Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at Community level of

Fluopyram

EC number: NA
CAS number: 658066-35-4

CLH-O-0000001412-86-46/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

04 December 2014

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),

Annex VI, Part 2

Substance Name: **N-{2-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide (ISO name Fluopyram)**

EC Number: **n.a.**

CAS Number: **658066-35-4**

Index Number: **n.a.**

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Version number: 2 **Date: September 2013**

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***N-{2-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide*** ***(ISO name Fluopyram)***
EC number:	***n.a.***
CAS number:	***658066-35-4***
Annex VI Index number:	***n.a.***
Degree of purity:	***≥ 96 %***
Impurities:	***No impurity is considered relevant for the classification of the substance Fluopyram***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	Regulation (EC) No 1272/2008 (CLP Regulation)	**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	Not Classified	Not Classified
Current proposal for consideration by RAC	Carc. 2; H351 Aquatic Chronic 2; H411	Carc. Cat. 3, R40; N; R51/53 C ≥ 25 %: N; R51/53 2.5 ≤ C < 25 %: R52/53
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 2; H351 Aquatic Chronic 2; H411	Carc. Cat. 3; R40; N; R51/53 C ≥ 25 %: N; R51/53 2.5 ≤ C < 25 %: R52/53

1.3 Proposed harmonised classification and labelling based on CLP Regulation and/or DSD criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	none		none	conclusive but not sufficient for classification
2.2.	Flammable gases	none		none	Data lacking
2.3.	Flammable aerosols	none		none	Data lacking
2.4.	Oxidising gases	none		none	Data lacking
2.5.	Gases under pressure	none		none	Data lacking
2.6.	Flammable liquids	none		none	Data lacking
2.7.	Flammable solids	none		none	conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	none		none	Data lacking
2.9.	Pyrophoric liquids	none		none	Data lacking
2.10.	Pyrophoric solids	none		none	Data lacking
2.11.	Self-heating substances and mixtures	none		none	Data lacking
2.12.	Substances and mixtures which in contact with water emit flammable gases	none		none	Data lacking
2.13.	Oxidising liquids	none		none	Data lacking
2.14.	Oxidising solids	none		none	conclusive but not sufficient for classification
2.15.	Organic peroxides	none		none	Data lacking
2.16.	Substance and mixtures corrosive to metals	none		none	Data lacking
3.1.	Acute toxicity - oral	none		none	conclusive but not sufficient for classification
	Acute toxicity - dermal	none		none	conclusive but not sufficient for classification
	Acute toxicity - inhalation	none		none	conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	none		none	conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	none		none	conclusive but not sufficient for classification
3.4.	Respiratory sensitisation	none		none	Data lacking
3.4.	Skin sensitisation	none		none	conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	none		none	conclusive but not sufficient for classification
3.6.	Carcinogenicity	Carc. 2; H351			
3.7.	Reproductive toxicity	none		none	conclusive but not sufficient for classification
3.8.	Specific target organ toxicity – single exposure	none		none	conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	none		none	conclusive but not sufficient for classification
3.10.	Aspiration hazard	none		none	Data lacking
4.1.	Hazardous to the aquatic environment	Aquatic Chronic 2; H411	none	none	
5.1.	Hazardous to the ozone layer	none		none	Data lacking

[1)] Including specific concentration limits (SCLs) and M-factors

[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Table 4: Proposed labelling according to the CLP Regulation

	Labelling	Wording
Pictograms	GHS08 GHS09	
Signal Word	Warning	
Hazard statements	H351 H411	Suspected of causing cancer Toxic to aquatic life with long lasting effects
Precautionary statements	(P102) P260 P273 P281 P308 + P313 P363 P391 P405 P501	(Keep out of reach of children) Do not breathe dust/ spray Avoid release to the environment Use personal protective equipment as required IF exposed or concerned: Get medical advice/ attention Wash contaminated clothing before reuse Collect spillage Store locked up Dispose of contents/container to …

Proposed notes assigned to an entry:

none

Table 5: Proposed classification according to DSD

Hazardous property	Proposed classification	Proposed SCLs	Current classification [1)]	Reason for no classification [2)]
Explosiveness	none		none	conclusive but not sufficient for classification
Oxidising properties	none		none	conclusive but not sufficient for classification
Flammability	none		none	conclusive but not sufficient for classification
Thermal stability	none		none	conclusive but not sufficient for classification
Acute toxicity	none		none	conclusive but not sufficient for classification
Acute toxicity – irreversible damage after single exposure	none		none	conclusive but not sufficient for classification
Repeated dose toxicity	none		none	conclusive but not sufficient for classification
Irritation / Corrosion	none		none	conclusive but not sufficient for classification
Sensitisation	none		none	conclusive but not sufficient for classification
Carcinogenicity	Carc. Cat. 3; R40		none	
Mutagenicity – Genetic toxicity	none		none	conclusive but not sufficient for classification
Toxicity to reproduction – fertility	none		none	conclusive but not sufficient for classification
Toxicity to reproduction – development	none		none	conclusive but not sufficient for classification
Toxicity to reproduction – breastfed babies. Effects on or via lactation	none		none	conclusive but not sufficient for classification
Environment	N; R51/53	C[3)] ≥ 25 %: N; R51/53 2.5 ≤ C < 25 %: R52/53	none	

[1)] Including SCLs

[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

[3)] C is the concentration of Fluopyram in the preparation

Table 6: Proposed labelling according to DSD

	Labelling	Wording
Hazard Symbols, Indications of danger	Xn N	Harmful Dangerous for the environment
R-phrases	R40 R51/53	Limited evidence of a carcinogenic effect Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment
S-phrases	(S2) S22 S36/37 S60 S61	Keep out of the reach of children Do not breathe dust Wear suitable protective clothing and gloves This material and its container must be disposed of as hazardous waste Avoid release to the environment. Refer to special instructions/ Safety data sheets.

2 BACKGROUND TO THE CLH PROPOSAL

Fluopyram is a new active substance in the meaning of Directive 91/414/EEC (new: Regulation (EC) No 1107/2009) and as such has not been discussed before for a CLH proposal.

Germany received an application from Bayer CropScience for inclusion in Annex I to Directive 91/414/EEC. The RMS Germany provided its initial evaluation of the dossier on fluopyram in the Draft Assessment Report (DAR), which was received by the EFSA on 30 August 2011. The DAR was prepared in a joint review together or under discussion with US, Canada and Australia.

The EU peer review was initiated on 12 September 2011 by dispatching the DAR for consultation of the Member States and the applicant Bayer CropScience. EFSA conducted an expert consultation in the areas of mammalian toxicology, residues, fate and behaviour and ecotoxicology and EFSA adopted a conclusion on whether fluopyram can be expected to meet the conditions provided for in Article 5 of Directive 91/414/EEC, in accordance with Article 8 of Commission Regulation (EU) No 188/2011 (EFSA Journal 2013:11(4):3052).

In accordance with Article 36(2) of the CLP Regulation, fluopyram should now be considered for harmonised classification and labelling.

No registration dossiers were available for floupyram until 4th September 2013.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Fluopyram is an active substance in the meaning of Regulation (EC) 1107/2009 replacing Directive 91/414/EEC. Following article 36(2) or Regulation (EC) 1272/2008 such substances should normally be subject to harmonised classification.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

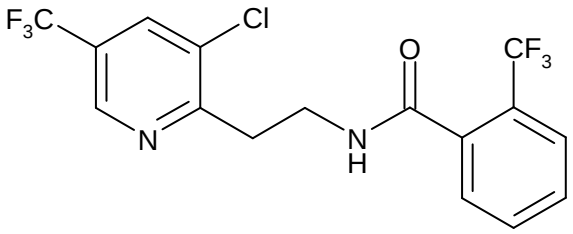
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 7: Substance identity

EC number:	n.a.
EC name:	n.a.
CAS number (EC inventory):	n.a.
CAS number:	658066-35-4
CAS name:	Benzamide, N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)-
IUPAC name:	N-{2-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide *N*-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-*o*-toluamide *N*-{2-[3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide (ACD/ChemSketch) Fluopyram (ISO name)
CLP Annex VI Index number:	n.a.
Molecular formula:	$C_{16}H_{11}ClF_6N_2O$
Molecular weight range:	396.7 g/mol

Structural formula:



1.2 Composition of the substance

The confidential information can be found in the “Confidential Annex” or the technical dossier.

Table 8: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Fluopyram	Min. ≥ 96.0 %		

Current Annex VI entry: None

Table 9: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
confidential			

Current Annex VI entry: None

Table 10: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
confidential				

Current Annex VI entry: -

1.3 Physico-chemical properties

Table 11: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Solid, white powder	Draft Assessment Report (DAR)	measured (Eyrich and Bogdoll, 2007)
Melting/freezing point	117.5 °C (99.8 %)		measured (Smeykal, 2008)
Boiling point	318 °C – 321 °C (correlated range) under decomposition (99.8 %)		measured (Smeykal, 2008; Moeller, 2008)
Relative density	1.53 (99.8 %)		measured (Bogdoll and Strunk, 2007)
Vapour pressure	1.2 x 10^{-6} Pa (20 °C) 3.1 x 10^{-6} Pa (25 °C) 2.9 x 10^{-4} Pa (50 °C) (99.8 %)		measured (Smeykal, 2005)
Surface tension	59.4 mN/m (90 % saturated aqueous solution; 20 °C)		measured (Eyrich and Bogdoll, 2007)
Water solubility	16 mg/L (distilled water) 15 mg/L (pH 4) 16 mg/L (pH 7) 15 mg/L (pH 9) at 20 °C, 99.8 %		measured (Gueldner, Hoppe, 2007)
Partition coefficient n-octanol/water	log $P_{o/w}$ = 3.3 (20 °C)		measured (Eyrich and Bogdoll, 2006)
Flash point	n.a.		
Flammability	Fluopyram technical was determined to be not highly flammable (97.5 %)		measured (Moeller, 2007 EEC A10)
Explosive properties	Not explosive (heat: Koenen; shock: fall hammer; friction: friction test apparatus) (97.5 %)		measured (Moeller, 2007 EEC A14)
Self-ignition temperature	No self-ignition up to 400 °C. Test substance did not ignite below or at the melting point of 118 °C. (97.5 %)		measured (Moeller, 2007 EEC A16)
Oxidising properties	Not oxidising under test conditions of EEC A 17 (97.5 %)		measured (Moeller, 2007 EEC A17)
Granulometry	no data available		
Stability in organic solvents and identity of relevant degradation products	solubility in organic solvents: acetone > 250 dichlorethane > 250 dimethyl sulfoxide> 250 ethyl acetate > 250 *n*-heptane 0.66 methanol> 250 toluene 62.2 all values in g/L at 20 °C no data on stability available		measured (Bogdoll and Lemke, 2006)

Dissociation constant	No pK_a value could be detected in the range of 2 < pK_a < 12. (screening method) pK_a = 0.5 (corresponding acid) [23 °C]		measured (Bogdoll and Lemke, 2006)
Viscosity	no data available		

2 MANUFACTURE AND USES

2.1 Manufacture

Confidential information, see Appendix I.

2.2 Identified uses

Fluopyram is a fungicide. Preparations containing fluopyram will be applied by foliar spray and are proposed for use in the areas agriculture, horticulture, and viticulture.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 12: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference
EEC A 10 flammability (solids)	not highly flammable	none	Draft Assessment Report (DAR)
EEC A 16 relative self-ignition temperature for solids	No self-ignition up to 400 °C	none	Draft Assessment Report (DAR)
UN Bowes-Cameron-Cage-test	Test substance did not ignite below or at the melting point of 118 °C.	none	Draft Assessment Report (DAR)
EEC A 14 explosive properties	not explosive (heat, shock, friction)	none	Draft Assessment Report (DAR)
EEC A 17 oxidising properties	not oxidising	none	Draft Assessment Report (DAR)

No classification and labelling based on physico-chemical properties of Fluopyram.

RAC evaluation of physical hazards

Summary of the Dossier submitter's proposal

Summaries of tests for flammability, explosivity and oxidising properties were tabulated in the CLH report and it was concluded that no classification for these hazard classes is warranted.

Comments received during public consultation

No comments were received for this hazard class.

Assessment and comparison with the classification criteria

The substance did not reveal any physical hazard properties relevant for classification and RAC agrees with the view of the Dossier submitter (DS) that based on the data presented no classification is warranted.

4 HUMAN HEALTH HAZARD ASSESSMENT

Fluopyram is currently evaluated as a new PPP active ingredient under directive 91/414/EEC. In this procedure Germany acts as rappoteur member state (RMS). Studies submitted under this procedure as well as the DAR prepared for this procedure were used to prepare the present CLP report.

The assessment prepared by Germany under the PPP prodecure is currently not publically available.

Changed criteria according to Commission Regulation (EU) No 286/2011 were taken into account, when assessing the study results.

There were no registration dossiers available for fluopyram by 28/01/2013.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

Five different studies were performed and are summarised in this section: two ADME studies with the two different labels, two quantitative whole body autoradiography studies with the two different labels and an organ depletion study with [pyridyl-2,6-^{14}C]fluopyram.

The overall results of the toxicokinetic studies show that fluopyram was quickly adsorbed and excreted within 168 h for approx. 90 %. There was evidence of significant enterohepatic circulation. Because of a rather higher log P_{OW} value of 3.3 and relatively high total residues after 168 hours in the single dose studies, a certain potential for bioaccumulation was suspected. However, toxicokinetics in general was not different in a study with repeated administration suggesting a low, if any, risk for accumulation. In particular, elimination kinetics was not significantly altered. Only minor sex specific differences in toxicokinetics have been observed although systemic exposure was higher and enterohepatic circulation more pronounced in females. In contrast, there were remarkable differences in the toxicokinetic data depending on the part of the molecule that had been radiolabeled (see Table 13). These results were explained by molecular cleavage of fluopyram and a different biokinetic behaviour of the two labels especially in terms of urinary excretion in an additional document provided upon request of BfR (Neumann, 2009; ASB 2009-3169 und ASB 2009-10702). In addition, routes of excretion were slightly different depending on the label. When labeled in the phenyl ring, excretion via the faeces accounted for 47 % - 64 % whereas renal excretion ranged from 35 % - 45 % in both sexes. With the pyridyl-label, excretion was 53 % faecal and 45 % urinary in male rats but 39 % faecal and 60 % urinary in females. Exhalation was negligible in all tests.

Following absorption from the GIT, fluopyram was widely distributed. Organs with highest residues were the liver, the kidneys, Harderian gland and, in some studies and to a lesser extent, erythrocytes, adrenals, thyroid, ovaries and carcass.

The analysis of metabolism revealed a number of metabolites including fluopyram-7-hydroxy and -8-hydroxy, fluopyram-pyridyl-acetic acid and fluopyram-benzamid being the most abundant. The benzamid metabolite and a fluopyram-olefine are of potential toxicological concern. The main metabolic transformation reactions were:

- hydroxylation of the ethyl linking group of the parent compound forming fluopyram-7-hydroxy and -8-hydroxy metabolites;
- further oxidation of fluopyram-7-hydroxy and -8-hydroxy metabolites leading to fluopyram-enol;
- hydroxylation of the phenyl ring giving fluopyram-phenol and fluopyram-7-OH-phenol;
- conjugation of the hydroxylated metabolites mainly with glucuronic acid and to a lesser extent with sulfate;
- molecular cleavage yielding fluopyram-benzamide;

- subsequent oxidation, hydroxylation and conjugation of fluopyram-benzamide to fluopyram-benzoic acid and various fluopyram-benzamide and fluopyram-hydroxybenzamide conjugates;
- conjugation of the phenyl ring moiety with glutathione followed by further degradation to fluopyram-7-OH-methyl-sulfone, fluopyram-BA-methyl-sulfoxide and fluopyram-BA-methyl-sulfone;
- elimination of water from compounds hydroxylated in the ethylene bridge leading to fluopyram-Z-olefine and E-olefine (E- and Z-olefine can isomerise into each other), because the double bond of the olefine may be a target for epoxidation and a 7-8-dihydroxy-metabolite which could result from hydrolysis of an epoxid by epoxid hydroxylase was observed, the olefine was considered to be of potential toxicological relevance;
- molecular cleavage to fluopyram-pyridyl-hydroxyethyl;
- hydrolytic cleavage and subsequent oxidation to mainly fluopyram-pyridyl-acetic acid (PAA), fluopyram-ethyl-diol and fluopyram-pyridyl-carboxylic acid (PCA).

Table 13: Overview of studies on adsorption, distribution, excretion and metabolism in mammals

Study type	Species / strain	Vehicle	Comments	Results	Reference
Absorption, Distribution, Excretion and Metabolism in the Rat	Rat, Wistar Hsd/Cpb: WU	0.5 % aqueous Tragacanth solution	[Phenyl-UL-^{14}C] fluopyram	See table below	Klempner, A. (2008 a) ASB2008-5536
Absorption, Distribution, Excretion and Metabolism in the Rat	Rat, Wistar Hsd/Cpb: WU	0.5 % aqueous tragacanth solution	[Pyridyl-2,6-^{14}C]-fluopyram	See table below	Klempner, A. (2008 b) ASB2008-5537
Distribution of the total radioactivity in male and female rats determined by quantitative whole body autoradiography	Rat, Wistar Hsd/Cpb: WU	0.5 % aqueous tragacanth solution	[Phenyl-UL-^{14}C] fluopyram	Widely distributed	Koester, J. (2008 a) ASB2008-5539
Distribution of the total radioactivity in male and female rats determined by quantitative whole body autoradiography	Rat, Wistar Hsd/Cpb: WU	0.5 % aqueous tragacanth solution	[Pyridyl-2,6-^{14}C]-fluopyram	Widely distributed	Koester, J. (2008 b) ASB2008-5540
Metabolism in organs and tissues of male and female rats	Rat, Wistar Hsd/Cpb: WU	0.5 % aqueous tragacanth solution	[Pyridyl-2,6-^{14}C]-fluopyram	Extensively metabolised	Koester, J. & Klempner, A. (2008) ASB2008-5541

4.1.2 Human information

No relevant submitted by the notifier.

4.1.3 Summary and discussion on toxicokinetics

Following oral intake, fluopyram is completely absorbed after oral administration and was rapidly excreted. Total elimination rate of radioactivity reached an amount of approx. 90 % within 7 d following treatment. The results indicated a low potential for accumulation of fluopyram, taking into

account also phys-chem properties (logPow = 3.3). Following absorption from the GIT, fluopyram was widely distributed. Organs with highest residues were the liver, the kidneys, Harderian gland and, in some studies and to a lesser extent, erythrocytes, adrenals, thyroid, ovaries and carcass. The analysis of metabolism revealed a number of metabolites including fluopyram-7-hydroxy and -8-hydroxy, fluopyram-pyridyl-acetic acid and fluopyram-benzamid being the most abundant.

Table 14 summarises basic toxicokinetic properties.

Table 14: Basic toxicokinetic parameters of fluopyram measured with phenyl- and pyridyl-labelled a.s. in male and female rats.

Sex **Label** **Dose level**	**Male phenyl LD**	**Male phenyl HD**	**Male pyridyl LD**	**Female phenyl LD**	**Female phenyl HD**	**Female pyridyl LD**
t_{max} [h]	24.0	48.0	0.7	8.0	48.0	3.0
C_{max} [µg/mL]	1.45	59.94	1.78	2.11	63.09	1.5
$AUC_{0-\infty}$ [mg/L h]	107	5680	22	148	7060	37
$t_{½\ abs}$ [h]	0.1	0.5	0.3	0.4	0.5	0.4
$t_{½\ elim\ initial}$ [h]	3.9	4.8	11.2	16.2	4.8	9.8
$t_{½\ elim\ terminal}$ [h]	30.9	23.6	55.9	53.0	29.0	72.9

LD – low dose (5 mg/kg bw); HD – high dose (250 mg/kg bw)

4.2 Acute toxicity

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

Title:	Eiben, R. (2005): AE C656948 - Acute toxicity in the rat after oral administration, AT02530, M-259398-01, ASB2008-5542.
Guidelines:	OECD 423 (2001), EEC 92/69- B1.
Deviations:	None (with regard to OECD 423).
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Material and Methods

Common name:	Fluopyram
Company code:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7%
Stability	Stable at 5 and 200 mg/mL at room temperature for at least 2 hours
Species	Rat
Strain	HsdCpb:Wu
Age/Weight	10 to 12 weeks approximately; body weight at dosing: 170 to 195 g
Source	Harlan/Winkelmann GmbH, 5960 AD Horst, Netherlands
Acclimat.	At least 5 days
Animal husbandry	Animals were caged in groups in polycarbonate cages on low dust wood granulate bedding. Husbandry was standardized with twelve hours of alternating light and dark cycles and approx. 10 air changes per h, 22 ± 2°C room temperature and 55 ± 5% mean relative humidity
Diet	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*
Water	Tap water, *ad libitum*
Vehicle	2% Cremophor EL in demineralized water

According to the “toxic class method”, fluopyram was examined for acute oral toxicity in a stepwise procedure with three female rats employed in each step. Following an overnight fast of 16 to 24 hours, the first group received a single dose of 2000 mg/kg bw by gavage at a volume of 10 mL/kg bw. The test substance had been dissolved in demineralized water with 2 % Cremophor EL. The rats were monitored for mortality and clinical signs several times on the day of administration and subsequently at least once daily for a 2-week post-observation period. Body weights were recorded on days 1, 8 and 15. On day 15, surviving animals were sacrificed, necropsied and examined for gross pathological changes. Because no deaths and no clinical signs occurred in the first group, the same procedure was repeated with the same limit dose level.

Findings

Mortality: No mortalities occurred at 2000 mg/kg bw, the only dose tested.

Clinical observations: No clinical signs were observed with the possible exception of an increased water intake observed in 3/6 animals (1st phase) from day 2 to 6. However, this finding was not confirmed in the second group and, thus, is considered rather equivocal.

Body weight: All rats gained weight throughout the study.

Necropsy: No abnormalities were observed at gross necropsy.

Conclusion

The dose of 2000 mg/kg bw was tolerated without mortalities, clinical signs, effects on weight gain or gross pathological findings. According to OECD guideline 423, the LD_{50} cut-off of fluopyram was ≥ 5000 mg/kg bw. Fluopyram does not warrant classification as being toxic or harmful on the basis of its acute oral toxicity.

4.2.1.2 Acute toxicity: inhalation

Title:	Folkerts, A. (2006): AE C656948 - Acute inhalation toxicity in rats, AT03464, M-283420-01, ASB2008-5544.
Guidelines:	OECD 403; Directive92/69/EEC, Annex V, Method B.2.(1992).
Deviations:	None (with respect to OECD 403).
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Material and methods

Common name:	Fluopyram
Company code:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7%
Stability	Stable until 04. May 2007 at room temperature
Species	Rat
Strain	HsdCpb:Wu
Age/Weight	2 month approximately; body weight at dosing: 170 to 196 g for males and 169 to 191 g for females.
Source	Harlan/Winkelmann GmbH, Borchen, Germany
Acclimat.	At least 5 days
Animal husbandry	Animals were individually caged in Makrolon® Type III_H cages. Husbandry was standardized with a twelve h alternating light and dark cycles and approx. 10 air changes per h.., 22 ± 2°C room temperature and

	50 ± 10% mean relative humidity
Diet	Provimi Kliba 3883= NAFAG 9441 pellets maintenance diet
	for rats and mice, Kaiseraugst Switzerland, *ad libitum*
Water	Tap water, *ad libitum*
Vehicle / Control	The test substance was an aerosol made from dry powder; conditioned dry air was used as a control

Animal assignment and treatment: Male and female rats were randomly assigned to the control and test groups listed in Table 15 below. Animals were exposed nose-only to the aerosolized test substance in Plexiglas exposure tubes for four hours. The rats were examined carefully several times on the day of exposure and at least once daily thereafter for 2 weeks. The following reflexes were tested: visual placing response, grip strength on wire mesh, abdominal muscle tone, corneal and pupillary reflexes, pinnal reflex, righting reflex, tail-pinch response, startle reflex with respect to behavioral changes stimulated by sounds (finger snapping) and touch (back). The rectal temperatures were measured shortly after cessation of exposure. Individual body weights were recorded before exposure and on days 3, 7 and 14. On day 15, all animals were sacrificed, necropsied and examined for gross pathological changes.

Generation of the test atmosphere / chamber description: Directed-flow nose-only inhalation chambers (TSE, 61348 Bad Homburg) were used. The method for dust generation employed a "Bayer Generator" system. The test substance concentration was determined by gravimetric analysis. Chamber samples were collected after the equilibrium concentration had been attained in hourly intervals. Two samples during each exposure were also taken for the analysis of the particle-size distribution using an Andersen cascade impactor.

The limit concentration of 5000 mg/m^3 was attained, however, at the expense of larger particles (because no cyclone was used) for test atmosphere generation. The actual analytically measured concentration was 5112.5 mg/m^3. At this concentration, the Mass Median Aerodynamic Diameter was 5.6 µm (SD = 2.02 µm) and 19.0% of the total particulate had an aerosol mass < 3 µm. Control animals were treated with conditioned dry air under exposure conditions similar to those of the treated animals.

Findings

Mortality: No mortality occurred up to 5112.5 mg/m^3, the maximum technically achievable concentration (Table 15). The 4 hour inhalation LC_{50} for both sexes was > 5112.5 mg/m^3.

Table 15: Doses and mortality / animals treated

N Group /sex	Target Concentration (mg/m³)	Results	Onset and duration of signs	Onset of mortality	Rectal temperature (°C)
1/m	0	0 / 0 / 5	--	--	38.3
2/m	5000	0 / 5 / 5	0d - 1d	--	36.1
1/f	0	0 / 0 / 5	--	--	38.4
2/f	5000	0 / 5 / 5	0d – 5d	--	34.3

N = group assignment, m = males, f = females, 0d: exposure day; Values given in the 'Toxicological results' column are: 1st = number of dead animals. 2nd = number of animals with signs after cessation of exposure.

3rd = number of animals exposed.

Clinical observations: All rats tolerated the exposure with some evidence of reversible signs. Clinical signs in both sexes exposed to fluopyram included: bradypnea, labored breathing patterns, reduced motility, piloerection, ungroomed hair-coat and limpness. A battery of reflex measurements was made on the first post-exposure day. Reflexes tested were normal in all males whereas one female showed a reduced tonus and vertical grip strength together with an impaired righting response. Rectal temperature

was lowered in both sexes after treatment. All clinical signs were fully reversible within 1 day in male rats and within 5 days in females.

Body weight: Comparisons between the control and the exposure groups revealed no toxicologically significant changes in body weight in both sexes.

Necropsy: There was nothing in particular to report at the necropsy.

Conclusion

Based on the results obtained in a nose-only inhalation system with 4-hour exposure, fluopyram proved to have essentially no inhalation toxicity and the LC_{50} was considered to be greater than 5112.5 mg/m^3 (> 5.1 mg/L) in male and female rats. Fluopyram does not warrant classification as being toxic or harmful on the basis of its acute inhalation toxicity.

4.2.1.3 Acute toxicity: dermal

Title:	Eiben, R. (2005): AE C656948 - Acute toxicity in the rat after dermal application, AT02500, M-259275-01, ASB2008-5543.
Guidelines:	OECD 402 (1987); EEC 67/548 Annex V- Method B.3.
Deviations:	None (with regard to OECD 402).
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Material and methods

Common name:	Fluopyram
Company code:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7%
Stability	Stable until 04. May 2007 at room temperature
Species	Rat
Strain	HsdCpb:Wu
Age/Weight	9 to 13 weeks approximately; Weight at dosing: 228 to 259 g for males and 212 to 222 g for females.
Source	Harlan/Winkelmann GmbH, 5960 AD Horst, Netherlands
Acclimat.	At least 5 days
Animal husbandry	Animals were caged in groups in polycarbonate cages on low dust wood granulate bedding. Husbandry was standardized with twelve hours of alternating light and dark cycles and approx. 10 air changes per h.., 22 ± 2°C room temperature and 55 ± 5% mean relative humidity
Diet	Provimi Kliba 3883.0.15 Maus/Ratte Haltung, Kaiseraugst Switzerland, *ad libitum*
Water	Tap water, *ad libitum*
Vehicle	None. Pure solid test material was transferred to wet gauze-layer

Animal assignment and treatment: Five animals per sex were employed for testing. Only a limit dose of 2000 mg/kg bw was administered. A control groups was not included. One day prior to treatment, the back and flanks of the rats were shorn (approximately 10% of the body surface). For each animal, the required amount of pure solid test substance was weighed and transferred to a wet gauze-layer (6.0 cm x 5.0 cm) of a "Cutiplast® steril" coated with air-tight, Leukoflex®. The gauze strip was placed on the rat's back and secured in place using "Peha®-half" cohesive stretch tape (8 cm x 23 cm) and additionally covered with a "Lomir biomedical Inc." rat jacket. After 24 hours the dressing was removed and the area rinsed with tepid water using soap followed by gently patting the area dry.

