, all studies were administered via the oral route by dietary administration. The studies are summarized in Table 3.12.1- 1.

Additional information is provided by the chronic / carcinogenicity studies in rats and mice (see Section 3.9.1), the findings in the parental animals and offspring in the 2-generation study and the developmental toxicity studies (see Section 3.10.1) and by the subchronic neurotoxicity study in rats (see Anonymous; 2002; M-208051-01-1, Section 3.12.1.9).

Table 3.12.1- 1: Summary of short-term toxicity studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Oral route				
Rat 28-day dietary study 0, 20, 200, 2,000 & 20,000 ppm	200 ppm [17.7 mg/kg/d (combined sexes)]	2,000 ppm [179 mg/kg/d (combined sexes)]	↓ bodyweight (F) ↑ water consumption (M) ↑ cholesterol in blood (M/F) ↑ incidence of centrilobular hepatocytic hypertrophy (M/F) ↑ severity of phloxine tartrazine-positive granulation (hyaline droplets) in kidneys (M)	Anonymous; 2000; M-199377-01-1
Mouse 28-day dietary study 0, 6, 64, 640 & 6,400 ppm	64 ppm [11.6 mg/kg bw/d (combined sexes)]	640 ppm [115 mg/kg bw/d (combined sexes)]	↑ ALT (M) ↑ rel. liver weight (F) ↑ incidence & severity of hypertrophy of centrilobular hepatocytes (M/F)	Anonymous; 2000; M-197343-01-1
Dog 28-day gavage study 0, 10, 100 and 1,000 mg/kg bw/d	1,000 mg/kg bw/d	-	No adverse effects observed	Anonymous; 2000; M-197350-01-1
Rat 90-day dietary study 0, 100, 1,400 or 20,000 ppm	100 ppm [7.9 mg/kg bw/d (combined sexes)]	1,400 ppm [114 mg/kg bw/d (combined sexes)]	↑ cholesterol in blood (M) ↑ epithelial cells in urinary sediment (M) ↑ urine volume & ↓ specific gravity (F) ↑ rel. liver weight (M) ↓ abs. and rel. spleen weight (F) ↑ rel. kidney weight (M) ↑ incidence hypertrophy of centrilobular hepatocytes (M) ↑ severity & incidence of trabecular hyperostosis of the bone joint (F) ↑ severity accumulation of hyaline droplets in the proximal kidney tubule and indications of degenerative and regenerative changes in kidney (M)	Anonymous; 2000; M-197622-01-1

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Mouse 90-day dietary study 0, 50, 200, 800 and 3,200 ppm	50 ppm [10.4 mg/kg bw/d (M)] [12.6 mg/kg bw/d (F)]	200 ppm [37.8 mg/kg bw/d (M)] [52.8 mg/kg bw/d (F)]	↓ cholesterol in blood (M/F)	Anonymous; 2006; M-205579-02-1
Mouse 90-day dietary study 0, 32, 320, 3,200 and 6,400 ppm	320 ppm [53 mg/kg bw/d (combined sexes)]	3,200 ppm [545 mg/kg bw/d (combined sexes)]	↓ body weight gain (F) ↑ALT (M/F) and AST (M) ↑ abs. & rel. liver weight (M/F) ↑ incidence hypertrophy of centrilobular hepatocytes (M/F) ↑ incidence of hepatocytic necrosis (F)	Anonymous; 2000; M-197623-01-1
Dog 90-day gavage study 0, 5, 70 or 1,000 mg/kg bw/d	70 mg/kg bw/d	1,000 mg/kg bw/d	↓ body weight gain (M/F) ↑ abs. & rel. liver weight (M/F)	Anonymous; 2000; M-199397-01-1
Dog 52-week gavage study 0, 70, 300 or 1,000 mg/kg/d	300 mg/kg bw/d	1,000 mg/kg bw/d	↓ bodyweight gain (M) ↑ cholesterol in blood (F) ↑ incidence of liver enlargement (M/F)	Anonymous; 2002; M-216694-01-1
Dermal route				
Subacute dermal toxicity study in rats 0, 100, 250, 500 & 1,000 mg/kg bw/d	1,000 mg/kg bw/d	-	No adverse effects observed	Anonymous; 2003; M-220782-01-1

M = male F = female

The liver was identified as target organ in all species following repeated oral exposure to fluopicolide. In rats the kidney was additionally affected, especially in male animals. Effects on other organs were only observed at high dose levels not relevant for STOT RE classification (e.g. effects on blood related parameters, bones and adrenals in the subchronic rat study). The dog was less sensitive than the rodents and generally showed no effects at dose levels relevant for STOT RE classification. No adverse effects were observed after subacute exposure by the dermal route in rats up to 1,000 mg/kg bw/day.

Histopathological changes observed in liver in a STOT RE classification relevant dose range described as minimal to slight centrilobular hepatocyte hypertrophy are indicative of adaptive reversible changes and not considered adverse. Kidney effects observed in male rats in a STOT RE classification relevant dose range were graded as minimal to slight and assumed to be caused by an non-human relevant mode of action.

Overall, therefore, the data are conclusive, but do **not warrant** a STOT RE classification according to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017).

3.12.1.1 Anonymous; 2000; M-199377-01-1

Study reference:

Anonymous; 2000; Rat 28-day dietary toxicity study - AE C638206; M-199377-01-1

Deviations: Deviations from the current OECD guideline (407, 2008):

- Prostate and seminal vesicles with coagulating glands were not weighed at termination
- Eye(s), seminal vesicles with coagulating glands and skeletal muscle were not sampled, fixed or examined histopathologically

However, seminal vesicles with coagulating glands were weighed at termination in the rat 2-generation study (Anonymous; 2003; M-232532-01-1) at dose levels of up to 2,000 ppm; whilst eyes, seminal vesicles with coagulating glands and skeletal muscle were all sampled, processed and examined histologically in the rat 90 day repeat dose study (Anonymous; 2000; M-197622-01-1) at doses up to 20,000 ppm. Therefore these deviations do not affect the overall acceptability of the study.

Executive Summary:

Groups of 5 male and 5 female Sprague Dawley CRL:(IGS)CDBR rats were administered fluopicolide (batch no., CDB234167-2; purity 99.9%) in the diet at dose levels of 0, 20, 200, 2,000 or 20,000 ppm (corresponding to achieved doses of approximately 1.78, 17.7, 179 and 1770 mg/kg bw/day for the combined sexes) for a minimum of 28 consecutive days.

There were no mortalities or clinical signs of toxicity. The neurotoxicity measurement did not show treatment effects.

At 20,000 ppm, overall body weight gain per day (Day 1-29) was reduced by 32% and 37% compared to control in males and females, respectively. At the end of treatment (Day 29) absolute body weight was reduced in males and females by 14% and 13% compared to control, respectively.

Food consumption for males and females was reduced by 41% and 28% when compared with controls during Week 1 of treatment, whilst food conversion ratios over the treatment period were reduced by 24% and 30%, in males and females, respectively. Water consumption was increased by 27% and 32%, in males and females, respectively when compared with controls.

Biochemical analysis revealed a slight increase in serum cholesterol levels of both sexes, and a slight decrease in ALT in males only. Absolute liver weight was increased by 24% and 13% in males and females, respectively, when compared to controls, and liver weight relative to body weight was increased by 47% in males and by 35% in females.

At necropsy, macroscopic examination revealed enlarged livers in 4/5 males, and both kidneys from 3/5 male animals were pale in color. Microscopic examination of the liver revealed minimal to slight centrilobular hepatocytic hypertrophy in 5/5 males and 2/5 females at 2,000 ppm and slight to moderate hepatocytic hypertrophy in 5/5 males and 5/5 females at 20,000 ppm. At 200 ppm, the same finding was seen in 2/5 males and 3/5 females. Due to the low incidence and minimal to slight severity at 200 ppm, the findings were considered a non-adverse adaptive change. Additionally, in the kidneys of males, the severity of phloxine tartrazine positive granulation was increased.

In conclusion, the No Observed Effect Level (NOEL) was considered to be 20 ppm, equivalent to 1.78 mg/kg bw/day of technical fluopicolide. The increase in the incidence of centrilobular hepatocyte hypertrophy at 200 ppm was not corroborated by any other indices at this dose level and is considered adaptive. Therefore, the No Observed Adverse Effect Level (NOAEL) is considered to be 200 ppm, equivalent to 17.7 mg/kg bw/day of technical fluopicolide for the combined sexes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.9% w/w
Batch no.:	CDB234167-2

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Sprague Dawley CRL: (IGS) CD BR
Age:	6 weeks of age
Weight at start:	179 g to 222 g for males and 151 g to 192 g for females
Source:	Charles River UK Ltd., Margate, Kent, UK
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib
Housing:	Caged in groups of five, by sex and dose group
Temperature:	21 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: January 19 to February 17, 1999

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Animals were killed on Study Day 29 (males) and Study Day 30 (females).

Table 3.12.1.1- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	5	5
2	20	5	5
3	200	5	5
4	2,000	5	5
5	20,000	5	5

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

Test diets were prepared weekly throughout the study. Diet for the highest concentration was prepared using a grinder and a Kenwood mixer with the appropriate quantity of laboratory rodent diet and blended in a Turbula mixer for approximately 20 minutes. Subsequent dietary concentrations were prepared by serial dilution.

Every week, aliquots of each concentration of the freshly prepared test diets were submitted to the Dose Analysis Section in the Toxicology Function of Aventis CropScience UK Ltd. (formally AgrEvo CropScience UK Ltd.), for analysis of the test material concentration. Samples were received in powder form in plastic bags and were stored deep frozen from time of receipt.

The mean results for the test diet samples analysed and prepared for dosing were within the range 92.9% to 100.9% of nominal (acceptable range +10% to -15% of nominal).

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 7% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning. They were also observed in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal behaviour or condition.

2. Body weight and food intake

The weight of the animals was recorded at receipt. Each animal was weighed at randomisation, at the start of treatment, weekly thereafter and at necropsy. For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on each animal prior to the start of treatment and on all animals of Groups 1 and 5 just prior to termination. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 0.5% w/v Mydriacyl.

4. Neurotoxicity assessments

Prior to the commencement of treatment, on Study Day 2, and weekly thereafter the appearance, behaviour and functional integrity of each animal was assessed using a Functional Observation Battery (FOB). The FOB was comprised of a combination of examinations that assess the reaction of animals to handling on removal from the cage and observations in an open field standard arena. In addition to the FOB, the grip strength of each animal was assessed.

The locomotor behaviour of all animals was measured during Week 4. Activity was monitored using Ethovision (Video tracking, Motion analysis and behaviour recognition system).

The cages were identified by animal number only and the observers were unaware of the experimental group to which the animal belonged. Any deviations from normal were recorded with respect to nature, and where appropriate, incidence or severity.

5. Laboratory investigations (hematology, clinical chemistry, urinalysis)

Blood samples for hematology and for biochemistry were collected on Study Day 28. All samples were obtained from the retro-orbital sinus of each animal, under isoflurane anaesthesia.

The following parameters were determined:

Table 3.12.1.1- 2: Hematology

Haematocrit (HCT)	Neutrophils (NEUT)
Haemoglobin (HB)	Lymphocytes (LYMP)
Red blood cells (RBC)	Monocytes (MONO)
Mean cell volume (MCV)	Eosinophils (EOS)
Mean cell haemoglobin (MCH)	Basophils (BASO)
Mean cell haemoglobin concentration (MCHC)	Large unstained cells (LUC)
	Reticulocyte count (RET)
Platelets (PLT)	Prothrombin time (PT)
White blood cells (WBC)	Activated partial thromboplastin time (APTT)

Table 3.12.1.1- 3: Biochemistry

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

4. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly, and macroscopic abnormalities were recorded.

The following organs from all animals were weighed at necropsy:

Adrenals	Kidneys	Spleen
Brain	Liver	Testes
Heart	Ovaries	Thymus
Epididymides		

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin:

Adrenals	Lymph nodes (cervical, mesenteric)	Bone (Sternum)
Bone marrow smear	Nerve (sciatic)	Stomach
Brain	Oesophagus	Testes
Caecum	Ovaries	Thymus
Colon	Oviducts	Thyroid (with parathyroid)
Duodenum	Pancreas	Trachea
Epididymides	Pituitary	Urinary bladder
Heart	Prostate	Uterus*
lleum	Rectum	Vagina
Jejunum	Spinal cord (3 levels)	Any other tissue showing macroscopic abnormalities
Kidneys	Spleen	
Liver		
Lungs		

* Uterine horns and cervix uteri examined

A bone marrow smear was taken from all animals, except decedants and fixed subsequently at staining. Pinnae were stored with formalin fixed tissues for animal identification.

5. Histopathology

Following fixation, nominal 5 µm sections of all tissues from animals in the control and highest dose groups, the liver from all animals, the kidneys from all male animals and any gross lesions, were prepared and stained with haematoxylin and eosin (except bone marrow smear which was fixed and stained with Wright's stain). In addition a Cryostat section of the liver from all animals was stained with Oil Red O to demonstrate lipid and sections of the kidneys from all male animals were stained with Phloxine Tartrazine to demonstrate the presence of hyaline droplets.

Tissues were examined for histopathological change with a light microscope by Finn International, One Eyed Lane, Weybread, Diss, Norfolk, UK. The data were entered directly onto a computer terminal using the Roelee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food and water intake

Body weight

At 20,000 ppm, overall body weight gain per day (Day 1-29) was reduced by 32% and 37% compared to control in males and females, respectively. At the end of treatment (Day 29) absolute body weight was reduced in males and females by 14% and 13% compared to control, respectively.

At 2,000 ppm, in females only, overall body weight gain per day (Day 1-29) was reduced by 30% and absolute body weight at the end of treatment (Day 29) was reduced by 11% compared to control.

At lower doses, body weight development remained unaffected by treatment with fluopicolide.

In general, body weight effects were more marked during the first week of the study.

Table 3.12.1.1- 4: Mean body weights and body weight gains per day

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Body weight [g] *(% difference to control)*					
Day 1	200	205 *(+2.5)*	197 *(-1.5)*	200 *(±0.0)*	198 *(-1.0)*
Day 8	254	261 *(+2.8)*	246 *(-3.1)*	240 *(-5.5)*	207* *(-18.5)*
Day 15	301	317 *(+5.3)*	300 *(-0.3)*	284 *(-5.6)*	250* *(-16.9)*
Day 22	333	358 *(+7.5)*	340 *(+2.1)*	322 *(-3.3)*	286* *(-14.1)*
Day 29	356	391* *(+9.8)*	368 *(+3.4)*	346 *(-2.8)*	305* *(-14.3)*
Body weight gain per day [g] *(% difference to control)*					
Day 1-8	7.8	8.1 *(+3.8)*	6.9 *(-11.5)*	5.8 *(-25.6)*	1.2* *(-84.6)*
Day 8-15	6.6	8.0 *(+21.2)*	7.7 *(+16.7)*	6.3 *(-4.5)*	6.2 *(-6.1)*
Day 15-22	4.6	5.9 *(+28.3)*	5.6 *(+21.7)*	5.4 *(+17.4)*	5.2 *(+13.0)*
Day 22-29	3.3	4.6 *(+39.4)*	4.1 *(+24.2)*	3.4 *(+3.0)*	2.7 *(-18.2)*
Day 1-29[#]	5.6	6.6 *(+17.9)*	6.1 *(+8.9)*	5.2 *(-7.1)*	3.8 *(-32.1)*
Females					
Body weight [g] *(% difference to control)*					
Day 1	174	173 *(-0.6)*	169 *(-2.8)*	170 *(-2.3)*	171*(-1.7)*
Day 8	206	202 *(-1.9)*	192 *(-6.8)*	192 *(-6.8)*	181 *(-12.1)*
Day 15	230	226 *(-1.7)*	212 *(-7.4)*	208 *(-9.6)*	195 *(-15.2)*
Day 22	247	246 *(-0.4)*	226 *(-8.5)*	225 *(-8.9)*	214 *(-13.7)*
Day 29	257	257 *(±0.0)*	238 *(-7.4)*	228 *(-11.3)*	224 *(-12.8)*
Body weight gain per day [g] *(% difference to control)*					
Day 1-8	4.6	4.2 *(-8.7)*	3.3 *(-28.3)*	3.2 *(-30.4)*	1.4* *(-69.6)*
Day 8-15	3.4	3.5 *(+2.9)*	3.0 *(-11.8)*	2.3 *(-32.4)*	2.0* *(-41.2)*
Day 15-22	2.4	2.7 *(+12.5)*	2.0 *(-16.7)*	2.4 *(±0.0)*	2.7 *(+12.5)*
Day 22-29	1.5	1.6 *(+6.7)*	1.7 *(+33.3)*	0.5 *(-66.7)*	1.5 *(±0.0)*
Day 1-29[#]	3.0	3.0 *(±0.0)*	2.5 *(-16.7)*	2.1 *(-30.0)*	1.9 *(-36.7)*

* $p < 0.05$; statistically different to controls using Dunnett's test

[#] No statistical analysis was performed for overall body weight gain per day (Day 1-29).

Food and water intake

At 20,000 ppm, food consumption during Week 1 of treatment was reduced by 41% and 28% for males and females respectively when compared with the controls. Thereafter, the food intake of treated and control animals was similar. Food conversion ratios over the treatment period were reduced by 24% and 30% in males and females, respectively, when compared with the controls. This change was most apparent during week 1 of treatment, when the ratios were decreased by 27% and 63% for males and females, respectively. At 2,000 ppm, food conversion ratios for females were slightly reduced when compared with controls, particularly during the first two weeks of treatment.

Table 3.12.1.1- 5: Mean food consumption (g/animal/day*) (% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Week 1	27.0	28.0 *(+3.7)*	25.2 *(-6.7)*	24.0 *(-11.1)*	15.9 *(-41.1)*
Week 2	26.7	28.5 *(+6.7)*	27.7 *(+3.7)*	26.0 *(-2.6)*	24.5 *(-8.2)*
Week 3	25.9	28.6 *(+10.4)*	27.4 *(+5.8)*	27.3 *(+5.4)*	25.6 *(-1.2)*
Week 4	25.0	28.0 *(+12.0)*	26.6 *(+6.4)*	24.7 *(-1.2)*	24.3 *(-2.8)*
Females					
Week 1	20.0	20.5 *(+2.5)*	18.9 *(-5.5)*	19.5 *(-2.5)*	14.4 *(-28.0)*
Week 2	20.2	20.4 *(+1.0)*	18.7 *(-7.4)*	18.1 *(-10.4)*	20.0 *(-1.0)*
Week 3	20.8	21.6 *(+3.8)*	19.6 *(-5.8)*	20.4 *(-1.9)*	20.0 *(-3.8)*
Week 4	21.1	21.2 *(+0.5)*	20.2 *(-4.3)*	20.1 *(-4.7)*	19.8 *(-6.2)*

Water consumption was measured during Week 3 of the study. At 20,000 ppm, water consumption was increased by 27% and 32%, in males and females, respectively, when compared with the controls. At 2,000 ppm in males only, water consumption was increased by 18% when compared with the controls.

Table 3.12.1.1- 6: Mean water consumption (g/animal/day) *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Week 3	27.2	28.1 *(+3.3)*	28.8 *(+5.9)*	32.2 *(+18.4)*	34.5 *(+26.8)*
Females					
Week 3	25.3	25.4 *(+0.4)*	25.4 *(+0.4)*	24.8 *(-2.0)*	33.3 *(+31.6)*

3. Ophthalmoscopic examinations

There were no treatment-related ophthalmic lesions.

4. Neurotoxicity assessments

No treatment-related effects were observed.

5. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 20,000 ppm, a slight increase in cholesterol was observed in animals of both sexes, and a slight decrease in alanine aminotransferase was observed in male animals only. At 2,000 ppm, a slight increase in cholesterol was observed in both sexes.

Table 3.12.1.1- 7: Mean cholesterol and ALT levels in males and females at termination *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Cholesterol (mmol/l)	1.61	1.57 *(-2.5)*	2.07 *(+28.6)*	2.42** *(+50.3)*	2.74*** *(+70.2)*
ALT (U/l)	79	75 *(-5.1)*	70 *(-11.4)*	67 *(-15.2)*	54* *(-31.6)*
Females					
Cholesterol (mmol/l)	1.85	2.03 *(+9.7)*	1.93 *(+4.3)*	2.38** *(+28.6)*	2.67*** *(+44.3)*
ALT (U/l)	68	69 *(+1.5)*	75 *(+10.3)*	80 *(+17.6)*	63 *(-7.4)*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ statistically different to controls using Bartlett's test

Other statistically significant differences were considered to be of no toxicological significance because their incidence was either not dose-related, or the values were within the normal range of historical controls.

6. Sacrifice and pathology

Macroscopic examination at necropsy revealed enlarged livers at 20,000 ppm in 4/5 males, and both kidneys from 3/5 male animals were pale in color. At 2,000 ppm, both kidneys of 3/5 male animals were recorded as pale.

At 20,000 ppm, absolute liver weight was increased by 24% and 13% in males and females, respectively, when compared to the controls. Liver weight relative to body weight was increased by 47% in males and by 35% in females, compared to the controls.

Table 3.12.1.1- 8: Liver weights in males and females following 4-week treatment period *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Terminal body weight (g)	352.2	385.4 *(+9.4)*	363.5 *(+3.2)*	337.6 *(-4.1)*	297.7* *(-15.5)*
Liver weight (g)	12.78	13.89 *(+8.7)*	13.82 *(+8.1)*	14.12 *(+10.5)*	15.91* *(+24.5)*
Liver relative to bodyweight	3.63	3.60 *(-0.8)*	3.78 *(+4.1)*	4.18 *(+15.2)*	5.35** *(+47.4)*
Females					
Terminal body weight (g)	260.0	258.4 *(-0.6)*	236.9 *(-8.9)*	233.2 *(-10.3)*	217.7 *(-16.3)*
Liver weight (g)	9.58	9.40 *(-1.9)*	8.52 *(-11.1)*	9.23 *(-3.7)*	10.79 *(+12.6)*
Liver relative to bodyweight	3.67	3.61 *(-1.6)*	3.59 *(-2.2)*	3.95 *(+7.6)*	4.95** *(+34.9)*

* $p < 0.05$; ** $p < 0.01$; statistically different to controls using Bartlett's test

7. Histopathology

At 20,000 ppm, centrilobular hepatocytic hypertrophy was recorded for all males and females. This finding was recorded as slight in all females and slight for 3/5 males and moderate for 2/5 males. At 2,000 ppm, all males and 2/5 females were observed to have centrilobular hepatocytic hypertrophy. This was recorded as minimal in both females and 1/5 males and slight for 4/5 males. At 200 ppm, centrilobular hepatocytic hypertrophy was seen in 2/5 males (one animal scored as minimal and the other as slight) and in 3/5 females (minimal).

Table 3.12.1.1- 9: Centrilobular hepatocyte hypertrophy – Number of animals affected

Severity	**Dose (ppm)**				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Minimal	0	0	1	1	0
Slight	0	0	1	4	3
Moderate	0	0	0	0	2
Total	**0**	**0**	**2**	**5**	**5**
Females					
Minimal	0	0	3	2	0
Slight	0	0	0	0	5
Total	**0**	**0**	**3**	**2**	**5**

At 20,000 ppm and 2,000 ppm, the severity of phloxine tartrazine positive granulation in the renal sections of male animals was increased from minimal to moderate/severe. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin. Since the α2μ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2μ-globulin accumulation are unlikely to occur in humans[27].

[27] Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of alpha 2μ-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ Health Perspect. 1993 Mar;99:313-49.

Table 3.12.1.1- 10: Kidney: Phloxine tartrazine-positive granulation in males – Number of animals affected

Severity	Dose (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Minimal	4	2	3	0	0
Slight	0	2	2	0	1
Moderate	1	0	0	3	3
Severe	0	0	0	2	1
Total	**5**	**4**	**5**	**5**	**5**

III. Conclusion

The NOAEL in the 28-dietary study in rats with fluopicolide was considered to be 200 ppm (equivalent to 17.8 mg/kg bw/day fluopicolide) for both sexes based on reduction in body weight gain in females, increased levels of cholesterol (suggestive of impaired liver function), increase in the absolute and relative liver weights in males, increased incidence and/or severity of centrilobular hepatocyte hypertrophy in both sexes and increased incidence and severity of phloxine tartrazine granulation at dose levels of ≥ 2,000 ppm (equivalent 179 mg/kg bw/day). The target organs for toxicity were the liver and kidney. The increase in the incidence of centrilobular hepatocyte hypertrophy at 200 ppm was not corroborated by any other indices at this dose level and is considered adaptive.

3.12.1.2 Anonymous; 2000; M-197343-01-1

Study reference:

Anonymous; 2000; Mouse 28-day dietary toxicity study - AE C638206; M-197343-01-1

Deviations: Deviations from the current OECD guideline (407, 2008):

- Prostate and seminal vesicles with coagulating glands were not weighed at termination
- Eye(s), seminal vesicles with coagulating glands and skeletal muscle were not sampled, fixed or examined histopathologically

Although performed in the rat, rather than the mouse, eyes, seminal vesicles and skeletal muscle were all sampled, processed and examined histologically in the rat 90- day repeat dose study (Anonymous; 2006; M-205579-02-1) at doses up to 3,200 ppm. Therefore these deviations do not affect the overall acceptability of the study.

Executive Summary:

Groups of 5 male and 5 female CD-1 mice were fed diet containing 0, 6, 64, 640 or 6,400 ppm of technical fluopicolide (equivalent to 1.07, 11.6, 115 and 1,111 mg/kg bw/day for the combined sexes) for a minimum of 28 consecutive days. At 6,400 ppm, biochemical analyses revealed a slight increase in alanine aminotransferase and alkaline phosphatase activities in male animals only. Absolute liver weight was increased by 33% and 50%, in males and females, respectively, when compared with the controls. Liver weight relative to body weight was also increased by 42% and 58%, in males and females, respectively, when compared with the controls. Histopathological examination of the liver indicated an increase in incidence and severity of hypertrophy of centrilobular hepatocytes. At 640 ppm, there was a slight increase in alanine aminotransferase activity for males and absolute and relative liver weights were increased in females only by 19% when compared with the controls. In addition, there was

an increase in severity of hypertrophy of centrilobular hepatocytes in males and females. There were no treatment-related effects at 64 and 6 ppm.

The NOAEL in the 28-dietary study in mice was 64 ppm, (equivalent to 10.4 and 12.9 mg/kg bw/day, in males and females, respectively) based on increased liver weight and centrilobular hepatocyte hypertrophy at dose levels of ≥ 640 ppm (equivalent 100 and 129 mg/kg bw/day in male and female rats respectively).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.0% w/w
Batch no.:	CDB234187-1

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	CRLCD-1 (ICR) BR
Age:	35 days of age
Weight at start:	27.6 g to 33.9 g for males and 21.4 g to 26.6 g for females
Source:	Charles River UK Ltd., Margate, Kent, UK
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib
Housing:	Housed in groups of five, by sex and dose group
Temperature:	21 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 1 to April 8, 1999

2. Animal assignment and treatment

The mice were randomized and assigned to the following test groups.

Table 3.12.1.2- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	5	5
2	6	5	5
3	64	5	5
4	640	5	5
5	6,400	5	5

Groups of 5 male and 5 female CRLCD-1 (ICR) BR mice were administered in the diet fluopicolide (batch N° CDB234187-1, purity 99.0%) at nominal concentrations of 0 (control), 6, 64, 640 or 6,400 ppm (equivalent 0, 0.98, 10.4, 100 and 980 mg/kg bw/day in males and 0, 1.19, 12.9, 129 and 1,242 mg/kg bw/day in females) for a minimum of 28 consecutive days. The animals were killed on Study Day 29 (males) and Study Day 30 (females).

3. Diet preparation and analysis

The test diet was prepared prior to the study with the appropriate quantity of test material in the diet using a Turbula T50 automatic mixer to produce diet containing the highest concentration. Subsequent dietary concentrations were prepared by serial dilution. Prior to administration, the study mixes were analysed to demonstrate that homogeneity and stability of the test material in the laboratory rodent diet were at acceptable levels for all required nominal concentrations.

Aliquots of each concentration of the test diets were submitted for analysis of the test material concentration. Samples were received in powder form in plastic bags and were stored deep frozen from time of receipt.

The mean results for the test diet samples analysed and prepared for dosing were within the range 90.5% to 101.0% of nominal (acceptable range +10% to -15% of nominal).

Homogeneity was shown to be satisfactory at all levels, i.e. mean values obtained for top, middle and bottom samples were within the acceptable range 90% to 110% of nominal and these mean % nominal values differed by < 10%.

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 12% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made each morning. Animals were also observed in the afternoon on Mondays to Fridays, except on public and company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

Each animal was weighed upon receipt, at randomisation, at the start of treatment, at weekly intervals thereafter and at termination.

For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Ophthalmoscopic examination

Not conducted in mice.

4. Laboratory investigations (hematology, clinical chemistry, urinalysis)

Samples for hematology were collected on Study Day 23 whilst samples for biochemistry were collected on the day of necropsy. All samples were obtained from the retro-orbital sinus under isoflurane anaesthesia.

The parameters listed below were measured or derived.

Table 3.12.1.2- 2: Haematology

Haematocrit (HCT)	White blood cells (WBC)
Haemoglobin (HB)	Neutrophils (NEUT)
Red blood cells (RBC)	Lymphocytes (LYMP)
Mean cell volume (MCV)	Monocytes (MONO)
Mean cell haemoglobin (MCH)	Eosinophils (EOS)
Mean cell haemoglobin concentration (MCHC)	Basophils (BASO)
Platelets (PLT)	Large unstained cells (LUC)
	Reticulocytes (RET)

Table 3.12.1.2- 3: Biochemistry

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

5. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

The following organs from all animals were weighed at necropsy:

Table 3.12.1.2- 4: Organ weights

Brain	Adrenals	Spleen
Kidneys	Liver	Epididymides
Ovaries	Thymus	
Heart	Testes	

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin.