The rats were observed for clinical signs and mortality several times on the day of administration and at least daily thereafter for an observation period of at least 14 days. Mortality and nature, duration and intensity of symptoms, if occurring, were recorded individually. The weight gain of the animals was

checked weekly. It was intended that each animal which died or was killed in moribund condition was weighed, autopsied and examined macroscopically. The surviving animals were sacrificed by carbon dioxide at the end of the study and examined macroscopically.

Findings

Mortality: No mortalities occurred at 2000 mg/kg bw, the only dose tested.

Clinical observations: No clinical signs were observed.

Body weight: All animals gained weight throughout the study.

Necropsy: No abnormalities were observed at gross necropsy.

Conclusions

Based on those results, the LD_{50} was greater than 2000 mg/kg bw for both males and females. Fluopyram does not warrant classification as being toxic or harmful on the basis of its acute dermal toxicity.

4.2.1.4 Acute toxicity: other routes

No data submitted by the notifier.

4.2.2 Human information

No data submitted by the notifier.

4.2.3 Summary and discussion of acute toxicity

The acute toxicity of fluopyram was low for all routes of exposure (oral, dermal and inhalative). The oral LD_{50} cut-off for rats was greater than or equal to 5000 mg/kg bw because no mortality, clinical signs or abnormalities at necropsy were reported at 2000 mg/kg bw in a study that was conducted according to the OECD Guideline 423. The acute dermal LD_{50} in the rat was > 2000 mg/kg body weight, with no mortality or clinical signs observed. The rat acute inhalation LC_{50} (4-hour) was > 5.1 mg/L which was the highest technically achievable concentration and did not cause mortality. Reversible clinical signs in the acute inhalation study included bradypnea, laboured breathing, piloerection, ungroomed hair-coat, reduced motility, high-legged gait and limpness. There were no effects on body weight gain in any of the acute studies.

Table 16: Summary table of relevant acute toxicity studies

Study type	Species / strain	Comments	Results m /f	Reference
LD_{50} oral	Rat, HsdCpb:Wu		No mortalities observed at 2000 mg/kg (limit dose)	Eiben, (2005a) ASB2008-5542
LD_{50} dermal	Rat		LD_{50} > 2000 mg/kg,	Eiben, (2005b) ASB2008-5543
LC_{50} inhalation	Rat	4h, nose only	LC_{50} >5.1 mg/L	Folkerts, (2006) ASB2008-5544

Fluopyram was shown not to be acutely toxic by oral, dermal or inhalation route.

4.2.4 Comparison with criteria

Table 17 presents the toxicological results in comparison with DSD and CLP criteria.

Table 17: Results of acute toxicity studies in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Oral LD_{50}, rat: > 2000 mg/kg	no classification LD_{50} > 2000 mg/kg (oral)	no classification LD_{50} > 2000 mg/kg (oral)
Inhalation LC_{50}, rat: > 5,1 mg/l (highest attainable conc., dust, 4-h, nose only)	no classification LC_{50} > 5 mg/litre/4h	no classification LC_{50} > 5.0 (dusts and mists)
Dermal LD_{50}, rat: > 2000 mg/kg	no classification LD_{50} > 2 000 mg/kg	no classification LD_{50} > 2 000 mg/kg (dermal)

4.2.5 Conclusions on classification and labelling

The acute oral toxicity of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute toxicity.

According to the criteria in Reg. 1272/2008, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute oral toxicity.

The acute inhalative toxicity of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute toxicity.

According to the criteria in Reg. 1272/2008, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute toxicity.

The acute dermal toxicity of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute toxicity.

According to the criteria in Reg. 1272/2008, based on the results of the acute oral toxicity studies fluopyram does not warrant classification and labelling for acute toxicity.

RAC evaluation of acute toxicity

Summary of the Dossier submitter's proposal

One oral, one dermal and one inhalation acute toxicity study, all in rats, were included in the CLH report. After oral and dermal exposure, no deaths occurred and no clinical signs of toxicity, effects on weight gain or gross pathological findings were seen at the only tested dose of 2000 mg/kg bw. In the acute inhalation toxicity study no deaths occurred at the highest technical achievable concentration of 5.11 mg/L. All exposed animals showed clinical signs such as bradypnoea, laboured breathing patterns, reduced motility and body temperature, piloerection, ungroomed hair-coat and limpness. In a battery of reflex measurements one female showed reduced tonus and vertical grip strength and impaired righting response. All clinical signs were fully reversible within 1 day (males) or 5 days (females).

Comments received during public consultation

No comments were received for this hazard class.

Assessment and comparison with the classification criteria

The substance induced no mortalities after either oral, dermal or inhalation exposure at

concentrations at or above the guidance values for classification. RAC thus agrees with the DS that no classification for acute toxicity is warranted for any of the exposure routes.

4.3 Specific target organ toxicity – single exposure (STOT SE)

4.3.1 Summary and discussion of Specific target organ toxicity – single exposure

No toxicity to a specific organ in the absence of lethality was observed in acute oral, inhalation or dermal toxicity studies in animals. Additionally, no acute organ toxicity was observed in short-term or long-term studies.

4.3.2 Comparison with criteria

Table 18 presents the toxicological results in comparison with DSD and CLP criteria.

Table 18: Classification criteria for Categories 1 and 2 of specific target organ toxicity-single exposure

Toxicological data on fluopyram	CLP criteria	
No effects observed; there were no findings in any of the acute studies at necropsy. Furthermore, short-term or long-term studies did not reveal effects pointing at specific target organ effects after single exposure.	Category 1	Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following single exposure - reliable and good quality evidence from human cases or epidemiological studies; or - observations from appropriate studies in experimental animals in which significant and/or severe toxic effects of relevance to human health were produced at generally low exposure concentrations.
	Category 2	Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following single exposure - observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

There is neither evidence from human experience/incidents nor from appropriate animal studies that specific target organ toxicity by single exposure occurs.

4.3.3 Conclusions on classification and labelling

The specific target organ toxicity after single exposure of fluopyram does not meet the CLP criteria.

According to the criteria in Reg. 1272/2008, based on the results of appropriate animal studies, fluopyram does not warrant classification for specific target organ toxicity after single exposure.

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

Summary of the Dossier submitter's proposal

No toxicity to a specific organ in the absence of lethality was observed in acute oral, dermal or inhalation toxicity studies in animals. Additionally, no acute organ toxicity was observed in short-term or long-term studies.

Comments received during public consultation

No comments were received for this hazard class

Assessment and comparison with the classification criteria

No effects that could lead to classification as STOT SE where reported. RAC thus agrees with the DS that no classification for STOT SE is warranted.

4.4 Irritation

4.4.1 Skin irritation

4.4.1.1 Non-human information

Title: Schuengel, M. (2005): AE C656948 - Acute skin irritation/corrosion on rabbits, AT02737, M-263302-01.

Guidelines: OECD 404 (1981); EEC 92/69- B4.

Deviations: None (with respect to OECD 404).

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Material and methods

Common name:	Fluopyram
Company code:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7%
Stability	Stability verified until 04. May 2007 at room temperature

This study was performed in healthy adult female albino rabbits. One day before application, the fur was shorn on the right and left side from the dorso-lateral area of the trunk. Fluopyram was applied to the skin of the animal under a gauze patch. The test substance (0.5 g) was moistened with *Aqua per injectionem* to ensure good contact with the skin. The treated area was approximately 2.5 cm x 2.5 cm. The patch was held in place with non-irritating tape for the duration of exposure period. As a first step, only one animal was used and 3 test patches were applied successively to this animal, for 3 minutes, 1 hour and 4 hours, respectively, with observation of reactions after one time-point before moving to a longer duration of exposure. Then, the test was completed with 2 additional animals.

Dermal irritation was scored at 1, 24, 48 and 72 hours after patch removal. In the case of an irritation reaction, animals were further monitored for reversibility of the effect on day 7 and day 14 (maximum) after patch removal. The degree of erythema/eschar or oedema formation was recorded and scored according to the Draize scheme. Any serious lesions or toxic effects other than dermal irritation were also recorded and described.

Findings

There was no systemic intolerance reaction to the test substance. None of the animals showed any irritation reaction throughout the study and the mean score according to the Draize scale was always 0.

Conclusion

Fluopyram is no skin irritant under the test conditions.

4.4.1.2 Human information

No information submitted by the notifier.

4.4.1.3 Summary and discussion of skin irritation

In the skin irritation study no erythema, eschar or oedema was observed at any time point in any treatment group.

Table 19: Summary table of relevant skin irritation studies

Study type	Species / strain	Comments	Results m /f	Reference
Skin irritation	New Zealand White rabbit		Not irritating	Schuengel, 2005, ASB2008-5545

4.4.1.4 Comparison with criteria

Fluopyram is compared to the criteria set in DSD and CLP in .

Table 20.

Table 20: Comparison of results for skin irritation with the criteria in DSD and CLP.

Toxicological result	DSD criteria	CLP criteria
Mean Score: erythema / eschar: 0.0 oedema: 0.0 no persistent inflammation	Not irritating to skin: No significant inflammation of the skin which persists for at least 24 hours after an exposure period of up to four hours; mean value of the scores for either erythema and eschar formation or oedema formation, calculated over all the animals tested, is less than 2	No irritant: Mean value of < 2,3 for erythema/eschar or for oedema in at least 2 of 3 tested animals from gradings at 24, 48 and 72 hours after patch removal or, no reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions; no inflammation that persists to the end of the observation period normally 14 days in at least 2 animals, particularly taking into account alopecia (limited area), hyperkeratosis, hyperplasia, and scaling; or no very definite positive effects related to chemical exposure in a single animal but less than the criteria above.

4.4.1.5 Conclusions on classification and labelling

The skin irritation potential of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the skin irritation studies fluopyram does not warrant classification and labelling for skin irritation.

According to the criteria in Reg. 1272/2008, based on the results of the skin irritation studies fluopyram does not warrant classification and labelling for skin irritation.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier submitter's proposal

One skin irritation study with fluopyram was included in the CLH report. In this study no systemic reaction to the test substance was observed. None of the three animals showed any irritation reaction throughout the study and the mean score according to the Draize scale was

always 0.

Comments received during public consultation
No comments were received for this hazard class.

Assessment and comparison with the classification criteria
In the reported skin irritation study there were no indications of irritation or corrosion. RAC thus agrees with the DS that no classification for skin corrosion/irritation is warranted.

4.4.2 Eye irritation

Fluopyram was tested for eye irritancy according to OECD 405 and was shown to have no eye irritating properties in New Zealand white rabbits under the test conditions.

Table 21: Summary table of relevant eye irritation studies

Study type	Species / strain	Comments	Results m /f	Reference
Eye irritation	New Zealand White rabbit		Non-Irritating	Schuengel, 2005, ASB2008-5546

4.4.2.1 Non-human information

Report: Schuengel, M. (2005): AE C656948 - Acute eye irritation on rabbits, AT02738, M-263277-01.

Guidelines: OECD 405 (2002); EEC 92/69- B5.

Deviations: None (with respect to OECD 405).

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Material and methods

Common name: Fluopyram

Company code: AE C656948

Description: Beige powder

Lot/Batch: Mix-Batch:08528/0002

Purity: 94.7%

Stability Stability verified until 04. May 2007 at room temperature

This study was performed in healthy adult female albino rabbits. One day prior to the application, both eyes of each animal were examined including fluorescein examination. On the day of treatment, 0.1 g of fluopyram was placed into the conjunctival sac of one eye of the first animal. The lids were then gently held together for about 1 second in order to prevent loss of test compound. The eye was not rinsed for at least 24 hours after instillation. The other eye remained untreated and served as control. Since no severe irritation was observed 1 hour after the treatment, 2 additional rabbits were used.

Eye irritation was scored and recorded approximately 1, 24, 48 and 72 hours post-application. In case of irritation, animals showing the effects were monitored usually on day 7, 14 and 21 (maximum) after application otherwise the study was finished after 72 hours. The degree of ocular lesions was recorded according to Draize. In addition, any serious lesions or toxic effects other than ocular lesions were also recorded and described.

Findings

There was no systemic intolerance reaction. Ocular findings were confined to conjunctival redness in two out of three rabbits on test after 24 hours that had disappeared one day later. A summary of the irritant effects is given in Table 22 below.

Table 22: Eye irritation scores

Animal	Irritation	1h	24h	48h	72h	Mean score	Response	Reversibility (days)
1	Corneal opacity	0	0	0	0	0.0	-	n.a.
	Iritis	0	0	0	0	0.0	-	n.a.
	Conjunctivae redness	1	1	0	0	0.3	-	2
	Conjunctivae chemosis	0	0	0	0	0.0	-	n.a.
2	Corneal opacity	0	0	0	0	0.0	-	n.a.
	Iritis	0	0	0	0	0.0	-	n.a.
	Conjunctivae redness	1	0	0	0	0.0	-	1*
	Conjunctivae chemosis	0	0	0	0	0.0	-	n.a.
3	Corneal opacity	0	0	0	0	0.0	-	n.a.
	Iritis	0	0	0	0	0.0	-	n.a.
	Conjunctivae redness	2	1	0	0	0.3	-	2
	Conjunctivae chemosis	0	0	0	0	0.0	-	n.a.

* due to the result 1h post application; n.a.: not applicable; Response: Corneal opacity: "-" if mean score < 2; Iritis: "-" if mean score < 1; Conjunctivae redness: "-" if mean score < 2.5; Conjunctivae chemosis: "-" if mean score < 2

Conclusion

Based on the results of this study, fluopyram was considered to be not irritating to the eyes.

4.4.2.2 Human information

No information submitted by the notifier.

4.4.2.3 Summary and discussion of eye irritation

Fluopyram was not irritating to eyes of New Zealand white rabbits.

4.4.2.4 Comparison with criteria

Table 23 presents the toxicological results in comparison with DSD and CLP criteria.

Table 23: Toxicological results of relevant eye irritation studies in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Mean Score: corneal opacity: 0.0 iris lesion: 0.0 conjunctival redness: 0.3 oedema of the conjunctivae (chemosis): 0.0	Not irritating to eyes: cornea opacity: < 2 iris lesion: < 1 redness of the conjunctivae: < 2,5 oedema of the conjunctivae (chemosis): < 2	Not irritating to eyes: corneal opacity: < 1 iritis: < 1 conjunctival redness: < 2 conjunctival oedema (chemosis): < 2

4.4.2.5 Conclusions on classification and labelling

The eye irritation potential of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the eye irritation studies fluopyram does not warrant classification and labelling for eye irritation.

According to the criteria in Reg. 1272/2008, based on the results of the eye irritation studies fluopyram does not warrant classification and labelling for eye irritation.

RAC evaluation of eye corrosion/irritation

Summary of the Dossier submitter's proposal

One eye irritation study with fluopyram was included in the CLH report. In this study no systemic intolerance reaction to the test substance was observed. Ocular findings were confined to conjunctival redness in two out of three rabbits after 24 hours. These findings had disappeared one day later.

Comments received during public consultation

No comments were received for this hazard class.

Assessment and comparison with the classification criteria

Only minor and reversible effects were seen in the reported eye corrosion/irritation study. RAC thus agrees with the DS that no classification for eye corrosion/irritation is warranted.

4.4.3 Respiratory tract irritation

4.4.3.1 Non-human information

In the acute (4-hour) inhalation toxicity study in rats with fluopyram no mortalities were recorded during the study. During the exposure, bradypnea was observed as well as labored breathing patterns, reduced motility, piloerection, ungroomed hair-coat and limpness. Within a few hours after exposure all animals were completely recovered and showed normal behaviour. No visible lesions were observed at gross necropsy (Folkerts, (2006) ASB2008-5544).

There is no evidence of respiratory tract irritation in the available studies.

4.4.3.2 Human information

No data submitted by the notifier.

4.4.3.3 Summary and discussion of respiratory tract irritation

There are no relevant human data. The only relevant findings with respect to respiratory tract irritation were clinical signs (bradypnea, labored breathing patterns, reduced motility, piloerection, ungroomed hair-coat and limpness) in an acute inhalation study. No gross necropsy findings were reported.

4.4.3.4 Comparison with criteria

Considering that the findings were restricted to clinical signs which were not corroborated by histological findings, it is concluded that the findings were not sufficient to classify fluopyram with STOT-SE 3.

4.4.3.5 Conclusions on classification and labelling

The respiratory tract irritation potential of fluopyram does not meet the CLP criteria.

According to the criteria in Reg. 1272/2008, based on the above considerations regarding respiratory tract irritation of fluopyram the substance does not warrant classification and labelling for respiratory tract irritation (STOT-SE 3).

4.5 Corrosivity

4.5.1 Non-human information

No data submitted by the notifier.

4.5.2 Human information

No data submitted by the notifier.

4.5.3 Summary and discussion of corrosivity

Due to physico-chemical properties and due to findings in studies on skin- and eye-irritation, both showing no irritating potential, it is reasonable to assume, that fluopyram is not corrosive.

4.5.4 Comparison with criteria

Comparison with criteria in DSD:

The criteria for classification with R35 (Causes severe burns) are:

"if, when applied to healthy intact animal skin, full thickness destruction of skin tissue occurs as a result of up to three minutes exposure, or if this result can be predicted."

The criteria for classification with R34 (Causes burns) are:

"if, when applied to healthy intact animal skin, full thickness destruction of skin tissue occurs as a result of up to four hours exposure, or if this result can be predicted […]"

No effects as described by these criteria were observed.

Comparison with criteria in CLP regulation:

According the Guidance to Regulation (EC) No 12772/2008 if destruction of skin tissue is observed in >1 of 3 animals. Subcategorization depends on duration of exposure and observation after which destructive effects (e.g. visible necrosis, ulcers or bleeding) are observed.

No effects indicating corrosivity were observed.

4.5.5 Conclusions on classification and labelling

The corrosive potential of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, fluopyram does not warrant classification as corrosive.

According to the criteria in Reg. 1272/2008, fluopyram does not warrant classification as corrosive.

4.6 Sensitisation

4.6.1 Skin sensititsation

4.6.1.1 Non-human information

Title: Repetto-Larsay, M. (2006): AE C656948 - Evaluation of potential dermal sensitization in the local lymph node assay in the mouse, SA06320, M-281845-01.

Guidelines: OECD guideline 429 (2002).

Deviation: In the group treated with the test substance at a concentration of 5 %, one animal (QT5F4141) did not receive the intravenous injection of tritiated thymidine.

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Materials and methods

Common name:	Fluopyram
Company code:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7%
Stability:	Stability verified until 04. May 2007 at room temperature
Species:	Mouse (females only)
Strain:	CBA/J
Age/Weight:	8 Weeks (at least)
Source:	R. Janvier, Le Genest St Isle, France
Acclimat.:	5 days
Control:	Positive: alpha-hexylcinnamaldehyde (HCA)
	Negative: Dimethylformamide (DMF)

Fluopyram was topically applied to the dorsal surface of each ear of mice. Groups of 5 mice/dose were dosed once daily for 3 consecutive days with 25 µL of formulation of fluopyram at concentrations of 0.5, 1.0, 2.5 and 5% in Dimethylformamide (DMF). These dose levels were chosen on the basis of preliminary results showing that concentrations of 10, 25, 50 and 100 % caused excessive cellular toxicity. Two additional groups received the vehicle (DMF) or alpha-hexylcinnamaldehyde (HCA, 25 %) to serve as negative and positive controls, respectively.

Animals were checked for mortality and clinical signs at least daily during the study. In particular, the site of application was examined for signs of local irritation. Individual bodyweights were measured at study start and at scheduled sacrifice. On day 5, each mouse was placed in a retention box, intravenously injected via the tail vein with 250 µL of NaCl (0.9%) containing 20 µCi of 3H methyl thymidine and then placed in a plastic cage for 5 hours. Then the mice were sacrificed and the auricular lymph nodes removed. A single cell suspension (free of connective tissue) was obtained by placing the lymph nodes in an individual tube containing physiological saline and crushing them with a plastic

piston. Cell suspensions were washed with 5 ml of physiological saline, centrifuged and then resuspended in 2 ml of 5% trichloroacetic acid (TCA) and stored overnight at 4°C. After a final centrifugation, the cell pellets were resuspended in 1 ml of physiological saline and placed in an ultrasonic bath for 25 min to ensure thoroughly dispersed suspension. Cell suspensions were then added to scintillation pots containing 10 ml of scintillation fluid and assayed in a beta-counter to evaluate the incorporation of ^{3}H methyl thymidine. The results were expressed as disintegrations per minute (DPM) per node. Stimulation indices (SI) were then calculated by the ratio of DPM in treated group to DPM in control group according to the formula: SI = DPM of treated group / DPM of control group. A test substance is regarded as a skin sensitizer when one concentration results in a 3-fold or more increase in ^{3}H methyl thymidine incorporation compared to the control in the absence of skin irritation and when a dose-response is observed.

Findings

No mortality or clinical signs were observed during the study. In particular, no cutaneous reactions were observed at the application site. Bodyweight changes were comparable between control and treated groups. The results of the proliferation assay are presented in the Table 24 below.

Table 24: Lymph node DPM values and stimulation indices

Test group	DPM/node	Stimulation index	Response
Control (DMF)	581.4	1.0	-
Fluopyram 0.5% in DMF	543.4	0.9	-
Fluopyram 1% in DMF	591.6	1.0	-
Fluopyram 2.5% in DMF	647.5	1.1	-
Fluopyram 5% in DMF	519.0	0.9	-
HCA 25% in DMF	3330.3	5.7	+

There were no confounding effects of irritation or toxicity. Negative responses were observed at all dose levels of fluopyram. The results of the positive control demonstrated the validity of the assay.

Conclusion

Fluopyram did not induce any changes in the local lymph node stimulation index. The positive control substance gave the expected result. On the basis of this study, fluopyram did not show any sensitization potential and does not warrant classification and labeling as being a skin sensitizer.

4.6.1.2 Human information

No information submitted by the notifier.

4.6.1.3 Summary and discussion of skin sensitisation

Fluopyram did not induce any changes in the local lymph node stimulation index. The positive control substance gave the expected result. On the basis of this study, fluopyram did not show any sensitization potential and does not warrant classification and labeling as being a skin sensitizer.

Table 25: Summary table of relevant skin sensitisation studies

Study type	Species / strain	Comments	Results m /f	Reference
LLNA	Mouse (f, CBA/J)		Non-Sensitising	Repetto-Larsay, M. (2006); ASB2008-5547

4.6.1.4 Comparison with criteria

Table 26 presents the toxicological results in comparison with DSD and CLP criteria.

Table 26: Results of LLNA test in comparison with DSD and CLP criteria

Toxicological result	DSD criteria	CLP criteria
Local lymphnode assay Stimulation index 1.0	LLNA not considered in DSD (usually a SI > 3 is considered a positive result)	**Local lymphnode assay** Positive, if stimulation index ≥ 3

4.6.1.5 Conclusions on classification and labelling

Fluopyram does not warrant classification as a skin sensitizer according to the criteria set in DSD and CLP.

4.6.2 Respiratory sensitisation

No data were submitted by the notifier.

RAC evaluation of skin sensitisation

Summary of the Dossier submitter's proposal

One local lymph node assay (LLNA) was presented in the dossier. No mortality or clinical signs of toxicity were observed during the study. In particular, no cutaneous reactions were observed at the application site. Fluopyram did not induce any changes in the local lymph node stimulation index.

Comments received during public consultation

No comments were received for this hazard class.

Assessment and comparison with the classification criteria

No signs of sensitisation were seen in the LLNA study presented. RAC thus agrees with the DS that no classification for skin sensitisation is warranted.

4.7 Repeated dose toxicity

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

Title: Kennel, P. (2004): AE C656948 - Exploratory 28-day toxicity study in the rat by dietary administration, SA 03332, M-085510-01.

Oral administration (28–day study) in the rat (range finding study)

Guidelines:	Not applicable, range finding study.
Deviations:	n.a.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable as a range finding study.

Materials and methods

Test Material:	AE C656948
Description:	Pale yellow powder
Lot/Batch:	FLH 999
Purity:	98,6 %
Vehicle or positive control:	None
Stability of test compound:	Stable in rodent diet for a period covering the study duration
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age / weight at dosing:	7 weeks approx.; Males: 255 g to 283 g; Females: 171 to 193 g at time of dosing
Source/breeder:	R. Janvier, Le Genest St Isle, France
Acclimatization:	7 to 8 days
Identification:	Labeling by cage cards and water-insoluble spots on the tail
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

There were 5 animals of each sex per dose group. Animals were assigned to dose groups randomly by body weight. Fluopyram was administered in the diet for 28 days to Wistar rats at the following doses – 0, 50, 400 and 3200 ppm (equating to 4.0, 31.0 and 254 mg/kg/day in males and 4.6, 36.1 and 263

mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals.

Diet preparation and analysis: Fluopyram was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The stability was demonstrated during the course of the study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the study. Fluopyram concentrations were verified to be within 99-105 % of nominal. Homogeneity at the lowest and highest dietary concentrations were within the range 99-115 %.

Table 27: Study design

Test group	Concentration in diet (ppm)	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	5	5
2	50	4.0	4.6	5	5
3	400	31.0	36.1	5	5
4	3200	254	263	5	5

Statistics: Data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett's test on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test if the Kruskal-Wallis was significant. For some parameters, when data were not homogenous, they were transformed (log transformation or square root transformation), then reanalyzed as above. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5 % and 1 % levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Observations: The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

Body weights were recorded three times during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment periods. Diet-fasted animals were weighed before necropsy.

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 4 was calculated for each sex.

Clinical pathology: On study day 29, prior to necropsy, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet-fasted overnight prior to blood sampling and anesthetized by inhalation of Isoflurane. Blood was collected on EDTA for hematology, on lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters.

The following hematology parameters were assayed using an Advia 120 (Bayer Diagnostics, Puteaux, France): red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear of Wright's stain was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, aspartate aminotransferase, alanine

aminotransferase, alkaline phosphatase activities, total protein and albumin concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Meylan, France).

Sacrifice and pathology: On study day 29 a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation, than exsanguinated before necropsy. All animals were diet-fasted prior to scheduled sacrifice. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, kidney, liver, ovary, spleen, testis, thyroid gland (with parathyroid gland) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

Tissues samples were fixed by immersion in neutral buffered 10 % formalin with the exception of the testes which were fixed in Davidson's fixative. The following tissues were sampled: adrenal gland, brain, liver, kidney, lung, ovary, pituitary, spleen, testis, and thyroid (including parathyroid).

Histopathological examinations were performed on all tissues from all the animals in the control and high dose groups. The liver, kidney, lung, pituitary gland and thyroid gland, were also examined in all animals in the intermediate doses in the study.

At final necropsy, the remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Results were compared to those generated with well known reference compounds.

Findings

Observations: There was no mortality in any group. There were no clinical signs evident in any group.

Body weight and food consumption: Body weight gain was reduced at 3200 ppm in both sexes, by 12-28 % in males (week 1 and 3) and by 16-29 % in females (weeks 1, 3 and 4). However, overall body weight gain was comparable to controls in males, whereas it was 14 % lower in females. Body weight was unaffected at 400 or 50 ppm in either sex.

At 3200 ppm, food intake was reduced by 4 to 10 % in females throughout the study compared to control values. There was no impact on food consumption in either sex at dietary concentrations of 400 or 50 ppm. The achieved doses in the study were 0, 4.6, 36.1, and 263 mg/kg/day for females, and 0, 4.0, 31.0, and 254 mg/kg/day in males.

Blood analyses: Hematological examination showed an increase of 30 % in platelets and of 34 % in prothrombin time in males at 3200 ppm.

Table 28: Changes in hematology parameters in the 28-day rat study with fluopyram

Dosage level (ppm)	0	50	400	3200
Males				
Platelet count (10^9 / l)	1183 ± 193	1331 ± 105	1341 ± 150	1539 ± 126**
Prothrombin time (s)	13.4 ± 1.5	14.0 ± 0.7	15.0 ± 0.6	18.0 ± 2.9*

*: p≤0.05; **: p≤0.01

Clinical chemistry findings included higher total cholesterol and triglyceride concentrations together with slightly lower aspartate aminotransferase and alkaline phosphatase activities in both sexes as much as decreased glucose levels in males. No relevant changes were observed at 400 or 50 ppm in either sex (Table 29).

Table 29: Changes in clinical chemistry parameters in the 28-day rat study with fluopyram

Dosage level (ppm)	0	50	400	3200
Males				
Total cholesterol (mmol/L)	1.45 ± 0.17	1.56 ± 0.28	1.52 ± 0.28	2.64 ± 0.52**
Triglycerides (mmol/L)	0.81 ± 0.24	0.83 ± 0.25	0.76 ± 0.23	2.01 ± 0.38**
Aspartate aminotransferase (IU/L)	86 ± 23	80 ± 14	67 ± 5	63 ± 7*
Alkaline phosphatase (IU/L)	127 ± 36	113 ± 13	124 ± 11	84 ± 10
Females				
Total cholesterol (mmol/L)	1.90 ± 0.19	2.09 ± 0.38	2.21 ± 0.32	3.50 ± 0.47**
Triglycerides (mmol/L)	0.40 ± 0.05	0.47 ± 0.10	0.40 ± 0.05	1.01 ± 0.41**
Aspartate aminotransferase (IU/L)	76 ± 7	70 ± 4	74 ± 16	63 ± 5
Alkaline phosphatase (IU/L)	90 ± 25	86 ± 21	95 ± 24	54 ± 8*

*: p≤0.05; **: p≤0.01

Sacrifice and pathology: There were no changes in terminal body weights at any dose in either sex. Liver weights were increased in the 400 and 3200 ppm dose groups in both sexes compared with controls, the effect being slightly more pronounced in females. At 3200 ppm, the increase was more than 50 % compared to controls. This increased weight was associated with enlarged and dark livers at macroscopic examination and with minimal to moderate centrilobular hepatocellular hypertrophy in most animals in both sexes. Similar effects were observed at 400 ppm, however with lower magnitude, incidence and severity.

In addition to the liver effects, the thyroid gland weights (absolute and relative) were higher in males at 3200 ppm and 400 ppm. In association, follicular cell hypertrophy was observed in 3/5 high-dose males. Thyroid gland weight was not affected in females although 2/5 animals showed a minimal follicular cell hypertrophy.

In males, increased kidney weights were also seen at 3200 and 400 ppm in association with microscopic changes of hyaline droplet nephropathy (basophilic tubules, hyaline droplets in the proximal tubule and granular cast in the medulla). This typical nephropathy is a well known lesion occurring in male rat kidney and which is linked to accumulation of $\alpha_{2\mu}$-globulin in the proximal tubule. This protein is naturally reabsorbed and accumulated in the renal tubular epithelium of young rats. The accumulation is dependent upon the interaction between a chemical and the $\alpha_{2\mu}$-globulin specifically in the rat. As humans secrete only trace amounts of this globulin this mechanism is generally accepted as being non-relevant for humans. No other treatment-related effects were observed in other organs.

Table 30: Organ weight changes in the 28-day rat study with fluopyram

Dosage level (ppm)	0	50	400	3200
Males				
Liver weight				
Absolute (g)	11.13 ± 0.93	11.18 ± 0.93 (+0.4 %)	12.45 ± 1.15 (+12 %)	17.11 ± 1.76** (+54 %)
Bodyweight-relative	2.864 ± 0.210	2.811 ± 0.137 (-2 %)	3.055 ± 0.176 (+7 %)	4.401 ± 0.239** (+54 %)
Kidney weight				
Absolute (g)	2.63 ± 0.09	2.49 ± 0.18 (-5 %)	3.16 ± 0.24** (+20 %)	3.13 ± 0.20** (+19 %)
Bodyweight-relative	0.676 ± 0.016	0.627 ± 0.053 (-7 %)	0.777 ± 0.052 (+15 %)	0.801 ± 0.094 (+18 %)
Thyroid weight				
Absolute (g)	0.0176 ± 0.0040	0.0197 ± 0.0023 (+12 %)	0.0222 ± 0.0026 (+26 %)	0.0252 ± 0.0033** (+43 %)
Bodyweight-relative	0.00456 ± 0.00118	0.00499 ± 0.00085 (+9 %)	0.00548 ± 0.00086 (+20 %)	0.00653 ± 0.00109** (+41 %)
Females				
Liver weight				
Absolute (g)	5.84 ± 0.36	6.24 ± 0.32 (+7 %)	6.76 ± 0.46 (+16 %)	9.64 ± 1.00** (+65 %)
Bodyweight-relative	2.665 ± 0.200	2.773 ± 0.064 (+4 %)	3.072 ± 0.151 (+15 %)	4.599 ± 0.349** (+73 %)

*: p≤0.05; **: p≤0.01; Figures in parentheses are % differences from control

Induction of microsomal liver enzymes: Changes that were observed are described in Table 31. A dose-related slight increase in total cytochrome P-450, BROD and PROD activities was observed in both sexes at 3200 and 400 ppm. No significant effects were observed at 50 ppm.

Table 31: Results of the special hepatotoxicity testing in the 28-day rat study with fluopyram

Dosage level (ppm)	0	50	400	3200
Males				
Total P-450 (nmol/mg prot.)	1.19 ± 0.19	1.24 ± 0.25 (-)	1.43 ± 0.11 (+20 %)	1.63 ± 0.25 (+37 %)
BROD (pmol/min/mg prot.)	8.90 ± 1.00	9.86 ± 2.89 (-)	71 ± 20.66 (+698 %)	171.82 ± 29.02 (+1831 %)
EROD (pmol/min prot.)	59.24 ± 6.94	46.80 ± 4.14 (-)	66.78 ± 2.26 (-)	79.23 ± 8.22 (-)
PROD (pmol/min/mg prot.)	6.56 ± 1.30	4.68 ± 1.00 (-)	29.54 ± 11.80 (+350 %)	68.31 ± 6.05 (+941 %)
Females				
Total P-450 (nmol/mg prot.)	0.85 ± 0.07	0.89 ± 0.01	0.98 ± 0.23 (+15 %)	1.20 ± 0.13 (+41 %)
BROD (pmol/min/mg prot)	2.79 ± 0.54	4.32 ± 0.63 (-)	26.27 ± 10.94 (+842 %)	86.55 ± 37.92 (+3002 %)
EROD (pmol/min prot.)	52.16 ± 3.31	54.4 ± 3.92 (-)	68.57 ± 8.61 (-)	87.90 ± 5.33 (-)
PROD (pmol/min/mg prot.)	2.81 ± 0.52	3.49 ± 0.64 (-)	12.92 ± 4.87 (+360 %)	45.68 ± 24.43 (+1526 %)

No statistical analysis performed. (%) as compared to control. (-) no relevant change

Conclusion

The NOEL in this study was 50 ppm (4.0 and 4.6 mg/kg/day for males and females, respectively) based on changes in liver and kidney weights and hepatic enzyme induction in the next higher dose. As this study was not conducted in a guideline conform manner, it was not used for deriving a NOAEL.

Oral 90-day study in the rat

Title:	Kennel, P. (2005): AE C656948 - 90-day toxicity study in the rat by dietary administration, SA04048, M-250946-01.
Guidelines:	OECD 408.
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.
Materials and methods	
Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	PFI 0304
Purity:	99.0 %
Vehicle or positive control	None
Stability of test compound	Stable at 20 and 10000 ppm at room temperature for 105 days
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age / weight at dosing:	6 weeks approx.; Males: 182 g to 221 g; Females: 159 to 190 g at time of dosing
Source/breeder:	R. Janvier, Le Genest St Isle, France
Acclimation period	7 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions :	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: There were 10 animals of each sex per dose group. Animals were assigned to dose groups randomly by weight. Fluopyram was administered in the diet for at least 90 days at the following doses – 0, 50, 200, 1000, and 3200 ppm (equating approximately to 0, 3.06, 12.5, 60.5 and 204 mg/kg/day in males and 0, 3.63, 14.6, 70.1, and 230 mg/kg/day in females). An additional

10 males and 10 females fed either 0 or 3200 ppm of test diet for at least 90 days were maintained for a minimum of 28 days to examine the reversibility of any effects seen. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 32: Study design

Test group	Concentration in diet of fluopyram (ppm)	Dose per animal (study averages)		Animals assigned	
		Males (mg/kg bw/day)	Females (mg/kg bw/day)	Males	Females
1	0	0	0	10 + 10*	10 + 10*
2	50	3.06	3.63	10	10
3	200	12.5	14.6	10	10
4	1000	60.5	70.1	10	10
5	3200	204	230	10 + 10*	10 + 10*

* These additional 10 males and 10 females in control and high dosed groups were sacrificed following one month of recovery after termination of treatment.

Diet preparation and analysis: Fluopyram was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There were two preparations of each concentration for the whole study. The stability was demonstrated before the start of the study.

Statistics: For the 90-day phase, continuous data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett test (2-sided) on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogenous, they were transformed using a log transformation or square root transformation, then reanalyzed as above. For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis test which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance.

For the recovery phase, continuous data were analyzed by the F test for homogeneity of variances. When the data were homogeneous, a t-test (2-sided) was performed. When the data were not homogeneous, a modified t-test was performed. For some parameters, when data were not homogenous, they were transformed using a log transformation or square root transformation, then reanalyzed using the F test. For urine analysis (pH), group means were compared using the non-parametric Mann-Whitney test. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5 % and 1 % levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Observations: The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period. On study weeks 11 to 12, a neurotoxicity assessment was performed for all animals (except for animals of the recovery groups) by observers who were blind with respect to the dose level. Animals were tested individually for motor activity using an automated photocell recording apparatus designed to measure quantitatively spontaneous exploratory motor activity in a novel environment. Motor activity was recorded for 90 minutes with data being collected at 15-minute intervals throughout the session. For sensory reactivity, the following reflexes and responses were recorded: pupillary reflex, surface righting reflex, corneal reflex, flexor reflex, auditory startle response and tail pinch response. The fore- and hindlimb grip strength of all animals were measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge. The mean of three successive measurements was determined for both fore- and hindlimb grip strength.

Body weight, food consumption and compound intake: Body weights were recorded twice during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment and recovery periods and before necropsy.

Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 13 was calculated for each sex.

Ophthalmic examination: During the acclimatization period all animals were subjected to an ophthalmologic examination. After instillation of an atropinic agent (Mydriaticum, Merk Sharp and Dohme) each eye was examined by means of an indirect ophthalmoscope. During Week 13, all surviving animals from control and high dose groups were re-examined as no significant findings were observed at the end of the dosing phase, no further ophthalmologic examination was performed at the end of the recovery phase.

Hematology and clinical chemistry: On study days 91, 92, 93, or 94, and on recovery phase days 29 and 30, prior to necropsy, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood was collected on EDTA for hematology, on lithium heparin for plasma and clot activator for serum for clinical chemistry and on sodium citrate for coagulation parameters. The following hematology parameters were assayed using an Advia 120 (Bayer Diagnostics, Puteaux, France): red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte count, white blood cell count and differential count evaluation and platelet count. A blood smear was prepared and stained with Wright stain. It was examined when results of Advia 120 determinations were abnormal. Prothrombin time was assayed on an ACL 3000 (Instrumentation Laboratory, Paris, France).

Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples using an Hitachi 911 (Roche Diagnostics, Meylan, France).

Urinalysis: In the morning, overnight urine samples were collected on study Days 85, 86, or 87 from the exposure phase animals and on recovery Day 25 from the surviving extra animals in the control and high dose groups allocated to the recovery phase. Food and water were not accessible during urine collection. Any significant change in the general appearance of the urine was recorded. The urine volume was measured. pH was assayed using a Clinitek 200+ and Ames Multistix dipsticks (Bayer Diagnostics, Puteaux, France). Urinary refractive index was measured using a RFM320 refractometer (Bioblock Scientific, Illkirch, France). The following semi-quantitative parameters were assayed using a Clinitek 200+ and Ames Multistix dipsticks: glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen. Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

Hormone analysis: During weeks 3 and 13 of the treatment phase, and week 5 of the recovery phase, blood samples were collected from the retro-orbital venous plexus of all surviving animals diet fasted overnight. Blood was collected in heparin, and plasma separated and frozen until used for hormone analyses. Thyroid hormones, including T3, T4, and TSH, were assayed by radioimmunoassay kit (Amersham for TSH, and Backman-Coulter for T3/T4).

Sacrifice and pathology: On study days 91, 92, 93, and 94 and on recovery days 29 or 30, a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by inhalation of Isoflurane, and then exsanguinated before necropsy. All animals were fasted prior to scheduled sacrifice. All animals, either 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. Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, cecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina.

A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues 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. Histopathological examinations were performed on all tissues from all the animals in the control and high dose groups and all decedents in all groups. The liver, kidney, lung, thyroid gland and significant gross findings were examined in all animals in the study. For the reversibility phase, liver, kidney, lung, thyroid gland and macroscopic observations were processed and liver, lung, kidney and thyroid gland were examined. In addition, immunohistochemical staining for $\alpha_{2\mu}$-globulin was performed on male kidney sections in all dose groups.

Findings

Diet preparation and analysis: Diet samples of 20 and 10000 ppm were found to be stable over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature. The homogeneity of the diet was verified on the first preparation at 50 and 3200 ppm and was within a range of 93-97 % of the nominal concentrations. Concentration was checked for each concentration for both dietary preparations. Measured concentrations for the four dose groups were within the range of 92-98 % of the nominal concentration. Hence all values were within the in-house target range of 85-115 % of the nominal concentration.

Table 33: Homogeneity and concentration of test material

	Nominal concentrations (ppm)	Analysed concentrations (ppm)	% of nominal concentration
Homogeneity (first preparation)	50	H1 48.1	96
		H2 48.1	96
		H3 48.4	97
		M1 47.6	95
		M2 48.4	97
		M3 47.6	95
		B1 48.0	96
		B2 47.7	95
		B3 48.0	96
	3200	H1 3048	95
		H2 3036	95
		H3 3051	95
		M1 3081	96
		M2 3062	96
		M3 3091	97
		B1 3075	96
		B2 2991	93
		B3 3032	95
Concentration (first preparation)	50	48.0	96
	200	184	92
	1000	959	96
	3200	3052	95
	3200 (second load)*	3066	96
Concentration (second preparation)	50	46.7	93
	200	192	96
	800	953	95
	3200	3114	97
	3200 (second load)*	3128	98

H = surface; M = middle; B = bottom of the preparation. Samples 1, 2 and 3 for samples H, M or B: three different samples taken at the same level of the preparation. *: two loads were prepared at this dose level

Clinical signs of toxicity and neurotoxicity assessment: There were no treatment-related clinical signs in either sex throughout the study. Sensory reactivity was unaffected by treatment. The few changes in the tail pinch response noted in the male high dose groups were considered to reflect inter-individual variations rather than any treatment-related effect.

At 3200 ppm, fore- and hindlimb grip strength were essentially comparable to control values in males and slightly decreased by 16 % and 23 %, respectively, in females (not statistically significant), compared to controls. This slight effect in females was considered most likely to be due to a decrease in body weight, rather than to be a specific treatment-related neurotoxic effect. At 1000, 200 and 50 ppm in both sexes, fore- and hindlimb grip strength were essentially comparable to control values. The few changes noted in females at 1000 ppm were considered to reflect inter-individual variations rather than any treatment-related effect. Motor activity was indistinguishable between the treated and control groups.

Mortality: Two males were sacrificed prematurely for humane reasons on Days 37 and 57. One male (1000 ppm) was sacrificed on Day 37 having had a distended abdomen between Days 22 and 37 and general pallor between Days 27 and 37. This animal was noted to have a pale appearance and an enlarged, irregular and red mottled liver at the macroscopic examination. One male (50 ppm) was sacrificed on Day 57 having been noted to have labored and noisy respiration, a wasted appearance, piloerection and ocular discharge on both eyes on the day of sacrifice, together with a body weight loss of 8.2 g/day and a food consumption reduced to 18 g/day during the week prior to sacrifice. The condition of this animal was attributable to an accidental trauma, as macroscopic examination revealed

soiled fur around both eyes and a fracture of the nasal cavity. These two premature sacrifices were considered not to be treatment-related. There were no mortalities during the recovery phase of the study.

Body weight and food consumption: At 3200 ppm, mean body weight was decreased by between 4 % and 6 % in males ($p \leq 0.01$ or $p \leq 0.05$) and 4 % and 8 % in females ($p \leq 0.01$ or $p \leq 0.05$ on most occasions) throughout the course of the study, compared to controls. The effect on body weight was primarily due to an initial decrease in mean body weight gain per day during the first week of treatment in males and females (-26 and -29 %, respectively, $p \leq 0.01$), compared to controls. Thereafter, mean body weight gain per day was essentially comparable to the controls in both sexes, but mean overall body weight gain was reduced by 9 % in males ($p \leq 0.05$) and 17 % in females ($p \leq 0.01$). The effect on mean body weight was still observed after 4 weeks of recovery in both sexes, as the magnitude of the decrease was similar to that observed at the end of the treatment phase. At 1000 ppm, mean body weight parameters were unaffected by the treatment in either sex, with the exception of a slight decrease of 15 % (not statistically significant) in mean body weight gain per day in females during the first week of treatment, compared to controls. Other minor differences (reaching statistical significance) from controls were noted but they were considered to reflect inter-individual variation rather than a treatment-related effect. At 200 and 50 ppm, no effect on mean body weight and mean body weight change was noted in either sex.

At 3200 ppm, mean food consumption in males was essentially comparable to the control values and in females was slightly decreased by between 5 and 12 % from Days 29 to 90 ($p \leq 0.01$ or $p \leq 0.05$ on most occasions), compared to the controls. No treatment-related effect on mean food consumption was noted in either sex during the recovery phase. The slight decrease of 8 % in mean food consumption noted in males during the last two weeks of the recovery phase was considered to be incidental. At 1000 ppm, mean food consumption was similar to the control values in males and slightly decreased by between 4 and 9 % on most occasions in females (not statistically significant), compared to controls. At 200 and 50 ppm, no effect on food consumption was noted in either sex. The mean achieved dosage intake per group was as follows:

Table 34: Mean achieved dietary intake of fluopyram (Weeks 1 - 13)

Diet concentration ppm	Males mg/kg/day	Females mg/kg/day
50	3.06	3.63
200	12.5	14.6
1000	60.5	70.1
3200	204	230

Ophthalmologic examination: No treatment-related ocular abnormalities were observed at the ophthalmoscopic examination.

Hematological findings: Dosing Phase: Higher mean prothrombin time was noted at 3200 ppm in males only (+74 %, $p \leq 0.01$), when compared to the control values. Slightly lower mean hemoglobin concentrations were noted at 3200 ppm in both sexes and at 1000 ppm in males only. These variations were associated with lower mean hematocrit in males and lower mean corpuscular volume and mean corpuscular hemoglobin in females at 3200 ppm. The magnitude and statistical significance relative to the control groups were as follows:

Table 35: Hematological findings (dosing phase)

Dose levels (ppm)	0	50	200	1000	3200
Males					
Hemoglobin (g/dL)	15.5 ± 0.3	15.4 ± 0.3	15.2 ± 0.6	14.9 ± 0.5	14.6 ± 0.7** (-6 %)
Hematocrit (%)	47.6 ± 0.9	47.6 ± 0.9	47.0 ± 1.9	45.7 ± 1.2*	45.4 ± 1.9**
Females					
Hemoglobin (g/dL)	15.0 ± 0.5	15.2 ± 0.5	15.4 ± 0.3	15.1 ± 0.6	14.4 ±0.6* (-4 %)
Mean corpuscular volume (fl)	53 ± 1	54 ± 1	54 ± 2	53 ± 1	49 ± 1** (-8 %)
Mean corpuscular hemoglobin (pg)	17.8 ± 0.5	17.8 ± 0.4	17.9 ± 0.7	17.5 ± 0.5	16.3 ± 0.4** (-8 %)

* = p≤0.05. **= p≤0.01

Additionally, higher mean platelet (+24 %, p≤0.01) and reticulocyte (+50 % for absolute count and +42 % for percentage, p≤0.01) counts were noted at 3200 ppm in females. There was no evidence of treatment-related changes at 1000 ppm in females or at 200 and 50 ppm in either sex.

Recovery Phase: After 1 month of recovery in the high dose group, there was a tendency towards reversibility although mean hemoglobin concentration was still lower after the recovery period in males and females (-4 %, p≤0.01 and -3 %, not statistically significant, respectively), compared to controls. However these variations were lower than at the end of the dosing phase. Male prothrombin time and female platelet and reticulocyte variations observed at the end of the dosing phase were considered to be reversible, as after the 1-month recovery period, no significant differences were noted between the high dose and the control groups.

Clinical chemistry findings: Dosing Phase: At 3200 and/or 1000 ppm, several treatment-related variations were observed in males and/or females. The magnitude and statistical significance relative to the control groups were as follows:

Table 36: Clinical chemistry findings (dosing phase) male

Dose levels (ppm)	0	50	200	1000	3200
Males					
Total cholesterol (mmol/L)	1.87 ± 0.5	1.73 ± 0.25	2.23 ± 0.57 (+ 19 %)	2.72 ± 0.84* (+45 %)	2.95 ± 0.47** (+58 %)
Total bilirubin (µmol/L)	2.0 ± 0.4	1.7 ± 0.2	1.8 ± 0.5	1.4 ± 0.2** (-30 %)	1.3 ± 0.3** (-35 %)
γ-glutamyltransferase (IU/L)	0 ± 1	0 ± 0	0 ± 0	0 ± 1	3 ± 1** (+625 %)
Total protein (g/L)	72 ± 3	73 ± 3	72 ± 3	73 ± 4	78 ± 4** (+8 %)
Globulin (g/L)	28 ± 2	28 ± 1	29 ± 2	29 ± 2	31 ± 2
Creatinine (µmol/L)	35 ± 3	35 ± 4	33 ± 2	39 ± 5* (+11 %)	40 ± 3** (+14 %)
Urea (mmol/L)	5.14 ± 0.64	4.96 ± 0.39	4.86 ± 0.44	5.45 ± 0.79	6.01 ± 0.57** (+17 %)
Glucose (mmol/L)	6.64 ± 1.04	7.05 ± 1.38	6.87 ± 1.15	5.85 ± 0.49	4.88 ± 0.32** (-27 %)
Inorganic phosphorus (mmol/L)	1.79 ± 0.09	1.86 ± 0.15	1.86 ± 0.18	1.96 ± 0.14* (+9 %)	2.11 ± 0.15** (+18 %)
Calcium (mmol/L)	2.71 ± 0.07	2.71 ± 0.05	2.73 ± 0.06	2.81 ± 0.07* (+4 %)	2.86 ± 0.08** (+6 %)
Chloride (mmol/L)	103 ± 2	103 ± 1	102 ± 1	100 ± 1** (-3 %)	100 ± 1** (-3 %)

= p≤0.05. **= p≤0.01

Table 37: Clinical chemistry findings (dosing phase) female

Dose levels (ppm)	0	50	200	1000	3200
Females					
Total cholesterol (mmol/L)	1.88 ± 0.17	2.13 ± 0.39	2.11 ± 0.43 (+12 %)	2.79 ± 0.65** (+48 %)	3.78 ± 0.78** (+101 %)
Triglycerides (mmol/L)	0.44 ± 0.12	0.48 ± 0.12	0.45 ± 0.04	0.50 ± 0.08	0.85 ± 0.33** (+102 %)
Total bilirubin (µmol/L)	2.6 ± 0.4	2.1 ± 0.3**	1.9 ± 0.5** (-27 %)	1.6 ± 0.3** (-38 %)	1.7 ± 0.04** (-35 %)
γ-glutamyltransferase (IU/L)	0 ± 0	0 ± 0	1 ± 1 (+480 %)	1 ± 1 (+480 %)	9 ± 4** (+4300 %)
Alkaline Phosphatase (IU/L)	47 ± 10	45 ± 9	44 ± 10	42 ± 9	31 ± 6** (-34 %)
Total protein (g/L)	72 ± 3	70 ± 4	72 ± 5	75 ± 4	78 ± 7* (+8 %)
Globulin (g/L)	24 ± 2	25 ± 2	27 ± 2	27 ± 2	30 ± 3** (+25 %)
Albumin/Globulin ratio	1.96 ± 0.26	1.82 ± 0.15	1.74 ± 0.06	1.78 ± 0.10	1.64 ± 0.10** (-16 %)
Inorganic phosphorus (mmol/L)	1.46 ± 0.13	1.45 ± 0.13	1.48 ± 0.11	1.58 ± 0.18	1.68 ± 0.23* (+15 %)
Calcium (mmol/L)	2.67 ± 0.07	2.65 ± 0.06	2.70 ± 0.05	2.74 ± 0.07	2.80 ± 0.12** (+5 %)
Chloride (mmol/L)	104 ± 1	103 ± 1	103 ± 2	103 ± 1	101 ± 1** (-3 %)

* = p≤0.05. **= p≤0.01

Recovery Phase: After 1 month of recovery in the high dose group, there was a general tendency towards reversibilty. Nevertheless, statistically significant differences were still noted in mean total cholesterol, globulin concentrations and albumin/globulin ratio in females (+19 %, +14 % and -14 % respectively, p≤0.05), compared to the control values. However, the variations were lower than at the end of the dosing phase. The other changes observed at the end of the dosing phase were considered to be reversible, as after the 1-month recovery period, no significant differences were noted between the high dose and the control groups. The slightly higher mean alkaline phosphatase activity seen in females was considered not to be biologically or toxicologically relevant.

Urinalysis: Dosing Phase: The incidence and severity of cellular casts in urine were increased in all groups of fluopyram treated males in a dose-related manner. The presence of casts in the urine is to be seen in connection to the hyaline droplet nephropathy observed at histopathology examinations. Other changes were considered to be incidental and not treatment-related.

Table 38: Incidence and severity of cellular casts in urine at the end of the treatment phase

Sex	Males				
Dietary level (ppm)	0	50	200	1000	3200
Number of animals examined	10	9	10	9	10
Slight	1	4	2	0	1
Moderate	0	2	1	3	2
Severe	0	0	2	5	7
Total	1	6	5	8	10

Recovery Phase: After 1 month of recovery, cellular casts in urine were still observed in male high dose group. However, the incidence and severity were lower than at the end of the dosing phase supporting a progression towards reversibility.

Table 39: Incidence and severity of cellular casts in urine at the end of the recovery phase

Sex	Males	
Dietary level (ppm)	0	3200
Number of animals examined	10	10
Slight	0	5
Moderate	0	3
Severe	0	0
Total	0	8

Hormone Analysis: Dosing Phase: At 3200 ppm, an increase in mean TSH level was noted at Week 3 in both sexes (+63 %, p≤0.05 and +71 %, p≤0.01, respectively), together with an increase in mean T3 and T4 levels in females (+24 %, p≤0.05 and +54 %, p≤0.01, respectively), whereas at Week 13, only an increase in mean TSH and T3 levels was noted in males (+88 and +40 %, respectively, p≤0.01). At 1000 ppm, mean TSH level was increased by 54 % (not statistically significant) and T4 level was significantly increased by 43 % in males on Week 13. A dose dependent increase of T3, T4 and TSH was observed in both sexes at most time points and for most parameters at all dose levels when compared to controls, however, without being significant or of relevant magnitude at the lower two dose levels of 200 and 50 ppm.

Recovery Phase: All changes observed at the end of the dosing phase were considered to be reversible, as after the 1-month recovery period, no statistically significant differences were noted between the high dose and the control groups. There were however, slight changes in thyroid hormone levels.