Adrenals	Lungs (inflated)	Testes
Bone - sternum	Mesenteric I. node	Thymus
Brain	Nerve (sciatic)	Thyroid (with
Caecum	Oesophagus	parathyroid)
Cervical L. node	Ovaries	Trachea
Colon	Oviducts	Urinary Bladder
Duodenum	Pancreas	Uterus*
Epididymides	Pituitary	Vagina
Heart	Prostate	Any other tissue showing
lleum	Rectum	macroscopic abnormalities
Jejunum	Spleen	
Kidneys	Spinal cord	
Liver	Stomach	

* Uterine horns and cervix examined.

A bone marrow smear was taken from all animals and fixed subsequently at staining. Pinnae were stored with formalin fixed tissues for animal identification.

6. Histopathology

Following fixation, all tissues from animals in the control and highest dose groups (with the exception of pinnae), and sections of the liver from all intermediate dose groups, were prepared and stained with haematoxylin and eosin (except bone marrow smear which was fixed and stained with Wright's stain). An additional cryostat section of liver was stained with Oil red 0 to demonstrate lipid from all dose groups. After at least three weeks in fixative, the brain and spinal cord from all animals were processed to wax block.Tissues were examined for histopathological change with a light microscope by Peter Finn of Finn International, One Eyed Lane, Weybread, Diss, Norfolk, IP21 5TT, UK. The data were entered directly into a computer terminal using the Roelee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food intake

Body weight

There were no treatment-related effects on body weight.

Table 3.12.1.2- 5: Mean body weights (g)

	Dose level (ppm)								
	0	**6**	***%[a]***	**64**	***%[a]***	**640**	***%[a]***	**6,400**	***%[a]***
Males									
Day 1	31.1	30.1	*(-3.2)*	29.8	*(-4.2)*	30.2	*(-2.9)*	31.5	*(+1.3)*
Day 8	34.7	33.4	*(-3.7)*	33.5	*(-3.5)*	32.6	*(-6.1)*	33.7	*(-2.9)*
Day 15	37.5	34.6	*(-7.7)*	35.7	*(-4.8)*	34.2	*(-8.8)*	35.0	*(-6.7)*
Day 22	39.2	35.9	*(-8.4)*	37.3	*(-4.8)*	35.8	*(-8.7)*	37.1	*(-5.4)*
Day 29	40.1	35.6	*(-11.2)*	35.8*	*(-10.7)*	36.3	*(-9.5)*	37.9	*(-5.5)*
Females									
Day 1	23.0	23.4	*(+1.7)*	23.4	*(+1.7)*	22.8	*(-0.9)*	23.7	*(+3.0)*
Day 8	25.1	25.7	*(+2.4)*	26.6	*(+6.0)*	25.3	*(+0.8)*	24.6	*(-2.0)*
Day 15	26.3	26.8	*(+1.9)*	26.4	*(+0.4)*	26.3	*(±0.0)*	25.7	*(-2.3)*
Day 22	28.1	27.8	*(-1.1)*	28.9	*(+2.8)*	27.7	*(-1.4)*	26.3	*(-6.4)*
Day 29	28.2	27.7	*(-1.8)*	28.8	*(+2.1)*	28.3	*(+0.4)*	26.0	*(-7.8)*

[a] % difference compared to control

* $p < 0.05$; statistically different to controls using Dunnett's test

Food intake

There were no treatment-relatedeffects on food consumption. Food conversion ratios were reduced in most of the treatment groups. However, food conversion ratios were highly variable and no consistent dose-dependent effect was observed.

Table 3.12.1.2- 6: Mean food consumption (g/animal/day)

	Dose level (ppm)								
	0	**6**	***%[a]***	**64**	***%[a]***	**640**	***%[a]***	**6,400**	***%[a]***
Males									
Week 1	5.7	5.8	*(+1.8)*	5.8	*(+1.8)*	5.5	*(-3.5)*	5.1	*(-10.5)*
Week 2	5.8	5.6	*(-3.4)*	5.9	*(+1.7)*	5.3	*(-8.6)*	5.4	*(-6.9)*
Week 3	5.9	5.6	*(-5.1)*	5.8	*(-1.7)*	5.6	*(-5.1)*	5.9	*(±0.0)*
Week 4	5.7	5.1	*(-10.5)*	5.5	*(-3.5)*	5.3	*(-7.0)*	5.6	*(-1.8)*
Females									
Week 1	5.1	5.4	*(+5.9)*	5.3	*(+3.9)*	4.9	*(-3.9)*	4.9	*(-3.9)*
Week 2	5.1	5.3	*(+3.9)*	5.4	*(+5.9)*	5.3	*(+3.9)*	4.8	*(-9.8)*
Week 3	5.2	5.2	*(±0.0)*	5.9	*(+13.5)*	5.9	*(+13.5)*	5.3	*(+1.9)*
Week 4	5.1	5.5	*(+7.8)*	5.6	*(+9.8)*	5.6	*(+9.8)*	4.9	*(-3.9)*

[a] % difference compared to control

Table 3.12.1.2- 7: Mean food conversion (%)

	Dose level (ppm)				
	0	**6**	**64**	**640**	**6,400**
Males					
Week 1	8.9	8.2	9.2	6.2	6.3
Week 2	6.8	2.9	5.2	4.3	3.4
Week 3	4.3	3.4	4.0	4.0	5.1
Week 4	2.0	-0.9	-3.9	1.5	2.1
Mean (week 1-4) *(% difference to control)*	5.5	3.4 *(-38.2)*	3.6 *(-34.6)*	4.0 *(-27.3)*	4.2 *(-23.6)*
Females					
Week 1	6.0	6.2	8.6	7.2	2.8
Week 2	3.3	2.7	-0.5	2.8	3.0
Week 3	4.8	2.8	6.0	3.4	1.7
Week 4	0.3	-0.1	-0.3	1.3	-0.9
Mean (week 1-4) *(% difference to control)*	3.6	2.9 *(-19.4)*	3.5 *(-2.8)*	3.7 *(+2.8)*	1.7 *(-52.8)*

3. Ophthalmoscopic examinations

Not performed.

4. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 640 and 6,400 ppm, a slight increase in alanine aminotransferase was observed. At 6,400 ppm, alkaline phosphatase was slightly and statistically non-significantly increased in males only.

Any other statistically significant differences were considered not to be treatment-related because their incidence was either not dose-related or the values were within the normal range of historical control data.

Table 3.12.1.2- 8: Clinical chemistry

Parameter	**Dose level (ppm)**								
	0	**6**	***%[a]***	**64**	***%[a]***	**640**	***%[a]***	**6,400**	***%[a]***
Males									
ALT (U/L)	42	41	*(+2.4)*	46	*(+9.5)*	76**	*(+81.0)*	104**	*(+147.6)*
ALP (U/l)	58	71	*(+22.4)*	66	*(+13.8)*	81	*(+39.7)*	134	*(+131.0)*
Females									
ALT (U/L)	35	31	*(-11.4)*	35	*(±0.0)*	52**	*(+48.6)*	54**	*(+54.3)*
ALP (U/l)	98	80	*(-18.4)*	97	*(-1.0)*	87	*(-11.2)*	88	*(-10.2)*

[a] % difference compared to control

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

5. Sacrifice, pathology

There were no treatment-related findings at necropsy.

6. Organ weights

At 6,400 ppm, absolute liver weight was increased by 33% and 50%, in males and females, respectively when compared with controls. Liver weight relative to body weight was also increased by 42% and 58%, in males and females, respectively, when compared with controls.

At 640 ppm, liver absolute and body weight relative liver weights were increased in females only by 19%, when compared with the controls.

There were no other treatment-related effects on organ weights.

Table 3.12.1.2- 9: Liver weights in males and females

	Dose level (ppm)								
	0	**6**	***%[a]***	**64**	***%[a]***	**640**	***%[a]***	**6,400**	***%[a]***
Males									
Terminal body weight (g)	38.2	33.6*	*(-12.0)*	33.9	*(-11.3)*	34.8	*(-8.9)*	35.8	*(-6.3)*
Liver weight (g)	1.91	1.69	*(-11.5)*	1.73	*(-9.4)*	1.94	*(+1.6)*	2.54**	*(+33.0)*
Liver relative to bodyweight	5.01	5.02	*(+0.2)*	5.13	*(+2.4)*	5.60	*(+11.8)*	7.11**	*(+41.9)*
Females									
Terminal body weight (g)	26.9	27.0	*(+0.4)*	27.4	*(+1.9)*	27.0	*(+0.4)*	25.7	*(-4.5)*
Liver weight (g)	1.33	1.32	*(-0.8)*	1.52	*(+14.3)*	1.58	*(+18.8)*	2.00**	*(+50.4)*
Liver relative to bodyweight	4.92	4.88	*(+0.8)*	5.50	*(+11.8)*	5.86**	*(+19.1)*	7.77**	*(+57.9)*

[a] % difference compared to control

* $p < 0.05$; ** $p < 0.01$; statistically different to controls using Dunnett's test

7. Histopathology

At 6,400 ppm, there was an increase in the incidence (5/5 males and 5/5 females) and severity (mainly moderate: 4/5 males, 5/5 females) of hypertrophy of centrilobular hepatocytes in the liver compared to control animals. At 640 ppm, there was also an increase in the incidence (5/5 males and 4/5 females) and severity (mainly slight: 3/5 animals each sex) of hypertrophy of centrilobular hepatocytes in the liver.

At the high dose level of 6,400 ppm, the animals remained unaffected by treatment with fluopicolide regarding the indicence of focal coagulative necrosis in the liver. At 640 ppm, slight focal coagulative necrosis was observed in 1/5 females. At 64 ppm, only minimal focal coagulative necrosis was seen in 1/5 males.

The finding of focal coagulative necrosis in the liver was not considered treatment-related given the low incidence, lack of dose response and the missing evidence of progression of severity with increasing dose levels.

Table 3.12.1.2- 10: Selected histopathological liver findings – Number of animals affected

Finding / Severity	Dose level (ppm)				
	0	**6**	**64**	**640**	**6,400**
Centrilobular hepatocyte hypertrophy					
Males					
Minimal	3	3	3	1	0
Slight	0	0	1	3	1
Moderate	0	0	0	1	4
Total	**3**	**3**	**4**	**5**	**5**
Females					
Minimal	1	3	0	1	0
Slight	0	0	0	3	0
Moderate	0	0	0	0	5
Total	**1**	**3**	**0**	**4**	**5**
Focal coagulative necrosis					
Males					
Minimal	0	0	1	0	0
Slight	0	0	0	0	0
Total	**0**	**0**	**1**	**0**	**0**
Females					
Minimal	0	0	0	0	0
Slight	0	0	0	1	0
Total	**0**	**0**	**0**	**1**	**0**

III. Conclusion

The main target organ in mice was the liver. The NOAEL in the 28-dietary study in mice was 64 ppm, (equivalent to 10.4 and 12.9 mg/kg bw/day, in males and females, respectively) based on increased liver weight and centrilobular hepatocyte hypertrophy at dose levels of ≥ 640 ppm (equivalent 100 and 129 mg/kg bw/day in male and female rats respectively).

3.12.1.3 Anonymous; 2000; M-197350-01-1

Study reference:

Anonymous; 2000; Dog 28-day oral toxicity study Code: AE C638206 00 1C99 0005; M-197350-01-1

Deviations:	This is a 28-day study, serving primarily as a dose range finding (DRF) study for a subsequently conducted 90-day study performed according to OECD TG 409 (1998). As such, it is considered that the study was performed to the principles of OECD 409 (1998) with appropriate adjustments for a study of this type – i.e. a DRF. Therefore, listing deviations is not considered appropriate.

Executive Summary:

Groups of 2 male and 2 female beagle dogs were treated by gavage, at dose levels of 0, 10, 100 and 1,000 mg/kg bw/day of technical fluopicolide, for at least 28 consecutive days. The test substance was administered in a vehicle of 1.0% w/v methyl cellulose in distilled water at a constant volume of 5 mL/kg bw. Controls received the vehicle alone. There were no mortalities or clinical signs of toxicity.

At 1,000 mg/kg bw/day, biochemistry analyses revealed a slight increase in cholesterol concentration on Study Days 14 and 29 for one male (number 947). This animal also had an increase in absolute liver weight and liver weight relative to body weight of 34% and 44%, respectively, when compared to controls. There were no treatment-related effects for males at 100 and 10 mg/kg bw/day or treated females.

In the absence of histopathological correlates and based upon the fact that the minor hepatic changes (slight increase in cholesterol and increased absolute and relative liver weight) at 1,000 mg/kg bw/day were observed in a single male only, it is considered that the NOAEL under the conditions of this study was 1,000 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9% w/w
Batch no.:	T/035/99

2. Vehicle and/or positive control

Vehicle:	1.0% w/v methyl cellulose in distilled water

3. Test animals

Species:	Dogs
Strain:	Beagle
Age:	6 – 7 months old
Weight at start:	10.5 to 11.9 kg for males, and from 8.3 to 9.8 kg for females
Source:	Harlan Interfauna, Wyton, Huntingdon, Cambridgeshire, UK
Acclimation period:	Yes
Diet:	Modified SQC Expanded Pellet Laboratory Diet A, supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib. through automatic valves
Housing:	The dogs were housed in pairs (except during feeding and dosing when they were housed individually), by sex and dose group, under controlled environmental conditions, in a solid floor pen, measuring approximately 1 m x 4.5 m, with wood chips as bedding
Temperature:	18 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: June 10 to August 5, 1999

2. Animal assignment and treatment

The dogs were randomized and assigned to the following test groups.

Table 3.12.1.3- 1: Study design

Group no.	Dose (mg/kg bw/day)	Number of males	Number of females
1	0	2	2
2	10	2	2
3	100	2	2
4	1,000	2	2

Dose levels were 0, 10, 100 and 1,000 mg/kg bw /day. The test substance was suspended in 1.0% w/v methyl cellulose in distilled water, and administered at a constant volume of 5 mL/kg bw. Males were treated for 29 consecutive days, females for 30 consecutive days.

3. Preparation and analysis of dosing suspensions

Prior to the start of treatment, a procedure was developed to reliably prepare homogeneous mixtures of the test material in the vehicle, 1.0% w/v methyl cellulose in distilled water, at the required nominal concentrations of 2, 20 and 200 mg/mL (equivalent to 10, 100 and 1000 mg/kg bw/day dose levels, respectively).

Throughout the study dosing suspensions were generally prepared one day in advance of dosing, except for suspensions dosed over the weekend and on Monday, which were made up on the preceding Friday.

On each occasion, the required volume of dosing suspension was prepared by gradually adding the vehicle to the appropriate quantity of test material and thoroughly mixing them. Initially, a smooth paste was prepared which was made up to volume with more vehicle and the mixture was then homogenised.

On one occasion every week, aliquots of each concentration of the freshly prepared dosing suspensions were submitted to the Dose Analysis Section of Aventis CropScience UK Limited (formerly AgrEvo UK Limited, Chesterford Park, for analysis of test material concentration.

Homogeneity and stability were analysed at all dose levels from the trial mix samples stored at room temperature for 0, 1, 2 and 4 days. The study mix samples from Days 1 and 22 were analysed at all dose levels.

The mean results for the test suspension samples analysed were within the range of 82.9% to 102.8% of nominal (acceptable range +20% to -20% of nominal).

Homogeneity was shown to be satisfactory in the trial mix, i.e. mean values were within the acceptable range of 80% to 120% of nominal, and standard deviation values were less than 10%.

Re-analysis of the trial mix after storage at 4 °C for 0, 1, 2 and 4 days indicated that the suspensions were stable over 4 days at this temperature.

4. Statistics

Not conducted – only 2 animals per sex per dose group.

C. Methods

1. Observations

Each dog was given a thorough clinical examination prior to the start of and at the end of the treatment period. Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning. They were also observed in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of each dog was recorded upon receipt, at randomisation, on Study Days -14 and -7, and at the start of treatment. Thereafter, each animal was weighed weekly throughout the treatment period and at necropsy.

Food consumption for each animal was measured daily for both 1 week prior to the start of treatment, and throughout the treatment period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on each dog prior to the start of treatment, and during the last week of treatment. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 1.0% w/v Mydriacyl.

4. Laboratory investigations (Haematology, clinical chemistry, urinalysis)

Blood samples for hematology and biochemistry were collected from the jugular vein prior to treatment (Study Days -14 and -1) and on Days 14 and 29 of treatment.

Samples for urinalysis were collected directly from the bladder at necropsy.

The parameters listed below were measured or derived:

Table 3.12.1.3- 2: Haematology

Haematocrit (HCT) Haemoglobin (HB) Red blood cells (RBC) Mean cell volume (MCV) Mean cell haemoglobin (MCH) Mean cell haemoglobin concentration (MCHC) Platelets (PLT) White blood cells (WBC) Neutrophils (NEUT)	Lymphocytes (LYMP) Monocytes (MONO) Eosinophils (EOS) Basophils (BASO) Large unstained cells (LUC) Erythrocyte sedimentation rate (ESR) Reticulocytes (RET) Activated partial thromboplastin time (APTT) Prothrombin time (PT)

Table 3.12.1.3- 3: Biochemistry

Total protein (PROT) Albumin (ALB) Total globulin (GLOB) A/G ratio (A/G) Calcium (CA) Phosphate (PO4) Sodium (NA) Potassium (K) Urea (UREA) Creatinine (CREA)	Glucose (GLUC) Total cholesterol (CHOL) Total bilirubin (TBIL) Chloride (CL) Aspartate aminotransferase (AST) Alanine aminotransferase (ALT) Alkaline phosphatase (AP) G-glutamyl transpeptidase (GGT) Creatine kinase (CPK)

Table 3.12.1.3- 4: Urinalysis

Appearance (APP) PH (PH) Protein (PROT) Glucose (GLUC) Specific gravity (SG) Bacteria (BACT) Red blood cells (RBC) Epithelial cells (EPTH) Sperm (SPER)	Ketones (KET) Urobilinogen (UBIL) Bilirubin (BIL) Blood (BLD) Phosphate crystals (PO4) Casts (CAST) Urate (URAT) White blood cells (WBC) Spun deposit colour (SDEP)

5. Sacrifice and pathology

All animals were killed by exsanguination via the carotid artery whilst under deep pentobarbitone anaesthesia. Males were killed on Study Day 30 and females on Study Day 31. One dog was necropsied in turn from each dose group in the following sequence: Group 1, Group 4, Group 2, Group 3. Within each dose group, dogs were necropsied in animal number order.

All dogs were examined thoroughly and macroscopic abnormalities recorded.

6. Organ weights

The following organs from all animals were weighed at necropsy:

Table 3.12.1.3- 5: Organ weights

Adrenals	Pituitary
Brain	Spleen
Ovaries	Lungs
Kidneys	Heart
Liver	Thyroid
Testes	

The following organs and tissues from all animals were fixed intact or sampled and fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Table 3.12.1.3- 6: Tissue sampling

Adrenals	Liver	Skin + subcutis[3]
Articulated surface and shaft of femur[3]	Lungs	Spinal cord (3 levels)[3]
Aorta[3]	Lymph nodes (mandibular, Mesenteric)[3]	Spleen
Brain	Mammary gland[3]	Sternum
Caecum	Oesophagus	Stomach
Colon	Optic nerve[3]	Testes
Diaphragm[3]	Ovaries + oviducts	Thymus
Duodenum	Pancreas	Thyroid + parathyroid[3]
Epididymides[3]	Pinnae[1]	Tongue[3]
Eyes[3]	Pituitary	Tonsils[3]
Gall bladder[3]	Prostate	Trachea[3]
Heart	Rectum	Urinary bladder
lleum	Salivary glands (parotid, mandibular + sublingual)[3]	Uterus[2]
Jejunum	Sciatic nerve[3]	Vagina
Kidneys	Skeletal muscle[3]	Any other tissue showing Macroscopic abnormalities
Lacrimal gland[3]		

[1] The ear with tattoo and ID implant was taken for identification purposes only, and was not examined.
[2] Cervix uteri and uterine horns examined
[3] These tissues were collected but not examined microscopically.

A bone marrow smear was taken from all animals and fixed at staining

5. Histopathology

Following fixation, nominal 5 µm sections of a range of selected organs and tissues from each animal were prepared and stained with haematoxylin and eosin (except bone marrow smear which was stained with Wright's stain). An additional frozen section of the liver from all animals was stained with Oil Red 0 to evaluate the presence of fat.

Tissues were examined for histopathological change with a light microscope by Peter Finn of Precision Histology International, One Eyed Lane, Weybread, Diss, Norfolk, UK. The data were entered directly onto a computer terminal using the Roelee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food intake

There were no consistent treatment-related effects on body weight or food consumption.

Table 3.12.1.3- 7: Mean body weights (g)

	Dose level (mg/kg bw/day)			
	0	**10**	**100**	**1,000**
Body weight [g] *(% difference to control)*				
Males				
Day 1	11.2	11.3 *(+0.9)*	11.3 *(+0.9)*	10.9 *(-2.7)*
Day 8	11.6	11.3 *(-2.6)*	11.4 *(-1.7)*	11.2 *(-3.4)*
Day 15	11.8	11.5 *(-2.5)*	11.6 *(-1.9)*	10.9 *(-7.6)*
Day 22	12.2	11.9 *(-2.5)*	11.7 *(-4.1)*	10.9 *(-10.7)*
Day 29	12.2	11.9 *(+2.5)*	11.8 *(-3.3)*	11.1 *(-9.0)*
Females				
Day 1	9.0	9.4 *(+4.4)*	8.5 *(-5.6)*	9.1 *(+1.1)*
Day 8	8.9	9.4 *(+5.6)*	8.4 *(-5.6)*	9.1 *(+2.2)*
Day 15	9.2	9.6 *(+4.3)*	8.5 *(-7.6)*	9.2 *(±0.0)*
Day 22	9.3	9.8 *(+5.4)*	8.5 *(-7.5)*	9.4 *(+1.1)*
Day 29	9.4	9.8 *(+4.3)*	8.5 *(-9.6)*	9.7 *(+3.2)*

Table 3.12.1.3- 8: Mean food consumption (g/animal/day) (% difference to control)

	Dose level (mg/kg bw/day)			
	0	**10**	**100**	**1,000**
Males				
Week 1	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 2	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 3	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 4	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Females				
Week 1	273	278 *(+1.8)*	284 *(+4.0)*	313 *(+14.7)*
Week 2	322	307 *(-4.7)*	302 *(-6.2)*	318 *(-1.2)*
Week 3	321	325 *(+1.2)*	314 *(-2.2)*	344 *(+7.2)*
Week 4	300	318 *(+6.0)*	305 *(+1.7)*	351 *(+17.0)*

3. Ophthalmoscopic examinations

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 1,000 mg/kg bw/day, there was a slight increase in cholesterol concentration in one male animal (number 947; bold print) on Study Days 14 and 29 when compared with pre-treatment and control values.

There were no other treatment-related effects observed.

Table 3.12.1.3- 9: Cholesterol measurements (mmol/L)

	Dose level (ppm)															
	Males								Females							
	0		10		100		1,000		0		10		100		1,000	
Animal No.	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956
Day -1	4.31	2.22	2.07	3.44	3.65	2.53	3.90	3.17	3.20	3.46	3.37	2.86	3.40	2.43	2.87	3.45
Day 14	3.93	2.38	2.45	3.90	4.02	2.65	5.56	3.99	3.22	3.41	3.36	2.68	3.76	2.36	3.55	3.44
Day 29	4.21	2.80	2.50	3.97	4.01	2.62	7.72	3.79	3.50	3.34	3.47	2.82	3.80	2.22	3.50	3.35

Urinalysis:

There were no treatment-related findings.

5. Sacrifice, pathology

At 1,000 mg/kg bw/day, an enlarged liver was noted for one male only (number 947).

6. Organ weights

At 1,000 mg/kg bw/day in one male only (number 947), absolute liver weight and liver weight relative to body weight was increased by 34% and 44%, respectively, when compared to the mean values for the controls

7. Histopathology

There were no treatment-related histopathological changes.

III. Conclusion

In the absence of histopathological correlates and based upon the fact that the minor hepatic changes (slight increase in cholesterol and increased absolute and relative liver weight) were observed at 1,000 mg/kg bw/d in a single male only, it is considered that the NOAEL under the conditions of this study was 1,000 mg/kg bw/day.

3.12.1.4 Anonymous; 2000; M-197622-01-1

Study reference:

Anonymous; 2000; AE C638206 - Rat 90-day dietary toxicity study with 4 week off-dose period; M-197622-01-1

Deviations: Deviations from the current OECD guideline (408, 2018):

- Blood/plasma samples were not taken for possible analysis of thyroid hormones
- At termination, an evaluation of oestrus cycle was not performed

Oestrous cyclicity was evaluated in the rat two-generation study (Anonymous; 2003; M-232532-01-1) at dose levels of up to 2,000 ppm (equivalent to at least 100 mg/kg bw/day in F0/F1 females).

These deviations do not affect the overall acceptability of the study.

Executive Summary:

Groups of 10 male and 10 female Sprague Dawley rats were fed diet containing 0, 100, 1,400 or 20,000 ppm (equivalent to 0, 7.9, 114 or 1671 mg/kg bw/day for the combined sexes, respectively) of technical fluopicolide for at least 13 weeks. An additional 10 males and 10 females, fed either 0 or 20,000 ppm for 13 weeks, were maintained on control diet for a further 4 weeks to examine the reversibility of any effects seen.

At 20,000 ppm, treatment-related clinical signs included hair loss in both sexes, body soiling and loss of coat condition in males only, and a soiled urogenital region in females. Absolute body weight after 13 weeks of treatment was reduced by 30% and 18% compared to the controls in males and females, respectively. Body weight gain over the course of treatment (Day 1-92) was reduced by 41% in males and 29% in females, whilst mean food consumption was reduced by 17% in males and 15% in females and mean food conversion ratios (Week 1-13) were reduced by 28% and 19% compared to control in males and females, respectively. Water intake was increased by 44% during Week 4 of treatment in females only. Haematological investigations indicated slight decreases in haematocrit, haemoglobin, mean cell haemoglobin and mean cell haemoglobin concentration in both sexes, and APTT was slightly increased in males only. There was a slight increase in cholesterol, total protein and GGT in both sexes.

Urinalysis revealed a slight increase in the number of epithelial cells in the urinary sediment of males only and a slight increase in urinary volume and a slight decrease in specific gravity for females only.

Absolute liver weight was increased by 22% in females only and liver weight relative to body weight was increased by 51% in males and 49% in females compared to control. Absolute spleen weight was decreased by 45% and 40% in males and females, respectively, and spleen weight relative to body weight was decreased by 24% in males and 29% in females, when compared to controls. Kidney weight relative to body weight was increased by 15% in males only when compared to controls. Macroscopic examination at necropsy revealed a speckled appearance in both kidneys of 4/10 males. Microscopic examination showed an increase in the severity and incidence of hypertrophy of the zona glomerulosa in the adrenal, trabecular hyperostosis of the bone joint and decreased cellularity of the bone marrow for males and females. Centrilolubar hepatocytic hypertrophy, scored as minimal to moderate, was seen in the livers of 9/10 males and 8/10 females. Males only had a number of kidney effects, consisting of an increase in the severity and incidence of accumulation of hyaline droplets in the proximal tubule, single cell death in the proximal tubule epithelium, foci of basophilic (regenerating) tubules and granular casts.

Following the four week off-dose period there was a complete or partial recovery of all treatment-related affects.

At 1,400 ppm, there was a slight increase in cholesterol and a slight increase in the number of epithelial cells in the urinary sediment of males only, and a slight increase in urinary volume and a slight decrease in specific gravity for females only.

Liver weight relative to body weight was increased by 15% in males only, when compared to controls. Absolute spleen weight was decreased by 10% and 16% in males and females, respectively, and spleen weight relative to body weight was decreased by 19% in females only, when compared to controls. Kidney weight relative to body weight was increased by 11% in males only when compared to controls.

Macroscopic examination at necropsy revealed a speckled appearance in both kidneys of 3/10 males, with a further 2/10 and 1/10 males having a speckled appearance in the right and left kidneys, respectively. Microscopic examination showed minimal to slight hypertrophy of centrilobular hepatocytes in 8/10 males only and an increase in the severity and incidence of trabecular hyperostosis of the bone joint in females. Kidney effects, in males only, consisted of an increase in severity of accumulation of hyaline droplets in the proximal tubule, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 100 ppm no effects of treatment were seen.

The NOAEL in the 90-day dietary study in rats was 100 ppm (7.4 or 8.4 mg/kg bw/day in male and female rats respectively) based on treatment-related haematological (reduced haemoglobin and haematocrit in male rats), clinical chemistry (increased cholesterol in male rats at 1,400 ppm), urinalysis (increased urine volume and specific gravity in females at ≥ 1,400 ppm), organ weight (increase in relative liver and kidney weight in male rats and in relative spleen weight in females at 1,400 ppm) and histopathological changes in the liver and kidneys at 1,400 ppm (equivalent 109 or 119 mg/kg bw/day in males and females respectively).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9 and 97.5%
Batch no.:	1C990005

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Sprague-Dawley Crl: CD IGS BR strain
Age:	28-35 days at receipt 34-41 day at day 1 (start of treatment)
Weight at start:	74-94 g for males, 72-92 g for females at receipt 119-153 g for males, 104-144 g for females at day 1 (start of treatment)
Source:	Charles River UK Ltd., Margate, Kent, UK
Acclimation period:	6 days
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex
Water:	Water ad lib
Housing:	Animals were housed in groups of five, by sex and dose group under controlled environmental conditions.
Temperature:	19 – 23 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: July 6 to November 10, 1999

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 3.12.1.4- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Treatment phase			
1	0	10	10
2	100	10	10
3	1,400	10	10
4	20,000	10	10
Recovery phase			
1	0	10	10
4	20,000	10	10

After 13 weeks of continuous dietary exposure, animals from the main groups were killed and those off-dose (recovery) were fed untreated (control) diet for a further 4 weeks and then killed (after a total of 17 weeks on study.