Organ weight: Dosing phase: Mean terminal body weight at 3200 ppm was lower than in controls in males and females (-5 %, not statistically significant and -9 %, p≤0.05, respectively).

Mean absolute and relative liver weights were statistically significantly higher at 3200 and 1000 ppm in both sexes, when compared to controls. A tendency towards higher liver weights was also noted at 200 ppm in both sexes (not statistically significant).

Table 40: Liver weight changes after 90 days of treatment

Dietary level (ppm)	0	50	200	1000	3200
Males					
Mean absolute liver Weight (g)	10.94 ± 0.86	10.82 ± 0.63 (-1 %)	12.02 ± 1.19 (+10 %)	13.63 ± 1.13** (+25 %)	16.77 ± 1.70** (+53 %)
Mean liver to body weight ratio	2.282 ± 0.096	2.277 ± 0.101 (0 %)	2.400 ± 0.151 (+5 %)	2.736 ± 0.188** (+20 %)	3.669 ± 0.223** (+61 %)
Females					
Mean absolute liver Weight (g)	5.74 ± 0.58	5.94 ± 0.32 (+3 %)	6.40 ± 0.43 (+11 %)	7.09 ± 0.58** (+24 %)	9.09 ± 1.55** (+58 %)
Mean liver to body weight ratio	2.123 ±0.096	2.235 ± 0.105 (+5 %)	2.366 ± 0.071 (+11 %)	2.697 ± 0.172** (+27 %)	3.699 ± 0.503** (+74 %)

* = p≤0.05. **= p≤0.01

Mean absolute and relative kidney weights were statistically significantly higher at 3200 and 1000 ppm in males, when compared to controls. A tendency towards higher kidney weights was also observed at 200 ppm in males (not statistically significant). At 3200 ppm in females, mean kidney to body weight ratio was statistically significantly higher than controls, but this change was attributable to lower terminal body weight and was thus considered not to be toxicologically relevant.

Table 41: Kidney weight changes after 90 days of treatment

Dietary level (ppm)	0	50	200	1000	3200
Males					
Mean absolute kidney weight (g)	2.81 ± 0.21	2.89 ± 0.20 (+3 %)	3.10 ± 0.30 (+10 %)	3.65 ± 0.32** (+30 %)	3.60 ± 0.38** (+28 %)
Mean kidney to bodyweight ratio	0.587 ± 0.045	0.608 ± 0.029 (+4 %)	0.620 ± 0.049 (+6 %)	0.732 ± 0.043** (+25 %)	0.788 ± 0.069** (+34 %)
Females					
Mean absolute kidney weight (g)	1.65 ± 0.14	1.74 ± 0.13 (+5 %)	1.73 ± 0.15 (+5 %)	1.73 ± 0.18 (+5 %)	1.70 ± 0.17 (+3 %)
Mean kidney to bodyweight ratio	0.614 ± 0.044	0.655 ± 0.041 (+7 %)	0.640 ± 0.030 (+4 %)	0.659 ± 0.062 (+7 %)	0.693 ± 0.069** (+13 %)

**= p≤0.01

There was a tendency towards higher thyroid weights at 3200 ppm in both sexes.

Table 42: Thyroid weight changes after 90 days of treatment (% change when compared to controls)

Dietary level (ppm)	0	50	200	1000	3200
Males					
Mean absolute thyroid weight (g)	0.0272 ± 0.0052	0.0224 ± 0.0058 (-18 %)	0.0269 ± 0.0048 (-1 %)	0.0282 ± 0.0060 (+4 %)	0.0299±0.0051 (+10 %)
Mean thyroid to bodyweight ratio	0.00568 ± 0.00107	0.00471 ± 0.00123 (-17 %)	0.00496 ± 0.00081 (-13 %)	0.00565 ± 0.00106 (-1 %)	0.00657 ± 0.00121 (+16 %)
Females					
Mean absolute thyroid weight (g)	0.0185 ± 0.0021	0.0203 ± 0.0040 (+10 %)	0.0164 ± 0.0042 (-11 %)	0.0184 ± 0.0038 (-1 %)	0.0206 ± 0.0026 (+11 %)
Mean thyroid to bodyweight ratio	0.00689 ± 0.00093	0.00767 ± 0.00163 (+11 %)	0.00610 ± 0.00156 (-11 %)	0.00696 ± 0.00116 (0 %)	0.00841 ± 0.00097* (+22 %)

* = p≤0.05

Recovery phase: After 1 month of recovery in the high dose groups, mean terminal body weight was still lower than in controls in males and females (-9 %, p≤0.01and -7 %, not statistically significant, respectively). Mean liver to body weight ratio in the high dose male and females groups was still statistically significantly higher than in controls. Mean absolute and relative kidney weights in the high dose male group were still statistically significantly higher than in controls. However, the magnitude of variation compared to control was clearly lower than at the end of the dosing phase.

Table 43: Kidney weight changes after 1 month of recovery (% change when compared to controls)

Dietary level (ppm)	0	3200
Males		
Mean absolute kidney weight (g)	2.89 ± 0.16	3.08 ± 0.22* (+7 %)
Mean kidney to body weight ratio	0.554 ± 0.024	0.645 ± 0.044** (+16 %)

* = p≤0.05. **= p≤0.01

Mean absolute thyroid weight and mean thyroid to body weight ratio in the female high dose group were statistically significantly higher than in controls.

Gross and histopathology: Dosing phase: Enlarged and dark liver and/or prominent lobulation of the liver were observed at 3200 and 1000 ppm. These findings corroborate the centrilobular hypertrophy noted at the microscopic examination.

Table 44: Incidence of treatment-related macroscopic changes in the liver after 90 days of treatment

Sex	Males					Females				
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200
Obviously large	0/10	0/9	2/10	6/9	10/10	0/10	0/10	1/10	7/10	10/10
Dark	0/10	0/9	0/10	2/9	9/10	0/10	0/10	0/10	0/10	9/10
Prominent lobulation	0/10	0/9	0/10	4/9	6/10	0/10	0/10	0/10	1/10	0/10

Enlarged and/or pale kidneys were found in males at 3200 ppm, 1000 ppm. This finding was associated with increased kidney weights and was thought to be treatment related.

Table 45: Incidence of treatment-related macroscopic changes in the kidney after 90 days of treatment

Sex	Males					Females				
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200
Obviously large	0/10	1/9	3/10	4/9	6/10	0/10	0/10	1/10	0/10	0/10
Pale	1/10	0/9	1/10	7/9	9/10	0/10	1/10	0/10	0/10	0/10

Recovery phase: After 1 month of recovery in the high dose group, enlarged kidneys were noted in 2/10 males.

Microscopic pathology: Dosing phase: Effects of treatment with fluopyram were seen in the liver, kidney, thyroid gland, adrenal gland and lung. In the liver, minimal to moderate centrilobular hepatocellular hypertrophy was observed with a dose-related increase in incidence and severity at 3200 and 1000 ppm in both sexes and minimal centrilobular hepatocellular hypertrophy was observed at 200 ppm in males. In addition, minimal to moderate periportal to midzonal hepatocellular macrovacuolation was observed in females at 3200 and 1000 ppm. Minimal periportal to midzonal hepatocellular macrovacuolation was also observed in 3/10 females at 200 ppm. Since the effect was not significant, occurred in only 3 animals and was of minimal extent, it was not considered to be adverse.

Table 46: Incidence of treatment-related microscopic changes in the liver after 90 days of treatment

Sex	Males					Females				
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200
Number of animals examined	10	9	10	9	10	10	10	10	10	10
Centrilobular hepatocellular hypertrophy: diffuse										
Minimal	0	0	2	5	0	0	0	0	5	3
Slight	0	0	0	4	2	0	0	0	2	5
Moderate	0	0	0	0	8	0	0	0	0	2
Total	0	0	2	9	10	0	0	0	7	10
Periportal to midzonal hepatocellular macrovacuolation : focal/multifocal										
Minimal	0	0	0	0	0	0	0	3	6	3
Slight	0	0	0	0	0	0	0	0	0	1
Moderate	0	0	0	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	0	3	6	5

In the kidney, hyaline droplet nephropathy (characterized by basophilic tubules, hyaline droplets in proximal tubules and granular casts in the medulla) and hyaline casts were higher at 3200 and 1000 ppm in males, in comparison with controls. Hyaline droplet nephropathy was also slightly higher at 200 ppm in males. Hyaline droplet nephropathy is a recognized lesion of the male rat kidney that is related to the accumulation of $\alpha_{2\mu}$-globulin in the proximal tubules of affected animals. Accumulation of $\alpha_{2\mu}$-globulin in the male kidney was confirmed with a protein-specific immunohistochemical determination, which showed a higher storage of $\alpha_{2\mu}$-globulin in the proximal convoluted tubules at 1000 ppm and above, and in the proximal straight tubules at 200 ppm and above, when compared to controls.

$\alpha_{2\mu}$-globulin is naturally reabsorbed and accumulated in the renal tubular epithelium of the young male rat. The sequence of renal events leading to its additional accumulation is dependent upon the interaction between a chemical and the $\alpha_{2\mu}$-globulin and is male rat-specific (G.C. HARD et al., *Susceptibility of the kidney to toxic substances*, in MOHR U. et al., *Pathobiology of the aging rat*, vol. 1, p. 252, ILSI Press, 1992, ASB2009-3176). As humans secrete $\alpha_{2\mu}$-globulin only in trace amounts, this mechanism is generally accepted as being not relevant to humans. Therefore, although this accumulation was considered to be treatment-related in the current study, it was considered not to be toxicologically relevant to man.

Table 47: Incidence of treatment-related microscopic changes in the kidney after 90 days of treatment

Sex	**Males**					**Females**				
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200
Number of animals examined	10	9	10	9	10	10	10	10	10	10
Hyaline droplets : proximal tubules										
Minimal	1	3	7	0	0	0	0	0	0	0
Slight	0	0	3	0	0	0	0	0	0	0
Moderate	0	0	0	9	1	0	0	0	0	0
Marked	0	0	0	0	9	0	0	0	0	0
Total	1	3	10	9	10	0	0	0	0	0
Basophilic tubules : focal/multifocal										
Minimal	2	1	3	3	1	0	0	0	0	0
Slight	0	1	0	6	8	0	0	0	0	0
Moderate	0	1	0	0	1	0	0	0	0	0
Total	2	3	3	9	10	0	0	0	0	0
Granular casts: medulla										
Minimal	0	0	1	2	6	0	0	0	0	0
Slight	0	0	0	6	2	0	0	0	0	0
Moderate	0	0	0	0	1	0	0	0	0	0
Total	0	0	1	8	9	0	0	0	0	0
Hyaline casts: focal/multifocal										
Minimal	1	0	0	4	6	0	1	1	0	2
Slight	1	0	0	0	0	0	0	0	0	0
Total	2	0	0	4	6	0	1	1	0	2

In the thyroid gland, a higher incidence of minimal to slight diffuse hypertrophy of follicular cells was seen at 3200 and 1000 ppm in both sexes compared to controls and internal historical control data.

Table 48: Incidence of treatment-related microscopic changes in the thyroid gland after 90 days of treatment

Sex	**Males**					**Females**				
Dietary level (ppm)	0	50	200	1000	3200	0	50	200	1000	3200
Number of animals examined	10	9	10	9	10	10	10	10	10	10
Follicular cell hypertrophy: diffuse										
Minimal	0	0	1	3	5	0	0	0	2	1
Slight	0	0	0	1	3	0	0	0	0	0
Total	0	0	1	4	8	0	0	0	2	1

In the adrenal gland zona fasciculate vacuolation was observed in male rats at 3200 ppm only. The incidence was increased (10 animals showed the effect as compared to 2 in the control). In the lung the only observation observed at increased incidence was increased accumulation of foamy macrophages (focal/multifocal) in 3200 ppm males.

Recovery phase: After 1 month of recovery in the high dose groups, basophilic tubules, hyaline droplets in proximal tubules, granular casts in the medulla and hyaline casts were persistent in the kidney of some males.

Table 49: Incidence of treatment-related changes in the kidney after 1 month of recovery

Sex	Males		Females	
Dietary level (ppm)	0	3200	0	3200
Number of animals examined	10	10	10	10
Hyaline droplets : proximal tubules				
Minimal	0	2	0	0
Total	0	2	0	0
Basophilic tubules : focal/multifocal				
Minimal	0	3	0	0
Slight	0	7	0	0
Total	0	10	0	0
Granular casts : medulla				
Minimal	0	5	0	0
Slight	0	2	0	0
Moderate	0	1	0	0
Total	0	8	0	0
Hyaline casts: focal/multifocal				
Minimal	1	9	0	1
Total	1	9	0	1

After the recovery period, liver and thyroid gland were comparable between the high dose and control groups, indicating that the changes noted after 90 days of treatment were reversible in these organs.

Conclusion

In the 90-day feeding study in rats the lowest dose level of 50 ppm represented the No Observed Effect Level (NOEL) in males and females (equating to 3.06 / 3.63 mg/kg body weight/day). At the next higher dose level of 200 ppm (12.5 / 14.6 mg/kg bw/d), however, treatment-related effects were confined to a rather adaptive liver weight increase due to hepatocellular hypertrophy that was associated with non-significant changes in clinical chemistry parameters. Because these findings were not considered adverse, the NOAEL for both sexes was set at that dose. It was based on much more pronounced organ weight, clinical chemistry and histopathological findings in liver, thyroid and kidneys at the next higher dose level of 1000 ppm (60.5 / 70.1 mg/kg bw/d).

Oral 28-day study - mouse

Title:	Kennel, P. (2004): AE C656948 - Preliminary 28-day toxicity study in the mouse by dietary administration, SA 04013, M-088486-01.
Guidelines:	None (range finding study).
Deviations:	None (range finding study).
GLP:	No.
Acceptability:	The study is considered to be supplementary (range finding study).

Materials and methods

Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	FLH 1046
Purity:	99,4 %
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm after 95 days frozen storage and 10 days at room temperature.
Species:	Mouse
Strain:	C57BL/6J
Age / weight at dosing:	6 weeks approx.; Males: 18.7 g to 21.6 g; Females: 15.1 to 18.3 g at time of dosing
Source/breeder:	Charles River, L'Arbresle, France
Acclimation period:	13 days
Identification:	Labeling by cage cards and water-insoluble spots on the tail
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions :	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: There were 5 animals/sex/group. Animals were randomly assigned to treatment groups by body weight, and were given fluopyram in the diet at concentrations of 0, 150, 1000, or 5000 ppm for 28 days. Animal housing and husbandry were in accordance with the regulations described above.

Diet preparation and analysis: Fluopyram was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation of each concentration for the whole study. The formulated diet was stored at approximately -18°C when not in

use. Fluopyram concentrations were shown in a previous study (SA 03332) to be stable at 20 and 10000 ppm for 95 days frozen followed by 10 days at room temperature.

Table 50: Study design and achieved doses

Test group	Achieved dose (mg/kg/day)	Animals assigned
Males		
Control	0	5
150	24.7	5
1000	162	5
5000	747*	5
Females		
Control	0	5
150	31.1	5
1000	197	5
5000	954	5

* Calculated for Weeks 1 to 3 only due to mortality or early sacrifice in this group.

Statistics: Statistics were performed as described above (28-d rat).

Observations: Observations, body weights and food consumption were recorded as described above (28-d rat).

Clinical chemistry: On study Day 29, blood samples were taken from all surviving animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood (0.6 ml) was collected on lithium heparin for plasma chemistry determinations. Plasma samples were analyzed for total bilirubin, urea, total protein, albumin, total cholesterol, AST, ALT, and alkaline phosphatase on a Hitachi 911 (Roche Diagnostics, Meylan, France).

Sacrifice and pathology: On study Day 29 a complete necropsy was performed on all surviving animals. Animals were deeply anesthetized by Isoflurane inhalation and sacrificed by exanguination. All animals were fasted prior to scheduled sacrifice. Macroscopic abnormalities were recorded, sampled and examined microscopically. Adrenal gland, brain, kidney, liver, spleen, testis and ovary were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following tissues were sampled: Adrenal gland, liver, kidney, lung, ovary, spleen, testis, and thyroid gland (including parathyroid). Tissue samples were fixed by immersion in neutral buffered 10 % formalin with the exception of the testis which was fixed in Davidson's fixative. These tissues were embedded in paraffin wax, and histological examinations were performed for all tissues in the control and high dose groups. Histological examinations were performed on the adrenal gland, liver, kidney, thyroid gland, lung and spleen, on all animals in all dose groups.

Findings

Mortality: All males and 3/5 females at 5000 ppm were humanely sacrificed between study Days 17 and 27. No mortalities occurred in the other dose groups.

Clinical signs: Clinical signs in the decedent animals comprised reduced motor activity, hunched posture, piloerection, wasted appearance and/or coldness to touch in both sexes together with labored respiration in 3/5 males and distended abdomen in 2/3 females. These clinical signs were noted mainly on the day of sacrifice or for a few days prior to sacrifice. A loss of body weight accompanied these signs, as did reduced food intake. In the surviving females at 5000 ppm, distended abdomen was noted between study Days 8 and 10 in one animal. There were no clinical signs in either the 1000 ppm or 150 ppm dose groups.

Body weight and food consumption: At 5000 ppm, in the surviving females, mean body weight and mean body weight change parameters were comparable to the control values. There was a slight decrease in the daily mean body weight gain during study Week 2 in males at 1000 ppm (0.03 g/day vs. 0.10 g/day in the control group, not statistically significant). No treatment-related effect on mean body weight and mean body weight change parameters was noted at 1000 ppm in females or 150 ppm in either sex. No effect on mean food consumption was noted in animals surviving to terminal sacrifice. Achieved doses are shown in Table 50.

Blood analysis: The two surviving females at 5000 ppm, had elevated total cholesterol (+218 %) and total protein (+16 %) concentrations, and alanine aminotransferase activities (+484 %).

At 1000 ppm, mean alanine aminotransferase activity was higher in males (+359 %, not statistically significant) and mean albumin concentration was lower in females (-12 %, p≤0.05). Lower mean total bilirubin and albumin concentrations were noted in males (-56 %, p≤0.05 and -10 %, p≤0.01, respectively, compared to controls). These changes were within historical control ranges according to the notifier.

At 150 ppm, lower mean total bilirubin and albumin concentrations were noted in males (-50 and -5 %, respectively, p≤0.05 compared to controls). These changes were within historical control ranges according to the notifier.

Sacrifice and pathology: Mean absolute and relative liver weights were higher at 5000 ppm in females and at 1000 and 150 ppm in both sexes. These changes were found to be dose-related. The magnitude of the changes relative to the controls and statistical significance between treated and control values were as follows:

Table 51: Liver weight changes at terminal sacrifice (% change when compared to controls)

Dose levels (ppm)	**0**	**150**	**1000**	**5000**
Males				
Liver weights				
Absolute (g)	0.80 ± 0.10	0.94 ± 0.05* (+18 %)	1.13 ± 0.08** (+41 %)	*a*
Body weight-relative	4.053 ± 0.495	4.896 ± 0.131 (+21 %)	5.905 ± 0.223** (+46 %)	*a*
Females				
Liver weights				
Absolute (g)	0.68 ± 0.05	0.79 ± 0.05 (+16 %)	0.94 ± 0.12** (+38 %)	1.66 ± 0.12# (+144 %)
Body weight-relative	4.502 ± 0.163	5.279 ± 0.294* (+17 %)	5.712 ± 0.594** (+27 %)	10.435 ± 0.43# (+132 %)

*: p≤0.05; **: p≤0.01 *a* = no surviving animals #: the statistical significance was not calculated as there were only two surviving animals.

All males and 3/5 females dosed at 5000 ppm were killed for humane reasons prior to the scheduled termination date. A pale pancreas was observed in all males and in 2/3 females. Rounded borders were observed in the liver in 3/5 males and 1/3 females. Dark livers were observed in 4/5 males and in all females, and enlarged livers were observed in 1/5 males and 2/3 females. The size of the thymus was clearly reduced in 4/5 males and 1/3 females, and distended abdomen was noted in 3/5 males. Red liquid was observed in the thoracic cavity in all males.

Enlarged livers were observed in both surviving females at 5000 ppm and in all males and 4/5 females at 1000 ppm. Dark livers were observed in 1/2 surviving females at 5000 ppm and in 3/5 males and 2/5 females at 1000 ppm.

In the decedent animals at 5000 ppm, treatment-related effects were seen in the adrenal glands, liver, lungs, spleen, thymus and thyroid gland. Hypertrophy, vacuolation and degeneration/necrosis of the zona fasciculata were seen in the adrenal glands in all animals, together with perivascular and

intra-alveolar hemorrhage and degeneration/inflammation of pulmonary veins in the lungs and erythroid extramedullary hematopoiesis in the spleen. Focal hemorrhage was seen in the thyroid gland in 3/5 males and decreased cellularity of the cortex and focal hemorrhage were seen in the thymus in all animals where examination was possible. In the liver, hypertrophy of hepatocytes (mainly centrilobular), hepatocellular eosinophilia, bile duct/oval cell hyperplasia, focal necrosis and single hepatocellular necrosis were seen in all animals, and centrilobular degeneration/necrosis in 1/5 males.

It was considered that premature sacrifice in all males and in 1/3 females dosed at 5000 ppm was associated with intrathoracic hemorrhage. The majority of decedent animals had areas of hemorrhage in the thoracic cavity, thyroid gland, lungs and thymus. Hemorrhaging into the thoracic cavity (and other tissues) was considered to have contributed to the clinical condition of most moribund animals. Hence, the increase in the incidence and severity of extramedullary hematopoiesis in the spleen most likely represents a reactive response to intrathoracic hemorrhaging in moribund animals. The lesions in the adrenal glands and decreased cellularity of the thymic cortex are consistent with stress as a non-specific reaction rather than a direct effect of treatment.

Hypertrophy of the zona fasciculata was seen in the adrenal glands in the two surviving females at 5000 ppm and in 3/5 females at 1000 ppm.

In animals surviving to terminal sacrifice, effects of treatment with fluopyram were seen in the liver in both sexes and in the adrenal glands in females only. Hypertrophy of centrilobular hepatocytes was seen in the liver of both females at 5000 ppm, in all animals at 1000 ppm and in all males and 2/5 females at 150 ppm, with evidence of a dose-response. Single cell hepatocellular necrosis was seen in 1/2 females dosed at 5000 ppm and in all males dosed at 1000 ppm. Focal necrosis was noted in 2/2 females dosed at 5000 ppm and in 3/5 males and 2/5 females dosed at 1000 ppm. Hepatocellular eosinophilia and bile duct/oval cell hyperplasia were noted in the two surviving females at 5000 ppm and in 1/5 females at 1000 ppm.

Table 52: Incidence of treatment related lesions in the liver

Sex	**Males**				**Females**			
Dose level of fluopyram (ppm)	0	150	1000	5000	0	150	1000	5000
Number examined	5	5	5	0*	5	5	5	2*
Hypertrophy, hepatocellular, centrilobular								
Minimal	0	3	0	-	0	2	1	0
Slight	0	2	0	-	0	0	4	0
Moderate	0	0	5	-	0	0	0	2
Total	0	5	5	-	0	2	5	2
Necrosis, hepatocellular, single cell								
Minimal	0	0	5	-	0	0	0	1
Necrosis, focal								
Minimal	0	0	3	-	0	0	2	0
Slight	0	0	0	-	0	0	0	2
Total	0	0	3	-	0	0	2	2
Eosinophilia, hepatocellular								
Minimal	0	0	0	-	0	0	1	2
Hyperplasia, bile ducts/oval cells								
Minimal	0	0	0	-	0	0	1	2

* 0/2 survivals in males/females, respectively

Hypertrophy of centrilobular hepatocytes is associated with the proliferation of sub-cellular organelles, although their identity cannot be confirmed in hematoxylin and eosin sections. The change is predominantly centrilobular but appears to be more diffuse in a few animals dosed at 5000 ppm. This finding in isolation is associated with adaptive changes rather than demonstrating evidence of toxicity and is consistent with an increase in liver weights at necropsy. Necrosis and eosinophilia of hepatocytes

and focal necrosis represent toxicity and hyperplasia of bile ducts/oval cells was considered to have occurred as a reactive change adjacent to areas of necrosis.

Conclusion

Because there were probably treatment-related effects at all dosages in this range finding study in mice, a NOAEL could not be established. The top dose level of 5000 ppm clearly exceeded the Maximum Tolerated Dose (MTD) due to the overt toxicity.

Oral 90-day study in the mouse

Title:	Kennel, P. (2005): AE C656948 - 90-day toxicity study in the mouse by dietary administration, SA 04052, M-251136-01.
Guidelines:	OECD 408; Directive 2001/59/EC, Method B.26.
Deviations:	Opthalmoscopic examinations have not been conducted.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	PFI 0304
Purity:	99.0 %
Stability of test compound	Stable at 20 and 10000 ppm at room temperature for 105 days
Species:	Mouse
Strain:	C57BL/6J
Age / weight at dosing:	6 weeks approx.; Males: 18.1 g to 22.3 g; Females: 14.5 to 17.9 g at time of dosing
Source/breeder:	Charles River Laboratories, L'Arbresle, France
Acclimation period:	7 days
Identification:	Labeling by cage cards and water-insoluble spots on the tail
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions :	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: There were 10 animals of each sex per dose group. Animals were assigned to dose groups using a randomization procedure by weight. Fluopyram was administered in the diet for at least 90 days to C57BL/6J mice at the following doses – 0, 30, 150 and 1000 ppm (equating approximately to 0, 5.4, 26.6 and 188 mg/kg/day in males and 0, 6.8, 32.0 and 216 mg/kg/day in females). A negative control group received plain diet. Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and "Le Guide du

Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986".

Table 53: Study design

Test group	Concentration in diet (ppm) of fluopyram	Dose per animal (study averages)		Animals assigned	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Male	Female
1	0	0	0	10	10
2	30	5.4	6.8	10	10
3	150	26.6	32.0	10	10
4	1000	188	216	10	10

Diet preparation and analysis: Fluopyram was ground to a fine powder before being incorporated into the diet by dry mixing to provide the required concentrations. There were two preparations of each concentration during the study. The stability had been demonstrated in a previous study where diet samples of 20 and 10000 ppm were found to be stable over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature.

Statistics: Statistics were performed as described above (rat 28-d).

Observations: The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed once during the acclimatization phase and at least weekly during the treatment period.

Body weight, food consumption and compound intake: Body weights were recorded twice during the acclimatization phase, on the first day of test substance administration, then at weekly intervals throughout the treatment period and before necropsy. Food consumption was recorded weekly; the weekly mean achieved dosage intake for each week and for weeks 1 to 13 was calculated for each sex.

Clinical chemistry: On study days 91, 92 or 93, in the morning, prior to necropsy, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane. Blood was collected on lithium heparin for plasma chemistry determinations. Any significant change in the appearance of the plasma was recorded and the following clinical chemistry parameters were measured on a Hitachi 911 (Roche Diagnostics, Meylan, France) for: total bilirubin, total protein, albumin, total cholesterol and urea concentrations, and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities.

Urinalysis was not performed.

Sacrifice and pathology: On study days 91, 92 or 93, a complete necropsy was performed on all animals. Animals were deeply anesthetized by isoflurane inhalation, and then exsanguinated before necropsy. All animals were fasted prior to scheduled sacrifice. The following organs were weighed fresh at scheduled sacrifice only: adrenal gland, brain, heart, kidney, liver, spleen, testis, thymus and uterus (including cervix). The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, oesophagus, exorbital (lachrymal) gland, eye and optic nerve, gall bladder, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. A bone marrow smear was prepared from femur, stained with May-Grünwald Giemsa, but not examined. Tissues 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. Histopathological examinations were performed on all tissues from all the animals

in the control and high dose groups and all decedents in all groups. The liver, kidney, lung and thyroid gland were examined in all animals in the study. In addition, the adrenal gland was also examined in the intermediate dose groups as necessary to identify the no-effect-level, as it was identified as a possible target organ. Significant macroscopic findings were also examined in all dose groups.

Findings

Diet preparation analysis: Results of the diet preparation analysis are presented in the table below. The homogeneity of the diet was verified on the first preparation at 30 and 1000 ppm and was within a range of 86-100 % of the nominal concentrations. Concentration was checked for all dose levels for each preparation and was within 92-98 % of the nominal concentration. Hence all values were within the target range of 85-115 % of the nominal concentration.

Table 54: Homogeneity and concentration of test material

	Nominal concentrations (ppm)	Analysed concentrations (ppm)	% of nominal concentration
Homogeneity (first load)	30	H1 29.3 H3 29.3 M1 29.9 M3 29.4 B1 29.6 B3 28.8	98 98 100 98 99 96
	1000	H1 1003 H3 940 M1 964 M3 932 B1 856 B3 906	100 94 96 93 86 91
Concentration (first load)	30	29.4	98
	150	147	98
	1000	934	93
Concentration (second load)	30	27.7	92
	150	140	93
	1000	976	98

H = surface; M = middle, B = bottom of the preparation.

Clinical signs of toxicity: There were no treatment-related clinical signs of toxicity observed in animals at any dose level.

Mortality: No treatment-related mortality occurred in the test animals at any dose level. One male at 30 ppm was found dead on Day 30 after a body weight loss of 6.9 g between Days 15 and 29 and reduced food consumption between Days 9 and 29. Clinical signs recorded prior to death for this animal were reduced motor activity on Days 22 and 23, together with wasted appearance and hunched posture from Days 22 to 29. Spontaneous hydrocephalus observed at the macroscopic examination and confirmed microscopically was considered to be the cause of death for this animal. Hence, the death of this animal was attributable to a spontaneous lesion and was not treatment-related. A second male, from the control group, was killed for humane reasons on Day 69 after an accidental trauma.

Body weight and food consumption: The body weight evolution of animals was unaffected by treatment in any dose group. Food consumption was unaffected by treatment at 1000 ppm in females or at 150 or 30 ppm in either sex. The mean achieved dosage intake of fluopyram per group is presented in Table 55.

Table 55: Mean achieved dietary intake of fluopyram (Weeks 1 - 13)

Dietary level ppm	Males mg/kg/day	Females mg/kg/day
30	5.4	6.8
150	26.6	32.0
1000	188	216

Blood analysis: At 1000 ppm, mean alanine aminotransferase activity was higher in males and females (+205 and +109 % after removal of an outlier in the female control values, respectively, p≤0.01), compared to the control groups. In addition in males, mean alkaline phosphatase activity was higher (+21 %, p≤0.01), mean albumin and mean total cholesterol concentrations were lower (- 12 and -40 %, respectively, p≤0.01) and a tendency towards higher values was noted in aspartate aminotransferase activity (+46 %, p≤0.05). In females, a tendency towards lower mean albumin concentration was also noted (-9 %, p≤0.01). At 150 treatment-related changes consisted in a lower mean total cholesterol concentration in both sexes (-41 and -30 %, respectively, p≤0.01) and lower total protein and albumin concentrations in males (-6 % and – 7 %, p≤0.01), associated with lower total bilirubin in males and females (- 43 %, significant only in males) compared to the control group. At 30 ppm the only observed finding was a significant decrease in total cholesterol in males (-30 %, p≤0.01).

Organ weight: There was no relevant change in terminal body weight in either sex. Mean absolute and/or relative liver weights were statistically significantly higher at 1000 and 150 ppm in both sexes, with a dose-related effect. At 30 ppm, mean absolute liver weight and relative liver to body weight ratio were statistically significantly higher in females only, but this change was considered not to be toxicologically relevant since it was not associated with any histological change.

Table 56: Liver weight changes at terminal sacrifice (% change when compared to controls)

Dietary level (ppm)	0	30	150	1000
Males				
Mean absolute liver Weight (g)	0.91 ± 0.08	0.94 ± 0.07	1.04 ± 0.15 (+14 %)	1.24 ± 0.14** (+36 %)
Mean liver to body weight ratio	3.973 ± 0.312	4.064 ± 0.640	4.590 ± 0.147** (+16 %)	5.497 ± 0.688** (+38 %)
Females				
Mean absolute liver Weight (g)	0.75 ± 0.09	0.84 ± 0.06* (+13 %)	0.96 ± 0.08** (+28 %)	1.09 ± 0.07** (+45 %)
Mean liver to body weight ratio	4.102 ± 0.452	4.566 ± 0.294* (+11 %)	5.117 ± 0.302** (+25 %)	5.681 ± 0.298** (+38 %)

* = p≤0.05. **= p≤0.01

Mean absolute and relative adrenal gland weights were increased by between 87 % and 92 % at 1000 ppm in males compared to controls, the difference being statistically significant for mean adrenal gland to body weight ratio only (p≤0.05).

Gross and histopathology: At 1000 ppm, enlarged livers were observed in 8/10 males and 9/10 females, and dark livers were observed in 5/10 males and 10/10 females.

Microscopic pathology: Effects of treatment with fluopyram were seen in the liver and adrenal gland in both sexes. In the liver, there was a minimal to moderate hypertrophy of centrilobular hepatocytes in both sexes at 1000 and 150 ppm. This finding was considered to be the explanation for the enlarged livers observed at necropsy in animals given 1000 ppm and the statistically significant increase in liver weight seen in animals given 1000 or 150 ppm. In addition in the liver, there was a greater incidence of minimal or slight focal necrosis in both sexes at 1000 ppm, when compared to controls. Minimal or slight focal necrosis was present in 3/10 males and 6/10 females given 1000 ppm, compared to one female in the control group.

Table 57: Incidence and severity of treatment-related changes in the liver at terminal sacrifice

Sex	Males				Females			
Dietary level of fluopyram (ppm)	0	30	150	1000	0	30	150	1000
Number of animals examined	9	9	10	10	10	10	10	10
Centrilobular hepatocellular hypertrophy								
Minimal	0	0	3	0	0	0	5	1
Slight	0	0	7	0	0	0	0	8
Moderate	0	0	0	10	0	0	0	1
Total	0	0	10	10	0	0	5	10
Focal necrosis								
Minimal	0	0	0	3	1	1	0	3
Slight	0	0	0	0	0	0	0	3
Total	0	0	0	3	1	1	0	6

In the adrenal glands at 1000 ppm, there was a lower incidence of cortical ceroid pigment in males and a greater incidence of minimal to slight cortical vacuolation in females, compared to controls. The change noted in males at 1000 ppm was considered to be slight compared to the magnitude of increase in adrenal gland weights seen in this sex. No treatment-related changes were observed in the adrenal glands at 150 or 30 ppm in either sex.

Table 58: Incidence and severity of treatment-related changes in the adrenal gland at terminal sacrifice

Sex	Males				Females			
Dietary level of fluopyram (ppm)	0	30	150	1000	0	30	150	1000
Number of animals examined	9	9	10	10	10	10	10	10
Cortical ceroid pigment								
Minimal	5	3	4	0	0	0	0	0
Slight	1	1	0	0	0	0	0	0
Total	6	4	4	0	0	0	0	0
Cortical vacuolation								
Minimal	0	0	0	0	3	1	2	9
Slight	0	0	0	0	0	0	0	1
Total	0	0	0	0	3	1	2	10

In the fore-stomach of males given 1000 ppm, there was a slightly greater incidence of focal epithelial hyperplasia than in controls. However, as these changes in males were only focal and isolated, they were considered not to be treatment-related. In females, the incidence and severity of this finding were comparable between controls and treated animals.

Conclusion

A dose level of 30 ppm fluopyram by dietary administration to the C57BL/6J mouse for 90 days represented the No Observed Effect Level (NOEL) in males and females (equating to 5.4 and 6.8 mg/kg body weight/day, respectively) based on a higher organ weight and hypertrophy in the liver at the next higher dose level. Because these findings were rather considered adapative than toxic, this dose of 150 ppm (equating to 26.6 and 32.0 mg/kg bw/d in males and females respectively) was considered the NOAEL in this study. More pronounced liver effects (hypertrophy, necrosis, changes in related clinical chem. parameters) were observed at the next higher dose of 1000 ppm (ca 118 or 216 mg/kg bw/d for males and females, respectively).

Oral 28-day study in the dog

Title:	Kennel, P. (2004): AE C656948 - Preliminary 28-day toxicity study in the dog by gavage, SA 04049, M-242097-01.
Guidelines:	Not applicable, range finding study.
Deviations:	Not applicable.
GLP:	No.
Acceptability:	The study is considered to be acceptable as range finding study.

Materials and methods

Description:	AE C656948	
Lot/Batch number:	PFI 0304	
Purity:	99.0 %	
Appearance:	Beige powder	
Test material Stability:	Stable at 25 ± 5°C (room temperature)	
Species:	Dog	
Strain:	Beagle	
Age / weight at study initiation (Day 1):	Approximately 8-10 months old; 6.9 to 8.5 kg for males; 6.4 to 8.1 kg for females at the start of treatment	
Source:	Marshall farms (now Marshall BioResources), North Rose, New-York, USA.	
Housing:	Animals were housed individually in stainless steel kennels with a floor surface area of 1.2 m^2.	
	Supervised exercise in inside runs was permitted daily for dogs of the same sex and treatment group throughout acclimatization and treatment periods except on weekends and public holidays. At the end of working day, dogs were pair housed overnight by opening the partition between 2 animals of the same sex and dose group.	
Diet:	Certified canine meal 153C3 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France).	
	Each animal received approximately 300 g of diet daily approximately 2.5 hours after gavage administration for 1.5 hours.	
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.	
Environmental conditions (target ranges):	Temperature:	18-21 °C
	Humidity:	40-70 %
		Target of 10-15/hr
	Air changes:	12 hrs dark/ 12 hrs light (7 am - 7 pm)
	Photoperiod:	
Acclimat. period:	At least 21 days	

Animal assignment and treatment: Shortly after arrival, all dogs were examined by a veterinarian for signs of ill-health and were subjected to a detailed clinical examination to assess their physical and behavioral status. Body weight was recorded and food intake determined. All animals were subjected to an ophthalmologic examination, hematology and clinical chemistry investigations and urinalysis once during the acclimatization phase. Animals were assigned to dosage groups using a computerized randomization procedure in order to ensure a similar body weight distribution among groups of each sex. Test groups were as described inTable 59.

Table 59: Test groups and dose levels

Test Group (Group number)	**Sex : Number of animals**	**Dose levels (mg/kg bw/day)**
Control (1)	Male: 2 Female: 2	0
Low (2)	Male: 2 Female: 2	30
Mid (3)	Male: 2 Female: 2	150
High (4)	Male: 2 Female: 2	750

Treatment and dosage formulation: Fluopyram was administered orally by gavage at a dosage volume of 5 mL/kg bw for at least 28 days. The dosing formulations were prepared by suspending the test substance in aqueous solution of 0.4 % methylcellulose 400. Four preparations of 3 concentrations (6, 30 and 150 g/L) were prepared during the course of the study. Stability in solution, homogeneity and concentration were checked.

Statistics: Not performed due to the low number of animals per group. The results of body weight parameters, food consumption and clinical pathology parameters were compared individually with the respective pre-test values, each animal serving as its own control.

Observations: All study animals were observed at least twice daily for ill-health, moribundity, mortality and clinical signs of toxicity (except once daily on weekends and holidays). Daily examination of the kennels was also carried out for vomitus, diarrhea or blood.

Clinical examinations: Detailed clinical observations for clinical signs of toxicity were performed on all animals at treatment initiation (study day 0) and on a weekly basis thereafter. The physical examination included but was not necessarily restricted to the following examinations: fur and skin, eyes, ears, teeth, gum, mucous membranes, rectal temperature, gait, stance, general behavior, chest, including heart and respiratory rate, abdomen including palpitation, external genitalia and mammary glands.

Body weight and food consumption: Each animal was weighed at least weekly before the gavage administration during the acclimatization and treatment periods and prior to necropsy. Food intake was measured for a minimum of 5 consecutive days immediately before start of treatment and daily throughout the study.

Ophthalmic examination: During the acclimation period and at the end of treatment, ophthalmic examinations were conducted on all animals by means of an indirect ophthalmoscope after instillation of an atropinic agent (mydriaticum).

Hematology and clinical chemistry: Clinical chemistry and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance (day -9) and on day 27. Animals were diet fasted overnight prior to blood collection, which was drawn via jugular vein puncture.

Urinalysis: Urinalysis was performed on all animals twice prior to administration of the test substance (Day -14 and -8) and on all animals on study Day 28. Urine volume was collected overnight.

Sacrifice and pathology: On study Days 29 to 30, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of acepromazine (50 µL/kg body weight) and then deeply anesthetized by intravenous injection of pentobarbital (i.v. injection of 60 mg/kg body weight). Animals were then exsanguinated and necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Organ weight measurements were performed for adrenals, brain, epididymis, heart, kidney, liver, ovary, pituitary, prostate, spleen, testis, thymus, thyroid gland, and uterus.

For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Grünwald Giemsa, but not examined. Samples were fixed by immersion in neutral buffered 10 % formalin with the exception of the eye, optic nerve, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in all dose groups.

Findings

Test material analysis: Stability of fluopyram in aqueous methylcellulose suspensions was demonstrated at 0.0868 and 250 g/L for 33 days under storage conditions used in this study. The homogeneity analysis showed that test material had 99-103 % of nominal concentration (checked at 6 and 150 g/L). Concentration Analysis showed that 93-101 % of nominal concentration (checked at 30 g/L) was reached. Results were within the in-house target range of 90 to 110 % of nominal concentration and were therefore considered to be acceptable for use on the current study.

Clinical signs of toxicity: The only clinical sign observed at the high dose was liquid feces that were observed on a few occasions in 2 animals at the high dose. On 1 or 2 occasions only, liquid feces was also observed in 2/4 animals at 150 mg/kg/day and in 1/4 animals at 30 mg/kg/day. However, as liquid feces is occasionally observed in control animals and there was no evidence of a treatment-related increase in this finding over a 90-day period at a dose level of up to c. 330 mg/kg/day, this finding at 150 and 30 mg/kg/day was considered to be incidental. No abnormal findings were noted at the detailed physical examinations throughout the study. Rectal temperature was within the normal range.

Mortality: There were no mortalities throughout the study.

Body weight and food consumption: There was no treatment-related effect on body weights or body weight gains at any dose level in either sex. Mean food consumption was not affected throughout the study.

Ophthalmic examination: No treatment-related ocular abnormalities were observed at ophthalmic examination.

Hematological findings: The 2 males at 750 mg/kg/day showed a lower erythrocyte count, lower hemoglobin and a lower hematocrit values compared to their respective pre-test values. No change was observed in any other group.

Clinical chemistry findings: A high alkaline phosphatase activity and a low albumin concentration (and consequently a low albumin/globulin ratio) were observed in 1 male and 1 female at the high dose. This female also showed a high δ-glutamyltransferase activity and triglyceride concentration. No relevant changes were observed at 150 or 30 mg/kg/day.

Urinalysis: There were no treatment–related findings in either sex at any dose level.

Organ weight: Liver weights (absolute and relative) were clearly higher in both sexes at 750 mg/kg/day. This weight increase was associated with histopathological findings (see below). Liver weights were also slightly higher in both sexes at 150 mg/kg/day and in males at 30 mg/kg/day but were not associated with any histopathological findings. They were therefore considered as non adverse. There were no other organ weight changes that were considered to be treatment-related.

Gross and histopathology: At microscopic examination, minimal to slight centrilobular to panlobular hepatocellular hypertrophy and eosinophilic inclusion bodies were observed in most animals at 750 mg/kg/day. No other treatment-related changes were observed.

Table 60: Histopathological changes in the 28-day dog study with fluopyram

Dosage level (mg/kg/day)	**0**	**30**	**150**	**750**
Males				
Liver				
Centrilobular to panlobular hepatocellular hypertrophy: diffuse				
Minimal	0	0	0	1
Slight	0	0	0	1
Total	0/2	0/2	0/2	2/2
Eosinophilic inclusion bodies: focal/multifocal				
Minimal	0	0	0	1
Slight	0	0	0	0
Total	0/2	0/2	0/2	1/2
Females				
Liver				
Centrilobular to panlobular hepatocellular hypertrophy: diffuse				
Minimal	0	0	0	0
Slight	0	0	0	2
Total	0/2	0/2	0/2	2/2
Eosinophilic inclusion bodies: focal/multifocal				
Minimal	0	0	0	1
Slight	0	0	0	1
Total	0/2	0/2	0/2	2/2

Conclusion

The NOEL was found to be 150 mg/kg/day in both sexes. However, this was a non-guideline range finding study with a limited set of parameters investigated.

Oral 90-day toxicity study in dogs

Title: Kennel, P. (2006): AE C656948 - 90-day toxicity study in the dog by dietary administration, SA05046, M-276047-01.

Guidelines: OECD 409 (1998); E.E.C. Directive 2001/59/EC, Method B.27.

Deviations: None.

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Materials and methods

Description:	AE C656948
Lot/Batch number:	Mix-Batch:08528/0002
Purity:	Min. 94.6 %
Appearance:	Beige powder
Test material Stability:	Stable at 25 ± 5°C / room temperature
Species:	Dog
Strain:	Beagle
Age / weight at study initiation	Approximately 8 months old

(Day 1):	6.3 to 9.0 kg for males; 4.5 to 7.1 kg for females at the start of treatment
Source:	Marshall BioResources, North Rose, New-York, USA.
Housing:	Animals were housed individually in stainless steel kennels with a floor surface area of 1.2 m^2. When possible, they were pair housed overnight by temporary opening of the partitions between 2 dogs from the same sex and dose group. Supervised exercise in inside runs was permitted daily for dogs of the same sex and treatment group throughout the acclimatization and treatment periods except on weekends and public holidays. Additional supervised exercise was permitted in inside runs for an extended time of approximately 1 hour at least once per week for dogs of the same sex and treatment group.
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France) including 2% E.A.125 (S.A.F.E, diet enhancer). Three hundred grams of diet moistened with 450 ml of water at the time of distribution was given daily to each animal for approximately 1.5 hours each morning, except on study Day 337 when animals were fed in the afternoon due to a change in planning. In addition, due to a low food consumption observed on several consecutive days and/or to a marked body weight loss, a thin layer of commercialized dog food (Beef pâté from Pedigree®) was spread over the normal dietary ration of 2 animals (PT4M1291 and PT4F1295) to stimulate their appetite as described in the following table:

Animal	Study days	Beef pâté added to thediet
PT4M1291	16	200 g
	17 to 19	100g
PT4F1295	16	200 g
	17 to 21	100 g
	66 to 69	100g
	90	400g

Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.	
Environmental conditions (target ranges):	Temperature:	18-21 °C
	Humidity:	40-70 %
	Air changes:	Target of 15/hr
	Photoperiod:	12 hrs dark/ 12 hrs light (7 am - 7 pm)
Acclimatization:	20 days	

Animal assignment and treatment: Shortly after arrival, all dogs were examined by a veterinarian for signs of ill-health and were subjected to a detailed clinical examination to assess their physical and behavioral status. Body weight was recorded and food intake determined. All animals were subjected to an ophthalmological examination, hematology and clinical chemistry investigations and urinalysis once during the acclimatization phase. Animals were allocated to dosage groups using a randomization procedure in order to ensure a similar body weight distribution among groups of each sex. Test groups were as described in Table 61. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 61: Test groups, dose levels and achieved dosages

Test Group (Group number)	Sex : Number of animals	Concentration of fluopyram in Diet (ppm)	Achieved dose to animal (Weeks 1-13) (mg/kg/day)
Control (1)	Male: 4 Female: 4	0	0
Low (2)	Male: 4 Female: 4	800	Male: 28.5 Female: 32.9
Mid (3)	Male: 4 Female: 4	5000	Male: 171 Female: 184
High (4)	Male: 4 Female: 4	20000 (Day 1 to 14) 10000 (Day 15 to sacrifice)	Male: 332 Female : 337

Diet preparation and analysis: The appropriate amount (weight/weight concentration) of test substance was incorporated into the ground diet to provide the required dietary concentrations of 800, 5000 or 10000 or 20000 ppm. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. One preparation at 20000 ppm and three preparations at 800, 5000 and 10000 ppm were performed to provide the treated diet required for the study.

Homogeneity of the test substance in the diet was checked at 800, 10000 and 20000 ppm on the 1st formulation. Concentrations were checked for each dose levels and all preparations. Stability of the preparations under storage conditions was determined at 800 and 20000 ppm. In addition, stability in the moistened diet distributed to the dogs was determined for a period covering the time for food preparation and distribution.

Statistics: Continuous data were analyzed by the Bartlett's test for homogeneity of variances. When the data were homogeneous, an ANOVA was performed followed by Dunnett test (2-sided) on parameters showing a significant effect by ANOVA. When the data were not homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Dunn test (2-sided) to identify statistical significance between groups. For some parameters, when data were not homogenous, they were transformed (log transformation or square root transformation), then reanalyzed as above. For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis test which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance. When one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5 % and 1 % levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Cage side observations: All study animals were observed at least twice daily for ill-health, moribundity, mortality and clinical signs of toxicity (except once daily on weekends and holidays). This included but was not limited to changes in general behavior and appearance, skin and fur, teeth and gum, eyes, ears, mucous membranes. Any deviations from normal were recorded in respect to the nature and severity. Daily examination of the kennels was also carried out for vomitus, diarrhea or blood.

Clinical examinations: Clinical examinations were performed as described above (dog 28-day study).

Body weight: Body weights were recorded weekly throughout the treatment. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios.

Food consumption: Food intake was measured for a minimum of 4 consecutive days immediately before the start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded. The group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for Weeks 1 to 13.

Ophthalmic examination: Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals after instillation of an atropinic agent (mydriaticum). Ophthalmic examinations were also conducted on all animals just prior to termination of the study.

Hematology and clinical chemistry: Clinical chemistry and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance and on days 55-57 and 86-87. Animals were diet fasted overnight prior to blood collection, which was drawn via jugular vein puncture.

Urinalysis: Urinalysis was performed on all animals once prior to administration of the test substance and on all animals on study days 49-50 and 84. Urine volume was collected overnight.

Sacrifice and pathology: On study days 91 to 94, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of acepromazine (50 µl/kg body weight) and then deeply anesthetized by intravenous injection of pentobarbital (0.5 mL/kg body weight). Animals were then exsanguinated and necropsied. The necropsy included the examination of all major organs, tissues and body cavities according to guideline requirements. Macroscopic abnormalities were recorded, sampled and examined microscopically.

For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Grünwald Giemsa, but not examined. Samples were fixed by immersion in neutral buffered 10 % formalin with the exception of the eye, optic nerve, epididymis and testis that were fixed in Davidson's fixative. Histopathological examinations were performed on all tissues from all the animals in all dose groups. After the examination by the study pathologist, an independent review by a second pathologist was conducted. The results presented in the report are a consensus view of the 2 pathologists.

Findings

Diet preparation analysis: Homogeneity analysis showed that 87-115 % of the nominal concentration was reached and concentration analysis showed that 89-109 % of the nominal concentration was reached. Results were within the in-house target range of 85 to 115 % of nominal concentration and were therefore considered to be acceptable for use on the current study. In the stability analysis, fluopyram was found to be stable in the diet at 800 and 20000 ppm over a period that covered the usage and storage conditions used in this study and for at least 4 additional hours, which covered the time of food preparation and distribution.

Clinical signs of toxicity: The only clinical sign observed at the high dose was a wasted appearance noted for 1 male and 2 females. This sign was observed in correlation with a reduced food consumption attributed to a lack of palatability of fluopyram at this dietary level. Animals at 5000 and 800 ppm had no treatment-related clinical signs. There was no treatment-related change in rectal temperature.

Mortality: There were no mortalities throughout the study.

Body weight and body weight gain: At 20000 ppm, there was a mean body weight loss noted in both sexes during the first 2 weeks. This was observed together with a reduced food consumption in animals of these groups which was attributed to a lack of palatability of the test substance in the diet. Thereafter, the dietary level was reduced from 20000 ppm to 10000 ppm, however, mean body weight gain

remained reduced in comparison to controls on most occasions. Overall, a body weight loss of 0.8 kg and 1.1 kg was noted in males and females, respectively compared to a body weight gain of 1.0 kg in controls which leaded to an 18 % and 29 % reduction in final body weight for males and females, respectively compared to controls. At 5000 ppm, mean absolute weight gains were slightly reduced in both sexes compared to controls. Overall, body weight gains of 0.6 kg and 0.2 kg were noted for males and females, respectively compared to 1.0 kg in both male and female control groups. This corresponded to 7 % and 11 % reduction in final body weight in males and females respectively, compared to controls. At 800 ppm, body weight parameters were comparable to controls in males while there was a slight reduction in body weight gain in females. At the end of the study, mean female body weight was 8 % lower than controls. In all cases, effects on body weight parameters were concomitant with a lower food consumption of comparable magnitude due to a lack of palatability of the test substance in the diet.

Table 62: Body weight and weight gains (kg) in the 90-day dog study with fluopyram

Dosage level (ppm)	**0**	**800**	**5000**	**20000/10000**
Males				
Initial BW (%C)	7.4 ± 0.8	7.4 ± 0.4 (100 %)	7.2 ± 0.7 (97 %)	7.7 ± 0.3 (104 %)
BWG Week 1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	-0.3 ± 0.1**
BWG Weeks 1-4	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.3	-0.7 ± 0.4**
BWG Weeks 1-8	0.9 ± 0.2	0.8 ± 0.4	0.4 ± 0.6	-0.8 ± 0.4**
BWG Weeks 1-13	1.0 ± 0.3	1.0 ± 0.7	0.6 ± 0.8	- 0.8 ± 0.5**
Final BW (%C)	8.4 ± 0.8	8.4 ± 0.7 (100 %)	7.8 ± 1.1 (93 %)	6.9 ± 1.0 (82 %)
Females				
Initial BW (%C)	5.5 ± 0.6	5.6 ± 1.1 (102 %)	5.6 ± 0.6 (102 %)	5.7 ± 0.7 (104 %)
BWG Week 1	0.3 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	-0.4 ± 0.2**
BWG Weeks 1-4	0.7± 0.1	0.5 ± 0.3	0.1 ± 0.4	-0.8 ± 0.3**
BWG Weeks 1-8	0.8 ± 0.2	0.4 ± 0.4	0.0 ± 0.5	-1.1 ± 0.6**
BWG Weeks 1-13	1.0 ± 0.3	0.4 ± 0.5	0.2 ± 0.5	-1.1 ± 0.5**
Final BW (%C)	6.5 ± 0.6	6.0 ± 1.2 (92 %)	5.8 ± 0.8 (89 %)	4.6 ± 0.7 (71 %)

*: p≤0.05; **: p≤0.01; BWG: body weight gain; (%) = % versus control

Food consumption: At 20000 ppm, there was a marked reduction of food consumption in both sexes (-25 to 53 % and - 53 to 58 % in males and females, respectively) during the first 2 weeks of treatment compared to controls. Thereafter, although the dietary level of fluopyram was reduced to 10000 ppm, the food consumption was still reduced by 9-38 % in males and 28-53 % in females. To a lesser extent the food consumption was also reduced at 5000 ppm in both sexes (overall, 7 % and 22 % reduction in males and females, respectively compared to controls). In addition there was still a slightly reduced food consumption in females at the low dose (-9 % overall in comparison to controls). The decrease in food consumption was attributed to a lack of palatability of the test substance when incorporated into the diet. Hence, lower dietary concentrations were associated with better food intake and the use of commercialized dog food moistened with the diet to improve the palatability of the diet and to stimulate appetite of the dogs resulted in an improved food intake.

Table 63: Food consumption (g/day) in the 90-day dog study with fluopyram

Dosage level (ppm)	0	800	5000	20000/10000
Males				
Week 1 (%C)	641	671 (105 %)	548 (85 %)	478* (75 %)
Weeks 1-13 (%C)	701	722 (103 %)	653 (93 %)	528 (75 %)
Females				
Week 1 (%C)	638	524 (82 %)	490 (77 %)	299** (47 %)
Weeks 1-13 (%C)	676	612 (91 %)	527 (78 %)	368 (54 %)

*: $p \leq 0.05$; **: $p \leq 0.01$; (%) = % versus control.

Ophthalmic examination: No treatment-related ocular abnormalities were observed at ophthalmic examination.

Hematological findings: Changes in haematology parameters were limited to the high dose group where higher mean platelet counts were noted throughout the study, although the effect was less pronounced at the end of the study (Week 13) than at week 8. In addition, lower erythrocyte counts, haemoglobin concentrations and haematocrit values were observed in females.