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare homogeneous and suitably stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

Test diets were prepared weekly throughout the study. On each occasion, for each dose level, a pre-mix was made using a grinder and food processor. This in turn was then mixed with remaining laboratory rodent diet and blended in a turbula mixer to produce the required dietary concentration. Subsequent concentrations were prepared by serial dilution.

Every week, aliquots of each concentration (i.e. 100, 1,400 and 20,000 ppm) of the freshly prepared test diets were submitted to Dose Analysis in the Toxicology Function of Aventis CropScience UK Limited (formerly AgrEvo UK Limited) at Chesterford Park for analysis of the test material concentration. Samples were received in powder form in plastic bags and stored deep frozen from time of receipt.

Samples (approx. 200 g) were supplied for analysis from:

i) A trial mix at 100, 1,400 and 20,000 ppm for homogeneity testing and for stability testing after storage at room temperature for 0,1, 8 and 15 days.

ii) The mixes prepared for weeks 1 to 14 of the study.

All the homogeneity samples from the trial mix were analysed. The trial mix stability samples from days 0, 8 and 15 were analysed from the 100 ppm level and the trial mix stability samples from days 0 and 15 were analysed from the 1,400 and 20,000 ppm levels. The week 1, 5, 10 and 14 mixes were analysed at all levels.

The mean results for the test diet samples analysed were within the range 94.3% to 105.9% of nominal (acceptable range +10% to -15% of nominal).

Homogeneity was shown to be satisfactory at all levels, i.e. mean values obtained for top, middle and bottom samples were within the acceptable range 90% to 110% of nominal and these mean % nominal values differed by < 10%.

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 7% over 15 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

Body weight, food consumption, water consumption, organ weights, motor activity and assessments from the functional observations battery were analysed for homogeneity of group variances using Bartlett's test. If not significant at the 5 % level, the data were then analysed by one way analysis of variance to establish the significance of variability amongst the groups. If significant, pair-wise comparisons of each treated group in turn with the controls were made using Dunnett's test of significance. Where group variances were heterogeneous using Bartlett's test, pair-wise comparisons were made using a modified t tesf. For pair-wise comparisons, significance was tested at the 5 and 1% levels ($p < 0.05$, $p < 0.01$).

Observations from the functional observations battery where there are ranked assessments that are scored into a limited number of categories (e.g. scored 0, 1, 2, 3) were assessed using the Mann-Whitney test (otherwise known as the Wilcoxon rank sum test) for pain/vise comparisons. In the functional observation battery where asessments were scored as either present (0) or absent (1) the number of animals in which a condition is present or absent will be recorded for each group at each observation time, in the form of a 2x2 contingency table. Fisher's Exact Test was used to compare each test article treated group with the vehicle control group. Statistical analyses on functional observations were only conducted where positive findings were seen in 3/5 or more animals per group.

Haematology and clinical chemistry data were subjected to the following statistical evaluation:

Bartlett´s test was used to determine homogeneity of variance between groups; if significant at the 5 % level, a log transformation was applied to the data to attempt to remove the heterogeneity.

If homogeneity of variance was demonstrated on either the untransformed or transformed data, parametric tests to detect significant difference between control and test groups at the 5, 1 or 0.1% level comprised:

- One-Way Analysis of Variance to establish the significance of variability among all groups and
- Student's "t" Test based on a pooled variance estimate, for intergroup comparisons, i.e. control vs each test group.

If significant heterogeneity of variance was indicated, even with transformation, then non-parametric analysis was by the Kruskal-Wallis Rank Test to detect any significant group differences at the 5, 1 or 0.1% level.

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning and, on Mondays to Fridays except public and company holidays, also in the afternoon. More frequent observations were made as necessary. Detailed observations were conducted once weekly prior to weighing.

Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

Animals killed in extremis were subjected to necropsy as soon as possible.

2. Body weight, food and water intake

Each animal was weighed upon receipt, at randomisation, at the start of treatment, at weekly intervals thereafter and at necropsy. During Weeks 4, 8 and 11 of treatment, and at the end of the off-dose period (Week 17 of the study), water consumption was measured over a 4-day period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on all animals prior to the start of treatment and in Week 13, on animals from the control and highest dose groups scheduled for necropsy after 13 weeks of treatment. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 0.5% w/v Mydriacyl.

4. Neurotoxicity assessments

During Week 11 of the study, the appearance, behaviour and functional integrity of all animals scheduled for termination after 13 weeks of treatment were assessed using a Functional Observation Battery (FOB).

The FOB comprised a combination of examinations that assessed the reaction of animals to handling on removal from the cage and proceeded to observations in an open field standard arena. In addition to the FOB, the grip strength of each animal was assessed during Week 12.

The cages were identified by animal number only and tie observers were unaware of the experimental group to which the animal belonged. Any deviations from normal were recorded with respect to nature, and where appropriate, incidence or severity.

Functional Observations Battery (FOB):

A clear polycarbonate arena was used for examination in the open field, following parameters were assessed:

Table 3.12.1.4- 2: FOB parameters

Ease of removal from cage (EREM)	Clonic convulsions (CLON)
Ease of handling (HAND)	Tonic convulsions (TONN)
Body temperature (TEMP)	Tonic involuntary leaping (TONL)
Exophthalmus (EXOS)	Rearing (REAR)
Palpebral closure (PALP)	Defaecation frequency (DEFC)
Pupil response - right eye (PUPR)	Urination frequency (URIN)
Pupil response - left eye (PUPL)	Alertness (ALER)
Lachrymation (LACH)	Approach response (APPR)
Salivation (SALN)	Righting reflex (RRFX)
Vocalisations (VOCS)	Right/left tilt (TILT)
Mobility (MOBI)	Startle response (STRT)
Posture (sways/lurches) (SWAY)	Landing foot splay (LFSM)
Hind limbs splayed (SPLY)	Grip strength - fore limb (GRPF)
Hind limbs dragging (DRAG)	Grip strength - hind limb (GRPH)
Hunched posture (HNCH)	Tail pinch (TPCH)
Ataxia (ATAX)	

General observations - included any other relevant information, e.g. dehydration, emaciation etc.

Motor activity:

The locomotor behaviour of all animals scheduled for termination after 13 weeks of treatment was measured during week 12. Activity was monitored using Ethovision™, (Video tracking, Motion analysis and behaviour recognition system). The experimental apparatus consisted of one video camera (Panasonic WV-CP412E) suspended from the ceiling above a group of cages, which were held within a black wooden template. This was connected to a separate video recorder (Panasonic AG-5700b) which in turn was attached to a single colour monitor (Panasonic VWCM1450).

In the first session the first two animals numerically from each group were monitored. In each consecutive period the next animals from each group were monitored in numerical order. The video recorder was activated to record animals over a 60 minute period. This procedure was repeated for each session. The analogue video images from each tape were translated into digital information using the Ethovision™ software, and then analysed to derive the total distance moved (cm) by each rat during the whole 60 minute period.

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Samples for haematology andclinical chemistry analyses were collected during week 13 and at the end of the off-dose period (week 17 of the study) from the retro-orbital sinus of each animal whilst under isoflurane anaesthesia.

Urine samples were obtained from each animal overnight during Weeks 12 and 17.

Animals were housed in individual urine collecting cages and all the urine voided overnight was collected. Animals were deprived of food during the collection period.

The parameters listed below were measured or derived:

Table 3.12.1.4- 3: Haematology

Haematocrit (HCT)	White blood cells (WBC)
Haemoglobin (HB)	Neutrophils (NEUT)
Red blood cells (RBC)	Lymphocytes (LYMP)
Mean cell volume (MCV)	Monocytes (MONO)
Mean cell haemoglobin (MCH)	Eosinophils (EOS)
Mean cell haemoglobin concentration (MCHC)	Basophils (BASO)
	Large unstained cells (LUC)
Platelets (PLT)	Reticulocyte count (RET)
Pro-thrombin time (PT)	Activated partial thromboplastin time (APTT)

Table 3.12.1.4- 4: Biochemistry

Total protein (PROT)	Total cholesterol (CHOL)
Albumin (ALB)	Total bilirubin (TBIL)
Total globulin (GLOB)	Chloride (CL)
A/G ratio (A/G)	Aspartate aminotransferase (AST)
Calcium (CA)	Alanine aminotransferase (ALT)
Phosphate (PO4)	Alkaline phosphatase (AP)
Sodium (NA)	G-glutamyl transpeptidase (GGT)
Potassium (K)	Creatine kinase (CPK)
Urea (UREA)	
Creatinine (CREA)	
Glucose (GLUC)	

Table 3.12.1.4- 5: Urinalysis

PH (PH)	Bacteria (BACT)
Protein (PROT)	Red blood cells (RBC)
Glucose (GLUC)	Epithelial cells (EPTH)
Ketones (KET)	Phosphate crystals (P04)
Urobilinogen (UBIL)	Urate crystals (URAT)
Bilirubin (BIL)	Casts (CAST)
Blood (BLD)	White blood cells (WBC)
Appearance (APP)	Sperm (SPER)
Volume (VOL)	Spun deposit colour (SDEP)
Specific gravity (SG	

6. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

Organ weights:

The following organs from all animals were weighed at necropsy:

Table 3.12.1.4- 6: Organ weights

Liver	Brain
Kidneys	Adrenals
Spleen	Heart
Testes	Thymus
Ovaries	Uterus
Epididymides	

Tissue sampling:

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin except eyes which were fixed in Davidson's fluid:

Table 3.12.1.4- 7: Tissue sampling and processing

Adrenals Aorta Brain Caecum Coagulating gland Colon Diaphragm Duodenum Epididymides Eyes Femur, joint Harderian gland Head (fixed only) Heart lleum Jejunum Kidneys Lacrimal gland Larynx	Liver Lungs Lymph nodes (cervical & Mesenteric) Mammary gland (females) Nose/turbinates Oesophagus Optic nerve Ovaries Oviducts Pancreas Pharynx Pinnae[1] Pituitary Prostate Rectum Salivary glands (parotid, sublingual + submaxillary)	Sciatic nerve Skeletal muscle Skin + subcutis Spinal cord (3 levels) Spleen Sternum Stomach Seminal vesicle Testes Thymus Thyroid + parathyroid Tongue Trachea Urinary bladder Uterus[2] Vagina Any other tissue showing macroscopic abnormalities

[1] Taken for identification purposes only; not examined
[2] Uterine horns and cervix uteri examined

A bone marrow smear was taken from all animals and fixed subsequently at staining.

7. Histopathology

Tissue processing:

Following fixation, nominal 5 µm sections of all organs and tissues from all main study animals in the control and high dose groups were prepared and stained with haematoxylin and eosin. Cryostat liver sections were stained with Oil Red O to identify and quantify the presence of any fat deposits.

In addition, the liver and liver Oil Red O sections, kidneys, lungs, adrenals, bone joint and bone marrow from the femur and sternum, respectively, were examined from all animals in the low and intermediate dose groups. The caecum of females in all the dose groups was also examined. Any macroscopic abnormalities were examined from all dose groups except off-dose animals.

Microscopic examination:

Tissue sections were examined for histopathological changes by using a light microscope. Findings were entered directly onto a computerised histopathology database, which subsequently generated summary tables.

Sections from the caecum, bone (stifle joint, femur and tibia), and sternal bone marrow were peer reviewed by J.P. Finn of Precision Histology International, London Road, Harleston, Norfolk, UK.

II. Results and Discussion

A. Results

1. Clinical results

At 20,000 ppm, treatment-related clinical signs included hair loss in both sexes, body soiling and loss of coat condition in males only, and a soiled urogenital region in females. There were no treatment-related clinical signs at the lower dose levels.

Treatment-related clinical signs that persisted during the off-dose period were hair loss in both sexes and urogenital soiling in females. Two mortalities in this study were incidental and unrelated to treatment.

2. Body weight and food and water intake

Body weight

At 20,000 ppm, absolute body weight after 13 weeks of treatment (Day 92) was reduced by 30% and 18% compared to the controls in males and females, respectively. Body weight gain over the course of treatment (Day 1-92) was reduced by 41% in males and 29% in females, when compared to the controls. This effect was most severe during week 1 of treatment (Day 1-8: -98% in males, -95% in females).

There were no relevant effects on body weight or body weight gain at the lower dose levels.

At the end of the recovery period (Day 120), body weight gain of both sexes was considerably greater than the controls (+111% for males, +300% for females).

Table 3.12.1.4- 8: Mean body weight and body weight gain (g)

	Dose (ppm)			
	0	**100**	**1,400**	**20,000**
MAIN PHASE				
Males				
Body weight [g] *(% difference to control)*				
Day 1	134	137 *(+2.2)*	136 *(+1.5)*	135 *(+0.7)*
Day 8	192	194 *(+1.0)*	183 *(-4.7)*	136** *(-29.2)*
Day 15	252	254 *(+0.8)*	238 *(-5.6)*	175** *(-37.7)*
Day 22	307	308 *(+0.3)*	293 *(+4.6)*	212** *(-30.9)*
Day 29	346	346 *(±0.0)*	323* *(-6.6)*	235** *(-32.1)*
Day 36	385	381 *(+1.0)*	365 *(-5.2)*	268** *(-30.4)*
Day 43	418	414 *(-1.0)*	396 *(-5.3)*	290** *(-30.6)*
Day 50	443	436 *(-1.6)*	419 *(-5.4)*	306** *(-30.9)*
Day 57	461	463 *(+0.4)*	443 *(-3.9)*	325** *(-29.5)*
Day 64	483	480 *(-0.6)*	458 *(-5.2)*	334** *(-30.8)*
Day 71	501	500 *(-0.2)*	475 *(-5.2)*	342** *(-31.7)*
Day 78	520	520 *(±0.0)*	492 *(-5.4)*	358** *(-31.2)*
Day 85	528	523 *(-0.9)*	495 *(-6.3)*	366** *(-30.7)*
Day 92	543	533 *(-1.8)*	512 *(-5.7)*	378** *(-30.4)*

	Dose (ppm)			
	0	**100**	**1,400**	**20,000**
Body weight gain [g] *(% difference to control)* +				
Day 1-8	58	57 *(-1.7)*	47 *(-19.0)*	1 *(-98.3)*
Day 1-15	118	117 *(-0.8)*	102 *(-13.6)*	40 *(-66.1)*
Day 1-22	173	171 *(-1.2)*	157 *(-9.2)*	77 *(-55.5)*
Day 1-92	409	396 *(-3.2)*	376 *(-8.1)*	243 *(-40.6)*
Females				
Body weight [g] *(% difference to control)*				
Day 1	125	125 *(±0.0)*	124 *(-0.8)*	123 *(-1.6)*
Day 8	164	162 *(-1.2)*	160 *(-2.4)*	125** *(-23.8)*
Day 15	190	189 *(-0.5)*	187 *(-1.6)*	153## *(-19.5)*
Day 22	211	212 *(+0.5)*	211 *(±0.0)*	176** *(-16.6)*
Day 29	229	230 *(+0.4)*	228 *(-0.4)*	193** *(-15.7)*
Day 36	248	247 *(-0.4)*	248 *(±0.0)*	208** *(-16.1)*
Day 43	261	259 *(-0.8)*	260 *(-0.4)*	220** *(-15.7)*
Day 50	270	270 *(±0.0)*	267 *(-1.1)*	227** *(-15.9)*
Day 57	282	282 *(±0.0)*	280 *(-0.7)*	235** *(-16.7)*
Day 64	287	287 *(±0.0)*	283 *(-1.4)*	237** *(-17.4)*
Day 71	293	292 *(-0.3)*	288 *(-1.7)*	240## *(-18.1)*
Day 78	299	300 *(+0.3)*	298 *(-0.3)*	246## *(-17.7)*
Day 85	302	300 *(-0.7)*	297 *(-1.7)*	247## *(-18.2)*
Day 92	306	304 *(-0.7)*	300 *(-2.0)*	251## *(-18.0)*
Body weight gain [g] *(% difference to control)* +				
Day 1-8	39	37 *(-5.1)*	36 *(-7.7)*	2 *(-94.9)*
Day 1-15	65	64 *(-1.5)*	63 *(-3.1)*	30 *(-53.8)*
Day 1-22	86	87 *(+1.2)*	87 *(+1.2)*	53 *(-38.4)*
Day 1-92	181	179 *(-1.1)*	176 *(-2.8)*	128 *(-29.3)*
RECOVERY PHASE				
Males				
Body weight [g] *(% difference to control)*				
Day 99	564	-	-	406** *(-28.0)*
Day 106	579	-	-	423** *(-26.9)*
Day 113	582	-	-	436** *(-25.1)*
Day 120	583	-	-	446** *(-23.5)*
Body weight gain [g] *(% difference to control)* +				
Day 99-120	19	-	-	40 *(+110.5)*
Females				
Body weight [g] *(% difference to control)*				
Day 99	321	-	-	258** *(-19.6)*
Day 106	326	-	-	263** *(-19.3)*
Day 113	326	-	-	265** *(-18.7)*
Day 120	323	-	-	266** *(-17.6)*
Body weight gain [g] *(% difference to control)* +				
Day 99-120	2	-	-	8 *(+300)*

* / **: $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet´s test
# / ##: $p < 0.05$ / $p < 0.01$, statistically different to control using modified t-test
+*:* No statistical analyses were performed for body weight gains; - *:* Dose levels not analysed during recovery phase

Food and water intake

At the end of the treatment period (Week 13), mean food consumption of the 20,000 ppm dose group animals was reduced by 17% in males and 15% in females, when compared to controls. This effect was more severe during the first week of treatment, when food consumption was reduced by 54% and 48% compared to controls for males and females, respectively.

At the lower dose levels, no effects on food consumption were observed.

At the end of the recovery period (Week 17), the mean food intake of males of the 20,000 ppm dose group was slightly reduced (-4% compared to control) whilst the mean food intake of females was similar to that of the control animals.

Table 3.12.1.4- 9: Mean food consumption (g/animal/day) (% difference to control)

	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
MAIN PHASE				
Males				
Week 1	26	26 *(±0.0)*	24* *(-7.7)*	12** *(-53.8)*
Week 4	30	29 *(-3.3)*	29 *(-3.3)*	24** *(-20.0)*
Week 8	29	28 *(-3.4)*	28 *(-3.4)*	24** *(-17.2)*
Week 13	29	27 *(-6.9)*	28 *(-3.4)*	24** *(-17.2)*
Females				
Week 1	21	21 *(±0.0)*	21 *(±0.0)*	11** *(-47.6)*
Week 4	21	21 *(±0.0)*	21 *(±0.0)*	18** *(-14.3)*
Week 8	21	21 *(±0.0)*	21 *(±0.0)*	18** *(-15.0)*
Week 13	20	21 *(+5.0)*	20 *(±0.0)*	17** *(-15.0)*
RECOVERY PHASE				
Males				
Week 17	24	-	-	23 *(-4.2)*
Females				
Week 17	17	-	-	17 *(±0.0)*

* $p < 0.05$, ** $p < 0.01$, statistically different to control using Dunnett's test

- : Dose levels not analysed during recovery phase

At 20,000 ppm, mean food conversion ratios over the course of the study (mean values Week 1-13) were reduced by 28% and 19% compared to controls in males and females, respectively.

Table 3.12.1.4- 10: -Mean food conversion (%)

	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Week 1	32.0	31.4	29.1	0.6
Week 2	29.5	29.8	27.9	26.1
Week 3	25.9	26.1	26.7	22.1
Week 4	18.8	18.3	14.8	13.2
Week 5	17.8	17.2	19.6	16.9
Week 6	16.4	17.0	16.0	13.4
Week 7	12.4	11.6	11.5	9.8
Week 8	8.6	13.6	12.4	11.2
Week 9	10.9	9.0	7.8	5.6
Week 10	8.7	10.4	8.4	5.2
Week 11	9.8	10.2	9.1	10.0
Week 12	3.7	2.0	1.3	5.0
Week 13	7.5	4.9	9.0	6.7
Mean (Week 1-13) *(% difference to control)*	**15.54**	**15.50** ***(-0.3)***	**14.89** ***(-4.2)***	**11.22** ***(-27.8)***
Females				
Week 1	26.3	25.4	24.4	2.6
Week 2	18.1	18.6	18.5	21.6
Week 3	15.4	16.3	16.5	17.4
Week 4	12.4	11.9	11.4	12.9
Week 5	12.2	10.8	12.7	10.9
Week 6	9.1	8.2	7.8	9.6
Week 7	6.2	7.0	4.9	5.2
Week 8	7.7	8.9	9.3	6.9
Week 9	3.7	3.5	2.1	1.3
Week 10	4.6	4.1	3.9	3.3
Week 11	4.4	5.4	6.5	5.3
Week 12	2.2	0.3	-0.7	0.6
Week 13	2.4	2.7	2.1	3.0
Mean (Week 1-13) *(% difference to control)*	**9.59**	**9.47** ***(-1.2)***	**9.18** ***(-4.3)***	**7.74** ***(-19.3)***

At 20,000 ppm, water intake of females was increased by 44% compared to control during Week 4 of treatment only. At lower doses (1,400 and 100 ppm), water consumption of females remained unaffected. In males, no effects on water consumption were observed at any dose level.

Table 3.12.1.4- 11: Mean water consumption (g/animal/day) (% difference to control)

	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Week 4	29	29 *(±0.0)*	32 *(+10.3)*	30 *(+3.4)*
Week 8	32	31 *(-3.1)*	39 *(+21.9)*	31 *(-3.1)*
Week 11	31	32 *(+3.2)*	38 *(+22.6)*	31 *(±0.0)*
Females				
Week 4	23	27 *(+17.4)*	28 *(+21.7)*	33** *(+43.5)*
Week 8	30	30 *(±0.0)*	32 *(+6.7)*	36 *(+20.0)*
Week 11	27	28 *(+3.7)*	30 *(+11.1)*	31 *(+14.8)*

* $p < 0.05$, ** $p < 0.01$; statistically different to control using Dunnett's test

3. Ophthalmoscopic examinations

There were no treatment-related ophthalmic lesions.

4. Neurotoxicity assessments

Functional Observations Battery:

There were no treatment-related effects on the functional integrity or behaviour of the animals.

Grip Strength:

There were no treatment-related findings.

Motor activity:

There were no treatment-related findings.

5. Laboratory investigations

Haematology:

At 20,000 ppm, haemoglobin concentration, haematocrit, mean cell haemoglobin and mean cell haemoglobin concentration were slightly decreased in both sexes whilst activated partial thromboplastin time was slightly increased in males only when compared with controls.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or the values were within the normal range of historical controls.

After 4 weeks off-dose, the previously affected haematological values in both sexes were mostly comparable to controls.

Table 3.12.1.4- 12: Haematological results in males and females at termination and at the end of the recovery period *(% difference to control)*

	Dose level (ppm)						
	0	**100**		**1,400**		**20,000**	
Males							
Week 13							
Haemoglobin (g/L)	152	154	*(+1.3)*	145*	*(-4.6)*	143*	*(-5.9)*
Haematocrit (L/L)	0.45	0.45	*(±0.0)*	0.43*	*(-4.4)*	0.43*	*(-4.4)*
MCH (pg)	17.9	18.3	*(+2.2)*	17.5	*(-2.2)*	17.0**	*(-5.0)*
MCHC (G/L)	342	341	*(-0.3)*	338	*(-1.2)*	332**	*(-2.9)*
Recovery - week 17							
Haemoglobin (g/L)	152	-	-	-	-	147	*(-3.3)*
Haematocrit (L/L)	0.44	-	-	-	-	0.43	*(-2.3)*
MCH (pg)	17.6	-	-	-	-	17.4	*(-1.3)*
MCHC (G/L)	348	-	-	-	-	344*	*(-1.1)*
Females							
Week 13							
Haemoglobin (g/L)	151	152	*(+0.7)*	148	*(-2.0)*	137***	*(-9.3)*
Haematocrit (L/L)	0.43	0.44	*(+2.3)*	0.43	*(±0.0)*	0.41**	*(-4.7)*
MCH (pg)	18.8	18.7	*(-0.5)*	18.9	*(+0.5)*	17.1***	*(-9.0)*
MCHC (G/L)	349	350	*(+0.3)*	346	*(-0.9)*	340**	*(-2.6)*
Recovery - week 17							
Haemoglobin (g/L)	149	-	-	-	-	145	*(-3.4)*
Haematocrit (L/L)	0.42	-	-	-	-	0.42	*(±0.0)*
MCH (pg)	18.8	-	-	-	-	18.3*	*(-2.7)*
MCHC (G/L)	354	-	-	-	-	349*	*(-1.4)*

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, statistically different to control
- : Dose levels not analysed during recovery phase

Clinical chemistry:

At 20,000 ppm, there was a slight increase in cholesterol concentration, total protein and gamma-glutamyl transferase activity in males and females.

At 1,400 ppm, there was a slight increase in cholesterol concentration for males only.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or the values were within the normal range of historical controls.

After 4 weeks off-dose, all previously affected clinical chemistry values in both sexes were comparable to controls.

Table 3.12.1.4- 13: Selected biochemistry results at the end of the study and recovery period *(% difference to control)*

Week	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Week 13				
Total protein (g/L)	64.3	65.7 *(+2.2)*	66.0 *(+2.6)*	70.5** *(+9.6)*
Cholesterol (mmol/L)	1.63	1.94* *(+19.0)*	2.23*** *(+36.8)*	3.63*** *(+122.7)*
Recovery - Week 17				
Total protein (g/L)	63.8	-	-	62.6 *(-1.9)*
Cholesterol (mmol/L)	1.75	-	-	1.46* *(-16.6)*
Females				
Week 13				
Total protein (g/L)	68.4	67.0 *(-2.0)*	69.5 *(+1.6)*	73.5** *(+7.5)*
Cholesterol (mmol/L)	2.11	2.11 *(±0.0)*	2.26 *(+7.1)*	3.51*** *(+66.4)*
Recovery - Week 17				
Total protein (g/L)	64.6	-	-	66.6 *(+3.1)*
Cholesterol (mmol/L)	1.89	-	-	2.14 *(+13.2)*

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, statistically different to control
- : Dose levels not analysed during recovery phase

Urinalysis:

At 20,000 ppm, there was a slight increase in the number of epithelial cells in the urinary sediment of males only. There was a slight increase in urinary volume and a slight decrease in specific gravity in females only.

At 1,400 ppm there was a slight increase in the number of epithelial cells in the urinary sediment of males only. There was a slight increase in urinary volume and a slight decrease in specific gravity in females only.

There were no treatment-related effects at 100 ppm in either sex.

After 4 weeks off-dose, the urinary volume of females was still slightly increased when compared with conrols. All other previously affected urinalysis parameters were comparable to controls.

4. Sacrifice and pathology

Necropsy:

At 20,000 ppm, 4/10 males killed after 13 weeks had a speckled appearance in both kidneys. At 1,400 ppm, 3/10 males killed after 13 weeks had a speckled appearance in both kidneys, with a further 2/10 and 1/10 males having a speckled appearance in the right and left kidneys, respectively.

At 100 ppm, speckled kidney was observed in one male animal (right). Due to its isolated occurance and in the absence of any correlating histopathological findings this finding is considered incidental and not adverse. Therefore, no treatment-related macroscopic abnormalities were observed at this dose level.

In females, no necropsy kidney findings were observed at any dose level.

After 4 weeks off-dose 3/10 males previously given 20,000 ppm were found to have a speckled appearance in both kidneys, with a further 1/10 males having a speckled appearance in the right kidney.

Table 3.12.1.4- 14: Necropsy findings - kidney (13 weeks) – Number of animals affected

Finding	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Speckled kidney - left	0	0	4	4
Speckled kidney - right	0	1	5	4
Total	**0**	**1**	**9**	**8**
Females				
Speckled kidney - left	0	0	0	0
Speckled kidney - right	0	0	0	0
Total	**0**	**0**	**0**	**0**

Organ weights:

At 20,000 ppm, absolute liver weight was increased by 22% compared to control in females only whilst relative liver was increased by 51% in males and 49% in females compared to control. Absolute spleen weight was decreased by 45% and 40% in males and females, respectively, and relative spleen weight relative was decreased by 24% in males and 29% in females compared to control.

In addition, several absolute organs weights were statistically significantly decreased while relative weights were increased or not changed (adrenals, kidney, epididymis, testes, ovaries). Therefore, these organ weight changes are considered as an indirect effect of the markedly reduced bodyweight at the hightest dose level.

At 1,400 ppm, liver weight relative to body weight was increased by 15% in males only, when compared to controls. Absolute spleen weight was decreased by 10% and 16% in males and females, respectively whilst for females only, spleen weight relative to body weight was decreased by 19% compared to control. Relative kidney weight was increased by 11% in males only.

At 100 ppm, no effects on organ weight were noted in either sex.

After 4 weeks off-dose, there was a trend for a reversal of the effects on the liver and spleen weights of males and females and the kidney weights of males.