Table 64: Haematology results in the 90-day dog study with fluopyram

Dosage level (ppm)	0	800	5000	20000/10000	0	800	5000	20000/10000
Males	Week 8				Week 13			
Platelet count (10^9/L)	361 ± 77 -	381 ± 46 (+6 %)	396 ± 71 (+10 %)	548 ± 151 (+52 %)	379 ± 78 -	406 ± 32 (+7 %)	390 ± 84 (+3 %)	491 ± 103 (+30 %)
Females	Week 8				Week 13			
Platelet count (10^9/L)	370 ± 70 -	435 ± 47 (+18 %)	415 ± 164 (+12 %)	577 ± 233 (+56 %)	411 ± 82 -	470 ± 57 (+14 %)	492 ± 100 (+20 %)	570 ± 183 (+39 %)
Red blood cell count (10^{12}/L)	7.16 ± 0.57 -	7.06 ± 0.35 (-1 %)	6.93 ± 0.53 (-3 %)	6.45 ± 0.65 (-10 %)	7.27 ± 0.73 -	6.89 ± 0.50 (-5 %)	6.70 ± 0.47 (-8 %)	6.49 ± 0.82 (-11 %)
Haemoglobin concentration (g/dL)	16.9 ± 0.9 -	16.6 ± 1.0 (-2 %)	15.8 ± 0.9 (-7 %)	14.8 ± 1.3 (-12 %)	16.5 ± 1.4 -	15.8 ± 1.2 (-4 %)	15.0 ± 0.8 (-9 %.)	14.7 ± 2.0 (-11 %)
Haematocrit (l/L)	0.480 ± 0.028 -	0.476 ± 0.031 (-1 %)	0.456 ± 0.020 (-5 %)	0.429 ± 0.038 (-11 %)	0.479 ± 0.035 -	0.457 ± 0.041 (-5 %)	0.436 ± 0.023 (-9 %)	0.431 ± 0.061 (-10 %)

(%) = % variation versus control

Clinical chemistry findings: At 20000/10000 ppm, higher alkaline phosphatase and δ-glutamyl transferase activities were noted in both sexes, whereas aspartate and alanine aminotransferase activities were higher in males only when compared to controls. In addition, lower total bilirubin, mean albumin (and as a consequence albumin/globulin ratio) and mean total protein were observed in both sexes. To a lesser extent, the same parameters were also affected at 5000 ppm. No adverse effects were noted at 800 ppm in either sex.

Table 65: Changes in clinical chemistry in the 90-day dog study with fluopyram

Dosage level (ppm)	**0**	**800**	**5000**	**20000/ 10000**	**0**	**800**	**5000**	**20000/ 10000**
Males	Week 8				Week 13			
Alkaline phosphatase (IU/l)	97 ± 16 -	129 ± 15 (+33 %)	325 ± 150* (+235%)	461 ± 309** (+375 %)	93 ± 22 -	139 ± 15 (+49 %)	372 ± 172* (+300 %)	555 ± 420** (+497 %)
γ-glutamyl transferase (IU/l)	1 ± 1 -	2 ± 0 (+100 %)	3 ± 1 (+200%)	10 ± 7** (+900 %)	2 ± 1 -	3 ± 1 (+50 %)	3 ± 1 (+50 %)	13 ± 8** (+550 %)
Aspartate-amino-transferase (IU/l)	28 ± 5 -	25 ± 8 (-11 %)	25 ± 4 (-11 %)	49 ± 23 (+75 %)	28 ± 4	31 ± 7 (+11 %)	26 ± 5 (-7 %)	43 ± 20 (+54 %)
Alanine-amino-transferase (IU/l)	32 ± 12 -	27 ± 4 (-16 %)	34 ± 12 (+6 %)	115 ± 98 (+259 %)	31 ± 8 -	28 ± 6 (-10 %)	38 ± 15 (+23 %)	215 ± 189 (+594 %)
Total bilirubin (µmol/l)	4.4 ± 1.0 -	2.7 ± 0.4** (-39 %)	2.0 ± 0.3** (-55 %)	1.9 ± 0.5** (-57 %)	4.5 ± 1.0 -	3.1 ± 0.6 (-31 %)	2.2 ± 0.9* (-51 %)	2.8 ± 1.4 (-38 %)
Albumin (g/l)	36 ± 2 -	34 ± 1 (-6 %)	30 ± 3* (-17 %)	29 ± 3** (-19 %)	36 ± 1 -	33 ± 1 (-8 %)	28 ± 3** (-22 %)	27 ± 4** (-25 %)
Total protein (g/l)	57 ± 4 -	55 ± 1 (-4 %)	54 ± 3 (-5 %)	52 ± 3 (-9 %)	59 ± 4 -	55 ± 2 (-7 %)	53 ± 4 (-10 %)	52 ± 1* (-12 %)
Albumin/globulin ratio	1.72 ± 0.16 -	1.61 ± 0.08 (-6 %)	1.20 ± 0.16 ** (-30 %)	1.23 ± 0.22 ** (-28 %)	1.64 ± 0.16 -	1.45 ± 0.04 (-12 %)	1.09 ± 0.12** (-34 %)	1.11 ± 0.25** (-32 %)
Females	Week 8				Week 13			
Alkaline phosphatase (IU/l)	111 ± 21 -	190 ± 43 (+71 %)	299 ± 161 (+169%)	375 ± 73** (+238 %)	111 ± 28 -	190 ± 72 (+71 %)	355 ± 208 (+220 %)	383 ± 85* (+245 %)
γ-glutamyl transferase (IU/l)	2 ± 1 -	2 ± 1 (0 %)	4 ± 3 (+100%)	10 ± 5* (+400 %)	2 ± 2 -	3 ± 1 (+50 %)	5 ± 3 (+150 %)	14 ± 12** (+600 %)
Total bilurubin (µmol/l)	3.4 ± 0.7 -	2.8 ± 0.7 (-18 %)	1.9 ± 0.3* (-44 %)	2.2 ± 0.5* (-35 %)	3.3 ± 0.9 -	3.2 ± 1.1 (-3 %)	2.1 ± 0.6 (-36 %)	2.0 ± 0.9 (-39 %)
Albumin (g/l)	36 ± 2 -	36 ± 1 (0 %)	30 ± 1** (-17 %)	30 ± 2** (-17 %)	36 ±1 -	37 ± 1 (+3 %)	29 ± 1** (-19 %)	28 ± 2** (-22 %)
Total protein (g/l)	57 ± 2 -	58 ± 3 (+2 %)	53 ± 3 (-7 %)	51 ± 4* (-11 %)	58 ± 1 -	59 ± 2 (+2 %)	53 ± 3** (-9 %)	50 ± 1** (-14 %)
Albumin/globulin ratio	1.67 ± 0.28 -	1.71 ± 0.21 (+2 %)	1.32 ± 0.10 (-21 %)	1.41 ± 0.14 (-16 %)	1.61± 0.17 -	1.63 ± 0.27 (+1 %)	1.24 ± 0.04 * (-23 %)	1.28 ± 0.20 (-20 %)

*: p≤0.05; **: p≤0.01; (%) = % versus control

Urinalysis: There were no treatment–related findings in either sex at any dose level.

Organ weight: Absolute and relative liver weights were higher in both sexes at 20000/10000 ppm and 5000 ppm compared to controls. In addition in females, absolute and relative thymus weights were lower in comparison to control. These changes were associated with gross and histopathological changes (see below).

Table 66: Organ weight changes in the 90-day dog study with fluopyram

Dosage level (ppm)	0	800	5000	20000/10000
Males				
Liver weight				
Absolute (g)	280.9 ± 31.6	351.9 ± 20.4 (+25 %)	448.0 ± 67.4** (+59 %)	410.3 ± 60.9** (+46 %)
Body weight-relative	3.32 ± 0.38	4.18 ± 0.50* (+26 %)	5.61 ± 0.37** (+69 %)	5.91 ± 0.11** (+78 %)
Females				
Liver weight				
Absolute (g)	228.7 ± 15.8	295.8 ± 26.7 (+29 %)	308.2 ± 41.2* (+35 %)	307.8 ± 55.1* (+35 %)
Body weight-relative	3.55 ± 0.24	4.95 ± 0.69 (+39 %)	5.30 ± 1.11* (+49 %)	6.57 ± 0.90** (+85 %)
Thymus weight				
Absolute (g)	8.32 ± 2.40	6.13 ± 1.31 (-26 %)	6.97 ± 2.69 (-16 %)	2.38 ± 0.59** (-71 %)
Body weight-relative	0.128 ± 0.034	0.102 ± 0.018 (-20 %)	0.115 ± 0.034 (-10 %)	0.050 ± 0.008** (-61 %)

*: p≤0.05; **: p≤0.01; %C: % versus control.

Gross and histopathology: At the macroscopic examination, enlarged liver was noted in both sexes in 2/4 and 1/4 animals at the high and mid dose, respectively and generalized atrophy of thymus was noted in all females at the high dose. At the microscopic examination, minimal to slight hepatocellular hypertrophy and intracytoplasmic eosinophilic droplets were observed in all animals of both sexes at both 20000/10000 and 5000 ppm. In addition at these dose levels, hepatocellular single cell necrosis was observed in males. In the thymus, a slightly higher severity of thymic involution was observed in both sexes compared to controls. In addition in females at the high dose, disturbance of the estrous cycle was observed as only anestrus phase was seen. However, these 2 latter effects (thymus and estrous cycle) were attributed to a secondary effect linked to the decrease in food consumption and bodyweight or may have been incidental.

Table 67: Histopathological changes in the 90-day dog study with fluopyram (male)

Dosage level (ppm)	**0**	**800**	**5000**	**20000/10000**
Males				
Liver				
Hepatocellular hypertrophy: diffuse				
Minimal	0	0	3	1
Slight	0	0	1	3
Total	0/4	0/4	4/4	4/4
Intracytoplasmic eosinophilic droplets: multifocal				
Minimal	0	1	1	3
Slight	0	0	2	1
Moderate	0	0	0	0
Total	0/4	1/4	3/4	4/4
Hepatocellular single cell necrosis: focal/multifocal				
Minimal	0	0	2	3
Moderate	0	0	0	0
Total	0/4	0/4	2/4	3/4
Thymus				
Decreased size of cortex: involution				
Minimal	4	1	1	0
Slight	0	2	3	2
Moderate	0	0	0	1
Marked	0	1	0	1
Total	4	4	4	4

Table 68: Histopathological changes in the 90-day dog study with fluopyram (female)

Dosage level (ppm)	0	800	5000	20000/10000
Females				
Liver				
Hepatocellular hypertrophy: diffuse				
Minimal	0	0	3	4
Slight	0	0	1	0
Total	0/4	0/4	4/4	4/4
Intracytoplasmic eosinophilic droplets: multifocal				
Minimal	0	1	1	2
Slight	0	0	2	0
Moderate	0	0	1	0
Total	0/4	1/4	4/4	2/4
Hepatocellular single cell necrosis: focal/multifocal				
Minimal	0	0	0	0
Moderate	0	0	1	0
Total	0/4	0/4	1/4	0/4
Thymus				
Decreased size of cortex: involution				
Minimal	4	3	2	0
Slight	0	0	2	1
Moderate	0	0	0	2
Marked	0	0	0	0
Total	4/4	3/4	4/4	3/4
Estrus phases in the uterus				
Proestrus	1	1	4	0
Early metestrus	1	2	0	0
Midmetestrus	1	0	0	0
Anestrus	1	1	0	4

Conclusion

The NOAEL for this study was considered to be 800 ppm for both sexes (equivalent to 28.5 and 32.9 mg/kg bw/day for males and females, respectively) based on findings in the liver (increased absolute and relative liver weights, related histopathology findings like necrosis and related clinical chemistry like increased AP and GGT) in the next higher dose of 5000 ppm.

Oral 1-year toxicity study in dogs

Title:	Kennel, P. (2007): AE C656948 - Chronic toxicity study in the dog by dietary administration, SA 05047, M-294279-01.
Guidelines:	OECD 452 (1981); EEC Directive 88/302, Method B.30 (1992).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Description:	AE C656948
Lot/Batch number:	Mix-Batch08528/0002
Purity:	Min. 94.6 %
Appearance:	Beige powder
Test material Stability:	Stable at 25 ± 5°C / room temperature
Species:	Dog
Strain:	Beagle
Age / weight at study initiation (Day 1):	Approximately 8 months old 7.0 to 9.4 kg for males; 5.2 to 7.7 kg for females at the start of treatment
Source:	Marshall BioResources, North Rose, New-York, USA.
Housing:	Animals were housed individually in stainless steel kennels with a floor surface area of 1.2 m^2. When possible, they were pair housed overnight by temporary opening of the partitions between 2 dogs from the same sex and dose group. Supervised exercise in inside runs was permitted daily for dogs of the same sex and treatment group throughout the acclimatization and treatment periods except on weekends and public holidays. Additional supervised exercise was permitted in inside runs for an extended time of approximately 1 hour at least once per week for dogs of the same sex and treatment group.
Diet:	Certified canine meal 125C3-P1 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France). Three hundred grams of diet moistened with 450 ml of water at the time of distribution was given daily to each animal for approximately 1.5 hours each morning, except on study Day 337 when animals were fed in the afternoon due to a change in planning. Supplementary food ration: due to a decrease in body weight of between 0.8 to 1.3 kg during the study for animals PT1M6617 (control group), PT3M6632 (mid dose group), PT1F6620 and PT1F6621 (control group), despite the fact these animals ate their entire daily food ration, a supplementary untreated pelleted diet ration (153C3 from S.A.F.E.) was given to these animals starting on Day 149,

	130, 199 and 109, respectively, until study termination. Approximately 100 g per day of untreated pelleted diet was distributed to each dog after the measurement of its empty feeder of the dietary mixture, providing that the dog had eaten approximately its entire ration. The weight of the supplementary food supplied and that remaining was recorded on the day of distribution or the day after (supplemental food ration was left overnight where necessary on weekends and holidays). Data were kept in the study file.	
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.	
Environmental conditions (target ranges):	Temperature:	18-21 °C
	Humidity:	40-70 %
	Air changes:	Target of 15/hr
	Photoperiod:	12 hrs dark/ 12 hrs light (7 am - 7 pm)
Acclimat. period:	33 days	

Animal assignment and treatment: Shortly after arrival, all dogs were examined by a veterinarian for signs of ill-health and were subjected to a detailed clinical examination to assess their physical and behavioral status. During the acclimatization phase, animals were checked twice daily for moribundity and mortality. Clinical signs were recorded daily and a detailed physical examination was performed approximately weekly. All animals were weighed at least weekly before food distribution and food intake was measured for a minimum of 5 consecutive days before start of treatment. All animals were subjected to an ophthalmologic examination, hematology and clinical chemistry investigations and urinalysis once during the acclimatization phase. Animals were selected and randomized for use in the study on the basis of acceptable findings from physical and ophthalmologic examinations, body weight, food consumption, clinical chemistry, hematology analyses and urinalysis. Animals were allocated to dosage groups in order to ensure a similar body weight distribution among groups of each sex, whilst ensuring full siblings were not placed in the same treatment group.

Animals were randomly assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 69: Test groups, dose levels and achieved dosages

Test Group (Group number)	Sex : Number of animals	Concentration of fluopyram in Diet (ppm)	Achieved dose in animals (Weeks 1-52) (mg/kg/day)
Control (1)	Male: 4 Female: 4	0	0
Low (2)	Male: 4 Female: 4	100	Male: 3.0 Female: 3.8
Mid (3)	Male: 4 Female: 4	400	Male: 13.2 Female: 14.4
High (4)	Male: 4 Female: 4	2000	Male: 67.6 Female: 66.1

Diet preparation and analysis: The test substance formulations were prepared to cover the dietary requirements over approximately 6-week periods. The appropriate amount (weight/weight concentration) of test substance was incorporated into the ground diet to provide the required dietary concentrations of 100, 400 or 2000 ppm. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. Nine preparations at each dose level were performed to provide the treated diet required for the study. The stability of the test substance in the diet supplemented with 2 % of diet enhancer (E.A. 125 from S.A.F.E.) was determined in a previous study at concentrations of 800 and 20000 ppm for a period which covers the period of storage and usage for the current study. The stability of the test substance at the lowest and highest dosage levels was determined during the current study in the moistened diet distributed to the dogs after a 55 day period of storage of dry diet at room temperature.

The homogeneity of the test substance in the diet was verified before the start of the study for all concentrations on the first preparation from the first formulation to demonstrate adequate formulation procedures. In addition, the homogeneity of the test substance in diet was verified at the lowest and highest dietary levels on the first preparation from the fifth formulation. The mean values obtained from the homogeneity check were taken as measured concentration. The dietary level of the test substance in the diet was verified for all concentrations on all preparations from the first, third and fifth formulations and on the first preparation from the seventh formulation.

Statistics: Statistics were performed as described in the 90-day oral toxicity study (dog) above.

Cage side observations: All study animals were observed at least twice daily for clinical signs of toxicity (except once daily on weekends and holidays). This included but was not limited to changes in general behavior and appearance, skin and fur, teeth and gum, eyes, ears, mucous membranes. Any deviations from normal were recorded in respect to the nature and severity. Daily examination of the kennels was also carried out for vomitus, diarrhea or blood.

Clinical examinations: Clinical examinations were performed as described above (28-day toxicity study in the dog)

Body weight: Body weights were recorded weekly throughout the treatment. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios.

Food consumption: Food intake was measured for a minimum of five consecutive days immediately before start of treatment. The weight of food supplied to each animal and that remaining were recorded daily throughout the treatment period. From these records, the mean weekly consumption was calculated for each dog. Food spillage was also recorded. The group mean achieved dosage for each sex, expressed as mg/kg body weight/day, was calculated for each week and the overall mean subsequently derived for weeks 1 to 52.

Ophthalmic examination: Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals just prior to termination of the study.

Hematology and clinical chemistry: Clinical chemistry and complete blood count, including differentials, were performed on all animals once prior to administration of the test substance and during weeks 12, 23 and 51. Animals were diet fasted overnight prior to blood collection, which was drawn via jugular vein puncture. The following hematology parameters were assayed: Hematocrit, hemoglobin concentration, mean corpuscular hemoglobin concentration, MCH, MCV, red- and white blood cell count, platelet count, leukocyte differential count, reticulocyte count and % reticulocytes, activated partial thromboplastin time and prothrombin time.

The following clinical chemistry parameters were measured: Alkaline phosphatase, alanine- and aspartate aminotransferases, gamma glutamyltransferase, creatinine, albumin, total protein, total bilirubin, total cholesterol, triglycerides, glucose, urea and electrolytes (calcium, potassium, sodium, chloride, inorganic phosphorus).

Urinalysis: Urinalysis was performed on all animals once prior to administration of the test substance and on all animals during study weeks 14, 24 and 52. Urine volume was collected overnight. Parameters measured were: Appearance, volume, pH, sediment (microscopic), protein, glucose, ketones, bilirubin, urobilinogen, specific gravity/osmolality and blood (red blood cells).

Sacrifice and pathology: On study Days 365 to 368, all animals from all groups were sacrificed. All animals were tranquilized by intramuscular injection of acepromazine (50 µl/kg body weight) and then deeply anesthetized by intravenous injection of pentobarbital (60 mg/kg body weight). Animals were then exsanguinated and necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically. Organs or tissues were sampled and/or weighed at necropsy as described in the 90 day dog study except for gallbladder. In addition seminal vesicles, oviduct, lacrimal exorbital gland, Harderian gland and nasal cavities were collected. For sacrificed animals, a bone marrow smear was prepared from one rib and stained with May-Grünwald Giemsa, but not examined. 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. Histopathological examinations were performed on all tissues from all the animals in all dose groups.

Findings

Diet preparation analysis: In the homogeneity analysis diet was found to have 93-108 % of the nominal concentration. In the concentration analysis diet was found to have 93-107 % of nominal concentration. Results were within the in-house target range of 85 % to 115 % of nominal concentration and were therefore considered to be acceptable for use on the current study. In the stability analysis fluopyram was found to be stable in the diet at 100 and 2000 ppm over a 55-day day period of storage at room temperature and then moistened for 4 hours, which covered the time of food preparation and distribution.

Observations: No treatment-related clinical signs were noted in either sex. There were no mortalities throughout the study.

Body weight and body weight gain: At 2000 ppm during the 1st week of treatment, there was a mean body weight loss of 0.2 and 0.1 kg in males and females, respectively compared to controls. Thereafter body weight gains were comparable to controls in both sexes. The initial decrease in body weight gain corroborated with a lower food consumption in both sexes over this period. At 400 and 100 ppm, body weight and weight gain were not affected by the treatment in either sex.

Table 70: Body weight and weight gains in the 1-year dog study with fluopyram

Dosage level (ppm)	0	100	400	2000
Males				
Initial BW (%C)	8.3 ± 0.6	8.1 ± 0.6 (98 %)	8.2 ± 0.9 (99 %)	8.2 ± 0.3 (99 %)
BWG Week 1	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.1	-0.2 ± 0.2
BWG Weeks 1-52	0.4 ± 1.0	2.3 ± 1.3	1.3 ± 1.8	0.2 ± 1.1
Final BW (%C)	8.7 ± 0.8	10.4 ± 1.7 (120 %)	9.6 ± 1.3 (110 %)	8.5 ± 0.8 (98 %)
Females				
Initial BW (%C)	6.9 ± 0.6	6.6 ± 0.9 (96 %)	6.8 ± 0.8 (99 %)	6.7 ± 0.4 (97 %)
BWG Week 1	0.0 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	-0.1 ± 0.2
BWG Weeks 1-52	1.1 ± 0.6	1.4 ± 0.9	1.2 ± 0.6	1.0 ± 0.2
Final BW (%C)	8.0 ± 1.0	8.0 ± 1.3 (100 %)	8.1 ± 1.4 (101 %)	7.6 ± 0.3 (95 %)

BWG: body weight gain; (%C) = % versus control

Food consumption: During the 1st week of treatment, there was a 30 % and 24 % reduction in food consumption at 2000 ppm in males and females, respectively. This initial effect was most likely due to

lack of palatability of the test compound as already observed in previous study in dogs. Overall, food consumption was comparable to control in males whereas this parameter remained slightly lower in female throughout the study resulting in an overall 10 % reduction.

Table 71: Food consumption in the 1-year dog study with fluopyram

Dosage level (ppm)	0	100	400	2000
Males				
Week 1 (Days 1-8) (%C)	709	648 (91 %)	656 (93 %)	498 (70 %)
Weeks 1-52 (Days 1-364) (%C)	740	730 (99 %)	745 (101 %)	720 (97 %)
Females				
Week 1 (Days 1-8) (%C)	645	636 (99 %)	625 (97 %)	493 (76 %)
Weeks 1-52 (Days 1-364) (%C)	688	703 (102 %)	693 (101 %)	620 (90 %)

(%C) = % versus control.

Ophthalmic Examination: No treatment-related ocular abnormalities were observed at ophthalmic examination.

Hematological findings: There were no treatment-related changes in either sex at any dose level.

Clinical chemistry findings: The only consistent change throughout the study concerned the alkaline phosphatase activity which was at least doubled at 2000 ppm compared to controls in both sexes. Values were also higher than the pre-test values. The slightly elevated ALP activity seen in males at 400 ppm was judged not to be related to treatment since this elevation was mainly due to one animal only and since there was no other finding at this dose. No effect was seen at 100 ppm.

Table 72: Alkaline phosphatase activity (IU/L) in the 1-year dog study with fluopyram

Dosage level (ppm)	0	100	400	2000
Males				
Pre-test	111 ± 19	142 ± 35	119 ± 16	119 ± 16
Month 3	120 ± 51 (+8 %)	107 ± 17 (-25 %)	162 ± 41 (+36 %)	256 ± 108* (+115 %)
Month 6	154 ± 121 (+39 %)	108 ± 31 (-24 %)	215 ± 122 (+81 %)	341 ± 135 (+187 %)
Month 12	117 ± 46 (+5 %)	81 ± 30 (-43 %)	176 ± 67 (+48 %)	299 ± 166 (+151 %)
Females				
Pre-test	121 ± 18	134 ± 41	135 ± 34	145 ± 51
Month 3	119 ± 27 (-2 %)	123 ± 30 (-8 %)	129 ± 41 (-4 %)	229 ± 62** (+58 %)
Month 6	147 ± 49 (+21 %)	134 ± 37 (0 %)	134 ± 24 (-1 %)	334 ± 123 (+130 %)
Month 12	140 ± 43 (+16 %)	135 ± 72 (+1 %)	161 ± 61 (+19 %)	285 ± 126 (+97 %)

(%) change compared to pre-test value. *: p≤0.05; ** p≤0.01

Urinalysis: There were no treatment–related findings in either sex at any dose level.

Organ weight: The only change in organ weight was a higher mean absolute and brain relative thyroid gland weight in females at 2000 ppm. However this change was considered not to be adverse since there was no associated histopathological effect.

Gross and histopathology: No treatment-related changes were observed at the macroscopic examination. At the microscopic examination, the liver was characterized by a minimal diffuse centrilobular hepatocellular hypertrophy in 3/4 males at 2000 ppm. This was the only treatment-related finding. A minimal diffuse hypertrophy of the follicular cells was observed in the thyroid gland of 2/4 and 1/4 males at 2000 and 100 ppm, respectively. In the absence of a dose relationship this minor change was judged to be incidental.

Table 73: Histopathological changes in the 1-year dog study with fluopyram

Dosage level (ppm)	0	100	400	2000
Males				
Liver : centrilobular hepatocellular hypertrophy (minimal, diffuse)	0/4	0/4	0/4	3/4
Thyroid gland : Follicular epithelium hypertrophy (minimal, diffuse)	0/4	1/4	0/4	2/4

Conclusion

The NOAEL for this study was 400 ppm for both sexes (equivalent to 13.2 and 14.4 mg/kg bw/day for males and females, respectively) based on liver effects (increased alkaline phosphatase activities and liver cell hypertrophy) at the next higher dose of 2000 ppm. Because the NOAEL of 800 ppm as obtained in the 90-day study was well below the LOAEL in this 1-yr study and because both studies were, in general, comparable with regard to test animals, experimental design and the observed changes, it appears reasonable to establish an overall NOAEL for subchronic toxicity in that species. Thus, 800 ppm (corresponding to a mean daily intake of 28.5 and 32.9 mg/kg bw by males and females, respectively) is considered an overall NOAEL.

4.7.1.2 Repeated dose toxicity: inhalation

No data submitted by the notifier.

4.7.1.3 Repeated dose toxicity: dermal

Title:	Eigenberg, D. A. (2007): A subacute dermal toxicity study in rats with technical grade AE C656948, 201617, M-293833-01.
Guidelines:	In compliance with OECD 410.
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Test material:	AE C656948	
Description:	Beige Powder	
Lot/Batch #:	Mix-batch:08528/0002	
Purity (% ai):	94.7	
Stability:	Stable at room temperature	
Vehicle:	For treated animals gauze pads were moistened with deionized water and the test substance was then applied to the gauze. The control animals were treated with gauze pads moistened with deionized water.	
Test animals:	Rat	
Strain:	Wistar Hanover CRL: WI [GLX/BRL/HAN] IGS BR (nulliparous and nonpregnant)	
Age / weight at treatment initiation:	11 weeks / males: 281.3-351.0 g & females: 203.1-237.6 g	
Source:	Charles River Laboratories, Incorporated, Raleigh, North Carolina, U.S.A.	
Housing:	Individually housed in stainless steel cages.	
Diet:	PMI Certified Rodent Diet 5002 in "meal" form provided continuously for *ad libitum* consumption, except when fasted prior to bleeding.	
Water:	Tap water provided continuously for *ad libitum* consumption. The water was sampled monthly by the KCMO Water Department and was periodically sampled and analyzed by a Continental Analytical Services, Inc., Salina, KS for a variety of potential impurities (e.g., aflatoxins, chlorinated hydrocarbons, heavy metals, etc.).	
Environmental conditions:	Temperature:	Daily average of 18 to 26°C (64 to 79°F).
	Humidity:	Daily average of 30 to 70 %.
	Air changes:	Average of at least 12.7 changes per hour.
	Photoperiod:	Approximately 12 hours of light alternating with 12 hours of darkness, except when lights were turned off for eye exams.
Acclimation period:	7 days prior to release for the study.	