Table 3.12.1.4- 15: Mean absolute and relative organ weights (% difference to control)

		Dose level (ppm)							
		0	**100**		**1,400**		**20,000**		
Males									
Week 13									
Terminal body weight (g)		527.0	526.5	*(-0.1)*	501.6	*(-4.8)*	355.5	*(-32.5)*	
Adrenals	abs. (g)	0.061	0.062	*(-1.6)*	0.053	*(-13.1)*	0.042**$	*(-31.1)*	
	rel. (%)	0.012	0.012	*(±0.0)*	0.011	*(-8.3)*	0.012	*(±0.0)*	
Liver	abs. (g)	16.44	15.99	*(-2.7)*	17.99	*(+9.4)*	16.75	*(+1.9)*	
	rel. (%)	3.13	3.02	*(-3.5)*	3.59**	*(+14.7)*	4.71**	*(+50.5)*	
Kidney	abs. (g)	2.82	2.77	*(-1.8)*	3.02	*(+7.1)*	2.20**$	*(-22.0)*	
	rel. (%)	0.54	0.53	*(-1.9)*	0.60*	*(+11.1)*	0.62**$	*(+14.8)*	
Spleen	abs. (g)	0.87	0.88	*(+1.1)*	0.78	*(+10.3)*	0.48**	*(-44.8)*	
	rel. (%)	0.17	0.17	*(±0.0)*	0.16	*(-5.9)*	0.13*	*(-23.5)*	
Testes	abs. (g)	3.73	3.66	*(-1.9)*	3.34*	*(-10.5)*	3.47	*(-7.0)*	
	rel. (%)	0.71	0.70	*(-1.4)*	0.67	*(-5.6)*	0.98**$	*(+38.0)*	
Epididymis	abs. (g)	1.285	1.257	*(-2.2)*	1.218	*(-5.2)*	1.142*$	*(-11.1)*	
	rel. (%)	0.245	0.241	*(-1.6)*	0.245	*(±0.0)*	0.322**$	*(+31.4)*	
Recovery – Week 17									
Terminal body weight (g)		575.9	-	-	-	-	442.2	*(-23.2)*	
Adrenals	abs. (g)	0.059	-	-	-	-	0.045**$	*(-23.7)*	
	rel. (%)	0.010	-	-	-	-	0.010	*(±0.0)*	
Liver	abs. (g)	16.98	-	-	-	-	13.98**$	*(-17.7)*	
	rel. (%)	2.95	-	-	-	-	3.15[#]	*(+6.8)*	
Kidney	abs. (g)	2.99	-	-	-	-	2.45**$	*(-18.1)*	
	rel. (%)	0.52	-	-	-	-	0.55	*(+5.8)*	
Spleen	abs. (g)	0.88	-	-	-	-	0.67**$	*(-23.9)*	
	rel. (%)	0.15	-	-	-	-	0.15	*(±0.0)*	
Testes	abs. (g)	3.47	-	-	-	-	3.56	*(+2.6)*	
	rel. (%)	0.61	-	-	-	-	0.81**$	*(+32.8)*	
Epididymis	abs. (g)	1.366	-	-	-	-	1.225	*(-10.3)*	
	rel. (%)	0.239	-	-	-	-	0.280*$	*(+17.2)*	

		Dose level (ppm)						
		0	**100**		**1,400**		**20,000**	
Females								
Week 13								
Terminal body weight (g)		301.1	301.9	*(±0.0)*	297.3	*(-1.3)*	247.7	*(-17.7)*
Adrenals	abs. (g)	0.072	0.074	*(+2.8)*	0.080	*(+11.1)*	0.060*$	*(-16.7)*
	rel. (%)	0.024	0.024	*(±0.0)*	0.027	*(+12.5)*	0.024	*(±0.0)*
Liver	abs. (g)	9.68	9.54	*(-1.4)*	10.03	*(+3.6)*	11.79**	*(+21.8)*
	rel. (%)	3.21	3.16	*(-1.6)*	3.37	*(+5.0)*	4.77**	*(+48.6)*
Kidney	abs. (g)	1.84	1.76	*(-4.3)*	1.86	*(+1.1)*	1.52**$	*(-17.4)*
	rel. (%)	0.62	0.58	*(+6.5)*	0.63	*(+1.6)*	0.61	*(-1.6)*
Spleen	abs. (g)	0.62	0.61	*(-1.6)*	0.52#	*(-16.1)*	0.37##	*(-40.3)*
	rel. (%)	0.21	0.20	*(-4.8)*	0.17**	*(-19.0)*	0.15**	*(-28.6)*
Ovaries	abs. (g)	0.125	0.150*	*(+20.0)*	0.130	*(+4.0)*	0.101*$	*(-19.2)*
	rel. (%)	0.042	0.050	*(+19.0)*	0.044	*(+4.8)*	0.041	*(-2.4)*
Uterus	abs. (g)	0.79	0.63	*(-17.7)*	0.90	*(+13.9)*	0.53	*(-32.9)*
	rel. (%)	0.27	0.21	*(-22.2)*	0.31	*(+14.8)*	0.21	*(-22.2)*
Recovery – Week 17								
Terminal body weight (g)		320.7	-	-	-	-	266.3	*(-17.0)*
Adrenals	abs. (g)	0.079	-	-	-	-	0.058**$	*(-26.6)*
	rel. (%)	0.025	-	-	-	-	0.022	*(-12.0)*
Liver	abs. (g)	10.04	-	-	-	-	9.26	*(-7.8)*
	rel. (%)	3.13	-	-	-	-	3.48**$	*(+11.2)*
Kidney	abs. (g)	1.90	-	-	-	-	1.65**$	*(-13.2)*
	rel. (%)	0.60	-	-	-	-	0.62	*(+3.3)*
Spleen	abs. (g)	0.61	-	-	-	-	0.51*$	*(-16.4)*
	rel. (%)	0.19	-	-	-	-	0.19	*(±0.0)*
Ovaries	abs. (g)	0.142	-	-	-	-	0.128	*(-9.9)*
	rel. (%)	0.045	-	-	-	-	0.048	*(+6.7)*
Uterus	abs. (g)	0.63	-	-	-	-	0.66	*(+4.8)*
	rel. (%)	0.20	-	-	-	-	0.25	*(+25.0)*

* / **: $p \leq 0.05$ / $p \leq 0.01$, statistically different from control (Dunnett's test)
# / ##: $p \leq 0.05$ / $p \leq 0.01$, statistically different from control (modified T-test)
$: considered secondary to decreased bodyweight
abs.: absolute organ weight
rel.: relative organ weight (body weight ratio, %)
- : Dose levels not analysed during recovery phase

6. Histopathology

Histopathological changes were seen in the adrenals, bone joint and bone marrow, liver and kidneys.

Adrenals:

At 20,000 ppm, there was an increase in the severity and incidence of hypertrophy of the zona glomerulosa of the adrenal cortex. There were no treatment-related histopathological changes in either sex at 100 or 1,400 ppm.

Following 4 weeks off-dose the severity of hypertrophy had decreased in both sexes.

Table 3.12.1.4- 16: Adrenal findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	**100**	**1,400**	**20,000**	**0**	**100**	**1,400**	**20,000**
Hypertrophy of the zona glomerulosa								
After 13 weeks:								
- minimal	1	0	0	0	1	1	3	3
- slight	0	0	0	7	0	0	0	7
Total	**1/10**	**0/10**	**0/10**	**7/10**	**1/10**	**1/10**	**3/10**	**10/10**
After off-dose period								
- minimal	0	-	-	8	1	-	-	4
- slight	0	-	-	0	0	-	-	1
Total	**0/10**	**-**	**-**	**8/10**	**1/10**	**-**	**-**	**5/10**

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Liver:

At 20,000 ppm, minimal to moderate hypertrophy of centrilobular hepatocytes was seen in 9/10 males and 8/10 females.

At 1,400 ppm minimal to slight hypertrophy of centrilobular hepatocytes was present in 8/10 males only.

There were no treatment-related histopathological changes in females at 1,400 ppm, or in either sex at 100 ppm.

Following 4 weeks off-dose, a complete reversibility of all liver effects was seen.

Table 3.12.1.4- 17: Liver findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	**100**	**1,400**	**20,000**	**0**	**100**	**1,400**	**20,000**
Centrilobular hepatocytic hypertrophy								
After 13 weeks:								
- minimal	0	0	3	1	0	0	0	4
- slight	0	0	5	0	0	0	0	4
- moderate	0	0	0	8	0	0	0	0
Total	**0/10**	**0/10**	**8/10**	**9/10**	**0/10**	**0/10**	**0/10**	**8/10**
After off-dose period								
- Not present	10	-	-	10	10	-	-	10

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Bone joint and bone marrow:

At 20,000 ppm, an increase in the severity and incidence of trabecular hyperostosis in the bone joint and a decreased cellularity in the bone marrow for 7/10 males and 9/10 females was recorded.

At 1,400 ppm, there was an increase in the severity and incidence of trabecular hyperostosis in the bone joint for females only.

There were no treatment-related histopathological changes in males at 1,400 ppm, or in either sex at 100 ppm.

Following 4 weeks off-dose, there was a full or partial recovery of the effects seen during the treatment period.

Table 3.12.1.4- 18: Bone joint and bone marrow findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	**100**	**1,400**	**20,000**	**0**	**100**	**1,400**	**20,000**
Trabecular hyperostosis in the bone								
After 13 weeks:								
- minimal	0	0	0	2	3	1	2	1
- slight	0	0	0	3	0	0	3	4
- moderate	0	0	0	2	0	0	3	4
Total	**0/10**	**0/10**	**0/10**	**7/10**	**3/10**	**1/10**	**8/10**	**9/10**
After off-dose period								
- minimal	0	-	-	0	0	-	-	3
- slight	0	-	-	0	1	-	-	3
- moderate	0	-	-	0	0	-	-	1
Total	**0/10**	**-**	**-**	**0/10**	**1/10**	**-**	**-**	**7/10**
Decreased cellularity in the bone marrow								
After 13 weeks:								
- minimal	0	1	0	3	3	0	0	4
- slight	0	0	0	4	0	0	0	5
Total	**0/10**	**1/10**	**0/10**	**7/10**	**3/10**	**0/10**	**0/10**	**9/10**
After off-dose period								
- minimal	0	-	-	3	4	-	-	2
- slight	0	-	-	0	0	-	-	3
Total	**0/10**	**-**	**-**	**3/10**	**4/10**	**-**	**-**	**5/10**

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Kidneys:

At 20,000 ppm, there were a number of kidney effects in the males consisting of an increase in incidence and severity of accumulation of hyaline droplets, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 1,400 ppm, in males only, there was an increase in incidence and severity accumulation of hyaline droplets, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 100 ppm, no treatment-related changes were observed in males.

In females, there were no treatment-related histopathological kidney changes at any dose level.

Following 4 weeks off-dose, there was a partial recovery of all effects seen previously.

Table 3.12.1.4- 19: Kidney findings – Number of animals affected

Finding / Severity	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Accumulation of hyaline droplets in the proximal tubule				
After 13 weeks:				
- minimal	1	0	0	0
- slight	5	0	0	0
- moderate	0	2	1	1
- severe	0	0	9	7
Total	**6/10**	**2/10**	**10/10**	**8/10**
After off-dose period				
- minimal	1	-	-	1
- slight	4	-	-	4
- moderate	2	-	-	2
Total	**7/10**	**-**	**-**	**5/10**
Single cell death – proximal tubule epithelium				
After 13 weeks:				
- minimal	5	0	0	1
- slight	0	2	1	1
- moderate	0	0	9	6
Total	**5/10**	**2/10**	**10/10**	**8/10**
After off-dose period				
- minimal	4	-	-	2
- slight	2	-	-	2
Total	**6/10**	**-**	**-**	**4/10**
Foci of basophilic (regenerating) tubules				
After 13 weeks:				
- minimal	6	3	0	1
- slight	0	3	4	7
- moderate	0	0	6	0
Total	**6/10**	**6/10**	**10/10**	**8/10**
After off-dose period				
- minimal	7	-	-	3
- slight	0	-	-	5
Total	**7/10**	**-**	**-**	**8/10**
Granular casts				
After 13 weeks:				
- minimal	0	0	1	1
- slight	0	0	0	3
Total	**0/10**	**0/10**	**1/10**	**4/10**
After off-dose period				
- Not present	10	-	-	10

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

III. Conclusion

The NOAEL in the 90-day dietary study in rats was 100 ppm (7.4 or 8.4 mg/kg bw/day in male and female rats respectively) based on treatment-related haematological (reduced haemoglobin and haematocrit in male rats), clinical chemistry (increased cholesterol in male rats at 1,400 ppm), urinalysis (increased urine volume and specific gravity in females at ≥ 1,400 ppm), organ weight (increase in relative liver and kidney weight in male rats and decreased of relative spleen weight in females at 1,400 ppm) and histopathological changes in the liver and kidneys at 1,400 ppm (equivalent 109 or 119 mg/kg bw/day in males and females respectively).

Following a 4-week off-dose period, there was a full or partial recovery of all treatment-related changes.

3.12.1.5 Anonymous; 2006; M-205579-02-1

Study reference:

Anonymous; 2006; AE C638206 - 90-Day toxicity study in the mouse by dietary administration; M-205579-02-1

Deviations:

Deviations from the current OECD guideline (408, 2018):

- Ophthalmological examinations were not conducted
- Sensory reactivity to stimuli were not performed
- Blood/plasma samples were not taken for possible analysis of thyroid hormones
- At termination, an evaluation of oestrus cycle was not performed
- Prostate and seminal vesicles with coagulating glands were not weighed at termination

Although performed in the rat, rather than the mouse, ophthalmoscopy and sensory reactivity to stimuli assessments were performed in the 90-day rat study (Anonymous; 2000; M-197622-01-1) at doses of up to 20,000 ppm (1,671 mg/kg bw/day, combined sexes); whilst seminal vesicles with coagulating glands were weighed at termination in the rat two-generation study (Anonymous; 2003; M-232532-01-1) as was an evaluation of oestros cycles at dose levels of up to 2,000 ppm (equivalent to approx. 100 mg/kg bw/day in F0 females throughout the 10-week pre-mating period and during gestation and lactation).

Therefore, these deviations do not affect the overall acceptability of the study

Executive Summary:

Fluopicolide (batch number OP2050046: a fine, beige colored powder, 95.9% purity) was administered continuously via the dietary to groups of C57BL/6JICO mice (10/sex/group) at concentrations of 50, 200, 800 and 3,200 ppm for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control. This additional 90-day mouse study was planned and performed as dose-range finder for the mouse carcinogenesis study with the same strain of mice (C57BL/6).

There were four animals found dead or sacrificed moribund between Day 6 and 73, one male from each of the dose groups 0, 200 and 3,200 ppm and one female at 800 ppm. In addition, one male at 0 ppm, two males at 800 ppm and one male at 3,200 ppm, died due to accidental trauma between Day 21 and 85. On Day 94, one control female and one female at 50 ppm died during anesthesia. All the unscheduled deaths were considered to be incidental and not related to treatment. There were no treatment-related clinical signs.

Overall body weight gain was slightly reduced in both males (-7%) and females (-14%) at 3,200 ppm. The effect on body weight gain was most marked during Week 1 for males (-88%; statistically significant) and during Week 1 and 2 for females (-87% at Week 1, -85% at Week 2; statistically significant). Absolute body weight after one week of treatment (Day 8) was statistically significantly reduced at the high dose level by 10% and 7% in males and females, respectively. At study termination (Day 90), the reduction was only 3% in both sexes.

There was no clear treatment-related effect on food consumption.

Clinical chemistry assessment revealed a slight decrease in total cholesterol concentration in both sexes at 3,200, 800 and 200 ppm. A very slight decrease in albumin concentration was observed in both sexes at 3,200 and 800 ppm. Mean alkaline phosphatase activity was increased in males at 3,200 ppm.

Terminal body weight was slightly, but statistically significantly reduced for males at 3,200 ppm (-5%). Mean absolute and relative liver weights were increased in a dose-related manner, in both sexes at 3,200 and 800 ppm compared to control.

Macroscopic examination of decedent animals revealed no treatment-related changes. At the terminal sacrifice dark liver was noted in 4/8 males and 9/10 females at 3,200 ppm. At microscopic examination, diffuse centrilobular hepatocellular hypertrophy was observed in one decedent male at 3,200 ppm and one decedent female at 800 ppmas well as in surviving in all high dose animals and the majority of animals at 800 ppm. The severity ranged from slight in one animal to mild in the reminder of animals at 3,200 ppm and slight in all animals at 800 ppm from the terminal sacrifice.

At the request of the Japanese Food Safety Commission, additional Proliferating Cell Nuclear Antigen (PCNA) staining on selected liver slides was conducted. The result of this additional evaluation showed that there was no relevant change in hepatocytic cell cycling for males or females at 3,200 ppm fluopicolide, when compared to controls.

The No Observed Adverse Effect Level in this 90-day dietary study in mice was 50 ppm (equivalent 10.4 and 12.8 mg/kg bw/day in males and females, respectively) based on apparent significant treatment-related reduction in cholesterol suggestive of impaired liver function at ≥ 200 ppm (equivalent 37.8 and 52.8 mg/kg bw/day in males and females, respectively). The liver was the main target organ in the study as indicated by reduced albumin and cholesterol levels, increased relative liver weights and centrilobular hepatocyte hypertrophy observed at dose levels of ≥ 800 ppm.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	C57BL/6JICO
Age:	5 weeks old
Weight at start:	12.7 to 19.5 g for males and 11.6 to 16.6 g for females
Source:	Iffa-Credo, St Germain-sur-L'Arbresle, France
Acclimation period:	Yes
Diet:	Certified rodent powder diet "M 20 controle" (Pietrement, Provins, France)
Water:	Water ad lib
Housing:	Mice were housed in suspended stainless steel wire mesh cages, in groups of 3 on the day of arrival until Day 6 of the pre-study, when they were housed individually
Temperature:	20 – 24 °C
Humidity:	40% - 70%
Air changes:	10 to 15 air changes per hour (average, not monitored)
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 6th 2000 to March 15th 2001

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 3.12.1.5- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Treatment phase			
1	0	10	10
2	50	10	10
3	200	10	10
4	800	10	10
5	3,200	10	10

On Study Days 91, 92, 93 or 94, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to sacrifice.

3. Diet preparation and analysis

The test substance was incorporated into the diet to provide the required concentrations. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. Diet formulations were prepared twice during the course of the study, for each concentration. When not in use the diet formulations were stored at below -15 °C.

The homogeneity and stability of the test substance in the diet were demonstrated in the pre-study mix of 25 and 10,000 ppm for stability where samples of this diets were kept either at ambient temperature up to 56 days or frozen for 7 weeks at below -15 °C and ambient temperature for 1 week. The homogeneity of fluopicolide in the diet was verified for the lowest and highest concentrations from study diet mix 1 to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified for each concentration.

Homogeneity of diet formulations with fluopicolide was found to be acceptable at concentrations of 25, 50, 3,200 and 10,000 ppm. Fluopicolide was found to be stable for a 7-week frozen period at below -15 °C and one week at ambient temperature. At 10,000 ppm, fluopicolide was also stably stored at ambient temperature for at least 56 days but the stability at 25 ppm stored at ambient temperature was poorer than for frozen storage. All concentration checks were in a range of 94% to 104% of the nominal values.

4. Statistics

The following variables were analysed:

- body weight parameters
- food consumptions
- clinical chemistry parameters
- organ weight parameters
- organ/body weight ratios
- organ/brain weight ratios

Statistical analysis:

Means and standard deviations (STD) were calculated for each sex separately for each group at each time period. Results of the clinical pathology and organ weight parameters were inter-compared for the treated and control groups by use of:

- Bartlett's test for homogeneity of variances between groups.
- When Bartlett's test indicated homogeneous variances, any significant differences were identified by using the combination of Analysis of Variance (ANOVA) and Dunnett's test.
- When Bartlett's test indicated heterogeneous variances, any significant differences were identified by using a modified t-test.

For body weight and food intake data Dunnett's test was used.

All tests were performed at 5% and 1% levels. All calculations and statistical analysis were performed with Xybion PathTox system (version 4.2.2).

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight and food intake

Each animal was weighed once during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period and before necropsy.

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The mean achieved dosage intake in mg/kg bw/day for each week and for Weeks 1 to 13 was calculated.

3. Laboratory investigations (clinical chemistry)

On Study Days 91, 92, 93 or 94, in the morning, prior to necropsy, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood (0.6 mL) was collected on lithium heparin for plasma chemistry determinations.

4. Biochemistry

Any significant change in the general appearance of the plasma was recorded. Total bilirubin, urea, total protein, albumin and total cholesterol concentrations and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using a Hitachi 911 (Roche Diagnostics, Meylan, France).

5. Sacrifice and pathology

On Study Days 91, 92, 93 or 94, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to sacrifice.

All animals, either found dead or killed by design, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded and sampled and examined microscopically.

<u>Organ weights:</u>

Brain, heart, kidneys, liver, spleen, thymus. adrenal gland and testes were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

Tissue sampling:

The following organs and tissues from all animals were sampled:

Table 3.12.1.5- 2: Organ weights

Adrenal gland	Ovary
Aorta	Pancreas
Articular surface (femoro-tibial)	Pituitary gland
Bone (sternum)	Prostate
Bone marrow (sternum)	Sciatic nerve
Brain	Seminal vesicle
Epididymis	Skeletal muscle
Esophagus	Skin
Eye and optic nerve	Spinal cord (cervical, thoracic, lumbar)
Gallbladder	Spleen
Harden an gland	Stomach
Heart	Submaxillary (salivary) gland
Intestine (duodenum, jejunum, ileum, cecum, colon, rectum)	Testis
	Thymus
Kidney	Thyroid (with parathyroid)
Larynx	Tongue
Liver	Trachea
Lung	Urinary bladder
Lymph nodes (submaxillary, mesenteric)	Uterus (including cervix)
Mammary gland	Vagina

A bone marrow smear was prepared from one femur, stained with May-Grünwald Giemsa, but not examined.

6. Histopathology

Tissue processing:

Tissue samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Tissues were sent to Propath U.K. Ltd., Willow Court, Netherwood Road, Hereford, HR2 6JU, England for slide preparation.

With the exception of larynx, all the above mentioned samples were embedded in paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared for all the organs from all the animals in the control and high dose groups as well as from all decedent animals in intermediate dose groups: additionally, sections from the liver, lung, kidney, adrenal gland and thyroid gland and from gross findings observed at necropsy were prepared for all the animals in all intermediate dose groups.

Microscopic examination:

Histopathological examinations were performed on all the tissues from all the animals in the control and high dose groups and all decedents in all groups. The liver, lung, kidney, adrenal gland and thyroid gland were examined in all the animals in the study. Macroscopic findings were also examined in all intermediate dose groups.

Following the initial histopathological examination by the study pathologist, a review pathologist undertook an independent "peer review" of representative slides and diagnoses. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

7. Proliferating cell nuclear antigen (PCNA) analysis

A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction included incubation with a monoclonal antibody raised against PCNA, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Hematoxylin counterstaining. The immunohistochemical staining for PCNA and determination of the labeling index were performed on all surviving animals in the control group and the Group 5 (3200 ppm fluopicolide). The zonal labeling index, expressed as the number of PCNA-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an automatic image analysis system (technical sheet included in the study file). The mean and standard deviation were calculated for each zone and each group.

II. Results and Discussion

A. Results

1. Clinical results

There were no treatment-related signs during the course of the study.

One male from each of the dose groups 0, 200 and 3,200 ppm and one female dosed at 800 ppm were found dead or moribund between Day 6 to 73. These deaths were considered to be incidental and not treatment-related. In addition, one male at 0 ppm, two males at 800 ppm and one male at 3,200 ppm, died as a result of accidental trauma between Day 21 and 85. One control female and one female at 50 ppm died during anesthesia prior to blood sampling on Day 94.

2. Body weight and food intake

Body weight

At 3,200 ppm, overall body weight gain (Day 1-90) was reduced by 7% in males and 14% in females compared to controls. The effect on body weight gain was most marked during Week 1 for males (-88%; statistically significant) and during Week 1 and 2 for females (-87% at Week 1, -85% at Week 2; statistically significant). Absolute body weight after one week of treatment (Day 8) was statistically significantly reduced at the high dose level by 10% and 7% in males and females, respectively. At study termination (Day 90), the reduction was only 3% in both sexes.

At 800 ppm, there was an apparent overall decrease in body weight gain of 14% in males (Day 1-90). However, since this effect does not occur in a dose-related manner and is not reflected in terms of absolute weight, it is thought to be attributable to the premature deaths of the two smallest animals in this group. Therefore, this apparent effect is not considered to be treatment-related. At this dose level, no effect on body weight development was observed in females.

At 200 and 50 ppm, no effect on body weight development was observed in either sex.

Table 3.12.1.5- 3: Mean body weight and body weight gain (g)

	Dose level (ppm)				
	0	**50**	**200**	**800**	**3,200**
Males					
Body weight [g] *(% difference to control)*					
Day 1	17.0	17.1 *(+0.6)*	17.3 *(+1.8)*	16.9 *(-0.6)*	17.3 *(+1.8)*
Day 8	19.4	18.9 *(-2.6)*	19.6 *(+1.0)*	19.4 *(±0.0)*	17.4** *(-10.3)*
Day 15	21.0	20.7 *(-1.4)*	21.2 *(+1.0)*	20.7 *(-1.4)*	18.9** *(-10.0)*
Day 22	21.9	21.8 *(-0.5)*	21.9 *(±0.0)*	21.4 *(-2.3)*	20.0** *(-8.7)*
Day 29	22.6	22.1 *(-2.2)*	22.3 *(-1.3)*	22.0 *(-2.7)*	20.7* *(-8.4)*
Day 36	22.9	22.8 *(-0.4)*	23.0 *(+0.4)*	22.2 *(-3.1)*	21.2** *(-7.4)*
Day 43	22.9	22.9 *(±0.0)*	23.1 *(+0.9)*	22.1 *(-3.5)*	21.4** *(-6.6)*
Day 50	24.1	24.2 *(+0.4)*	24.5 *(+1.7)*	23.5 *(-2.6)*	23.2 *(-3.7)*
Day 57	24.3	24.4 *(+0.4)*	24.8 *(+2.1)*	23.7 *(-2.5)*	23.4 *(-3.7)*
Day 64	24.5	24.5 *(±0.0)*	25.0 *(+2.0)*	24.0 *(-2.0)*	23.7 *(-3.3)*
Day 71	24.8	24.9 *(+0.4)*	25.1 *(+1.2)*	24.3 *(-2.0)*	24.4 *(-1.6)*
Day 78	25.6	25.2 *(-1.6)*	25.2 *(-1.6)*	24.6 *(-3.9)*	24.6 *(-3.9)*
Day 85	26.1	26.0 *(-0.4)*	26.0 *(-0.4)*	24.8 *(-5.0)*	25.0[#] *(-4.2)*
Day 90	26.1	26.0 *(-0.4)*	25.9 *(-0.8)*	25.2 *(-3.4)*	25.3 *(-3.1)*
Body weight gain [g] *(% difference to control)* [+]					
Day 1-8	2.5	1.8 *(-28.0)*	2.2 *(-12)*	2.5 *(±0.0)*	0.3** *(-88.0)*
Day 1-15	4.1	3.6 *(-12.2)*	3.8 *(-7.3)*	3.8 *(-7.3)*	1.9** *(-53.7)*
Day 1-22	5.0	4.7 *(-6.0)*	4.6 *(-8.0)*	4.2 *(-16.0)*	3.0* *(-40.0)*
Day 1-90	9.0	8.9 *(-1.1)*	8.5 *(-5.6)*	7.7 *(-14.4)*	8.4 *(-6.7)*
Females					
Body weight [g] *(% difference to control)*					
Day 1	14.6	15.0 *(+2.7)*	15.0 *(+2.7)*	14.4 *(-1.4)*	14.9 *(+2.1)*
Day 8	16.1	16.5 *(+2.5)*	16.5 *(+2.5)*	16.3 *(+1.2)*	15.0* *(-6.8)*
Day 15	16.6	17.6 *(+6.0)*	17.4 *(+4.8)*	16.5 *(-0.6)*	15.1* *(-9.0)*
Day 22	17.8	18.6 *(+4.5)*	18.3 *(+2.8)*	17.2 *(-3.4)*	16.1* *(-9.6)*
Day 29	18.8	19.6 *(+4.3)*	19.3 *(+2.7)*	18.6 *(-1.1)*	17.2** *(-8.5)*
Day 36	19.1	20.0 *(+4.7)*	19.5 *(+2.1)*	18.9 *(-1.0)*	17.9** *(-6.3)*
Day 43	19.4	19.9 *(+2.6)*	19.3 *(-0.5)*	19.0 *(-2.1)*	17.9** *(-7.7)*
Day 50	20.5	21.0 *(+2.4)*	20.6 *(+0.5)*	20.3 *(-1.0)*	19.3** *(-5.9)*
Day 57	20.1	21.0* *(+4.5)*	20.8 *(+3.5)*	20.4 *(+1.5)*	19.5 *(-3.0)*
Day 64	19.9	20.7 *(+4.0)*	20.5 *(+3.0)*	20.1 *(+1.6)*	19.3 *(-3.0)*
Day 71	20.5	21.4* *(+4.4)*	20.8 *(+1.5)*	20.6 *(+0.5)*	19.8 *(-3.4)*
Day 78	19.8	21.3[#] *(+7.6)*	21.2[#] *(+7.1)*	20.8 *(+5.1)*	19.9 *(+0.5)*
Day 85	20.9	21.5 *(+2.9)*	21.3 *(+1.9)*	21.0 *(+0.5)*	20.3 *(-2.9)*
Day 90	21.2	21.7 *(+2.4)*	21.7 *(+2.4)*	21.0 *(-0.9)*	20.5 *(-3.3)*
Body weight gain [g] *(% difference to control)* [+]					
Day 1-8	1.5	1.5 *(±0.0)*	1.5 *(±0.0)*	1.9 *(+26.7)*	0.2* *(-86.7)*
Day 1-15	2.0	2.6 *(+30.0)*	2.4 *(+20.0)*	2.0 *(±0.0)*	0.3** *(-85.0)*
Day 1-22	3.1	3.6 *(+16.1)*	3.3 *(+6.5)*	2.8 *(-9.7)*	1.3** *(-58.1)*
Day 1-90	6.6	6.7 *(+1.5)*	6.7 *(+1.5)*	6.7 *(+1.5)*	5.7 *(-13.6)*

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet´s test;
# / ## $p < 0.05$ / $p < 0.01$, statistically different to control using modified t-test;
[+] Mean absolute body weight gains according to calculation within study report

Food intake

Due to the excessive amount of food spillage in this study, an accurate assessment of food consumption could not be made for every time interval. However, there does not appear to be a clear treatment-related effect on food consumption.