Animal assignment: The rats were randomly assigned to dose groups, based on weight, using INSTEM DATATOX®. Weight variation of animals used were targeted not to exceed ±20 % of the mean weight for each sex.

Table 74: Study Design

Dose Group (mg/kg/day)	No. Animals/ Dose/Sex
0 (control)	10
100	10
300	10
1000	10

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. Frequency data that were examined statistically were evaluated using the Chi-Square and/or Fisher's Exact tests.

Preparation of animals: On study day -3, the hair was removed from the dorsal and lateral areas of the trunk of each rat using electric clippers. During the dosing period, the animals were shaved, as necessary, due to hair growth.

Application of the test substance: Individual doses of the test substance were weighed out for each animal and applied to a commercially-available adhesive bandage (2 in. x 4 in.; dose area 2 in. x 2 in.) that was moistened before dose application with 1 ml of deionized water. The bandage was placed on the shaved skin of the rat and the torso of the animal was then wrapped with porous tape to assure that the bandage remained in contact with the skin during the dosing interval. The same procedure was performed for control animals, except that only a bandage moistened with 1 mL of deionized water was applied to the dose site. The test substance/bandage 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 sacrificed (rats were not dosed on the day of sacrifice). Each day, the bandage and tape were removed and the dose site was wiped with deionized water-dampened and dry gauze to remove as much test substance residue as feasible without damaging the skin.

Observations: All animals were observed at least twice daily (AM and PM) for clinical signs of toxicity, except once daily on weekends. Findings were recorded when first observed and then at the beginning of each week thereafter.

Body weight and food consumption: Individual body weights were measured weekly throughout the study. Body weights were also taken immediately prior to necropsy to allow for calculation of organ-to-body weight ratios. Food consumption was measured weekly during the study.

Ophthalmologic examination: Following the acclimation period and prior to initiation of dosing, ophthalmic examinations were conducted on all animals. Ophthalmic examinations were also conducted on all animals prior to termination of the study.

Hematology & clinical chemistry: Clinical chemistry and a hematology, including differentials, were performed on all animals (days 28 and 29; prior to being euthanized). Animals were fasted overnight prior to the collection of blood from the orbital sinus of all rats, under anesthesia with Isoflurane. The following hematology parameters were measured: Hematocrit, hemoglobin concentration, mean corpuscular hemoglobin concentration (MCHC), MCH, MCV, red blood cell distribution width (RDW), hemoglobin distribution width (HDW), blood cell morphology, red- and white blood cell count, platelet count, leukocyte differential count, reticulocyte count, thromboplastin time and prothrombin time.

The following clinical chemistry parameters were measured: Alkaline phosphatase, alanine- and aspartate aminotransferases, gamma glutamyltransferase, creatinine kinase, lactic acid dehydrogenase, creatinine, albumin, globulin, A/G ratio, total protein, total bilirubin, total cholesterol, triglycerides, glucose, urea-nitrogen, uric acid and electrolytes (calcium, potassium, sodium, chloride, inorganic phosphorus).

Sacrifice and pathology: Animals were euthanized at the end of the study by asphyxiation with CO_2. A complete gross examination was performed on all animals. The necropsy consisted of a systematic gross examination of each animal's general physical condition, body orifices, external and internal organs and tissues. A list of the tissues collected and weighed at necropsy is presented in the table below (marked with an x). All tissues were processed, embedded in paraffin, sectioned, mounted, and stained with hematoxylin and eosin (H&E) for examination under the light microscope. Histopathologic evaluation was conducted on all protocol-required tissues from the control and high-dose group animals and from the liver of low- and mid-dose group males and females. Where appropriate, all findings were assigned a severity score of normal, 1 = minimal, 2 = mild or slight, 3 = moderate, and 4 = marked. The mean severity was determined by dividing the sum of the individual animal severity scores by the number of tissues examined in the group. At necropsy, organs were weighed and tissues collected for subsequent histopathological examination. An overview on the organs/tissues examined is given as follows.

Table 75: Overview on the organs/tissues

	DIGESTIVE SYSTEM		**CARDIOVASC./HEMAT.**		**GLANDULAR**
X	Cecum*	X	Aorta*	X	Adrenal gland*+#
X	Colon*	X	Bone marrow*	X	Exorbital/lacrimal gland@
X	Duodenum*	X	Heart*+#	X	Thyroid (with parathyroid)*
X	Esophagus*	X	Lymph node, mesenteric*		**NEUROLOGIC**
X	Ileum*	X	Lymph node, cervical*	X	Brain*+#
X	Jejunum*	X	Spleen*#	X	Cerebellum
X	Liver*+#	X	Thymus*+#	X	Cerebrum-Midbrain
X	Pancreas*		**UROGENITAL**	X	Medulla/Pons
X	Rectum*	X	Harderian gland@	X	Eyes*
X	Salivary glands*	X	Cervix@	X	Nerve, optic*
X	Stomach, glandular *	X	Clitoral gland@	X	Nerve, sciatic*
X	Stomach, non-glandular*	X	Epididymides*+#	X	Pituitary*
X	Tongue@	X	Kidneys*+#	X	Spinal cord, cervical*
X	Tooth	X	Mammary gland*	X	Spinal cord, thoracic*
		X	Ovary*+#	X	Spinal cord, lumbar*
	RESPIRATORY	X	Preputial gland@		**OTHER**
X	Larynx*	X	Prostate*	X	Bone, femur@
X	Lung*	X	Testicle*+#	X	Bone, sternum
X	Nasal structure*	X	Urinary bladder*	X	Gross lesions*
X	Nasopharynx*	X	Uterus*+#	X	Muscle, protocol@
X	Oral structure	X	Vagina@	X	Physical Identifier
X	Trachea*	X	Seminal vesicles*	X	Skin, treated*
		X	Zymbal's gland@	X	Skin, untreated*

* Recommended for 28-day dermal toxicity studies based on Guideline 870.3200. +Organ weight required in a 21/28-Day Dermal Study. #Organ weighed. @No histopathology performed

Findings

Observations: There were no compound-related clinical observations for males and females at any dose level. No animals were found dead or sacrificed *in-extremis* during the study.

Body weight and food consumption: There was no compound-related effect on body weight for males and females at any dose level. There was no compound-related effect on food consumption.

Ophthalmologic examination: There were no compound-related ophthalmic findings at any dose level.

Hematology: The only hematology parameter which was affected by compound administration was a statistically significant increase in prothrombin time for high-dose group males as compared to concurrent controls. In addition there was a statistically significant increase in large unstained cells (LUC) in high-dose group females as compared to controls. This was considered not to be compound-related by the notifier since it was within Bayer laboratory's historical control range.

Clinical chemistry: The only clinical chemistry parameter which was affected by compound administration was a statistically significant increase in total cholesterol values for high-dose group females as compared to the concurrent controls. There was also a decrease in potassium (K) values in high-dose group females as compared to controls. This was considered not to be treatment-related for the following reasons: they were not dose dependent and the decreased values were within Bayer laboratory's historical control range.

Organ weight: Terminal body weights were not affected by compound administration in males and females at any dose level. Statistically significant increases in 1000 mg/kg/day male liver weights (relative) and 1000 mg/kg/day female liver weights (absolute and relative) were considered to be compound-related. These findings were further substantiated by the presence of a minimal to mild degree of hypertrophy in the liver in this dose group. A statistically significant increase in mean kidney weights (relative) in 1000 mg/kg/day males and a statistically significant increase in mean liver weights (absolute and relative) in 100 mg/kg/day females were considered not to be compound-related for one or more of the following reasons: this difference from concurrent controls was not dose dependent.

Gross pathology: There were no compound-related gross pathology findings at any dose level.

Microscopic pathology: In the liver, statistically significant increases in the incidence of hypertrophy (centrilobular and mid-zonal) in 1000 mg/kg/day males and females were considered to be compound-related. However, hypertrophy was not observed in livers of 300 and 100 mg/kg/day males and females. The liver hypertrophy is probably attributed to hepatic enzyme induction, as hepatic enzyme induction was observed in a 28-day dietary rat study with this compound. All other microscopic observations were considered to be incidental and/or background and not related to compound administration.

Conclusion

Compound-related findings were only observed in the high-dose group and consisted in an increased cholesterol concentration in females, an increased prothrombin time in males and effect in the liver (increased liver weights for males and females associated with hepatic hypertrophy). Based on these findings, the No-Observed-Adverse Effect Level (NOAEL) was 300 mg/kg/day, for systemic effects. The NOAEL for local effects was 1000 mg/kg/day, as there were no local effects observed up to the highest dose level.

4.7.1.4 Repeated dose toxicity: other routes

No data submitted by the notifier.

4.7.1.5 Human information

Medical surveillance on manufactoring plant personnel did not reveal any signs of diseases or health impairments caused by fluopyram. For further details see 4.12.1.4 Human information.

4.7.1.6 Other relevant information

No data submitted by the notifier.

4.7.1.7 Summary and discussion of repeated dose toxicity

The short-term toxicity of the active ingredient fluopyram was investigated in subacute and subchronic oral studies in rats, mice, and dogs. In addition, a subacute study in rats using the dermal route is available. The short-term studies and their results are summarised in Table 76.

Table 76: Summary table of relevant repeated dose toxicity studies

Species, duration and route, dose levels; reference	NOAEL		LOAEL		Findings
	ppm (M/F)	mg/kg bw/d (M/F)	ppm (M/F)	mg/kg bw/d (M/F)	
Rat, 28-day, oral (dietary), 0, 50, 400, 3200 ppm; Kennel, 2004, ASB2008-5503	Not established: range finding study		400	31.0 / 36.1	↓ body weight gain (M/F); ↑ platelet count and prothrombin time (M); ↑ cholesterol and triglycerides conc. (M/F), ↓ aspartate aminotransferase and alkaline phosphatase , induction of microsomal liver enzymes;↑ liver weight, hepatocellular hypertrophy (M/F); ↑ thyroid weight with follicular cell hypertrophy (M); ↑ kidney weight and hyaline droplet nephropathy
Mouse, 28-day, oral (dietary), 0, 150, 1000, 5000 ppm; Kennel, 2004, ASB2008-5506	Not established: range finding study		150	24.7/31.1	Mortality at 5000 ppm (M/F), ↓ body weight gain (M); ↑ ALT (M); ↑ liver weight (M/F) associated with centrilobular hypertrophy, single cell and focal necrosis, hepatocellular eosinophilia and bile duct/oval cell hyperplasia (M/F); adrenal glands: hypertrophy of the *Zona fasciculata* (F),
Dog, 28-day, oral (gavage), 0, 30, 150, 750 mg/kg bw/d; Kennel, 2004, ASB2008-5507	Not established: range finding study		-	750	↓ erythrocyte count, hemoglobin, hematocrit (M); ↑ alkaline phosphatase (M/F), ↓ albumin conc. (M/F), ↑ δ-glutamyltransferase activity and triglyceride concentration (F); ↑ liver weight associated with hepatocellular hypertrophy (M/F)
Rat, 90-day, oral (dietary) with one month recovery in satellite groups, 0, 50, 200, 1000, 3200 ppm; Kennel, 2005, ASB2008-5548	200 (NOEL: 50 ppm)	12.5 / 14.6	1000	60.5 / 70.1	↓ body weight & food consumption; various hematological parameters affected; ↓ cholesterol conc & δ-glutamyltransferase activity, ↑ TSH, T3 & T4; ↑ organ weights of liver, thyroid gland & kidney, histological lesions in liver (centrilobular hypertrophy & periportal to midzonal hepatocellular macrovacuolation), thyroid (follicular cell hypertrophy) and kidney (hyaline droplets); effects only partly reversible after 1-mo recovery of rats receiving the highest dose
Mouse, 90-day, oral (dietary), 0, 30, 150, 1000 ppm; Kennel, 2005, ASB2008-5549	150 (NOEL: 30 ppm)	26.6 / 32.0	1000	188 / 216	↑ ALT & ↓ albumin conc. (M/F), ↓ cholesterol conc & ↑ alkaline phosphatase (M); ↑ liver weight (M/F), centrilobular hypertrophy and focal live cell necrosis (M/F); ↑ adrenal weight (M), ↓ ceroid pigment (M), ↑ cortical vacuolation (F) in adrenal glands

Dog, 90-day, oral (dietary), 0, 800, 5000, 20000/10000 ppm (highest dose reduced after 2 wk); Kennel, 2006, ASB2008-5550	800	28.5 / 32.9	5000	171 / 184	↓ body weight & food consumption; hematological parameters affected at high doses; ↑ alkaline phosphatase δ-glutamyltransferase activity, ALT & AST, ↓ albumin conc and bilirubin; ↑ liver weight associated with hepatocellular hypertrophy, intracytoplasmic eosinophilic droplets and single cell necrosis (M/F); ↓ thymus weight and cortex involution; estrous cycle disturbed
Dog, 1 yr, oral (dietary), 0, 100, 400, 2000 ppm; Kennel, 2007, ASB2008-5363	400	13.2/ 14.4	2000	67.6 / 66.1	↓ body weight & food consumption (M/F); ↑ alkaline phosphatase (M/F); centrilobular hypertrophy of liver cells; follicular epithelial hypertrophy in the thyroid (M, equivocal effect)
Rat, 28-day, dermal (6 h/d; 5 d/wk), 0, 100, 300, 1000 mg/kg bw/day: Eigenberg, 2007, ASB2008-5551	-	300 (syste-mic), 1000 (local)	-	1000 (systemic), > 1000 (local)	↑ cholesterol (F), ↑ prothrombin time (M); ↑ liver weights associated with hepatocellular hypertrophy

Body weight and food consumption were compromised in nearly all feeding studies at higher dose levels whereas further clinical signs and mortality occurred only in the 28-day study in mice at the top dose level of 5000 ppm after two to four weeks of treatment. In rats, body weight gain was severely affected at the beginning of treatment but returned to normal values with ongoing duration of the 90-day study. Palatability problems were noted in dogs.

The liver proved to be the main target organ in all three species. Hepatotoxicity became apparent by a dose-related increase in organ weight, alterations of clinical chemical parameters and histopathological findings such as hypertrophy or vacuolation. In addition, there was evidence for induction of microsomal liver enzymes as indicated by a dose-related increase in total cytochrome P-450, BROD and PROD activities in the 28-day feeding study in rats at the two upper dose levels of 400 and 3200 ppm. Liver effects were also observed following dermal administration of the limit dose of 1000 mg/kg bw/day to rats. Another common target organ was the blood with changes in red blood cell parameters suggesting slight anaemia whereas an extended prothrombin time and an increase in platelet count in the oral and dermal studies in rats are most likely related to liver toxicity.

The pathological changes in kidneys (organ weight increase associated with hyaline droplet nephropathy) were confined to the studies in rats. The nephropathy in male rats was characterised by basophilic tubules, hyaline droplets in the proximal tubule and granular cast in the medulla and was not reversible after one month of recovery. It was assumed to be due to accumulation of $\alpha_{2\mu}$-globulin in the proximal tubules, *i.e.*, a toxic mechanism that is rat-specific and of no relevance to man. In contrast, the mechanism causing thyroid changes (higher organ weight, follicular cell hypertrophy) in rats is not that clear because they were accompanied by an increase in TSH, T3 and occasionally also T4 levels. Usually, the rodent-specific mode of action of many chemicals is by enhanced catabolism of T3 and T4 (*i.e.*, lower plasma/serum levels) due to induced microsomal liver enzymes that, via a feed back mechanism, results in an increase in TSH production with subsequent stimulation and proliferation of the thyroid. A similar pathological finding in male dogs (diffuse follicular epithelial hypertrophy) was observed only in the one-year study and not after 90 days yet. The dose response was not convincing but because the highest incidence was seen at the top dose level, a treatment-related effect cannot be completely excluded.

Adrenal glands were affected only in mice and these findings (organ weight increase, decrease in ceroid pigmentation, hypertrophy of the *Zona fasciculata* and cortical vacuolation) were confined to the high dose levels.

Two effects specific to dogs receiving the highest dose in the 90-day study, *i.e.*, thymic involution and estrous cycle disturbance were attributed to the body weight loss at this dose level.

Liver and, in rats, kidney pathology turned out the most sensitive endpoints on which, in most studies, the NOAELs are based. In the dog, it was possible to establish an overall NOAEL of 800 ppm (28.5 mg/kg bw/d) for both the 90 day and the 1 year study.

In general, the adverse effects of fluopyram were more pronounced in rodents than in dogs. If the NOAELs and LOAELs in the different studies are taken into consideration, they were in the same magnitude for rats and mice but higher in the dog studies suggesting that this latter species might be less vulnerable. The lowest relevant NOAEL that should be used for human risk assessment was established at 12.5 mg/kg bw/day and was obtained in the 90-day feeding study in rats. It is based on liver and kidney effects at the next higher dose level of about 60.5 mg/kg bw/day.

4.7.1.8 Summary and discussion of repeated dose toxicity findings relevant for classification according to DSD

Potentially relevant organ effects were observed in short term toxicity studies in livers, kidneys (rats), thyroid (rats and dogs) and adrenals (mice).

Liver effects were observed in rats, dogs and mice and consisted of changes in organ weight, clinico-chemical parameters and histopathology. Effects were significant, adverse and occurred at dose levels of 24.7 mg/kg bw/d and above in the 28-d studies and at 60.5 mg/kg bw/d and above in the 90-d studies.

Kidney effects occurred in male rats only and were correlated to a mechanism of toxicity specific for male rats. They were assumed to be due to accumulation of $\alpha_{2\mu}$-globulin in the proximal tubules and of no relevance to man.

In contrast, the mechanism causing thyroid changes (higher organ weight, follicular cell hypertrophy) in rats is not that clear because these changes were accompanied by an increase in TSH, T3 and occasionally also T4 levels. Usually, the rodent-specific mode of action of many chemicals is by enhanced catabolism of T3 and T4 (*i.e.*, lower plasma/serum levels) due to induced microsomal liver enzymes that, via a feed back mechanism, results in an increase in TSH production with subsequent stimulation and proliferation of the thyroid. A similar pathological finding in male dogs (diffuse follicular epithelial hypertrophy) was observed only in the one-year study and not after 90 days yet. The dose response was not convincing but because the highest incidence was seen at the top dose level, a treatment-related effect cannot be completely excluded. Effects were significant and occurred at dose levels of 60.5 mg/kg bw/d and above. However, since mechanistic data showed non-relevance of long term thyroid effects to humans (see section 4.10), non-relevance of these effects is assumed for short-term toxicity too.

Adrenal effects consisted in a weight increase at a dose level of 188 mg/kg bw/d. This weight change was accompanied by a histopathological change in females only. However, even if these effects were significant, they occurred at rather high dose levels.

4.7.1.9 Comparison with criteria of repeated dose toxicity findings relevant for classification according to DSD

Comparison to criteria is presented in Table 77 below.

4.7.1.10 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification according to DSD

Since DSD requires ‘serious’ effects to classify a substance for “danger of serious damage to health by prolonged exposure”, these criteria are not met.

According to the criteria in Dir. 67/548, based on the results of the short-term oral toxicity studies fluopyram does not have to be classified as “harmfull” or “toxic” and the indication of “danger of serious damage to health by prolonged exposure” accordingly.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

4.8.1 Summary and discussion of repeated dose toxicity findings relevant for classification as STOT RE according to CLP Regulation

Significant liver effects were seen in rats, mice and dogs at moderate exposure concentrations after repeated dosing and are considered relevant for potential classification for STOT-RE. Kidney effects (seen in male rats only) are regarded as rat specific and not relevant to humans. For thyroid effects, even if evidence is less convincing, a rodent specific mechanism is assumed as well (see section 4.10). Significant adrenal effects (seen in mice) were confined to high doses and were therefore not regarded as relevant for a potential classification for STOT-RE.

Comparison with criteria of repeated dose toxicity findings relevant for classification as STOT RE

Table 77: Toxicological results in comparison with criteria of specific target organ toxicity – repeated exposure

Toxicological result	DSD criteria	CLP criteria
Liver: Rat, 90-d: Wt ↑, pathology (enlargement), histopathology (hypertrophy, vacuolation) and clinical chemistry (cholesterol, bilirubin, gGT, AP); LOAEL: 60.5 mg/kg bw/d Mouse, 90-d: Wt ↑, clin chem. (ALT↑, albumin ↓, cholesterol ↓, alkaline phosphatase (M) ↑) histo-pathology (centrilob. hypertrophy and focal live cell necrosis); NOAEL 26.5, LOAEL 188 (large dose spacing) Dog, 90-d: Wt ↑, clin chem. (alkaline phosphatase ↑ δ-glutamyltransferase activity ↑, ALT ↑, AST ↑, albumin ↓, bilirubin ↓); histopathology (hepatocellular hypertrophy, intra-cytoplasmic eosinophilic droplets, single cell necrosis); NOAEL: 28.5 mg/kg bw/d, LOAEL: 171 mg/kg bw/d (large dose spacing) 28-d range finding studies support these observations, but were considered supplementary. LOAEL for liver effects: 28-d rat: 31 mg/kg bw/d 28-d mouse: 24.7 mg/kg bw/d 28-d dog: 750 mg/kg bw/d	<u>Danger of serious damage to health by prolonged exposure</u> Serious damage (clear functional disturbance or morphological change which has toxicological significance) is likely to be caused by repeated or prolonged exposure by an appropriate route. Guide values can apply when severe lesions have been observed: Oral, rat: <u>Threshold for "harmful"</u> 28-day: ≤ 150 mg/kg bw/d 90-day: ≤ 50 mg/kg bw/d <u>Threshold for "toxic"</u> Substances and preparations are classified at least as toxic when these effects are observed at levels of one order of magnitude lower (i.e. 10-fold) than those set out above (for harmful)	<u>Category 1</u>: Substances that have produced significant toxicity in humans or that, on the basis of evidence from studies in experimental animals, can be presumed to have the potential to produce significant toxicity in humans following repeated exposure. Substances are classified in Category 1 for target organ toxicity (repeat exposure) on the basis of: reliable and good quality evidence from human cases or epidemiological studies; or observations from appropriate studies in experimental animals in which significant and/or severe toxic effects, of relevance to human health, were produced at generally low exposure concentrations. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 30 mg/kg bw/d 90-day: ≤ 10 mg/kg bw/d
		<u>Category 2</u>: Substances that, on the basis of evidence from studies in experimental animals can be presumed to have the potential to be harmful to human health following repeated exposure. Substances are classified in category 2 for target organ toxicity (repeat exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were produced at generally moderate exposure concentrations.

		Guidance dose/concentration values are provided below (see 3.9.2.9) in order to help in classification. In exceptional cases human evidence can also be used to place a substance in Category 2. Equivalent guidance values for 28-day and 90-day studies: Oral, rat: 28-day: ≤ 300 mg/kg bw/d 90-day: ≤ 100 mg/kg bw/d

4.8.2 Conclusions on classification and labelling of repeated dose toxicity findings relevant for classification as STOT RE

The short-term oral toxicity of fluopyram does not meet the CLP criteria, since significant liver effects occur at moderate exposure concentrations but are not regarded as being severe enough for classification.

According to the criteria in Reg. 1272/2008, based on the results of the short-term oral toxicity studies fluopyram does not warrant classification for "specific target organ toxicity after repeated exposure".

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

Summary of the Dossier submitter's proposal

A number of repeated dose studies were presented in the dossier.

Repeated dose oral studies

Oral exposure 28- and 90-day studies were performed in dogs, rats and mice. Additionally, a 1-year study in dogs was summarised in the dossier.

Rat:

In both rat studies (28 and 90 days exposure), treatment-related effects were found in the liver, kidneys and thyroid glands, characterised mainly as organ weight changes and hypertrophy (liver and thyroid), as well as hepatic enzyme induction. Hyaline droplet nephropathy was seen in males in both studies.

In the 28-day study fluopyram was administered in the diet at 0, 50, 400 or 3200 ppm (0, 4.0/4.6, 31.0/36.1, 254/263 mg/kg bw for m/f). A dose-related slight increase in total cytochrome P-450, benzyloxyresorufin O-dealkylation (BROD) and O-dealkylation of 7-pentoxyresorufin (PROD) activities, indicative of CYP2B activity, was observed in both sexes.

In the 90-day study fluopyram was administered in the diet at 0, 50, 200, 1000 or 3200 ppm (0, 3.06/3.63, 12.5/14.6, 60.5/70.1 and 204/230 mg/kg bw for m/f). A dose-dependent increase in T3, T4 and TSH was found in both sexes as well as a higher mean prothrombin time at the highest dose in males. After a 1 month recovery period there was partial reversibility, although some parameters remained statistically significantly different from controls.

In the 90-day study treatment-related effects at 200 ppm (adaptive liver weight increase due to minimal hepatocellular hypertrophy in 2/10 males, associated with non-significant changes in clinical chemistry parameters) were seen but these were not considered adverse. Centrilobular hepatocellular hypertrophy was found in all male rats and 7/10 and 10/10 females animals at 1000 ppm and 3200 ppm, respectively, with dose-related increases in severity associated with significant changes in blood biochemistry. Additionally, periportal to midzonal hepatocellular macrovacuolation was observed in 6/10 and 5/10 females at the mid and high dose,

respectively.
Therefore, the NOAEL was set at 200 ppm for both males and females (12.5/14.6 mg/kg bw/d), based on pronounced organ weight gain, clinical chemistry and histopathological findings in liver, thyroid and kidneys at the next higher dose level of 1000 ppm (60.5/70.1 mg/kg bw/d).

Mouse:
In the 28-day study in mice fluopyram was administered at 0, 150, 1000 or 5000 ppm (0, 24.7/31.1, 162/197, 747/954 mg/kg bw for m/f) in the diet. In those animals surviving to terminal sacrifice (3/5 females in the highest dose group were killed prior to terminal sacrifice for humane reasons), treatment-related effects were seen in the liver (organ weight changes, hypertrophy, single cell necrosis) and as hypertrophy in the adrenal glands, but only in females. The top dose level of 5000 ppm (747/954 mg/kg bw/d) clearly exceeded the MTD due to the overt toxicity.

Also in the 90-day study pronounced liver effects and changes in related clinical chemical parameters were observed. Liver weights were dose-relatedly increased at 150 and 1000 ppm (0, 5.4/6.8, 26.6/32.0, 188/216 mg/kg bw for m/f) (at the high dose in 5/10 males and 10/10 females) due to minimal to moderate hepatocellular hypertrophy in both sexes. Additionally, there was an increase in slight focal necrosis in 3/10 males and 6/10 females at 1000 ppm compared to the controls. Effects of treatment seen in the adrenal gland were increased organ weights (males) and slight cortical vacuolation in females. The NOAEL was set at 150 ppm for males and females (26.6/32.0 mg/kg bw/d) since the slight liver effects occurring at this dose were considered adaptive rather than toxic. The LOAEL was set at 1000 ppm for males and females (118/216 mg/kg bw/d).

Dog:
One 28-day (0, 30, 150 or 750 mg/kg bw/d), one 90-day (0, 800, 5000 or 10000/20000 ppm coresponding to 0, 28.5/32.9, 171/184 or 332/37 mg/kg bw for m/f) and one 1-year (0, 100, 400 or 2000 ppm coresponding to 0, 3.0/3.8, 13.2/14.4 or 67.6/66.1 mg/kg bw for m/f) study were summarized in the CLH report. In all three studies the main target organ was the liver.
Increases in liver weight (28-day and 90-day study) and hepatocellular hypertrophy (seen as an adverse effect starting at 750 mg/kg bw/d in the 28-day study and in 4/4 males and females at 5000 and 20000/10000 ppm in the 90-day study) was reported in combination with related clinical chemistry changes such as increased alkaline phosphatase (AP) (seen in the 90-day and 1-year studies). In the 90-day study, hepatocellular single cell necrosis was also observed in 2/4 males and one of four females at 5000 ppm and 3/4 males at the highest dose.

Thymic involution and estrous cycle disturbance observed in the 90-day study were attributed to the body weight loss observed.