The mean achieved dietary intake of fluopicolide (Weeks 1-13) is shown in Table 3.12.1.5- 4.

Table 3.12.1.5- 4: Mean achieved dietary intake of fluopicolide (Weeks 1-13)

	Dose level (ppm)							
	Males				Females			
	50	200	800	3,200	50	200	800	3,200
Concentration [mg/kg bw/day]	10.4	37.8	161	770[a]	12.6	52.8	207	965[b]

[a] No data recorded for weeks 5 and 7 due to spillage
[b] No data recorded for weeks 5, 6, 7 and 9 due to spillage

3. Laboratory investigations

Clinical chemistry:

Lower mean total cholesterol and albumin concentrations were observed in both sexes at 3,200 and 800 ppm, lower cholesterol concentrations were also noted in both sexes at 200 ppm. Mean alkaline phosphatase activity was higher in males at 3,200 ppm, only. The amplitude and statistical significance relative to controls were as follows:

Table 3.12.1.5- 5: Selected biochemistry results (% difference to control)

	Dose level (ppm)				
	0	50	200	800	3,200
Males					
Total cholesterol (mmol/L)	1.82	1.47 *(-19.2)*	1.36** *(-25.3)*	0.95** *(-47.8)*	0.91** *(-50.0)*
Albumin (g/L)	38	37 *(-2.6)*	36 *(-5.3)*	33** *(-13.2)*	33** *(-13.2)*
ALP (IU/L)	69	71 *(+2.9)*	69 *(±0.0)*	72 *(+4.3)*	89** *(+29.0)*
Females					
Total cholesterol (mmol/L)	1.51	1.43 *(-5.3)*	1.19** *(-21.2)*	1.16** *(-23.2)*	1.27** *(-15.9)*
Albumin (g/L)	38	37 *(-2.6)*	35 *(-7.9)*	34** *(-10.5)*	33** *(-13.2)*
ALP	99	102 *(+3.0)*	104 *(+5.1)*	109 *(+10.1)*	106 *(+7.1)*

** $p < 0.01$, significantly different from control using the Bartlett's test

4. Sacrifice and pathology

Necropsy:

Dark liver was observed in 4/8 males and in 9/10 females at 3,200 ppm.

Organ weights:

Mean terminal body weight was slightly, but statistically significantly lower (-5%) in males at 3,200 ppm when compared to controls.

Absolute liver weight was increased by 20% in males and 25% in females at 3,200 ppm, with relative liver weight increased by 30% in males and 34% in females. At 800 ppm, both absolute and relative liver weights were slightly increased by 10-14% for males and 13-16% for females at 800 ppm.

At 200 and 50 ppm, no effect on absolute and / or relative liver weight was observed in either sex.

Table 3.12.1.5- 6: Mean absolute and relative liver weights (% difference to control)

Parameter	Dose level (ppm)							
	0	**50**		**200**		**800**		**3,200**	
Males									
Terminal BW (g)	22.3	22.3	*(±0.0)*	22.2	*(-0.4)*	21.4	*(-4.0)*	21.2*	*(-4.9)*
Absolute liver weight (g)	1.0	1.0	*(±0.0)*	1.0	*(±0.0)*	1.1	*(+10.0)*	1.2**	*(+20.0)*
Relative liver weight (% of body weight)	4.3	4.3	*(±0.0)*	4.5	*(+4.7)*	4.9**	*(+14.0)*	5.6**	*(+30.2)*
Females									
Terminal BW (g)	17.7	18.0	*(+1.7)*	17.7	*(±0.0)*	17.7	*(±0.0)*	17.4	*(-1.7)*
Absolute liver weight (g)	0.8	0.8	*(±0.0)*	0.8	*(±0.0)*	0.9*	*(+12.5)*	1.0**	*(+25.0)*
Relative liver weight (% of body weight)	4.4	4.3	*(-2.3)*	4.7	*(+6.8)*	5.1**	*(+15.9)*	5.9**	*(+34.1)*

* p< 0.05 ; ** p< 0.01, significantly different from control using the Bartlett's test
BW: Body weight

5. Histopathology

Unscheduled deaths:

The decedent animals showed a range of incidental changes similar to those seen at terminal sacrifice. No cause of death could be determined, except for male KT1M2422 from the control group, which showed a suppurative encephalitis, considered to be contributive to the death.

The liver from animals KT5M2502 (3,200 ppm) and KT4F2496 (800 ppm) showed a diffuse centrilobular hepatocellular hypertrophy.

Terminal sacrifice:

A treatment-related change was only seen in the liver. There was an increase in the incidence and severity (slight to mild) of diffuse centrilobular hepatocellular hypertrophy in all animals at 3,200 ppm and in a proportion of animals at 800 ppm.

All other changes were considered to be incidental and unrelated to treatment.

Table 3.12.1.5- 7: Liver findings – Number of animals affected

Findings / Severity	Dose level (ppm)									
	Males					Females				
	0	**50**	**200**	**800**	**3,200**	**0**	**50**	**200**	**800**	**3,200**
Number of animals examined	8	10	9	8	8	9	9	10	9	10
Hypertrophy, hepatocellular, centrilobular, diffuse										
-slight	0	0	0	4	1	0	0	0	8	0
-mild	0	0	0	0	7	0	0	0	0	10
Total	**0**	**0**	**0**	**4**	**8**	**0**	**0**	**0**	**8**	**10**

6. Proliferating cell nuclear antigen (PCNA)

When compared to controls, there was no relevant change in hepatocytic cell cycling in either males or females dosed at 3,200 ppm.

Table 3.12.1.5- 8: PCNA measurements (mean ± SD)

Zone	Dose level (ppm)			
	Males		Females	
	0	**3,200**	**0**	**3,200**
Number of animals examined	8	8	9	10
Centrilobular	1.63 ± 1.35	2.21 ± 0.99	1.45 ± 0.60	1.37 ± 1.01
Perilobular	2.08 ± 1.72	1.68 ± 1.19	3.03 ± 2.02	3.22 ± 1.13
Total	**1.86 ± 1.29**	**1.93 ± 0.73**	**2.23 ± 1.24**	**2.22 ± 0.97**

SD: standard deviation

III. Conclusion

The No Observed Adverse Effect Level (NOAEL) in this 90-day dietary study in mice was 50 ppm (equivalent 10.4 and 12.8 mg/kg bw/day in males and females, respectively) based on apparent significant treatment-related reduction in cholesterol suggestive of impaired liver function at ≥ 200 ppm (equivalent 37.8 and 52.8 mg/kg bw/day in males and females, respectively). The liver was the main target organ in the study as indicated by reduced albumin and cholesterol levels, increased relative liver weights and centrilobular hepatocyte hypertrophy observed at dose levels of ≥ 800 ppm.

3.12.1.6 Anonymous; 2000; M-197623-01-1

Study reference:

Anonymous; 2000; AE C638206 - Mouse 90-day dietary toxicity study; M-197623-01-1

Deviations:

Deviations from the current OECD guideline (408, 2018):

- Ophthalmological examinations were not conducted
- Sensory reactivity to stimuli were not performed
- Blood/plasma samples were not taken for possible analysis of thyroid hormones
- At termination, an evaluation of oestrus cycle was not performed
- Prostate and seminal vesicles with coagulating glands were not weighed at termination

Although performed in the rat, rather than the mouse, ophthalmoscopy and sensory reactivity to stimuli assessments were performed in the 90-day rat study (Anonymous; 2000; M-197622-01-1) at doses of up to 20,000 ppm (1,671 mg/kg bw/day, combined sexes); whilst seminal vesicles with coagulating glands were weighed at termination in the rat two-generation study (Anonymous; 2003; M-232532-01-1) as was an evaluation of oestrous cycles at dose levels of up to 2,000 ppm (equivalent to 100-200 mg/kg bw/day in F0/F1 females).

Therefore, these deviations do not affect the overall acceptability of the study.

Executive Summary:

Groups of 10 male and 10 female Crl: CD-1 (ICR) BR mice, aged approx. 28 days, were fed diet containing 0, 32, 320, 3,200 or 6,400 ppm (equivalent to 0, 4.7, 46, 461 or 944 mg/kg bw/day in males, and 0, 6.2, 60, 629 or 1,239 mg/kg bw/day in females) fluopicolide for 13 weeks. No mortalities occurred and no clinical signs of toxicity were seen. At 6,400 ppm, overall body weight gain was reduced by 20% and 32% for males and females, respectively. At 6,400 ppm, alanine aminotransferase activity was slightly increased in both sexes, slight increases in aspartate aminotransferase and alkaline phosphatase activity were observed in males and slight increases in cholesterol and creatinine concentration in females. At 6400 ppm, absolute liver weights in males and females were increased by 42% and 60%, respectively, and relative liver weights by 50% in males and 78% in females.

At necropsy, abnormal areas in the liver were seen in 2/10 males and 3/10 females, and enlarged liver was recorded for 1/10 males and 1/10 females of the highest dose group. At 6,400 ppm, microscopic examination revealed centrilobular hepatocyte hypertrophy in the livers of 10/10 males and 10/10 females, scored as slight to severe in males and slight to moderate in females. Minimal to moderate focal hepatocyte necrosis was also seen in 3/10 males and 2/10 females. At the same dose level, 6,400 ppm, females had a 32% reduction in body weight gain over the duration of the study, whilst males had a reduction of 20%.

At 3,200 ppm, overall body weight gain was reduced by 22% for females when compared to controls. There was a slight increase in alanine aminotransferase activity in both sexes and a slight increase in aspartate aminotransferase for males only. Absolute liver weights in males and females were increased by 33% and 44%, respectively and relative liver weights by 36% in males and 59% in females.

At necropsy, abnormal areas were noted in the livers of 3/10 males and 3/10 females and an enlarged liver was seen in 1/10 females. Microscopic examination revealed minimal to moderate centrilobular hepatocyte hypertrophy in 10/10 males and 9/10 females, slight focal hepatocyte necrosis was seen in 2/10 females only.

At 320 ppm, microscopic examination revealed minimal to slight centrilobular hepatocyte hypertrophy in 9/10 males, and minimal hypertrophy in 2/10 females. There were no treatment-related effects in either sex at 32 ppm.

Thus, the No Observed Effect Level (NOEL) was 32 ppm, equivalent to a daily intake of 4.7 and 6.2 mg/kg bw/day for males and females, respectively. Since the only finding at 320 ppm was an adaptive minimal to slight centrilobular hepatocyte hypertrophy, the No Observed Adverse Effect Level (NOAEL) is considered 320 ppm, equivalent to a daily intake of 46 and 60 mg/kg bw/day for males and females, respectively and 53 mg/kg bw/day for the combined sexes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9% (10 June 1999), 97.3% (16 August 1999)
Batch no.:	Code number AE C638206 00 1C99 0005

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	Crl: CD-1 (ICR) BR
Age:	Approx. 28 days old
Weight at start:	19.3 to 25.3 g (males), 18.6 to 25.3 g (females)
Source:	Charles River UK Ltd., Margate, Kent, UK
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1, supplied by Special Diet Services Ltd., Stepfield, Witham, Essex
Water:	Tap water ad lib
Housing:	Groups of five, by sex and dose group
Temperature:	19-23 °C
Humidity:	45-65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: August 3 to November 5, 1999

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups.

Table 3.12.1.6- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	32	10	10
3	320	10	10
4	3,200	10	10
5	6,400	10	10

After 13 weeks of continuous dietary exposure, the animals were killed.

3. Diet preparation and analysis

Test diets were prepared weekly throughout the study. On each occasion, for each dose level, a pre-mix was made using a food processor. This in turn was then mixed with remaining laboratory rodent diet and blended in a turbula mixer to produce the required dietary concentration. Subsequent concentrations were prepared by serial dilution.

Every week, aliquots of each concentration of the freshly prepared test diets (i.e. 32, 320, 3,200 and 6,400 ppm) were submitted for analysis of the test material concentration. Samples were received in powder form in plastic bags and stored deep frozen from time of receipt.

The mean results for the test diet samples analyzed were within the range 93.5 - 107.0% of nominal (acceptable range +10% to -15% of nominal), except for week 9 at the 32 ppm level which gave a mean result of 111.5% of nominal. This deviation was not considered to affect the interpretation of the study.

Stability was shown to be satisfactory in previous studies over the time of use of the diet (8 days) in the concentration range 6 – 20,000 ppm i.e. % nominal values declined by a maximum of 12% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Observations were made twice each day, once in the morning and once in the afternoon from Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary.

Detailed observations were conducted once weekly prior to weighing. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of about 20% of the animals was recorded at receipt. Each animal was weighed at randomisation, at the start of treatment, at weekly intervals thereafter and at necropsy.

For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Laboratory investigations (clinical chemistry)

Blood for haematology and biochemistry analyses was obtained from the retro-orbital sinus of each animal under isoflurane anaesthesia. Samples for haematology and clinical chemistry were collected during Study Weeks 13 and 14, respectively.

The parameters listed below were measured or derived:

<u>Haematology:</u>

Haematocrit (HCT)
Haemoglobin (HB)
Red blood cells (RBC)
Mean cell volume (MCV)
Mean cell haemoglobin (MCH)
Mean cell haemoglobin concentration (MCHC)
Platelets (PLT)
White blood cells (WBC)
Neutrophils (NEUT)
Lymphocytes (LYMP)
Monocytes (MONO)
Eosinophils (EOS)
Basophils (BASO)
Large unstained cells (LUC)
Reticulocyte count (RET)

<u>Biochemistry:</u>

Total protein (PROT)
Albumin (ALB)
Total globulin (GLOB)
A/G ratio (A/G)
Calcium (CA)
Phosphate (PO4)
Sodium (NA)
Potassium (K)
Urea (UREA)
Creatinine (CREA)
Total cholesterol (CHOL)
Total bilirubin (TBIL)
Chloride (CL)
Aspartate aminotransferase (AST)
Alanine aminotransferase (ALT)
Alkaline phosphatase (AP)
G-glutamyl transpeptidase (GGT)
Creatine kinase (CPK)
Glucose (GLUC)

4. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

Organ weights:

The following organs from animals surviving to scheduled termination were weighed at necropsy:

Liver	Testes	Brain	Thymus
Kidneys	Ovaries	Adrenals	Uterus
Spleen	Epididymides	Heart	

Tissue sampling:

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Adrenals	Liver	Seminal vesicles
Aorta	Lungs (inflated)	Skeletal muscle
Brain	Lymph nodes (cervical + mesenteric)	Skin + subcutis
Caecum	Mammary gland	Spinal cord (3 levels)
Colon	Sternum	Spleen
Diaphragm	Optic nerve	Stomach
Duodenum	Ovaries	Testes
Epididymides	Oviducts	Thymus
Eyes	Pancreas	Thyroid (with parathyroid)
Femur, joint	Pinnae[1]	Tongue
Gall bladder	Pituitary	Trachea
Harderian gland	Preputial gland	Urinary bladder
Head[3]	Prostate	Uterus[2]
Heart	Rectum	Vagina
lleum	Salivary glands (parotid, submaxillary + sublingual)	Any other tissue showing macroscopic abnormalities
Jejunum		
Kidneys		
Lacrimal gland	Sciatic nerve	

[1] Taken for identification purposes only; not examined.
[2] Cervix uteri and uterine horns examined
[3] Nose/turbinates examined

A bone marrow smear was taken from all animals and fixed subsequently at staining.

5. Histopathology

Tissue processing

Following fixation, nominal 5µm sections of all organs and tissues from all animals from the control and high dose groups were prepared and stained with haematoxylin and eosin.

Liver sections from the low and intermediate dose groups were also examined microscopically, and additional sections of liver from all dose groups were stained with Oil-Red-O and examined for the presence of fat.

Microscopic examination

The following organs/tissues were examined histopathologically:

Adrenal cortex (left)	Ovary (left)
Adrenal cortex (right)	Ovary (right)
Adrenal medulla (left)	Oviduct (left)
Adrenal medulla (right)	Oviduct (right)
Aorta	Pancreas
Bone	Parathyroid
Bone marrow smear	Parotid gland
Brain	Pituitary
Caecum	Preputial gland
Cervical lymph node	Prostate
Cervix uteri	Rectum
Colon	Salivary gland, sublingual
Diaphragm	Sciatic nerve
Duodenum	Seminal vesicle
Epididymis (left)	Skin
Epididymis (right)	Spinal cord, cervical
Eye (left)	Spinal cord, thoracic
Eye (right)	Spinal cord, lumbar
Gall bladder	Spleen
Harderian gland	Stomach (fore)
Heart	Stomach (glandular)
Ileum	Submaxillary gland
Jejunum	Testis (left)
Joint	Testis (right)
Kidney (left)	Thymus
Kidney (right)	Thyroid (left)
Lacrimal gland	Thyroid (right)
Liver	Tongue
Liver ORO	Trachea
Lung	Turbinates
Lymph node, mesenteric	Urinary bladder
Mammary gland	Uterus (body)
Muscle, skeletal	Uterus (left horn)
Oesophagus	Uterus (right horn)
Optic nerve (left)	Vagina
Optic nerve (right)	

II. Results and discussion

1. Clinical results

There were no treatment-related signs or deaths during the course of the study.

2. Body weight and food intake

Body weight

There were no statistically significant changes in body weight in either males or females at any dose level or time point measured during the study.

Over the duration of the study (Day 1-92) at 6,400 ppm, overall body weight gain was reduced by 20% and 32% compared to control in males and females, respectively. This effect was most severe during Week 1 of treatment (Day 1-8: -74% in males, -64% in females).

At 3,200 ppm and 320 ppm in females only, overall body weight gain was reduced by 22% and 18%, respectively.

The decreased body weight gain in females at 320 ppm is not considered toxicologically relevant, because effects on body weight itself were small and inconsistent (changes ranged from +2.3% to -5.8% compared to control).

Table 3.12.1.6- 2: Mean body weight and body weight gain (g)

	Dose level (ppm)				
	0	**32**	**320**	**3,200**	**6,400**
Males					
Body weight [g] *(% difference to control)*					
Day 1	24.5	25.9 *(+5.7)*	26.3 *(+7.3)*	26.0 *(+6.1)*	26.4 *(+7.8)*
Day 8	29.9	29.9 *(±0.0)*	29.2 *(-2.3)*	29.2 *(-2.3)*	27.8 *(-7.0)*
Day 15	32.1	32.8 *(+2.2)*	32.1 *(±0.0)*	31.9 *(-0.6)*	30.0 *(-6.5)*
Day 22	33.4	34.1 *(+2.1)*	33.7 *(+0.9)*	33.4 *(±0.0)*	31.6 *(-5.4)*
Day 29	34.9	36.0 *(+3.2)*	35.5 *(+1.7)*	34.8 *(-0.3)*	33.2 *(-4.9)*
Day 36	36.0	37.2 *(+3.3)*	36.3 *(+0.8)*	36.5 *(+1.4)*	34.8 *(-3.3)*
Day 43	36.8	38.0 *(+3.3)*	38.2 *(+3.8)*	36.9 *(+0.3)*	35.5 *(-3.5)*
Day 50	37.8	38.9 *(+2.9)*	39.0 *(+3.2)*	37.7 *(-0.3)*	36.0 *(-4.8)*
Day 57	39.0	40.0 *(+2.5)*	40.5 *(+3.8)*	39.0 *(±0.0)*	37.2 *(-4.6)*
Day 64	39.4	39.9 *(+1.3)*	40.6 *(+3.0)*	38.7 *(-1.8)*	37.1 *(-5.8)*
Day 71	38.9	40.7 *(+4.6)*	41.9 *(+7.7)*	39.1 *(+0.5)*	37.7 *(-3.1)*
Day 78	40.0	41.1 *(+2.8)*	42.4 *(+6.0)*	39.6 *(-1.0)*	38.6 *(-3.5)*
Day 85	41.4	41.6 *(+0.5)*	44.1 *(+6.5)*	41.1 *(-0.7)*	39.3 *(-5.1)*
Day 92	41.1	41.9 *(+1.9)*	43.6 *(+6.0)*	40.6 *(-1.2)*	39.7 *(-3.4)*
Body weight gain [g] *(% difference to control)* [+]					
Day 1-8	5.4	4.0 *(-25.9)*	2.9 *(-46.3)*	3.2 *(-40.7)*	1.4 *(-74.1)*
Day 1-15	7.6	6.9 *(-9.2)*	5.8 *(-23.7)*	5.9 *(-22.4)*	3.6 *(-47.4)*
Day 1-92	16.6	16.0 *(-3.6)*	17.3 *(+4.2)*	14.6 *(-12.0)*	13.3 *(-19.9)*

	Dose level (ppm)				
	0	**32**	**320**	**3,200**	**6,400**
Females					
Body weight [g] *(% difference to control)*					
Day 1	23.9	23.0 *(-3.8)*	23.7 *(-0.8)*	22.7 *(-5.0)*	23.8 *(-0.4)*
Day 8	25.0	24.7 *(-1.2)*	24.6 *(-1.6)*	23.8 *(-4.8)*	24.2 *(-3.2)*
Day 15	25.8	25.4 *(-1.6)*	25.3 *(-1.9)*	24.5 *(-5.0)*	25.7 *(-0.4)*
Day 22	27.4	27.0 *(-1.5)*	27.3 *(-0.4)*	26.1 *(-4.7)*	27.2 *(-0.7)*
Day 29	29.4	28.7 *(-2.4)*	28.8 *(-2.0)*	27.6 *(-6.1)*	28.5 *(-3.1)*
Day 36	29.9	29.6 *(-1.0)*	28.7 *(-4.0)*	28.1 *(-6.0)*	28.8 *(-3.7)*
Day 43	29.5	30.4 *(+1.7)*	29.7 *(+0.7)*	29.0 *(-1.7)*	29.7 *(+0.7)*
Day 50	31.3	31.3 *(±0.0)*	30.0 *(-4.2)*	28.7 *(-8.3)*	29.6 *(-5.4)*
Day 57	32.3	31.9 *(-1.2)*	31.3 *(-3.1)*	30.6 *(-5.3)*	30.7 *(-4.6)*
Day 64	30.7	31.3 *(+2.3)*	31.3 *(+2.3)*	28.9 *(-5.9)*	30.5 *(-0.7)*
Day 71	32.6	33.0 *(+1.2)*	31.7 *(-2.8)*	30.4 *(-6.7)*	30.5 *(-6.4)*
Day 78	33.8	33.0 *(-2.4)*	32.5 *(-3.8)*	30.6 *(-9.5)*	31.0 *(-8.3)*
Day 85	32.3	33.5 *(+3.7)*	32.6 *(+0.9)*	30.5 *(-5.6)*	31.0 *(-4.0)*
Day 92	34.2	33.7 *(-1.5)*	32.2 *(-5.8)*	30.7 *(-10.2)*	30.8 *(-9.9)*
Body weight gain [g] *(% difference to control)* [+]					
Day 1-8	1.1	1.7 *(+54.6)*	0.9 *(-18.2)*	1.1 *(±0.0)*	0.4 *(-63.6)*
Day 1-15	1.9	2.4 *(+26.3)*	1.6 *(-15.8)*	1.8 *(-5.3)*	1.9 *(±0.0)*
Day 1-92	10.3	10.7 *(+3.9)*	8.5 *(-17.5)*	8.0 *(-22.3)*	7.0 *(-32.0)*

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet´s test
# / ## $p < 0.05$ / $p < 0.01$, statistically different to control using modified t-test
[+] No statistical analyses were performed for body weight gains (only between each study day).

Food intake

Food consumption was not affected by treatment.

The mean achieved dietary intake of fluopicolide is shown in Table 3.12.1.6- 3.

Table 3.12.1.6- 3: Mean achieved dietary intake of fluopicolide

	Dose level (ppm)							
	Males				Females			
	32	**320**	**3,200**	**6,400**	**32**	**320**	**3,200**	**6,400**
Concentration [mg/kg bw/day]	4.7	46	461	944	6.2	60	629	1,239

3. Laboratory investigations

Haematology:

There were no treatment-related effects.

Clinical chemistry:

At 6,400 ppm, there was a slight increase in alanine aminotransferase activity and of creatinine in both sexes. Aspartate aminotransferase and alkaline phosphatase activity were slightly increased in males only, whilst slight increases in cholesterol concentration were observed in females.

At 3,200 ppm, there was a slight increase in alanine aminotransferase activity in both sexes, a slight increase in aspartate aminotransferase activity in males and an increase of creatinine in females.

There were no treatment-related effects at 320 or 32 ppm.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or values were within the normal range of historical controls.

Table 3.12.1.6- 4: Selected biochemistry results (% difference to control)

Parameter	Dose level (ppm)				
	0	**32**	**320**	**3,200**	**6,400**
Males					
ALT (U/L)	42	43 *(+2.4)*	48 *(+14.3)*	83** *(+97.6)*	75*** *(+78.6)*
AST (U/L)	61	78 *(+27.9)*	70 *(+14.8)*	85* *(+39.3)*	103*** *(+68.9)*
Alkaline phosphatase (U/L)	45	35 *(-22.2)*	36 *(-20.0)*	71 *(+57.8)*	93** *(+106.7)*
Cholesterol (mmol/L)	3.28	3.11 *(-5.2)*	3.53 *(+7.6)*	2.94 *(-10.4)*	2.97 *(-9.5)*
Creatinine (µmol/L)	38	37 *(-2.6)*	40 *(+5.3)*	42 *(+10.5)*	45*** *(+18.4)*
Females					
ALT (U/L)	32	33 *(+3.1)*	34 *(+6.3)*	69** *(+115.6)*	79*** *(+146.9)*
AST (U/L)	78	76 *(-2.6)*	82 *(+5.1)*	99 *(+26.9)*	86 *(+10.3)*
Alkaline phosphatase (U/L)	56	55 *(-1.8)*	75 *(+33.9)*	53 *(-5.4)*	67 *(+19.6)*
Cholesterol (mmol/L)	2.24	1.83 *(-18.3)*	2.07 *(-7.6)*	2.68 *(+19.6)*	3.11** *(+38.8)*
Creatinine (µmol/L)	34	32 *(-5.9)*	35 *(+2.9)*	42*** *(+23.5)*	48*** *(+41.2)*

* p<0.05; ** p< 0.01; *** p<0.001, statistically different to control

4. Sacrifice and pathology

Necropsy:

At 6,400 ppm, there were abnormal areas in the livers of 2/10 males and 3/10 females and enlarged livers for 1/10 males and 1/10 females.

At 3,200 ppm, there were abnormal areas in the livers of 3/10 males and 3/10 females and an enlarged liver for 1/10 females.

Organ weights:

At 6,400 ppm, absolute liver weight in males and females was increased by 42% and 60%, respectively, and liver weight relative to body weight was increased by 50% in males and 78% in females when compared with controls.

At 3,200 ppm, absolute liver weight in males and females was increased by 33% and 44%, respectively, and liver weight relative to body weight was increased by 36% in males and 59% in females when compared with controls.

There were no effects on organ weights in either sex at 320 or 32 ppm.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or values were within the normal range of historical controls.

Table 3.12.1.6- 5: Mean absolute and relative liver weights (% difference to control)

Parameter	Dose level (ppm)				
	0	**32**	**320**	**3,200**	**6,400**
Males					
Terminal body weight (g)	40.0	40.8 *(+2.0)*	42.5 *(+6.3)*	39.2 *(-2.0)*	37.8 *(-5.5)*
Absolute liver weight (g)	1.83	1.89 *(+3.2)*	2.05 *(+12.0)*	2.44** *(+33.3)*	2.60** *(+42.1)*
Relative liver weight (% of body weight)	4.57	4.63 *(+1.3)*	4.82 *(+5.5)*	6.22** *(+36.1)*	6.86** *(+50.1)*
Females					
Terminal body weight (g)	33.2	31.6 *(-4.8)*	30.9 *(-6.9)*	30.0 *(-9.6)*	30.0 *(-9.6)*
Absolute liver weight (g)	1.60	1.48 *(-7.5)*	1.61 *(+0.63)*	2.30** *(+51.3)*	2.56** *(+60.0)*
Relative liver weight (% of body weight)	4.81	4.67 *(-2.9)*	5.19 *(+7.9)*	7.65** *(+59.0)*	8.56** *(+78.0)*

* p<0.05; ** p< 0.01, statistically different to control (Dunnett´s test)

6. Histopathology

At 6,400 ppm, centrilobular hepatocyte hypertrophy was present in the livers of 10/10 males and 10/10 females, and was scored as slight to severe in males and slight to moderate in females. There was also minimal to moderate hepatocyte necrosis in 3/10 males and 2/10 females, respectively.

At 3,200 ppm, minimal to moderate centrilobular hepatocyte hypertrophy was present in the livers of 10/10 males and 9/10 females. Slight focal hepatocyte necrosis was seen in 2/10 females only.

At 320 ppm, minimal to slight centrilobular hepatocyte hypertrophy was seen in the livers of 9/10 males, and was also present at minimal severity in 2/10 females. There were no other treatment-related histopathological changes.

Table 3.12.1.6- 6: Liver findings – Number of animals affected

Findings / Severity	Dose level (ppm)									
	Males					Females				
	0	**32**	**320**	**3,200**	**6,400**	**0**	**32**	**320**	**3,200**	**6,400**
Centrilobular hepatocyte hypertrophy										
-minimal	0	1	6	1	0	1	0	2	2	0
-slight	0	0	3	5	2	0	0	0	6	7
-moderate	0	0	0	4	5	0	0	0	1	3
-severe	0	0	0	0	3	0	0	0	0	0
Total	**0/10**	**1/10**	**9/10**	**10/10**	**10/10**	**1/10**	**0/10**	**2/10**	**9/10**	**10/10**
Focal hepatocyte necrosis										
-minimal	0	0	0	0	1	0	0	0	0	0
-slight	0	0	0	1	0	1	0	0	2	2
-moderate	0	0	0	0	2	0	0	0	0	0
Total	**0/10**	**0/10**	**0/10**	**1/10**	**3/10**	**1/10**	**0/10**	**0/10**	**2/10**	**2/10**

III. Conclusion

In this study, overall body weight gain was reduced in males and females at 6,400 ppm and at 3,200 and 320 ppm in females only. However, the decreased bodyweight gain at 320 ppm in females is not considered toxicologically relevant, since is not reflected in terms of absolute body weight.

The main target organ was the liver based on increased transaminase activities, increased organ weight and adverse histopathological findings at both the 6,400 and 3,200 ppm dose levels.

The No Observed Effect Level (NOEL) was 32 ppm, equivalent to a daily intake of 4.7 and 6.2 mg/kg bw/day for males and females, respectively. Since the only finding at 320 ppm was an adaptive minimal to slight centrilobular hepatocyte hypertrophy, the No Observed Adverse Effect Level (NOAEL) is considered 320 ppm, equivalent to a daily intake of 46 and 60 mg/kg bw/day for males and females, respectively and 53 mg/kg bw/day for the combined sexes.

3.12.1.7 Anonymous; 2000; M-199397-01-1

Study reference:

Anonymous; 2000; AE C638206 - Dog 90-day oral toxicity study; M-199397-01-1

Deviations:	Deviations from the current OECD guideline (409, 1998): None

Executive Summary:

Groups of 4 male and 4 female beagle dogs were treated by gavage with 0, 5, 70 or 1,000 mg/kg bw/day of fluopicolide daily for at least 13 consecutive weeks. The test substance was administered in a vehicle of 1% w/v methyl cellulose in distilled water at a constant volume of 5 mL/kg bw. Controls received the vehicle alone. Animals were observed daily for clinical signs. Body weight was recorded weekly, whilst food consumption was measured daily. A detailed clinical examination and ophthalmoscopy were carried out pre-treatment and prior to necropsy. Haematology and biochemistry investigations were conducted twice prior to treatment, during Week 7, and prior to termination. Urinalysis was conducted prior to treatment, during Week 6, and at termination. At necropsy, the weights of selected organs were recorded, and subsequently a range of tissues was examined histopathologically.

There were no mortalities or clinical signs of toxicity observed during the study. In addition, there were no treatment-related ophthalmic changes and no effect on food intake, haematology, biochemistry, urinalysis, macroscopic pathology or histopathology. There were no statistically significant effects on absolute body weight or body weight gain in either sex. However, body weight gain was reduced at the highest dose level by 27% and 33% in males and females.

At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, compared to controls. Liver weight relative to bodyweight ratio was increased by 28% and 43% in males and females, respectively, when compared to controls. Organ weights were unaffected at 5 or 70 mg/kg bw/day.

The NOAEL in the 90-day dietary study in dogs was 70 mg/kg bw/day based on the increased absolute and relative liver weight at 1,000 mg/kg bw/day for both sexes. Dogs are noted to be a non-rodent species and the large increase in liver weight is therefore considered toxicologically relevant.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Technical AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v methyl cellulose in distilled water

3. Test animals

Species:	Dogs
Strain:	Outbred beagle dogs
Age:	6 to 7 month old
Weight at start:	9.5-11.5 kg (males), 6.7-12.9 kg (females)
Source:	Cambell Farms Ltd., Abbots Ripton Road, Wyton, Huntingdon, Cambs., UK
Acclimation period:	Yes
Diet:	Harlan Teklad 9682, supplied by Harlan Teklad Ltd., Shaw's Farm, Bicester, Oxon, UK
Water:	Water ad lib
Housing:	Housed in pairs (except during feeding and dosing, or on specific veterinary advice, when they were housed individually), by sex and dose group, under controlled environmental conditions, in a solid floor pen, measuring approximately 1 m x 4.5 m, with wood chips as bedding
Temperature:	18 ± 2 °C
Humidity:	45 - 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 9th 1999 to March 10th 2000

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups:

Table 3.12.1.7- 1: Study design

Group no.	Dose (mg/kg bw/day)	Number of males	Number of females
Treatment phase			
1	0	4	4
2	5	4	4
3	70	4	4
4	1,000	4	4

Dose levels were 0, 5, 70 and 1,000 mg/kg bw/day by gavage (see Table 3.12.1.7- 1). Dose levels were selected on the basis of previous toxicity data in the dog.

Males were treated for at least 91 consecutive days, and females for at least 93 consecutive days.

3. Dose preparation and analysis

Prior to the start of treatment, a procedure was developed to reliably prepare homogeneous and suitably stable mixtures of the test material in the vehicle, 1% w/v methyl cellulose in distilled water, at the required nominal concentrations of 1, 14 and 200 mg/mL (equivalent to dose levels of 5, 70 and 1,000 mg/kg bw/day, respectively).

Throughout the study dosing suspensions were generally prepared one day in advance of dosing, except for dosing over the weekends, the following Mondays and bank holidays, in which instances doses for these days were prepared up to 4 days in advance.

On each occasion, the required volume of dosing mixture was prepared by gradually adding the vehicle to the appropriate quantity of test material and thoroughly mixing them. Initially, a smooth paste was prepared which was made up to volume with more vehicle and the mixture was then homogenised.

Samples from Days 1, 29, 57 and 85 of treatment were analysed at all levels. The mean results for the test suspensions analysed were 87% to 107.6% of nominal (acceptable range +20% to -20% of nominal). Homogeneity was within acceptable limits, and stability was also shown to be satisfactory after four days storage at 4 °C.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The convention employed to indicate statistical significance at the levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$ is indicated below the relevant tables.

C. Methods

1. Observations

Each dog was given a thorough clinical examination, including measurement of rectal temperature, prior to the start of and at the end of the treatment period.

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning.

They were also checked in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary.

Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of each dog was recorded upon receipt, at randomisation, on Study Days -14 and -7, and at the start of treatment. Thereafter, each animal was weighed weekly throughout the treatment period and at necropsy.

Food consumption for each animal was measured daily for two weeks prior to the start of the study, and then daily throughout the treatment period.

3. Ophthalmoscopy

Ophthalmological examinations were conducted by a consultant veterinarian on all dogs prior to the start of treatment. During the last week of treatment only the eyes of animals in the control and high dose groups were examined. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 1.0% w/v Mydriacyl.

4. Laboratory investigations (clinical chemistry)

Blood samples for haematology and biochemistry were collected from the jugular vein of all animals on two occasions prior to the start of treatment (Study Days -15 and -7), and during weeks 7 and 13 of treatment. Samples for urinalysis were collected by catheterisation on Study Days -5 and during week 6 of treatment, and were taken directly from the bladder at necropsy.

The parameters listed below were measured or derived:

Haematology:

Table 3.12.1.7- 2: Haematology parameters

Haematocrit (HCT)	Lymphocytes (LYMP)
Haemoglobin (HB)	Monocytes (MONO)
Red blood cells (RBC)	Eosinophils (EOS)
Mean cell volume (MCV)	Basophils (BASO)
Mean cell haemoglobin (MCH)	Large unstained cells (LUC)
Mean cell haemoglobin concentration (MCHC)	Erythrocyte sedimentation rate (ESR)
Platelets (PIT)	Reticulocytes (RET)
White blood cells (WBC)	Prothrombin time (PT)
Neutrophils (NEUT)	Activated partial thromboplastin time (APTT)

Biochemistry:

Table 3.12.1.7- 3: Biochemistry parameters

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

Urinalysis:

Table 3.12.1.7- 4: Urinalysis parameters

Appearance (APP)	Bacteria (BACT)
Specific gravity (SG)	Red blood cells (RBC)
Protein (PROT)	Epithelial cells (EPTH)
Bilirubin (BIL)	Phosphate crystals (PO4)
pH (PH)	Urate crystals (URAT)
Glucose (GLUC)	Casts (CAST)
Urobilinogen (UBIL)	White blood cells (WBC)
Ketones (KET)	Sperm (SPER)
Blood (BLD)	Spun deposit colour (SDEP)

5. Sacrifice and pathology

All animals were killed by exsanguination of the carotid artery whilst under deep anaesthesia induced by intravenous injection of pentobarbitone sodium. Males were killed on Study Days 92 and 93, and females on Study Days 94 and 95. Where possible, one dog was necropsied in turn from each dose group in the following sequence: Group 1, Group 4, Group 2, Group 3 where possible. Within each dose group, dogs were necropsied in animal number order.

All dogs were examined thoroughly and any macroscopic abnormalities recorded.

Organ weights:

The following organs were weighed:

Table 3.12.1.7- 5: Organ weights

Adrenals	Prostate
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Thyroid
Liver	Uterus + oviducts
Ovaries	
Pituitary	

Tissue sampling:

The following organs and tissues from all animals were fixed intact or sampled and fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Table 3.12.1.7- 6: Tissue sampling

Adrenals	Larynx[3]	Sciatic nerve
Articulated surface and shaft of femur	Liver	Skeletal muscle
Aorta	Lungs	Skin + subcutis
Brain	Lymph nodes (mandibular, mesenteric)	Spinal cord (3 levels)
Caecum	Mammary gland	Spleen
Colon	Nose[3]	Sternum
Diaphragm	Oesophagus	Stomach
Duodenum	Optic nerve	Testes
Epididymides	Ovaries + oviducts	Thymus
Eyes	Pancreas	Thyroid + parathyroid
Gall bladder	Pharynx[3]	Tongue
Head[3]	Pinnae[1]	Tonsils
Heart	Pituitary	Trachea
lleum	Prostate	Urinary bladder
Jejunum	Rectum	Uterus[2]
Kidneys	Salivary glands (parotid, mandibular + sublingual)	Vagina
Lacrimal gland		Any other tissue showing macroscopic abnormalities

[1] The ear with tattoo and ID implant was taken for identification purposes only, and was not examined.
[2] Cervix uteri and uterine horns examined.
[3] The head, pharynx, larynx and nose were taken but not examined. Bone marrow smears were taken from the 7th thoracic rib of all animals and fixed at staining.

6. Histopathology

Tissue processing:

Following fixation, nominal 5 µm sections of all organs and tissues from each animal were prepared and stained with haematoxylin and eosin, except eyes (Davidson's fixative) and bone marrow smears (fixed with Wright's stain). An additional frozen section of the liver from all animals was stained with Oil Red O to evaluate the presence of fat.

Microscopic examination:

Tissues were examined for histopathological change with a light microscope. The data were entered directly onto a computer terminal using the Roelee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities or treatment-related clinical signs.

2. Body weight and food intake

Body weight

There were no statistically significant effects on absolute body weight or body weight gain in either sex. However, overall body weight gain (day 1-92) was reduced at the highest dose level by 27% and 33% in males and females.

Table 3.12.1.7- 7: Mean body weight and body weight gain (kg)

	Dose level (mg/kg bw/day)			
	0	**5**	**70**	**1,000**
Males				
Body weight [kg] *(% difference to control)*				
Day 1	10.3	10.2 *(-0.1)*	10.2 *(-0.1)*	10.0 *(-0.3)*
Day 8	10.5	10.5 *(±0.0)*	10.4 *(-1.0)*	10.3 *(-1.9)*
Day 15	10.9	10.8 *(-0.9)*	10.7 *(-1.8)*	10.5 *(-3.7)*
Day 22	11.2	11.1 *(-0.9)*	10.9 *(-2.7)*	10.7 *(-4.5)*
Day 29	11.3	11.2 *(-0.9)*	11.1 *(-1.8)*	10.9 *(-3.5)*
Day 36	11.6	11.5 *(-0.9)*	11.3 *(-2.6)*	11.2 *(-3.4)*
Day 43	12.0	11.9 *(-0.8)*	11.6 *(-3.3)*	11.4 *(-5.0)*
Day 50	12.1	12.0 *(-0.8)*	11.7 *(-3.3)*	11.7 *(-3.3)*
Day 57	12.2	12.2 *(±0.0)*	11.8 *(-3.3)*	11.8 *(-3.3)*
Day 64	12.3	12.4 *(+0.8)*	11.7 *(-4.9)*	11.9 *(-3.3)*
Day 71	12.5	12.6 *(+0.8)*	12.1 *(-3.2)*	12.0 *(-4.0)*
Day 78	12.6	12.6 *(±0.0)*	12.1 *(-4.0)*	11.9 *(-5.6)*
Day 85	12.6	12.9 *(+2.4)*	12.1 *(-4.0)*	12.0 *(-4.8)*
Day 92	12.8	13.1 *(+2.3)*	12.2 *(-4.7)*	11.9 *(-7.0)*
Body weight gain [kg] *(% difference to control)*				
Day 1-92	2.6	2.9 *(+11.5)*	2.0 *(-23.1)*	1.9 *(-26.9)*

	Dose level (mg/kg bw/day)			
	0	**5**	**70**	**1,000**
Females				
Body weight [kg] *(% difference to control)*				
Day 1	8.9	9.6 *(+7.9)*	9.5 *(+6.7)*	9.1 *(+2.2)*
Day 8	9.0	9.7 *(+7.8)*	9.7 *(+7.8)*	9.1 *(+1.1)*
Day 15	9.3	9.8 *(+5.4)*	9.8 *(+5.4)*	9.1 *(-2.2)*
Day 22	9.5	9.9 *(+4.2)*	9.9 *(+4.2)*	9.2 *(-3.2)*
Day 29	9.6	10.0 *(+4.2)*	10.1 *(+5.2)*	9.5 *(-1.0)*
Day 36	9.8	10.3 *(+5.1)*	10.4 *(+6.1)*	9.6 *(-2.0)*
Day 43	10.1	10.6 *(+0.5)*	10.5 *(+4.0)*	9.9 *(-2.0)*
Day 50	10.2	10.6 *(+3.9)*	10.5 *(+2.9)*	9.9 *(-2.9)*
Day 57	10.3	10.6 *(+2.9)*	10.7 *(+3.9)*	9.9 *(-3.9)*
Day 64	10.3	10.8 *(+4.9)*	10.9 *(+5.8)*	9.9 *(-3.9)*
Day 71	10.6	10.8 *(+1.9)*	10.8 *(+1.9)*	10.1 *(-4.7)*
Day 78	10.6	11.2 *(+5.7)*	10.9 *(+2.8)*	10.2 *(-3.8)*
Day 85	10.6	11.2 *(+5.7)*	11.0 *(+3.8)*	10.2 *(-3.8)*
Day 92	10.7	11.3 *(+5.6)*	11.0 *(+2.8)*	10.3 *(-3.7)*
Body weight gain [kg] *(% difference to control)*				
Day 1-92	1.8	1.7 *(-5.6)*	1.6 *(-11.1)*	1.2 *(-33.3)*

* / ** p < 0.05 / p < 0.01, statistically different to control using Dunnet´s test
# / ## p < 0.05 / p< 0.01, statistically different to control using modified t-test

Food intake

There was no treatment-related effect on food consumption in either sex. There were also no consistent differences in food conversion ratios between treated and control groups.

Table 3.12.1.7- 8: Mean food consumption (g/animal/day) (% difference to control)

	Dose level (mg/kg bw/day)			
	0	**5**	**70**	**1,000**
Males				
Week 1	390	398 *(+2.1)*	401 *(+2.8)*	397 *(+1.8)*
Week 2	393	400 *(+1.8)*	389 *(-1.0)*	400 *(+1.8)*
Week 3	378	393 *(+4.0)*	396 *(+4.8)*	388 *(+2.6)*
Week 4	391	400 *(+2.3)*	400 *(+2.3)*	400 *(+2.3)*
Week 5	390	400 *(+2.6)*	391 *(+0.3)*	398 *(+2.1)*
Week 6	398	400 *(+0.5)*	400 *(+0.5)*	397 *(-0.3)*
Week 7	392	401 *(+2.3)*	400 *(+2.0)*	384 *(-2.0)*
Week 8	396	401 *(+1.3)*	400 *(+1.0)*	382 *(-3.5)*
Week 9	375	401 *(+6.9)*	396 *(+5.6)*	380 *(+1.3)*
Week 10	395	400 *(+1.3)*	387 *(-2.0)*	379 *(-4.1)*
Week 11	377	400 *(+6.1)*	385 *(+2.1)*	368 *(-2.4)*
Week 12	390	401 *(+2.8)*	400 *(+2.6)*	368 *(-5.6)*
Week 13	400	400 *(±0.0)*	394 *(-1.5)*	388 *(-3.0)*

	Dose level (mg/kg bw/day)			
	0	**5**	**70**	**1,000**
Females				
Week 1	330	358 *(+8.5)*	383 *(+16.1)*	298 *(-10.0)*
Week 2	342	361 *(+5.6)*	384 *(+12.3)*	332 *(-2.9)*
Week 3	343	344 *(+0.3)*	341 *(-0.6)*	313 *(-9.6)*
Week 4	356	359 *(+0.8)*	382 *(+7.3)*	345 *(-3.1)*
Week 5	363	376 *(+3.6)*	387 *(+6.6)*	348 *(-4.1)*
Week 6	369	383 *(+3.8)*	381 *(+3.3)*	344 *(-6.8)*
Week 7	362	376 *(+3.9)*	359 *(-0.8)*	330 *(-8.8)*
Week 8	372	382 *(+2.7)*	386 *(+3.8)*	329 *(-11.6)*
Week 9	358	375 *(+4.7)*	374 *(+4.5)*	348 *(-2.8)*
Week 10	350	378 *(+8.0)*	375 *(+7.1)*	336 *(-4.0)*
Week 11	351	381 *(+8.5)*	374 *(+6.6)*	317 *(-9.7)*
Week 12	353	366 *(+3.7)*	377 *(+6.8)*	331 *(-9.1)*
Week 13	347	377 *(+8.6)*	372 *(+7.2)*	314 *(-9.5)*

* / ** p < 0.05 / p < 0.01, statistically different to control using Dunnet´s test
# / ## p < 0.05 / p< 0.01, statistically different to control using modified t-test

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Hematology:

There were no treatment-related effects.

Clinical chemistry:

There were no treatment-related effects.

Urinalysis:

No treatment-related abnormalities in the urine were detected.

5. Sacrifice and pathology

Necropsy:

No treatment-related macroscopic abnormalities were seen.

Organ weights:

At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, when compared to controls. Liver weight relative to body weight was increased by 28% and 43% in males and females, respectively, when compared to controls.

There was no effect on organ weights in either sex at 70 or 5 mg/kg bw/day.

Table 3.12.1.7- 9: Mean absolute and relative liver weights (% difference to control)

Parameter	Dose level (ppm)					
	0	5		70		1,000	
Males						
Terminal BW (kg)	12.74	12.88	*(+1.1)*	12.16	*(-4.5)*	11.80	*(-7.4)*
Absolute liver weight (g)	368.1	351.1	*(-0.2)*	350.6	*(-4.8)*	438.8*	*(+19.2)*
Relative liver weight (% of body weight)	2.9	2.7	*(-6.9)*	2.9	*(±0.0)*	3.7*	*(+27.6)*
Females						
Terminal BW (kg)	10.64	11.15	*(+4.8)*	11.13	*(+4.6)*	10.19	*(-4.2)*
Absolute liver weight (g)	300.1	322.3	*(+7.4)*	318.6	*(+2.1)*	396.4	*(+32.1)*
Relative liver weight (% of body weight)	2.8	2.9	*(+3.6)*	2.9	*(+3.6)*	4.0*	*(+42.9)*

* $p < 0.05$, significantly different from control
BW: Body weight

6. Histopathology

No treatment-related effects were observed.

Minor changes observed were considered to be spontaneous in origin and of no toxicological significance.

III. Conclusion

There were no mortalities or clinical signs of toxicity observed during the study. In addition, there were no treatment-related ophthalmic changes and no effect on body weight, food intake, haematology, biochemistry, urinalysis, macroscopic pathology or histopathology. At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, compared to controls. Liver weight relative to bodyweight ratio was increased by 28% and 43% in males and females, respectively, when compared to controls.

Thus, the NOAEL in the 90-day dietary study in dogs was 70 mg/kg bw/day based on the increased absolute and relative liver weight at 1000 mg/kg bw/day for both sexes. Dogs are noted to be a non-rodent species and the large increase in liver weight is therefore considered toxicologically relevant.

3.12.1.8 Anonymous; 2002; M-216694-01-1

Study reference:

Anonymous; 2002; AE C638206 - 52-week toxicity study by oral route (gavage) in beagle dogs; M-216694-01-1

Deviations: Deviations from the current OECD guideline (452, 2018):

- Harderian gland, oesophagus, seminal vesicles with coagulating glands and trachea were not sampled, fixed or examined histopathologically

These deviations do not affect the overall acceptability of the study.

Executive Summary:

A total of 40 beagle dogs (20 males and 20 females) of average body weight 8.9 kg for the males and 8.2 kg for the females at the start of the experiment, were divided into four groups of 5 animals/sex/group which received doses of 0, 70, 300 or 1,000 mg/kg bw/day of fluopicolide by the oral (gavage) route. The test substance was administered as a suspension in 1% aqueous methylcellulose solution, at a constant dosage-volume of 5 mL/kg bw/day. The control group was given the same dose-volume of the vehicle by the same route on the same occasions.

The animals were checked at least twice daily for mortality, morbidity and clinical signs were observed once a day. A detailed clinical examination was performed once prior to the start of treatment, and once weekly throughout the treatment period. The body weights were recorded at least once before the start of the study, on the first day of treatment and then at weekly intervals. Food consumption was recorded daily, starting 7 days before treatment and then throughout the treatment period. An ophthalmological examination was performed once before treatment and during Weeks 12, 25 and 51. Haematological, blood biochemical and urinalysis investigations were performed prior to the treatment period and during weeks 13, 26 and 51. On completion of treatment, the animals were sacrificed. All animals were subjected to a complete macroscopic post-mortem examination. Selected organs were weighed and specified tissues preserved. Selected tissues from all animals were examined microscopically.

At 1,000 mg/kg bw/day, the males had no body weight gains and an increased incidence of regurgitation and salivation occasions was observed. At the same dose, an increase in cholesterol levels in females in Week 51 (+42%, $p<0.05$) was measured which may suggest possible impairment of some liver functions.

At 300 mg/kg bw/day also a slight increased incidence of regurgitation and salivation occasions was seen, whereas at 70 mg/kg bw/day no treatment-related effects occurred.

Gross examination at necropsy showed liver enlargement in single animals at ≥300 mg/kg bw/day. However, organ weights did not reveal any statistically significant intergroup differences. In addition, microscopic examination did not reveal any changes in organs and tissues. Therefore, the gross necropsy findings are considered adaptive and not toxicologically adverse.

In conclusion, the test substance, fluopicolide caused reduced body weight gain of males and increased cholesterol levels in females at 1,000 mg/kg bw/day as the main treatment-related effects. Thus, considering that regurgitation and salivation were secondary effects due to the viscous dosage form at high concentration gavage application, the No Observed Adverse Effect Level (NOAEL) was established at 300 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	1% aqueous methylcellulose solution

3. Test animals

Species:	Dogs
Strain:	Beagle dogs
Age:	6 months old
Weight at start:	8.95 kg (range: 7.90 to 10.1 kg) for the males and 8.18 kg (range: 6.70 to 10.4 kg) for the females
Source:	Marshall Farms, North Rose, New York, USA
Acclimation period:	Yes
Diet:	UAR 125 C2 pelleted diet, batch Nos. 10201, 10308,10403,10509, 10530, 10716, 10731, 10629, 10927 and 11010 and from day 275 UAR 125 C3 pelleted diet, batch Nos. 11102, 11205, 20109, 20208 and 20320 (UAR, Villemoisson, Epinay-sur-Orge, France)
Water:	Water ad lib
Housing:	Individually housed in pens containing wood shavings (SICSA, Alfortville, France) for bedding material, except when a urine sample was required. The dogs were group-housed once a week by sex and group after the last recording of clinical signs (afternoon) until the next morning
Temperature:	20 ± 5 °C
Humidity:	50 ± 20%
Air changes:	12 cycles/hour of filtered, non-recycled air
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 26th 2001 to April 15th 2002

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups.

Table 3.12.1.8- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Treatment phase			
1	0	5	5
2	70	5	5
3	300	5	5
4	1,000	5	5

The dosage forms were administered daily for a period of 52 weeks. Day 1 corresponds to the first day of treatment.

3. Dose preparation and analysis

The oral route was selected since it is a possible route of exposure in man.

The dosage forms were administered by gavage using a plastic syringe fitted with a plastic esophageal tube, once a day, at approximately the same time. The quantity of dosage formulation administered to each animal was adjusted according to the most recently recorded body weight. A constant dosage-volume of 5 mL/kg bw/day was used. The dosage forms were stirred continuously throughout the dosing procedure.

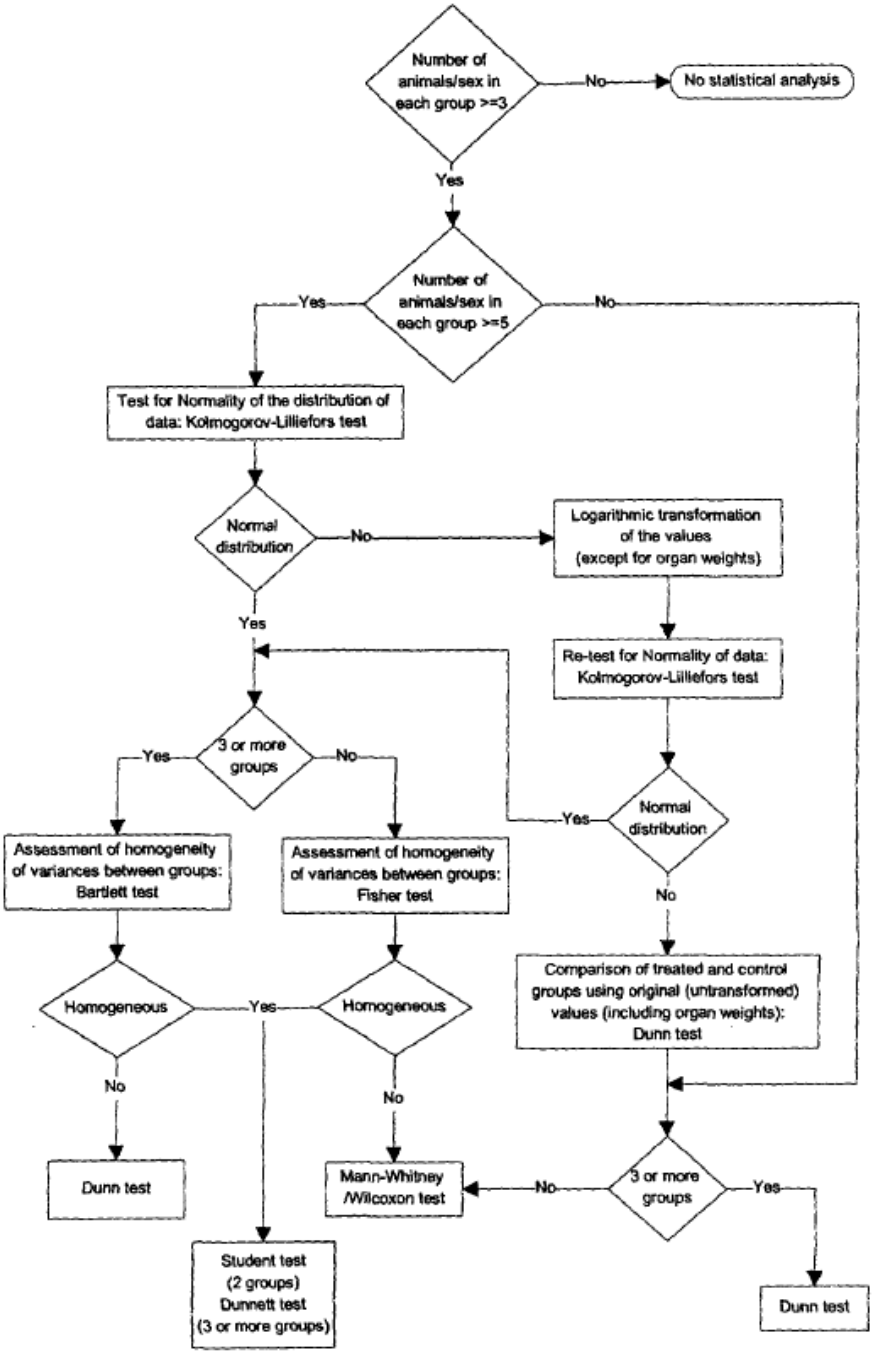
The results of the analyses demonstrated the homogeneity of each dosage form analyzed before the study (14 and 200 mg/mL).

The results of the analyses demonstrated the stability of dosage forms prepared at 14 and 200 mg/mL over a six hours period at room temperature (away from light).

Throughout the study, a satisfactory agreement was observed between the nominal and actual concentrations of the test item in the administered dosage forms since the deviations from nominal concentrations were in the acceptable range of ± 10%.

4. Statistics

The following sequence was used for the statistical analyses of body weight, food consumption, hematology, blood biochemistry, urinalysis and organ weight data:



C. Methods

1. Observations

Each animal was observed at least once a day, at approximately the same time, for the recording of clinical signs.

Each animal was checked at least twice a day, including during weekends and public holidays, for mortality or signs of morbidity. A macroscopic post-mortem examination was performed on the found dead female X50163 of dose group 3 and the required tissues preserved for a microscopic examination.

A detailed clinical examination was performed on all animals before the beginning of the treatment period and once weekly during the study.

These observations were made outside the kennels and included (but not limited to) evaluation of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. salivation, lacrimation, piloerection, pupil size, unusual respiratory pattern).

Changes in gait, posture and response to handling as well as presence of clonic or tonic movements, stereorypies (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) were recorded.

Due to the low weight of males of the high dose group, a detailed clinical examination was performed by a veterinarian on these animals in week 32, for health status.

2. Body weight and food intake

The body weight of each animal was recorded once before allocation of the animals to groups, on the first day of treatment, and then once a week until the end of the study.

The quantity of food consumed by each animal was recorded daily by weighing the quantity given and that remaining the next morning. Food intake per animal and per day was calculated 7-days before the beginning of the treatment period and then throughout the study. Whenever fasting was required, the food was removed at the end of the day and calculation of food consumed was made at that time (over a period of approx. 5 hours for that day).

3. Ophthalmoscopy

Ophthalmological examination was performed on all animals before the beginning of the treatment period and one occasion during Weeks 12, 25 and 51.

Pupillary light and blink reflexes were evaluated first. The pupils of the animals were then dilated with tropicamide (Mydriaticum®, Merck Sharp & Dohme-Chibret, Paris, France), and the appendages, optic media and fundus were examined by indirect ophthalmoscopy. Anterior segment and the lens, were also examined by a slit-lamp biomicroscope.

4. Laboratory investigations (clinical chemistry)

Blood samples were taken from a jugular vein without anesthesia (before the daily treatment) and collected into tubes containing the appropriate anticoagulant (see below).

Prior to blood sampling and during urine collection, the animals were deprived of food for an overnight period of at least 14 hours. The urine was collected in the presence of thymol crystals.

<u>Haematology:</u>

The following parameters were determined for all animals once before the beginning of the treatment period and during weeks 13, 26 and 51. In addition, a complete haematological examination for a clinical diagnostic was performed on the male X50118 of group 4 group which presented respiration distress (abdominal breathing) and lateral decubitus on day 95 approx. 30 minutes after dosing.

Table 3.12.1.8- 2: Haematology parameters

Mean cell haemoglobin (MCH)	Eosinophils (E)
Thrombocytes (PLAT)	Basophils (B)
Differential white blood cell count with cell morphology[a]	Reticulocytes (RET)[b]
Neutrophils (N)	Prothrombin time (PT)
Lymphocytes (L)	Activated partial thromboplastin time (APTT)
Monocytes (M)	Fibrinogen (FIB)

(a): blood smears were prepared from all sampled animals. If the samples were not accepted by the HI Analyzer, a microscopic evaluation was performed after May Grunwald Giemsa staining.

(b): blood smears were prepared from all sampled animals. As no anemia was observed, the reticulocyte count was not determined.

Bone marrow:

Bone marrow smears were prepared from the sternum of all animals killed on completion of the treatment period for examination of the bone marrow and differential cell count.

As no relevant abnormalities were observed in haematological investigations, the bone marrow differential cell count was not determined.

Biochemistry:

The following parameters were determined for all animals once before the beginning of the treatment period and during weeks 13, 26 and 51. In addition, a complete blood biochemical investigation was performed on the male X50118 of group 4 for a clinical diagnostic.

Table 3.12.1.8- 3: Biochemistry parameters

Total protein (PROT)	Cholesterol (CHOL)
Albumin (ALB)	Triglycerides (TRIG)
A/G ratio (A/G)	Total bilirubin (TOT.BIL)
Calcium (Ca++)	Chloride (Cl-)
Inorganic phosphorus (I.PHOS)	Aspartate aminotransferase (ASAT)
Sodium (Na+)	Alanine aminotransferase (ALAT)
Potassium (K+)	Alkaline phosphatase (AP)
Urea (UREA)	G-glutamyl transferase (GGT)
Creatinine (CREAT)	Creatine kinase (CK)
Glucose (GLUC)	
Total Proteins (PROT)	

Urinalysis:

The following parameters were determined for all animals once before the beginning of the treatment period and during Weeks 13, 26 and 51.

Table 3.12.1.8- 4: Urinalysis parameters

Appearance (APP)	Cytology of sediment:
Color (COLOR)	Leucocytes (WBC)
Volume (VOLUME)	Erythrocytes (RBC)
Specific gravity (SP.GRAV)	Cylinders (CYLIN)
pH (pH)	Magnesium ammonium phosphate crystals (AMM.PH)
Protein (PROT)	Calcium phosphate crystals (CAL.PH)
Bilirubin (BILI)	Calcium oxalate crystals (CAL.OX.)
Glucose (GLUC)	cells (CELLS)
Urobilinogen (UROB)	
Ketones (CETO)	
Blood (BLOOD)	

4. Sacrifice and pathology

Macroscopic examination:

On completion of the treatment period, after at least 14 hours fasting, all surviving animals were anesthetized by an intravenous injection of thiopental sodium and killed by exsanguination.

A complete macroscopic post-mortem examination was performed on all study animals. This included examination of the external surfaces, all orifices, the cranial cavity, the external surface of the brain and spinal cord, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues.

Organ weights:

The body weight of all animals killed at the end of the treatment period was recorded before sacrifice, and the organs specified in the Tissue Procedures Table were weighed wet as soon as possible after dissection.

The ratio of organ weight to body weight (recorded immediately before sacrifice) and the ratio of organ weight to brain weight were calculated.

Table 3.12.1.8- 5: Organ weights

Adrenals	Ovaries
Brain	Spleen
Epididymides	Testes
Heart	Thyroids with parathyroids
Kidneys	Uterus (horn and cervix)
Liver	

Tissue sampling:

For all study animals, the tissues specified in the Tissue Procedures Table were preserved in 10% buffered formalin (except for the eyes with optic nerve which were fixed in Davidson's fixative and the testes and epididymides which were preserved in Bouin's fluid).

Table 3.12.1.8- 6: Tissue sampling

Macroscopic lesions	Lungs with bronchi	Spinal cord (cervical, thoracic and lumbar)
Adrenals	Lymph nodes (mandibular, mesenteric)	Spleen
Aorta	Mammary glands/area	Sternum with bone marrow
Brain	Nasal cavities	Stomach
Caecum	Optic nerve	Testes
Colon	Ovaries	Thymus
Duodenum	Pancreas	Thyroids with parathyroids
Epididymides	Pharynx	Tongue
Eyes	Pituitary	Trachea
Femoral bone with articulation	Prostate	Urinary bladder
Gall bladder	Rectum	Uterus (horn and cervix)
Heart	Salivary glands (parotid and submandibular)	Vagina
lleum	Sciatic nerve	
Jejunum	Skeletal muscle	
Kidneys	Skin	
Larynx		
Liver		

5. Histopathology

Tissue processing:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned at thickness of approximately four microns and stained with haematoxylin-eosin. This work was performed at Histotox (La Rochelle, France).

In addition, Oil red O stainings were performed on frozen sections of liver of all animals from the control and high dose groups killed at the end of the treatment period and for the female that died.

Microscopic examination:

A microscopic examination was performed on all tissues listed in Table 3.12.1.8- 7 for all study animals.

Table 3.12.1.8- 7: Microscopic examination

Macroscopic lesions Adrenals Aorta Brain Caecum Colon Duodenum Epididymides Eyes Gall bladder Heart lleum Jejunum Kidneys Larynx Liver Lungs with bronchi	Lymph nodes (mandibular, mesenteric) Mammary glands/area Nasal cavities Optic nerve Ovaries Pancreas Pharynx Pituitary Prostate Rectum Salivary glands (parotid and submandibular) Sciatic nerve Skeletal muscle Skin	Spinal cord (cervical, thoracic and lumbar) Spleen Sternum with bone marrow Stomach Testes Thymus Thyroids with parathyroids Trachea Urinary bladder Uterus (horn and cervix) Vagina

II. Results and Discussion

A. Results

1. Clinical results and Mortality

There was no treatment-related mortality during the study. One female (X50163) of the 300 mg/kg bw/day dose group died. No specific indices for death were identified. After a review of clinical signs prior to death and macroscopic and microscopic post-mortem examinations, accidental aspiration of stomach contents into the lungs after regurgitation was suggested by the investigators as a possible cause of death.

Excessive salivation, vomiting and regurgitation were the most frequently observed clinical signs in treated animals. All these signs were seen transiently and sometimes noted among control animals.

The slightly higher incidence of excessive salivation recorded in the 1,000 mg/kg bw/day groups (3/5 males on 12 occasions; 3/5 females on 34 occasions) was considered to be due to the viscous dosage form at that high concentration (200 mg/mL). Male X50118 of this group presented respiration distress (abdominal breathing) and lateral decubitus on day 95 approx. 30 minutes after dosing. This was considered to be caused by difficult administration of the viscous dosage form at that concentration leading to regurgitation and probable accidental aspiration of stomach contents into the lungs. A relationship to toxicity of the test substance was ruled out.

The other recorded clinical signs were occasional, of low incidence, noted in control animals, without obvious dose-relationship and/or commonly noted in beagle dogs. Therefore, they were not considered to be related to treatment.

Table 3.12.1.8- 8: Clinical observations

Finding	Dose level (mg/kg bw/day)							
	Males				Females			
	0	**70**	**300**	**1,000**	**0**	**70**	**300**	**1,000**
Number of animals examined	5	5	5	5	5	5	5	5
Excessive salivation	0	1	2	3	2	1	2	3
Abdominal breathing	1	0	0	1	1	0	0	0
Lateral decubitus	0	0	0	1	0	0	0	0
Vomiting								
normal	0	0	1	0	0	0	0	0
colorless	1	0	0	0	0	1	0	0
whitish	0	0	1	1	0	1	0	0
yellowish	1	0	0	0	0	0	0	0
brown	0	0	0	1	0	1	0	0
brownish	1	0	1	2	0	0	0	2
Regurgitation	0	1	3	2	1	0	1	1

Detailed clinical examination:

No abnormalities were noted in any group. Males given 1,000 mg/kg bw/day were in healthy condition as observed in week 32.

Consequently, there were no treatment-related clinical signs in any groups.

2. Body weight and food intake

Body weight

The body weight development of males given 70 or 300 mg/kg bw/day was similar to that of controls. Treatment with the test item at 1,000 mg/kg bw/day affected the body weight gain of males: after 52 weeks of treatment, males given 1,000 mg/kg bw/day did not gain any weight, and few animals (X50116, X50119 and X50120) had body weight loss on some occasions (values of body weight loss varying from -0.4 to -1.2 kg).

The body weight gain of females treated at 70, 300 and 1,000 mg/kg bw/day was not affected by treatment with the test item.

Table 3.12.1.8- 9: Mean body weight and body weight gain (kg)

	Dose level (mg/kg bw/day)			
	0	**70**	**300**	**1,000**
Males				
Body weight [kg] *(% difference to control)*				
Week -3	8.7	8.8 *(+1.2)*	8.8 *(+1.2)*	8.7 *(±0.0)*
Week 1	9.0	8.9 *(-1.1)*	9.1 *(+1.1)*	8.8 *(-2.2)*
Week 4	8.9	9.1 *(+2.2)*	9.2 *(+3.4)*	8.9 *(±0.0)*
Week 8	9.2	9.5 *(+3.3)*	9.5 *(+3.3)*	8.7 *(-5.4)*
Week 12	9.4	9.8 *(+4.3)*	9.7 *(+3.2)*	9.0 *(-4.3)*
Week 16	9.5	10.0 *(+5.3)*	9.9 *(+4.2)*	8.9 *(-6.3)*
Week 20	9.5	10.0 *(+5.3)*	9.8 *(+3.2)*	8.8 *(-7.4)*
Week 24	9.5	10.2 *(+7.4)*	10.1 *(+6.3)*	8.8 *(-7.4)*
Week 28	9.6	10.3 *(+7.3)*	10.1 *(+5.2)*	8.7 *(-9.4)*
Week 32	9.5	10.4 *(+9.5)*	10.0 *(+5.3)*	8.7 *(-8.4)*
Week 36	9.5	10.4 *(+9.5)*	10.1 *(+6.3)*	8.7 *(-8.4)*
Week 40	9.8	10.7 *(+9.2)*	10.4 *(+6.1)*	8.9 *(-9.2)*
Week 44	9.9	10.8 *(+9.1)*	10.4 *(+5.1)*	8.9 *(-10.1)*
Week 48	10.0	10.9 *(+9.0)*	10.5 *(+5.0)*	9.1 *(-9.0)*
Week 52	9.8	10.6 *(+8.2)*	10.5 *(+7.1)*	8.8 *(-10.2)*
Body weight gain [kg] *(% difference to control)* [+]				
Week 1-52	0.8	1.7 *(+112.5)*	1.4 *(+75.0)*	0.0 *(-100.0)*
Females				
Body weight [kg] *(% difference to control)*				
Week -3	8.0	7.6 *(-5.0)*	7.8 *(-2.5)*	7.7 *(-3.7)*
Week 1	8.4	8.0 *(-4.8)*	8.3 *(-1.2)*	8.0 *(-4.8)*
Week 4	8.4	8.1 *(-3.6)*	8.4 *(±0.0)*	8.2 *(-2.4)*
Week 8	9.1	8.6 *(-5.5)*	9.1 *(±0.0)*	8.9 *(-2.2)*
Week 12	9.2	8.7 *(-5.4)*	9.3 *(+1.1)*	9.1 *(-1.1)*
Week 16	9.5	8.9 *(-6.3)*	9.4 *(-1.1)*	9.3 *(-2.1)*
Week 20	9.5	9.1 *(-4.2)*	9.4 *(-1.1)*	9.5 *(±0.0)*
Week 24	9.7	9.0 *(-7.2)*	9.3 *(-4.1)*	9.3 *(-4.1)*
Week 28	9.5	9.2 *(-3.2)*	9.6 *(+1.1)*	8.9 *(-6.3)*
Week 32	9.3	9.1 *(-2.2)*	9.5 *(+2.2)*	9.0 *(-3.2)*
Week 36	9.2	9.2 *(±0.0)*	9.6 *(+4.3)*	8.8 *(-4.3)*
Week 40	9.6	9.5 *(-1.0)*	10.0 *(+4.2)*	9.1 *(-5.2)*
Week 44	9.4	9.4 *(±0.0)*	9.7 *(+3.2)*	9.2 *(-2.1)*
Week 48	9.6	9.3 *(-3.1)*	9.7 *(-1.0)*	9.4 *(-2.1)*
Week 52	9.7	9.7 *(±0.0)*	10.1 *(+4.1)*	9.3 *(-4.1)*
Body weight gain [kg] *(% difference to control)* [+]				
Week 1-52	1.3	1.7 *(+30.8)*	1.8 *(+38.5)*	+1.3 *(±0.0)*

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet´s test

[+] No statistical analyses were performed for body weight gains

Food intake

Statistically significant changes in food consumption were recorded occasionally throughout the study in both males and females. However, since the effects were inconsistent and the overall amount of food consumed by treated animals was comparable to controls, these are considered to be unrelated to treatment.

Table 3.12.1.8- 10: Mean food consumption (g) (% difference to control)

Parameter	Dose level (mg/kg bw/day)			
	0	70	300	1,000
Males				
Week 1-52	299	292 *(-2.3)*	293 *(-2.0)*	291 *(-2.7)*
Females				
Week 1-52	282	275 *(-2.5)*	263 *(-6.7)*	262 *(-7.1)*

No statistical analyses were performed for mean food consumption for the whole study period

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

When compared to the mean control or pre-dose values, there were no treatment-related differences in the haematological parameters in any group.

Clinical chemistry:

Slightly increased cholesterol levels were noted in females given 1,000 mg/kg bw/day in Week 51, as indicated in Table 3.12.1.8- 11.

Some differences occasionally attaining statistical significance were noted in treated animals when compared with controls. However these were not considered to be related to treatment with the test item since they were slight, not necessarily dose-related and/or individual values were within the range of the laboratory historical background data.

Table 3.12.1.8- 11: Cholesterol data (mean ± SD)

	Dose level (mg/kg bw/day)							
	Males				Females			
	0	70	300	1,000	0	70	300	1,000
Cholesterol concentration (mmol/L)								
Pre-dose	3.0± 0.5	3.2± 0.7	2.9± 0.6	2.6± 0.5	3.2± 0.4	2.8± 0.2	2.8± 0.1	2.9± 0.3
Week 13	2.9± 0.4	3.6± 0.6	3.4± 0.6	3.3± 1.1	3.5± 0.9	3.8 ± 0.9	4.6 ± 1.3	5.0 ± 1.6
Week 26	3.0± 0.6	3.6± 0.5	3.3± 0.7	2.8± 0.9	3.8 ± 0.4	4.4 ± 1.6	3.8 ± 0.7	4.4 ± 0.8
Week 51	2.9± 0.6	3.5± 0.8	3.5± 0.6	3.6± 2.1	3.8 ± 0.7	4.4 ± 1.2	3.9 ± 0.7	5.4*± 0.8

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control
SD: standard deviation
Historical control range for plasma cholesterol level in females: 2.3-4.8 mmol/L

Urinalysis:

There were no changes in any qualitative or quantitative urinary parameters that could be attributed to treatment with the test item.

The increased urine volume noted in treated males during week 51 was not considered to be of toxicological importance since this difference was due to the abnormal low values of control males at this period.

5. Sacrifice and pathology

Necropsy:

Animals found dead:

At necropsy of the female (X50163) found dead in group 3, the main findings were foamy contents in the trachea, dilated/colored lungs together with several greyish/whitish area, reddish liquid contents hi the thoracic cavity and dilated stomach with yellowish liquid contents.

Terminal sacrifice:

The liver was enlarged in 1/5 males and 1/5 females given 300 mg/kg bw/day and in 3/5 males given 1,000 mg/kg/day. The kidneys were enlarged in 1/5 males given 300 mg/kg bw/day. As these necropsy findings were not associated with relevant histopathological changes, they were considered to be of no toxicological adversity.

The few other macroscopic findings noted were those which are commonly encountered in the untreated laboratory beagle dog kept under laboratory condition and thus were considered to be unrelated to treatment.

Organ weights:

Some changes in relative liver, thyroid and spleen weights to body weight or brain weight were observed in treated dogs compared to controls. However, they are considered to be of no toxicological significance since the differences were slight, without statistical significance and without any relevant histopathological changes.

The other differences noted in organ weights were minor, without any dose-relationship, and/or without the same trend in the two sexes. They were therefore not considered to be related to treatment.

Table 3.12.1.8- 12: Selected absolute and relative organ weights (% difference to control)

Parameter	Dose (mg/kg bw/day)			
	0	**70**	**300**	**1,000**
Males				
Liver				
Absolute weight (g)	303.8	298.2 *(-1.8)*	336.8 *(+10.9)*	354.3 *(+16.6)*
Relative to body weight (%)	3.08	2.82 *(-8.4)*	3.20 *(+3.9)*	4.01 *(+30.2)*
Relative to brain weight (%)	413.4	422.9 *(+2.3)*	458.7 *(+11.0)*	489.2 *(+18.3)*
Spleen				
Absolute weight (g)	29.46	28.91 *(-1.9)*	26.71 *(-9.3)*	23.99 *(-18.6)*
Relative to body weight (%)	0.296	0.267 *(-9.8)*	0.255 *(-13.9)*	0.273 *(-7.8)*
Relative to brain weight (%)	40.21	40.91 *(+1.7)*	36.33 *(-9.6)*	33.12 *(-17.6)*

Parameter	Dose (mg/kg bw/day)			
	0	70	300	1,000
Thyroid				
Absolute weight (g)	0.826	0.857 *(+3.8)*	0.871 *(+5.4)*	0.804 *(-2.7)*
Relative to body weight (%)	0.0081	0.0079 *(-2.5)*	0.0082 *(+1.2)*	0.0092 *(+13.6)*
Relative to brain weight (%)	1.13	1.22*(+8.0)*	1.19 *(+5.3)*	1.11 *(-1.8)*
Females				
Liver				
Absolute weight (g)	261.5	279.9 *(+7.0)*	279.0 *(+6.7)*	273.1 *(+4.4)*
Relative to body weight (%)	2.74	3.01 *(+9.8)*	2.91 *(+6.2)*	3.06 *(+11.7)*
Relative to brain weight (%)	400.8	378.4 *(-5.6)*	385.9 *(-3.7)*	384.6 *(-4.0)*
Spleen				
Absolute weight (g)	28.02	32.09 *(+14.5)*	31.92 *(+13.9)*	24.89 *(-11.2)*
Relative to body weight (%)	0.291	0.351 *(+20.6)*	0.330 *(+13.4)*	0.278 *(-4.5)*
Relative to brain weight (%)	43.11	43.12 *(+0.02)*	44.09 *(+2.3)*	35.12 *(-18.5)*
Thyroid				
Absolute weight (g)	0.756	0.808 *(+6.9)*	0.798 *(+5.6)*	0.866 *(+14.6)*
Relative to body weight (%)	0.0079	0.0086 *(+8.9)*	0.0082 *(+3.8)*	0.0097 *(+22.8)*
Relative to brain weight (%)	1.16	1.09 *(-6.0)*	1.11 *(-4.3)*	1.23 *(+6.0)*

6. Histopathology

Unscheduled death:

The major microscopic findings in the found dead female (X50163) were marked desquamative interstitial pneumonitis, moderate foamy alveolar macrophages and subpleural fibrosis in the lungs, adrenal cortical cell hypertrophy and vascular ectasia (agonic) in the intestines and some internal organs and tissues. No factor contributing to the death could be clearly established.

Terminal sacrifice:

No treatment-related microscopic findings were noted.

All microscopic changes recorded were recognized as those commonly observed in the Beagle dog kept under laboratory conditions. Moreover, they were present with approximately similar incidence and severity in both control and treated animals.

In addition, the microscopic examination of the frozen liver sections stained with Oil red O, showed that except for one male dog from the top dose group (1,000 mg/kg bw/day) where strong positive reaction for Oil red O was found, in all other animals examined very weak to moderate positive reactions were noted with approximately similar incidence in both control and treated animals. Consequently, it was concluded that the treatment with the test item did not exert an effect on lipid deposition/distribution in the liver.

III. Conclusion

The liver appeared to be the main target organ in the one year oral toxicity study in dogs and was characterised by adaptive enlargement of the liver. Increased plasma cholesterol levels may suggest possible impairment of some liver functions. The NOAEL in the one year oral toxicity study was 300 mg/kg bw/day based on impaired body weight gain in males and slight increased cholesterol levels in females at 1,000 mg/kg bw/day.

3.12.1.9 Anonymous; 2002; M-208051-01-1

Study reference:

Anonymous; 2002; Neurotoxicity study by dietary administration to CD rats for 13 weeks AE C638206; M-208051-01-1

Deviations:	Deviations from the current OECD guideline (424, 1997): None.

Executive Summary:

Three groups of 10 male and 10 female CD rats received fluopicolide orally, via the diet, at concentrations of 0, 200, 1,400 or 10,000 ppm for a treatment period of 13 weeks.

There were no deaths. The appearance and behaviour of the animals was unaffected by treatment. Overall body weight gains were low for males and females receiving 10,000 ppm and for females receiving 1,400 ppm and food consumption was slightly low for males and females receiving 10,000 ppm. The amount of food scattered was generally high for males and females receiving 1,400 or 10,000 ppm, and for females receiving 200 ppm, compared with the controls. Food conversion efficiency was low for males and females receiving 10,000 ppm and slightly low for females receiving 1,400 ppm. The overall achieved dosages at dietary concentrations of 200, 1,400 and 10,000 ppm were 15, 107 and 781 mg/kg bw/day for males and 18, 125 and 866 mg/kg bw/day for females.

The functional observational battery did not reveal any findings of significance. Absolute brain weights and anotomical measurements made of the cerebral hemispheres were not affected by treatment. Macroscopic examination revealed no treatment-related findings. There were no histopathological changes in the tissues presented for neuropathological examination which were considered to be related to treatment with fluopicolide.

However, treatment-related were seen in the liver and kidneys. An increased incidence of centrilobular hepatocyte hypertrophy of the liver which is considered adaptive was observed in males and females given 10,000 ppm and in males given 1,400 ppm. In addition, there was an increased incidence and/or severity of hyaline droplets in the cortical tubules in the kidneys of males at dose levels ≥1,400 ppm which was associated with increased incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin in the cortical tubules of affected kidneys. The hyaline droplets in the cortical tubules of male rats are considered likely to represent accumulation of α2μ-globulin within the lysosomes. It is generally regarded that α2μ-globulin nephropathy is a male rat specific toxic response to the administration of certain types of chemicals (hydrocarbon nephropathy) and therefore not relevant for humans.

It is concluded that the administration of fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. Thus, the NOAEL for neurotoxicity was >10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females). Adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male rat-specific 'hydrocarbon nephropathy' occurred at 1,400 and 10,000 ppm. Therefore, the NOAEL for systemic toxicity in the 13-week neurotoxicity study was 200 ppm (15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females) based on impaired growth and histopathological changes in kidney of male animals at dose levels of ≥1,400 ppm (equivalent 106.6 mg/kg bw/day in males and 125.2 mg/kg bw/day in females).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	CD strain
Age:	42 days old at start of study
Weight at start:	178 to 241 g (males, 151 to 183 g (females)
Source:	Charles River UK Ltd., Margate, Kent, UK
Acclimation period:	Yes
Diet:	Powdered rodent diet, Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England
Water:	Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), via polycarbonate bottles fitted with sipper tubes
Housing:	Individually housed in modified RB3 cages from North Kent Plastic Limited, Erith, Kent, England, which were made of a plastic body with a stainless steel mesh lid and floor.
Temperature:	19 – 23 °C
Humidity:	40 to 70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: May 16 to December 12, 2001

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 3.12.1.9- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	200	10	10
3	1,400	10	10
4	10,000	10	10

The test substance was administered continuously via the diet throughout the treatment period. Animals did not have access to the formulated diet beyond its shelf life as determined by the stability assay.

Dietary concentrations of fluopicolide remained constant throughout the study.

Control animals received untreated diet at the same frequency, and from the same batch of basal diet, as treated animals. The duration of treatment was 13 weeks.

Treatment, and the recording of serial observations, continued for all surviving animals throughout the necropsy period.

3. Diet preparation and analysis

Fluopicolide was incorporated into the ground diet to provide the required dietary concentrations by dilution of an appropriate premix. An initial premix at a concentration of 32,500 ppm was prepared.

The required amount of test substance was mixed with fine sieved diet by gentle stirring. Further quantities of sieved diet were then added to the premix until approximately half the required final weight was achieved. At this stage the premix was ground in a coffee grinder to ensure homogeneity of the mix. Coarse diet was added to make the premix up to the final required amount and mixed in a Turbula Mixer for 100 revolutions to ensure the test material was dispersed in the diet. Aliquots of this premix were then diluted with plain RM1 diet to produce the required highest and intermediate concentrations (1,400 and 10,000 ppm) for feeding to the animals and a second premix (3,250 ppm). The second premix was used to prepare the lowest concentration test mix (200 ppm). Each batch of treated diet was mixed for a further 100 revolutions in the Turbula mixer.

Batches of the test diets were prepared weekly and issued in sealed metal containers. The unused residues were discarded at the end of each treatment week. All diets were prepared at Huntingdon Research Centre and transported to Eye Research Centre for feeding to the animals.

The analytical procedure validation, the formulation homogeneity and the formulation stability, during both ambient temperature and frozen storage for 22 days, were confirmed for fluopicolide in rodent diet formulations at nominal concentrations of 50 and 2,500 ppm during an associated study.

Before commencement of treatment the homogeneity and stability of the test substance at 10,000 ppm (the highest concentration) in the diet was determined by a trial preparation. The homogeneity was assessed by analysing samples taken from the top, middle and bottom of the mix. The stability was determined after storage for 8 and 22 days at ambient temperature (nominally 21 °C). The results indicate that the test substance was homogeneous and stable for 22 days at ambient temperature.

Samples of each diet prepared for administration in Weeks 1 and 12 of treatment were analysed for the test substance. The results of these analyses demonstrated satisfactory achieved concentrations; the mean concentrations of fluopicolide in test diet formulations were within ±4% of nominal concentrations.

4. Statistics

For organ weights and body weight changes, homogeneity of variance was tested using Bartlett's test.

Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

The functional observation battery numerical data were subjected to statistical analysis: activity and rearing in the standard arena, body temperature, bodyweight, landing footsplay, grip strength and motor activity.

The following statistical analyses were performed:

If the data consisted predominantly of one particular value (relative frequency of the mode exceeds 75%), the proportion of animals with different values from the mode was analysed using Fisher's Exact test. Otherwise, Bartlett's test was performed to test for variance heterogeneity between groups.

Where significant (1% level) heterogeneity was found, the data were logarithmically transformed and re-tested for heterogeneity. If no statistically significant heterogeneity of variance was detected (with or without logarithmic transformation), a one way analysis of variance was carried out. If the analysis of variance showed evidence (at the 5% level) of differences between the groups, Student's t-test was used to test for differences between treatment groups and the control group. If heterogeneity was significant and could not be stabilised by logarithmic transformation, the Kruskal-Wallis test on ranks was performed on the untransformed data. If the Kruskal-Wallis test showed evidence (5% level) of differences between the groups, the Wilcoxon Rank-Sum test was used to test for differences between the treatment groups and the control group.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the controls ($p > 0.05$).

C. Methods

1. Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill-health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

In addition, a more detailed weekly examination, which included palpation, was performed on each animal. Further observations were made as part of the neurobehavioural screening examinations.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose facees.

During the acclimatisation period, observations of the animals and their cages were recorded at least once per day.

2. Body weight and food intake

Each animal was weighed during the acclimatisation period (Week -1), on the day that treatment commenced (Week 0), at weekly intervals throughout the treatment period and before necropsy. Further weighings occurred as part of the neurobehavioural screening.

More frequent weighing was instituted, when appropriate, for animals displaying ill-health, so that the progress of the observed condition could be monitored.

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded for each week throughout the treatment period. From these records the weekly consumption per animal was calculated.

Food conversion efficiencies were calculated for each sex/group for each week of treatment.

The group mean achieved dosage for each sex, expressed as mg/kg bw/day, was calculated at the same frequency that bodyweight was recorded. This was calculated from the nominal dietary test substance concentration, food consumption and bodyweight data.

3. Neurobehavioural screening - functional observational battery

Before commencement of treatment and during Weeks 4, 8 and 13 of treatment all animals were subjected to the procedures detailed below. The functional observational battery and motor activity recordings were performed at approximately the same time of day on each occasion and the observer was unaware of the experimental group to which the animal belonged. The animals were not necessarily all tested on the same day but the number of animals were balanced across the groups on each day of testing. Any deviation from normal was recorded with respect to nature and, where appropriate, degree of severity.

4. Home cage observations

The animals were observed in the home cage for the following parameters:

- Convulsions, tremors, twitches
- Palpebral closure
- Posture
- Spontaneous vocalisations

5. In the hand and standard arena observations

Observations were performed in the hand and then during a three minute period in a standard arena.

After removal from the home cage the following parameters were assessed:

In the hand:	**Standard arena:**
Ease of removal from cage	Activity count
Exophthalmos	Arousal
Fur condition	Convulsion, tremors, twitches
Piloerection	Defecation count
Reactivity to handling	Gait
Salivation/lacrimation	Grooming
Vocalisation on handling	Palpebral closure
	Posture
	Rearing count
	Respiration
	Urination

6. Manipulations

The following measurements, reflexes and responses were recorded:

- Approach response
- Body temperature
- Body weight
- Grip strength - fore and hindlimbs
- Landing footsplay
- Pupil reflex
- Righting reflex
- Startle reflex
- Tail pinch response
- Touch response

7. Motor activity

Motor activity was measured by automated infra-red sensor equipment, recording individual animal activity over a one hour period.

8. Euthanasia

Animals were sacrificed by an overdose of barbiturate by intra-peritoneal injection and exposure of the heart to permit perfusion with glutaraldehyde/paraformaldehyde fixative via the aorta.

The sequence in which the animals were sacrificed after completion of the treatment period was selected to allow satisfactory inter-group comparison.

9. Pathology

Macroscopic pathology:

All animals were subjected to a detailed necropsy. The necropsy procedure included a review of the history of each animal and a detailed examination of the external features and orifices, the neck and associated tissues and the cranial, thoracic, abdominal and pelvic cavities and their viscera. The requisite organs were measured and weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Abnormalities and interactions were noted and the required tissue samples preserved in fixative.

Organ weights:

The brain, taken from each animal, was dissected free of adjacent fat and other contiguous tissue and the weight recorded.

Anatomical measurements:

The brain from each animal was dissected from the spinal cord above the first cervical spinal nerve and the olfactory lobes removed. Measurements were taken of the length between the rostral part of the cerebral hemispheres to the most caudal part of the cerebellum and also the width between the widest parts of the cerebral hemispheres.

Tissues preserved for histopathology:

Samples of the following tissues were preserved in glutaraldehyde (1.5%) : paraformaldehyde (4%) fixative by *in situ* perfusion followed by immersion:

Brain	Spinal cord
Dorsal root fibres - cervical and lumbar	Tibial nerve - at knee, right
Dorsal root ganglia - cervical and lumbar	Tibial nerve - calf muscle branch (es), right
Eyes	Ventral root fibres - cervical and lumbar
Optic nerves	Adrenals
Sciatic nerve, right	Liver
Kidneys	Bone sections - stifle joint, femur, tibia and sternum
Skeletal muscle - gastrocnemius, right	

Samples of any abnormal tissues were also retained for histopathological examination.

Tissues preserved, but not examined:

Samples of the tissues listed below were not processed histologically, but held in fixative against any future requirement for microscopic examination:

Sciatic nerve, left (*in situ*)
Skeletal muscle - gastrocnemius, left
Tibial nerve - at knee, left (*in situ*)
Tibial nerve - calf muscle branch (es), left (*in situ*)

Histology:

Tissue samples taken from the five male and five female rats with the lowest animal numbers from the control and high dosage groups (Groups 1 and 4) sacrificed on completion of the treatment period were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Brain:	Three cross sections of the forebrain. One cross section of each of the mid-brain, cerebellum and pons and medulla oblongata.
Spinal cord:	Transverse and longitudinal sections at cervical and lumbar swellings.
Dorsal root ganglia:	One cervical and one lumbar.
Dorsal root fibres:	One longitudinal section at one cervical level and at one lumbar level.
Ventral root fibres:	One longitudinal section at one cervical level and at one lumbar level.
Eyes (retina):	One longitudinal section of each.
Optic nerves:	One longitudinal section of each.
Skeletal muscle (gastrocnemius):	One transverse section.

Using resin embedding procedures, sectioned at approx. 2 micron thickness and stained with toluidine blue:

Sciatic nerve:	Longitudinal and transverse sections at the sciatic notch and the mid-thigh.
Tibial nerve:	Longitudinal and transverse sections at the knee and calf muscle branch(es)

The following tissue samples taken all animals sacrificed on completion of the treatment period were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Adrenals:	Cortex and medulla
Stifle joint, femur and tibia:	Longitudinal section through joint to include articularsurface, epiphyseal plate and bone marrow
Sternum:	Including bone marrow
Kidneys:	Including cortex, medulla and papilla regions
Liver:	Sections from all main lobes

Microscopy:

Microscopic examination was performed as follows:

- The brain, spinal cord, dorsal root ganglia, dorsal and ventral root fibres, eyes, optic nerves, skeletal muscle, sciatic nerve and tibial nerve were examined for the five male and five female rats with the lowest animal numbers from Groups 1 and 4.
- The adrenals, bone sections, kidney and liver were examined for all animals.

Findings were either reported as "Present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe.

The initial examination was undertaken by the Study Pathologist. The results of this examination were then subjected to a routine peer review by a second pathologist. The diagnosis reported here represents consensus opinions of both pathologists.

II. Results and Discussion

A. Results

1. Clinical results

The appearance and behaviour of the animals was unaffected by treatment. No animals died prematurely.

2. Body weight and food intake

Body weight

Overall body weight gains, when compared with the control, were significantly low for males and females receiving 10,000 ppm (81 and 72% of control values respectively; $p < 0.01$). Overall gains were also slightly low for females receiving 1,400 ppm (87% of control value, statistical significant).

Body weight gains for males and females receiving 200 ppm and for males receiving 1,400 ppm were not considered to be affected by treatment.

Table 3.12.1.9- 2: Selected body weight measurements (group means)

	Dose level (ppm)							
	Males				**Females**			
	0	**200**	**1,400**	**10,000**	**0**	**200**	**1,400**	**10,000**
Body weight [g] *(% difference to control)*								
Week 0	213	211 *(-1)*	207 *(-2)*	212 *(±0)*	167	171*(+2)*	166 *(-1)*	172 *(+3)*
Week 1	268	275 *(+3)*	259 *(-3)*	243 *(-9)*	194	200 *(+3)*	190 *(-2)*	189 *(-3)*
Week 4	397	398 *(±0)*	378 *(-5)*	355 *(-11)*	245	252 *(+3)*	235 *(-4)*	235 *(-4)*
Week 8	497	493 *(-1)*	469 *(-6)*	434 *(-13)*	288	290 *(+1)*	273 *(-5)*	263 *(-9)*
Week 13	569	562 *(-1)*	536 *(-6)*	499 *(-13)*	318	316 *(-1)*	297 *(-7)*	281 *(-12)*
Body weight gain [g] *(% difference to control)*								
Week 0-13	356	351 *(-1)*	328 *(-8)*	287** *(-19)*	151	145 *(-4)*	131* *(-13)*	109** *(-28)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

Food intake

Overall food consumption, when compared with the controls, was slightly low for males and females receiving 10,000 ppm (93 and 92% of control values respectively).

Food consumption for males and females receiving 200 or 1,400 ppm was not affected by treatment.

Table 3.12.1.9- 3: Selected food consumption measurements (g/animal) *(% difference to control)*

	Dose level (ppm)							
	Males				Females			
	0	**200**	**1400**	**10000**	**0**	**200**	**1400**	**10000**
Week 1	205	211 *(+3)*	205 *(±0)*	179 *(-13)*	152	158 *(+4)*	157 *(+3)*	139 *(-9)*
Week 4	218	209 *(-4)*	204 *(-6)*	199 *(-9)*	146	160 *(+10)*	146 *(±0)*	142 *(-3)*
Week 8	224	219 *(-2)*	207 *(-8)*	196 *(-12)*	161	164 *(+2)*	157 *(-2)*	141 *(-12)*
Week 13	213	214 *(±0)*	208 *(-2)*	201 *(-6)*	159	153 *(-4)*	142 *(-11)*	134 *(-16)*
Total (week 0-13)	2834	2840 *(±0)*	2739 *(-3)*	2643 *(-7)*	2060	2148 *(+4)*	2010 *(-2)*	1885 *(-8)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

The amount of food scattered was generally higher than the controls for males and females receiving 1,400 or 10,000 ppm and for females receiving 200 ppm; males receiving 200 ppm were unaffected.

Individually housed animals are known to scatter more food than group housed animals and within the groups there was wide variability between the cages.

Food conversion efficiency was low, when compared with controls, for males and females receiving 10,000 ppm and slightly low for females receiving 1,400 ppm. There was no effect on food conversion efficiency for males and females receiving 200 ppm and for males receiving 1,400 ppm.

The achieved test substance doses were as follows.

Table 3.12.1.9- 4: Test substance doses (mg/kg bw/day)

Group no.	Dose (ppm)	Males	Females
2	200	15.0	18.0
3	1,400	106.6	125.2
4	10,000	780.6	865.8

3. Neurobehavioural screening (functional observational battery report)

Home cage observations:

Home cage observations were unaffected by treatment.

In the hand observations:

Some inter-group variation occurred during in the hand observations but there were no differences considered associated with treatment.

Arena observations:

Arena observations showed some inter-group variation but there were no treatment-related changes.

Manipulations:

Body temperature values for females receiving 1,400 ppm were significantly increased compared with controls but as group mean values for females receiving 10,000 ppm were identical with controls on both occasions, this was not considered to be associated with treatment.

Table 3.12.1.9- 5: Body temperature measurements in Weeks 4, 8 and 13 (mean ± SD)

Parameter	Week	Dose level (ppm)							
		Males				Females			
		0	200	1,400	10,000	0	200	1,400	10,000
Number of animals	-	10	10	10	10	10	10	10	10
Body temperature (°C)	4	37.4 ± 0.4	37.5 ± 0.3	37.3 ± 0.2	37.3 ± 0.3	37.4 ± 0.2	37.6 ± 0.3	37.9*** ± 0.4	37.4 ± 0.2
	8	37.3 ± 0.3	37.4 ± 0.3	37.2 ± 0.2	37.3 ± 0.3	37.7 ± 0.4	37.7 ± 0.4	38.0 ± 0.5	37.5 ± 0.3
	13	37.3 ± 0.4	37.2 ± 0.2	37.1 ± 0.2	37.3 ± 0.3	37.6 ± 0.2	37.9 ± 0.4	38.1** ± 0.4	37.6 ± 0.3

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control
SD: Standard deviation

Motor activity:

Group mean motor activity scores showed no consistent dosage or treatment-related changes and none of the differences achieved statistical significance. It was therefore concluded that motor activity was unaffected by treatment.

Brain weights:

Absolute brain weights were not adversely affected by treatment.

Anatomical measurements:

The anotomical measurements made of the cerebral hemispheres did not indicate any differences between the controls and treated animals.

Macroscopic pathology:

Macroscopic examination of animals killed on completion of the treatment period revealed no treatment-related findings.

Microscopic pathology:

There were no changes in the tissues presented for neuropathological examination which were considered to be related to treatment with fluopicolide.

Findings considered related to treatment were seen in the fluopicolide target tissues liver and kidneys. An increased incidence of centrilobular hepatocytic hypertrophy of the liver, when compared with the controls, was observed in males and females given 10,000 ppm and in males given 1,400 ppm. Centrilobular hepatocytic hypertrophy is a common finding in the livers of rodents which have been administered xenobiotics and, as such, is considered to be an adaptive change of no toxicological significance.

In addition, an increased incidence and/or severity of hyaline droplets in the cortical tubules was observed in the kidneys of males given 1,400 or 10,000 ppm. This was associated with increased incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin in the cortical tubules of affected kidneys. Since the α2μ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2μ-globulin accumulation are unlikely to occur in humans[28].

The histopathological changes due to treatment are summarized in Table 3.12.1.9- 6.

Table 3.12.1.9- 6: Histopathological changes related to treatment

Findings	Score	Dose level (ppm)							
		Males				Females			
		0	200	1,400	10,000	0	200	1,400	10,000
Liver									
Number examined	-	10	10	10	10	10	10	10	10
Centrilobular hepatocytic hypertrophy	Slight	0	0	9	10	0	0	0	6
	Total	0	0	9***	10***	0	0	0	6*
Kidney									
Number examined	-	10	10	10	10	10	10	10	10
Cortical tubules with hyaline droplets	Minimal	3	3	0	0	0	0	0	0
	Slight	2	1	8	0	0	0	0	0
	Moderate	0	1	2	10	0	0	0	0
	Total	5	5	10*	10*	0	0	0	0
Interstitial inflammation	Minimal	2	2	3	3	0	0	1	0
	Slight	0	0	2	4	0	0	0	0
	Moderate	0	0	0	2	0	0	0	0
	Total	2	2	5	9**	0	0	1	0
Granular casts-medulla	Minimal	0	0	2	2	0	0	0	0
	Slight	0	0	0	3	0	0	0	0
	Moderate	0	0	0	5	0	0	0	0
	Total	0	0	2	10***	0	0	0	0
Cortical tubular dilatation	Present	0	0	0	7**	0	0	0	0
Hyaline tubular casts	Minimal	0	3	3	2	0	0	0	0
	Slight	0	0	0	2	1	0	0	0
	Total	0	3	3	4	1	0	0	0

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, statistically different to control

[28] Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of alpha 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ Health Perspect. 1993 Mar;99:313-49

III. Conclusion

There was no evidence of neurotoxicity following the continuous administration of fluopicolide to CD rats for 13 weeks at dietary concentrations up to 10,000 ppm. Neurobehavioural screening and macroscopic and histopathological examination of the associated tissues (including anatomical measurements of the brain) revealed no treatment-related findings. In addition, no unusual signs or patterns of behaviour were observed at all routine observations.

It is concluded that the administration of fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. Thus, the NOAEL for neurotoxicity was >10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females). Adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male rat-specific 'hydrocarbon nephropathy' occurred at 1,400 and 10,000 ppm. Therefore, the NOAEL for systemic toxicity in the 13-week neurotoxicity study was 200 ppm (15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females) based on impaired growth and histopathological changes in kidney of male animlas at dose levels of ≥1,400 ppm (equivalent 106.6 mg/kg bw/day in males and 125.2 mg/kg bw/day in females).

3.12.1.10 Anonymous; 2003; M-220782-01-1

Study reference:

Anonymous; 2003; A subacute dermal toxicity study in rats with AE C638206; M-220782-01-1

Deviations:	Deviations from the current OECD guideline (410, 1981): None.

Executive Summary:

Fluopicolide was administered dermally under a semi-occlusive covering to male and female Wistar rats five days/week for four weeks. The dose groups for this study were 0 (control group), 100, 250, 500, and 1,000 mg/kg bw. The dose was based on each animal's body weight on Days 0, 7, 14, 21, and 28.

During the study, the animals were evaluated for the effect of the test compound on body weight, food consumption, clinical signs, the eyes, clinical chemistry, and hematology. Gross necropsy evaluations were performed on all adults. Histopathologic evaluation of selected tissues was conducted on the control and high-dose groups.

There were no treatment-related deaths during the study, and no treatment-related effects on body weight or food consumption. There were no treatment-related effects on clinical chemistry values or haematological parameters. There were no treatment-related effects on organ weights, no treatment-related gross or histopathological changes in the organs and tissues.

The NOEL in the 28-day dermal toxicity study in rats was 1,000 mg/kg bw/day based on the absence of toxicity at the highest dose investigated.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	2050190//PP241024/2

2. Vehicle and/or positive control

Vehicle:	0.5% aqueous carboxymethylcellulose

3. Test animals

Species:	Rats
Strain:	Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR) rats
Age:	8 to 9 weeks old
Weight at start:	232.9-244.6 g for the males, 166.9-171.1 g for the females
Source:	Charles River Laboratories, Inc. (Raleigh, NC)
Acclimation period:	Yes
Diet:	PMI Certified Rodent Diet 5002 in "meal" form, St. Louis, MO
Water:	Tap water ad lib
Housing:	Individually housed in suspended stainless steel cages
Temperature:	19-25 °C
Humidity:	30-70%
Air changes:	10-15 cycles/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 24 to March 27, 2003

2. Animal assignment and treatment

The dermal route of exposure was employed in accordance with the test guidelines for a subacute dermal toxicity study. The dose groups for this study were 0 (control group), 100, 250, 500, and 1,000 mg/kg bw, applied dermally. The doses were based on each animal's body weight on Days 0, 7, 14, 21, and 28.

Rats were randomly assigned to dose groups, based on weight, using special computer software.

Table 3.12.1.10- 1: Study design

Group no.	Dose (mg/kg bw)	Number of males	Number of females
Treatment phase			
1	0	10	10
2	100	10	10
3	250	10	10
4	500	10	10
5	1,000	10	10

3. Dose preparation and analysis

Three days prior to dosing, the hair was removed from the dorsal and lateral areas of the trunk of each rat using electric clippers. The day of dosing and on subsequent study days, when required due to hair growth, the dose area was clipped with electric clippers.

The test material was weighed out for each animal and applied to a 2 inch by 2 inch two ply gauze pad, backed with a piece of plastic, which had been moistened with 2 mL of 0.5% aqueous carboxymethylcellulose, as water would not moisten the test compound. The gauze was placed on the shaved skin of the rat and secured with porous medical tape (Zonas®, by Johnson and Johnson). The torso of the animal was then wrapped with porous medical tape, to assure that the gauze remained in contact with the skin during the dosing interval. The same procedure was performed for control animals, except for only gauze moistened with approximately 2 mL of 0.5% aqueous carboxymethylcellulose was applied to the dose site.

The test substance/gauze was held in contact with the skin for a minimum of six hours/day for five consecutive days/week for four weeks. During the fifth week, the animals were dosed daily until the day the rat was to be sacrificed (rats were not dosed on the day of sacrifice). Each day the gauze and tape were removed and the dose site was gently wiped with water-dampened and dry paper towels to remove as much test substance residue as feasible without damaging the skin.

4. Statistics

Statistical significance was determined at $p < 0.05$ for all tests, with the exception of Bartlett's test in which a probability value of $p < 0.001$ was used. All tests were two-tailed, except for gross and histopathologic lesion evaluations that were one-tailed.

Continuous data was analyzed by Bartlett's test for homogeneity. If the data was homogeneous, an ANOVA was performed followed by Dunnett's t-test on parameters showing a significant effect by ANOVA. If the data was non-homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Mann-Whitney U-test to identify statistical significance between groups.

C. Methods

1. Observations

Animals were observed twice daily for moribundity and mortality (except once daily on weekends and holidays). Clinical examinations were conducted once a week.

2. Body weight and food intake

The weight of each animal was determined on Study Days 0, 7, 14, 21, and 28. A terminal body weight was obtained on animals that were found dead or sacrificed in extremis following the day of dosing.

Individual food consumption was determined once a week for all animals.

3. Ophthalmoscopy

Ophthalmic exams were conducted pre-dose and during study week four.

4. Laboratory investigations (clinical chemistry)

During the fourth week of the study, following an overnight fast, blood was collected from the orbital sinus of all rats, under anesthesia with isoflurane, and was analyzed for standard serum chemistry and hematological parameters. During the fifth week of the study, blood was collected from the orbital sinus, under anesthesia with isoflurane, and was analyzed for prothrombin time.

Haematology:

The following parameters were determined:

Table 3.12.1.10- 2: Hematology parameters

Blood Cell morphologies Erythroctyes (RBC) Hematocrit (HCT) Hemoglobin (Hgb) Leukocytes (WBC) (total & differential)	Mean corpuscular volume (MCV) Mean corpuscular hemoglobin (MCH) Mean corpuscular hemoglobin concentration Platelets (PLTS) Prothrombin time (PT)

Biochemistry:

The following parameters were determined:

Table 3.12.1.10- 3: Biochemistry parameters

Alanine aminotransferase (ALT) Albumin (ALB) Alkaline phosphatase (ALP) Aspartate aminotransferase (AST) Bilirubin, total (T-Bili) Blood urea nitrogen (BUN) Calcium (Calc) Cholesterol, total (Choi) Chloride (Cl) Creatinine (Great) Creatine phosphokinase (CK)	Gamma-glutamyltranspeptidase (GGT) Globulin (Glob) Glucose, fasting (Glue) Lactic dehydrogenase (LDH) Phosphate (Phos) Potassium (K) Protein, total (T-Prot) Sodium (Na) Triglycerides (Trig) Uric Acid (Uric-A) A/G ratio

5. Sacrifice and pathology

Macroscopic examination:

A complete gross examination was performed on all animals that died, were sacrificed moribund, or were sacrificed at study termination. The organs in Table 3.12.1.10- 4 were weighed. With the exception of the physical identifier (microchip), representative sections of the tissues were preserved in 10% buffered formalin.

Organ weights:

The following organ weights were determined:

Table 3.12.1.10- 4: Organ weights

Adrenal Brain Epididymis Heart Kidney Liver	Ovaries Spleen Testes Thymus Uterus

6. Histopathology

Tissue sampling:

The tissues collected from the control (0 mg/kg bw) and high-dose group (1,000 mg/kg bw) animals, which were examined microscopically (see Table 3.12.1.10- 5), were processed, embedded in paraffin, sectioned, mounted, and stained with haematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses, as necessary, to establish no-observed-effect levels.

Table 3.12.1.10- 5: Tissue sampling

Adrenal	Jejunum	Rectum
Aorta	Joint, fem/tib[a]	Salivary gland
Bone marrow	Kidney	Seminal vesicle
Bone, sternum	Larynx	Skin (treated and untreated)
Brain	Liver	Spinal cord
Cerebrum	Lung	Cervical
Midbrain	Lymph node, cervical	Thoracic
Cerebellum	Lymph node, mesenteric	Lumbar
Medulla	Mammary gland	Spleen
Cecum	Muscle[a]	Stomach, glandular
Cervix[a]	Nasal structure	Stomach, non-glandular
C literal gland[a]	Nasopharynx	Testicle
Colon	Nerve, sciatic (peripheral)	Thymus
Duodenum	Optic nerve	Thyroid/parathyroid
Epididymis	Oral structure	Tongue[a]
Esophagus	Ovary	Tootha
Exoribital/lacrimal gland[a]	Pancreas	Trachea
Eye	Parathyroid	Urinary bladder
Gross lesions	Physical identifier[b]	Uterus
Harderian gland[a]	Pituitary	Vagina[a]
Heart	Preputial gland[a]	Zymbal's gland[a]
Ileum	Prostrate	

[a] Preserved for possible micropathologic evaluation

[b] The identification chip implanted in each animal was collected at necropsy

II. Results and Discussion

A. Results

1. Clinical results

There were no treatment-related clinical signs in any groups.

One animal (female OV 4104) was sacrificed on Study Day 21, due to the skin accidentally being cut when the animal was unwrapped.

2. Body weight and food intake

Body weight

There was no compound-related effect on body weight.

Table 3.12.1.10- 6: Mean body weight (g)

Day	Dose level (mg/kg bw/day)									
	0	100		250		500		1,000		
Males										
Body weights [g] (*% difference to control*)										
Day 0	244.6	242.9	*(-1)*	243.9	*(±0)*	232.9	*(-5)*	242.2	*(-1)*	
Day 7	263.8	260.5	*(-1)*	261.2	*(-1)*	259.5	*(-2)*	260.5	*(-1)*	
Day 14	282.7	278.1	*(-2)*	280.5	*(-1)*	280.8	*(-1)*	278.5	*(-1)*	
Day 21	302.2	294.4	*(-3)*	297.3	*(-2)*	298.7	*(-1)*	296.3	*(-2)*	
Day 28	311.3	302.2	*(-3)*	301.9	*(-3)*	305.2	*(-2)*	303.7	*(-2)*	
Females										
Body weights [g] (*% difference to control*)										
Day 0	171.1	169.7	*(-1)*	166.9	*(-2)*	167.4	*(-2)*	166.9	*(-2)*	
Day 7	181.0	177.1	*(-2)*	179.9	*(-1)*	182.0	*(+1)*	174.1	*(-4)*	
Day 14	190.6	193.8	*(+2)*	190.9	*(±0)*	199.0	*(+4)*	188.3	*(-1)*	
Day 21	203.5	203.2	*(±0)*	207.1	*(+2)*	211.0	*(+4)*	196.7	*(-3)*	
Day 28	211.5	207.8	*(-2)*	211.8	*(±0)*	217.7	*(+3)*	202.0	*(-4)*	

Food intake

There was no compound-related effect on food consumption.

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

There were no compound-related effects on hematology parameters evaluated in this study. There were various parameters for males and prothrobin time for the low-dose group females that were statistically significantly different from the control group, but these findings were not dose related and were not biologically different from the control group.

Clinical chemistry:

There were no compound-related effects on the clinical chemistry parameters evaluated in this study. There were various parameters which were statistically significant from the control group, but these findings were not dose related and/or were not biologically different than the control group.

5. Sacrifice and pathology

Necropsy:

There were no compound-related gross observations. Miscellaneous lesions noted were decreased size of testis/epididymis, discoloration of the thymus, eye lesions, and skin lesions.

Organ weights:

There were no compound-related differences between the absolute and relative organ weights for control and treatment animals.

6. Histopathology

There were no compound-related micropathological lesions in the 1,000 mg/kg dose group. Minimal background changes, typically present in young Wistar rats, were seen in both sexes.

III. Conclusion

The No Observed Effect Level (NOEL) for this study was 1,000 mg/kg bw/day, the highest dose tested.

3.12.2 Human data

No human data.

3.12.3 Other data

No other data.

3.13 Aspiration hazard

No specific studies on aspiration hazard are available. However, on the basis of existing animal studies and expert judgment that takes into account surface tension, water solubility, boiling point, volatility and chemical structure (fluopicolide is not an hydrocarbon, primary alcohol or ketone) aspiration hazard is not expected.

3.13.1 Animal data

No specific data.

3.13.2 Human data

No human data

3.13.3 Other data

No other data

4 ENVIRONMENTAL HAZARDS

Environmental hazards are **not** assessed in this dossier. Only health hazards are assessed.

4.1 Degradation

4.1.1 Ready biodegradability (screening studies)

Please refer to Section 4.

4.1.2 BOD_5/COD

Please refer to Section 4.

4.1.3 Aquatic simulation tests

Please refer to Section 4.

4.1.4 Other degradability studies

Please refer to Section 4.

4.2 Bioaccumulation

4.2.1 Bioaccumulation test on fish

Please refer to Section 4.

4.2.2 Bioaccumulation test with other organisms

Please refer to Section 4.

4.3 Acute toxicity

4.3.1 Short-term toxicity to fish

Please refer to Section 4.

4.3.2 Short-term toxicity to aquatic invertebrates

Please refer to Section 4.

4.3.3 Algal growth inhibition tests

Please refer to Section 4.

4.3.4 *Lemna* sp. growth inhibition test

Please refer to Section 4.

4.4 Chronic toxicity

4.4.1 Fish early-life stage (FELS) toxicity test

Please refer to Section 4.

4.4.2 Fish short-term toxicity test on embryo and sac-fry stages

Please refer to Section 4.

4.4.3 Aquatic Toxicity – Fish, juvenile growth test

Please refer to Section 4.

4.4.4 Chronic toxicity to aquatic invertebrates

Please refer to Section 4.

4.4.5 Chronic toxicity to algae or aquatic plants

Please refer to Section 4.

4.5 Acute and/or chronic toxicity to other aquatic organisms

Please refer to Section 4.