The NOAEL of 800 ppm as determined in the 90-day study was well below the LOAEL from the 1-year study. Due to the similarity of both studies with regard to test animals, experimental design and the observed changes, one overall NOAEL for subchronic toxicity in the dog of 800 ppm (28.5/32.9 mg/kg bw/d for males and females, respectively) was derived. The LOAEL was set at 2000 ppm for males and females (67.6/66.1 mg/kg bw/d) from the 1-year study.

Repeated dose dermal studies
One 28-day study performed in rats was summarised in the CLH report.
The animals were treated for a minimum of 6h/d, 5d/week with 0, 100, 300 or 1000 mg/kg bw/d. Substance related findings consisted of an increased prothrombin time (males) and cholesterol concentration (females). Additionally, increased liver weights associated with hepatic hypertrophy were observed in both sexes. Based on these findings, the systemic NOAEL was 300 mg/kg bw/d and the NOAEL for local effects was 1000 mg/kg bw/d, which was identical to the systemic LOAEL.

Overall conclusion

Significant liver effects were seen in rats, mice and dogs at moderate exposures after repeated dosing. These effects were not regarded by the DS to be severe enough for classification. Kidney effects (seen in male rats only) were regarded as rat specific and not relevant to humans. Also, for the described thyroid effects a rodent specific mechanism is assumed. Significant adrenal effects (seen in mice) were confined to high doses and were not regarded as relevant for a potential classification for STOT RE.

Comments received during public consultation

No comments were received for this hazard class.

Assessment and comparison with the classification criteria

Potentially relevant effects were observed in repeated dose toxicity studies in liver, kidneys (rats only), thyroid (rats only) and adrenals (mice only). The thyroid and kidney effects are not considered to be of relevance to humans, due to a rodent specific mechanism, as discussed further in the carcinogenicity section. Substance related effects in the adrenal gland (cortical vacuolation) were seen in the mouse 90-day study (females only) and appeared only at 1000 ppm (216 mg/kg bw/d). Adrenal gland effects in other species were either not severe or appeared at concentrations above the guidance value in the CLP Regulation (for 28 day studies: ≤ 300 mg/kg bw/d). Therefore, findings in this organ are not considered relevant for classification. However, in the rat, macroscopic liver changes combined with statistically significant liver weight increases and microscopically minimal to slight hepatocellular effects were observed at doses relevant for classification. All effects showed a high tendency towards reversibility after a 1 month recovery period, although not all effects had fully reverted. In the dog, slight liver changes were seen at relevant doses. However, neither the effects in rats nor in dogs can be regarded as significant toxic effects. Therefore classification is not warranted.

4.9 Germ cell mutagenicity (Mutagenicity)

4.9.1 Non-human information

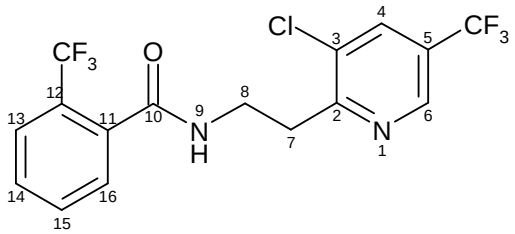
4.9.1.1 In vitro data

Gene mutation in bacterial cells

Title:	Wirnitzer, U. (2006): AE C656948 - Salmonella/microsome test plate incorporation and preincubation method, AT02911, M-269978-01.
Guidelines:	OECD 471 (1997).
Deviations:	2-AA was used as the only positive control for the S9 mix. However, the notifier has now provided data on metabolizing capacity of the S9 mix used in this study upon request (Herbold, B. 2009 a, b; ASB2009-3182/3).
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Chemical structure:



Common name:	Fluopyram	
Company code:	AE C656948	
CAS name:	Benzamide, N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)-(9CI)	
Empirical formula:	$C_{16}H_{11}ClF_6N_2O$	
Molecular weight:	396.72 g/mol	
Description:	Light brown solid powder	
Lot/Batch:	Mix-Batch:08528/0002	
Purity:	94.7 %	
Stability	Stable for the duration of the study	
Test organisms	Species	Salmonella typhimurium LT2 mutants
	Strains	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
	Source	Strains obtained from Prof. Bruce Ames in 1997 and stored in the laboratory since then.
Controls	Negative	Culture medium
	Solvent	DMSO
	Positive	Sodium azide (Serva) for TA 1535 at 10 µg/plate, Nitrofurantoin (Sigma) for TA 100 at 0.2 µg/plate, 4-Nitro-1,2-phenylene diamine (Merck-Schuchardt) for TA

1537 at 10 µg/plate and TA 98 at 0.5 µg/plate,

Mitomycin C (Fluka) for TA 102 at 0.2 µg/plate only in plate incorporation plate,

Cumene hydroperoxide (Sigma) for TA 102 in pre-incubation trials only at 50 µg/plate,

2-Aminoanthracene (Aldrich) as the only positive control for the activating effect of the S9 mix in all strains at 3 µg/plate.

Test compound concentrations:

Plate incorporation assay: For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate.

Pre-incubation assay: For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate.

Metabolic activation: The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats but was stored and had not been freshly prepared.

Study design: The experimental phase of the study was performed between November 30th and December 19th, 2005, at Bayer HealthCare AG (PH-PD P Health Care Toxicology). The Salmonella / microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups.

Plate incorporation assay: Fluopyram or the positive control material were dissolved in 0.1 mL of DMSO and were added to glass vessels with 0.1 mL of bacterial cultures grown overnight, 0.5 mL of S9 mix or buffer and 2 mL of soft agar. The mixture was placed in a water bath at 45°C for 30 seconds, shaken and overlaid onto Petri dishes containing solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colonies were scored using an automated colony counter. Three plates were used, both with and without S9 mix, for each strain and dose. The doses for the first trial were routinely determined on the basis of a standard protocol with a maximum dose of 5000 µg/plate and at least 5 additional doses. If less than three doses were used for assessment, at least two repeats were performed.

Pre-incubation assay: An independent repeat test was performed as pre-incubation of the previously described mixture in a water bath at 37°C for 20 minutes. At the end of the pre-incubation period, 2 mL of molten soft agar were added to the tubes, the content mixed and plated onto Petri dishes with solid agar. After 48 hours of incubation at 37°C, the numbers of revertant colonies were also scored using an automated colony counter.

Acceptance criteria: The negative controls had to be within the expected range. The positive controls had to show sufficient effects. Titer determinations had to demonstrate sufficient bacterial density in the suspension. For this purpose, the total bacterial counts were measured on two plates for each concentration studied with S9 mix.

Assessment criteria: A reproducible and dose-related increase in mutant colonies of at least one strain was considered to be positive. For TA 1535, TA 100 and TA 98, this increase should be about twice that of negative controls, whereas for TA 1537, at least a threefold increase should be reached. For TA 102, an increase of about 100 mutants should be reached. Otherwise, the result was considered as negative.

Findings

There was no indication of a bacteriotoxic effect of fluopyram at any dose up to and including 5000 µg/plate. From 1581 µg/plate onwards, the test substance precipitated but, nevertheless, assessment was still possible up to the highest dose of 5000 µg/plate.

Results are presented in the tables below. There was no relevant increase in mutant counts at any concentration of fluopyram compared to the negative controls both with and without metabolic activation (S9 mix). These results were confirmed in the 2nd experiment with a pre-incubation step. The positive controls caused the expected significant increase in the number of revertant colonies compared to the controls demonstrating the sensitivity of the system.

Table 78: Mutant colony counts presented as a ratio to the control (1st experiment)

Substance/concentration (µg/plate)	TA 1535	TA 100	TA 1537	TA 98	TA 102
Without S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	0.8	0.9	0.9	0.9	0.8
50	0.6	0.9	0.8	0.8	0.9
158	0.8	1.0	0.7	0.7	1.0
500	0.7	1.0	0.7	0.8	0.8
1581	0.7	0.8	0.7	0.7	0.7
5000	0.9	0.8	0.9	0.9	0.7
Na-azide	44.1*	-	-	-	-
NF	-	2.6*	-	-	-
4-NPDA	-	-	11.8*	6.2*	-
MMC	-	-	-	-	2.9*
With S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	1.0	1.0	0.7	0.9	1.0
50	0.9	0.9	0.7	0.8	1.0
158	0.8	1.2	0.7	0.8	1.0
500	0.8	0.9	1.0	0.9	0.9
1581	0.8	0.8	0.7	0.6	0.9
5000	0.7	0.9	0.5	0.8	0.8
2-AA	10.6*	11.6*	10.9*	29.8*	2.5*

= $p < 0.05$

Table 79: Mutant colony counts presented as a ratio to the control (2nd experiment)

Substance/concentration (µg/plate)	TA 1535	TA 100	TA 1537	TA 98	TA 102
Without S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	1.2	0.9	0.8	0.9	1.2
50	0.9	0.9	0.8	1.0	1.0
158	1.0	0.9	0.7	0.9	1.1
500	0.9	0.9	0.8	0.9	1.0
1581	0.8	0.9	0.8	0.8	0.8
5000	0.9	0.9	0.8	1.1	0.8
Na-azide	26.8*	-	-	-	-
NF	-	2.6*	-	-	-
4-NPDA	-	-	10.3*	6.2*	-
Cumene hydroperoxide	-	-	-	-	1.6*
With S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	0.8	0.9	1.0	0.8	0.9
50	0.9	0.9	0.7	0.7	1.0
158	0.9	0.9	1.0	0.7	1.0
500	1.0	0.9	1.0	0.6	1.0
1581	0.6	0.8	1.0	0.7	1.0
5000	0.8	0.8	0.8	0.6	0.8
2-AA	8.7*	10.7*	19.2*	18.0*	1.8*

* = $p < 0.05$

Conclusion

In this *in vitro* assessment of the mutagenic potential of fluopyram, histidine dependent auxotrophic mutants of *Salmonella typhimurium* were exposed to fluopyram. The test substance did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation. The positive control compounds produced the expected increases in the number of revertant colonies but the control substance for the activation experiment was not appropriate. Thus, efficacy of the S9 mix could not be proven in the original report. However, sufficient information on the efficacy of the S9 mix was provided by the notifier upon request. So it now can be concluded that fluopyram was not genotoxic in the plate incorporation as well as in the pre-incubation modifications of the Salmonella/microsome test with and without S9 mix.

Another study of this type was conducted under nearly identical conditions. This additional test was performed with a representative technical batch of the proposed specification as it is now. There were a few changes in the impurity profile of the test material compared to that one used in the initial assay and, thus, this second study might be of higher relevance than the previous one.

Title: Herbold, B. (2008): AE C656948 (project: Fluopyram) - Salmonella/microsome test - Plate incorporation and preincubation method, AT04419, M-298529-01, ASB2008-5554.

Guidelines: OECD 471 (1997).

Deviations: 2-AA was used as the only positive control for the S9 mix. However, the notifier has now provided data on metabolizing capacity of the S9 mix used in this study upon request (Herbold, B. 2009 c, ASB 2009-3186).

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Materials and methods

Common name:		Fluopyram
Company code:		AE C656948
Description:		Fine white powder
Lot/Batch:		2007-010986
Purity:		95.7 %
Stability		Stable for the duration of the study
Test organisms	Species	Salmonella typhimurium LT2 mutants
	Strain	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, TA 98 and TA 102
	Source	Strains obtained from Prof. Bruce Ames in 1997 and stored in the laboratory since then.
Control	Negative	Culture medium
	Solvent	DMSO
	Positive	Sodium azide (Serva) for TA 1535 at 10 µg/plate, Nitrofurantoin (Sigma) for TA 100 at 0.2 µg/plate, 4-Nitro-1,2-phenylene diamine (Merck-Schuchardt) for TA 1537 at 10 µg/plate and TA 98 at 0.5 µg/plate, Mitomycin C (Fluka) for TA 102 at 0.2 µg/plate only in plate incorporation plate, Cumene hydroperoxide (Sigma) for TA 102 in pre-incubation trials only at 50 µg/plate, 2-Aminoanthracene (Aldrich) for the activating effect of the S9 mix in all strains at 3 µg/plate.

Test compound concentrations:

Plate incorporation assay: For all strains with or without S9 mix: 16, 50, 158, 500, 1581 and 5000 µg/plate;

Pre-incubation assay: For all strains with or without S9 mix: 5, 16, 50, 158, 500 and 1581 µg/plate.

Metabolic activation: The S9 fraction was derived freshly from preparation isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats (protein content = 25 mg/mL)

Study design: The experimental phase of the study was performed between December 12th, 2007 and January 11th, 2008, at Bayer HealthCare AG (PH-PD P Health Care Toxicology). The Salmonella/microsome test is a screening method which detects point mutations caused by chemical agents *in vitro*. Auxotrophic mutants of Salmonella typhimurium are used to demonstrate this effect. For this purpose, the rate of reversion to prototrophy is evaluated in negative control and treated groups. Plate incorporation assay and pre-incubation assay were performed as described above (Wirnitzer, 2006, ASB2008-5552). Acceptance criteria and assessment criteria were set as described above (Wirnitzer, 2006, ASB2008-5552).

Findings

Concentrations of up to 158 µg/plate were not toxic to the bacteria. From 500 µg/plate onwards, fluopyram produced strain specific toxicity. Nevertheless, concentrations up to 1581 µg/plate could be used for reliable assessment.

The results are presented in the tables below. There was no relevant increase in mutant counts at any concentration of fluopyram compared to the negative controls both with and without metabolic activation (S9 mix). Those results were confirmed in the 2nd experiment with a pre-incubation step. The positive controls caused a significant increase in the number of revertant colonies compared to the controls demonstrating the sensitivity of the system.

Table 80: Mutant colony counts presented as a ratio to the control (1st experiment)

Substance/concentration (µg/plate)	TA 1535	TA 100	TA 1537	TA 98	TA 102
Without S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	0.9	0.9	1.0	0.9	1.0
50	1.0	0.8	0.8	0.8	1.1
158	0.8	0.7	0.8	0.6	1.1
500	0.7	0.7	0.9	0.7	1.0
1581	0.6	0.4	1.0	0.5	0.9
5000	0.4	0.3	0.5	0.1	0.8
Na-azide	20.1*	-	-	-	-
NF	-	3.1*	-	-	-
4-NPDA	-	-	10.6*	7.1*	-
MMC	-	-	-	-	3.3*
With S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
16	0.9	0.8	0.8	0.7	1.0
50	0.6	0.6	1.1	0.6	0.9
158	0.4	0.8	0.8	0.8	0.9
500	0.6	0.7	0.7	1.1	1.1
1581	0.4	0.6	0.7	0.8	0.6
5000	0.2	0.2	0.4	0.3	0.5
2-AA	8.8*	11.1*	20.1*	42.3*	2.3*

= $p < 0.05$

Table 81: Mutant colony counts presented as a ratio to the control (2nd experiment)

Substance/concentration (µg/plate)	TA 1535	TA 100	TA 1537	TA 98	TA 102
Without S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
5	0.9	0.9	1.2	1.0	1.0
16	1.0	1.0	1.2	1.0	1.0
50	1.1	1.2	1.0	1.1	1.0
158	1.3	0.8	1.2	0.9	0.9
500	1.0	0.8	0.8	0.9	0.9
1581	1.3	0.7	0.7	0.7	0.8
Na-azide	84.1*	-	-	-	-
NF	-	4.7*	-	-	-
4-NPDA	-	-	17.2*	6.7*	-
Cumene hydroperoxide	-	-	-	-	1.9*
With S9 mix					
Fluopyram 0 (DMSO)	1.0	1.0	1.0	1.0	1.0
5	1.1	1.0	0.9	1.0	1.1
16	0.9	0.9	0.9	0.9	1.1
50	0.8	1.1	0.6	1.0	1.2
158	1.1	0.9	0.8	0.9	1.2
500	0.9	0.8	0.8	0.8	1.2
1581	0.9	0.7	0.4	1.0	0.9
2-AA	11.5*	12.7*	17.6*	35.7*	2.2*

* = $p < 0.05$

Conclusion

In this *in vitro* assay, fluopyram did not cause any significant increase in the number of revertant colonies in either the presence or absence of metabolic activation. Again, the positive control substance for the activation experiment was not appropriate in the original report. However, sufficient information on the efficacy of the S9 mix was provided by the notifier upon request. So it now can be concluded that fluopyram was not genotoxic in the plate incorporation as well as in the pre-incubation modifications of the Salmonella/microsome test with and without S9 mix.

Clastogenicity in mammalian cells

Title: Nern, M. (2005): AE C656948 (Project: AE C656948) - In vitro chromosome aberration test with chinese hamster V79 cells, AT02798, M-266066-01, ASB2008-5555.

Guidelines: OECD 473 (1997); EEC 2000/32/EC Method B.10 (2000).

Deviations: None.

GLP: Yes.

Acceptability: The study is considered to be acceptable.

Materials and methods

Common name: Fluopyram

Company code: AE C656948

Description: Light brown solid powder

Lot/Batch: Mix-Batch:08528/0002

Purity: 94.7 %

Stability		Stable for the duration of the study
Test organisms	Cell line	Chinese hamster V79 lung cells
	Source	Cells obtained from Dr Utesch, Merck AG, Darmstadt/Germany, in 1993, stored in the laboratory since then.
Control	Negative	Culture medium
	Solvent	DMSO for fluopyram and Hanks's balanced salt solution for positive controls
	Positive	Mitomycin C (Fluka, batch 454188/2 44903116) without S9 mix at 0.1 µg/mL for a treatment period of 4 hours and 0.03 µg/mL for a treatment period of 18 hours.
		Cyclophosphamide (Endoxan 100 mg injection vials of dry substance, Baxter Oncology GmbH) with S9 mix at 2 µg/mL.

Test compound concentrations: Fluopyram was used with and without S9 mix at 30, 60, 120, 180 and 240 µg/mL.

Study design: The experimental phase of the study was performed from September 14th to December 2nd, 2005 at Bayer Healthcare AG (PH-PD P Health Care Toxicology). The *in vitro* cytogenetic test is a mutagenicity test system for the detection of chromosome aberrations in cultured mammalian cells. The test is designed to detect structural aberrations (chromatid and chromosome aberrations) in cells at their first post-treatment mitosis.

Determination of cytotoxicity: Duplicate cultures were exposed in a pre-test to fluopyram at concentrations ranging from 1 to 250 µg/mL for 4 hours (with S9 mix for metabolic activation) or for 18 hours (without S9 mix). The mitotic index was determined for all cultures. The number of mitotic cells among a total of 1000 cells per culture was determined using a light microscope at a magnification of about 630. All cells which were not in interphase were defined as mitotic.

Cytotoxicity was also examined in the main study in cell cultures which were incubated in the presence of S9 mix with the test compound at concentrations ranging from 30 or 120 to 240 µg/mL for 4 hours and harvested after 18 hours or 30 hours. Without S9 mix, additional cultures were exposed for 18 hours to concentrations of fluopyram ranging from 60 to 180 µg/mL. These cultures were harvested immediately at the end of the treatment period.

It was proven that concentrations of up to 4000 µg/mL fluopyram did not change the pH in the medium and that the osmolality in the medium was not altered up to a concentration of 250 µg/mL. Thus, at the concentration range employed in the main studies, these factors would have had no impact on the results.

Treatment protocol: Chinese hamster V79 cells were passaged on the day prior to treatment. Approximately 1x10^6 cells were seeded in 20 mL of medium per 75 cm^2 flasks and incubated at 37°C in a CO_2-incubator (5 % CO_2). Unless reported otherwise, the cells were grown in Eagle's minimal essential medium containing 10 % fetal calf serum. Immediately before treatment, the medium was removed from the cultures. For the trials without S9 mix, 20 mL of medium containing 2 % fetal calf serum and 0.2 mL of test substance solution were added to each flask. For the trials with S9 mix, 19 mL of medium containing 2 % fetal calf serum, 1 mL of S9 mix and 0.2 mL of test substance solution were added to each flask. Under activation conditions, the cells were incubated for 4 hours at 37°C. Afterwards, the medium was removed; the cells were washed with PBS and 20 mL of fresh medium containing 10 % fetal calf serum was added to the flasks. The flasks were placed in a CO_2-incubator for the remaining incubation time. In the cultures treated for 18 hours, the medium was not removed.

To each flask 0.2 mL of Colcemid-solution (40 µg/mL) was added 2 hours prior to the end of the 18-hour incubation period to arrest the cells in a metaphase-like stage of mitosis (c-metaphase).

Positive controls and solvent controls (0.2 mL of solvent per culture) were set up in parallel and handled as described for fluopyram-treated cultures. Untreated controls and solvent controls were used as negative controls.

Chromosome preparations: After the removal of the medium from each flask, the cells were trypsinized, suspended in medium and centrifuged for approximately 5 minutes at 700 rpm. The supernatant was removed and 1 to 2 mL of a hypotonic solution (0.4 % KCl; 37°C) was added to each tube. Within 4 minutes, the volume was brought to 6 mL with additional hypotonic solution and cells were resuspended. This suspension was centrifuged again and fixed with cold (4°C) fixative (ethanol/acetic acid 3:1) for 20 minutes at room temperature. This step was repeated before the pelleted cells were resuspended in a small volume of fixative and the suspension was dropped onto clean slides. The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes and stained for 15-20 minutes in 3 % Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered. At least two slides were generated per culture.

Evaluation criteria: Coded slides were evaluated using a light microscope at a magnification of about 1000. Chromosomes of approximately 200 metaphases per concentration (100 metaphases from each of two parallel cultures) were examined. Only metaphases containing the modal chromosome number (22) were analyzed unless exchanges were detected. The following aberrations were recorded: gaps (an achromatic lesion within a chromatid arm without dislocation of the chromatid end), break (a discontinuity of one chromatid with dislocation of the chromatid end), fragment (part of chromosome without centromer), deletion (result of a break with the terminal chromatid part of the chromosome missing within the metaphase under assessment), exchange (exchange of chromatid parts between different chromosomes or within the same chromosome), multiple aberration (when five or more structural changes occur within one metaphase). Observed polyploidy metaphases were recorded but not used for assessment.

Assessment criteria: An assay was acceptable, if there was a biologically relevant increase in chromosome aberrations induced by positive controls and if the numbers of aberrations for the negative controls were in the expected range.

A test was considered positive, if there was a relevant and statistically significant increase in the aberration rate over the negative control. However, an increased incidence of gaps without a concomitant increase of other aberration types was considered not to be an indication of a clastogenic effect.

A test was considered negative, if there was no such increase at any time interval or if there were statistically significant differences which were, however, within the range of historical negative controls.

A test was considered equivocal, if there was an increase above the range of historical negative controls which was statistically significant but considered not relevant, or if an increase occurred, which was considered relevant, but which was not statistically significant.

Statistics: The statistical analysis was performed by pair-wise comparison of fluopyram-treated and positive control groups to the respective solvent control group. The mitotic index was statistically analyzed (provided that it was reduced compared to the mean of the corresponding solvent control) using the one-sided Chi^2-test. The numbers of metaphases with aberrations (including and excluding gaps) and of metaphases with exchanges were compared (provided that these data superseded the respective solvent control). The one-sided Chi^2-test was used for the statistical evaluation. A difference was considered to be significant, if the probability of error was below 5 %.

Findings

Mitotic and survival indices: Fluopyram precipitation occurred in the medium at a concentration of 120 µg/mL and above (with and without S9 mix). In absence of S9 mix, the mitotic index was significantly reduced only at a concentration of 180 µg/mL after 18 hours of treatment. With S9 mix, there was no reduction of the mitosis rate. Survival indices were significantly reduced from 120 and 180 µg/mL onwards in the absence and in the presence of S9 mix, respectively.

Chromosome aberrations: Based of the results of cytotoxicity determinations, concentrations of 60, 120 and 180 µg/mL were selected for evaluation for both the 4-hour and 18-hour treatments. Metaphases examinations did not reveal any relevant or significant increase in numbers of metaphases with aberrations at any time points used in this study in presence or absence of metabolic activation. The sensitivity of the system was demonstrated by the significant increases in metaphases with aberrations caused by the positive controls used with and without S9 mix.

Conclusion

In this *in vitro* assessment of the clastogenic potential of fluopyram Chinese Hamster V79 cells were exposed to fluopyram in the absence and in presence of an Aroclor 1254-induced rat liver metabolic activation system (S9 mix). None of the cultures treated with fluopyram in the presence and in the absence of S9 mix showed biologically relevant or statistically significant increased numbers of aberrant metaphases. The positive controls mitomycin C and cyclophosphamide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the used S9 mix. Fluopyram was considered not to be clastogenic for mammalian cells *in vitro*.

Test for gene mutation in mammalian cells

Title:	Herbold, B. (2006): AE C656948 - V79/HPRT-test in vitro for the detection of induced forward mutations, AT02875, M-268775-01, ASB2008-5556.
Guidelines:	OECD 476 (1997), EEC B 17 (2000).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Common name:	Fluopyram	
Company code:	AE C656948	
Description:	Light brown solid powder	
Lot/Batch:	Mix-Batch:08528/0002	
Purity:	94.7 %	
Stability	Stable for the duration of the study	
Test organisms	Cell line	Chinese hamster V79 lung cells
	Culture	Incubation performed at 37°C in a humidified atmosphere with about 5 % CO_2.
	Source	Cells obtained from Prof. G. Speit, University of Ulm, Germany. These cells have since been recloned to maintain karyotypic stability. They have a modal chromosome number of 22 and a

		rapid population doubling time (10 to 14 hours)
Control	Negative	Culture medium [Eagle's minimal essential medium supplemented with 1 % L-glutamine, 1 % MEM-vitamins, 1 % MEM NEAA, 1 % penicillin/streptomycin and 10 % fetal calf serum (FCS)]
	Solvent	DMSO for AE C656948 and Dimethylbenzanthracene not exceeding 1 % (v/v) in the culture medium. No solvent needed for ethyl methanesulfonate as it is a liquid.
	Positive	Ethyl methanesulfonate (EMS), a directly alkylating agent, used at a final concentration of 900 µg/mL in non-activation trials.
		Dimethylbenzanthracene (DMBA), promutagen requiring a metabolic activation, used at a final concentration of 20 µg/mL for trials with S9 mix.

Test compound concentrations: Fluopyram was used at 8 concentrations ranging from 1.95 to 250 µg/mL in the clonal cytotoxicity assay and at 4, 8, 16, 32, 64, 128 and 256 µg/mL in the mutagenicity assays.

Metabolic activation: The S9 fraction was isolated from the livers of Aroclor 1254 induced male Sprague Dawley rats. The preparations dated from April 6, 2004 (protein content 22.2 mg/mL) and September 13, 2005 (protein content 24.0 mg/mL), and was kept frozen at -80°C. The batch was tested for contamination and cytotoxicity prior to use in the first study. Cofactors were freshly dissolved in sodium phosphate buffer (150 mM, pH 7.4)

Study design: The experimental phase of the study was performed from November 4th to December 15th, 2005 at Bayer HealthCare AG (PH-PD P Health Care Toxicology). The selection of V79 forward mutations is based on the resistance of induced mutants to the purine analogue 6-thioguanine (6-TG). This resistance is a result of a mutation at the X-chromosome-linked HPRT locus rendering the cells unable to use 6-TG for DNA synthesis. Therefore, cell colonies formed in the presence of 6-TG are considered to represent mutants at the HPRT gene.

Determination of cytotoxicity: Exponentially growing V79 cells were plated in 20 mL culture medium in a 75 cm^2 flask with a total volume of 275 mL ($4x10^6$ cells per flasks). For each concentration, one culture was available. After attachment (16 to 24 hours later), cells were exposed without S9 mix to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL medium containing 2 % FCS. In experiments with metabolic activation, 1 mL of medium was replaced by 1 mL of S9 mix. Thereafter, each cell monolayer was washed with PBS, trypsinized and replated in 5 mL culture medium at a density of 200 cells into 3 Petri dishes (diameter of 60 mm). These dishes were incubated for 6 to 8 days to allow colony development. Thereafter, colonies were fixed with 95 % methanol, stained with Giemsa (Merck; stock solution diluted 1:5 with deionized water) and counted automatically using an Artek counter if there was no interference by precipitation or coloration on the plates. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

Treatment protocol without metabolic activation: Exponentially growing V79 cells were plated in 20 mL culture medium in two 75 cm^2 flasks per concentration ($4x10^6$ cells per flask) including all control groups. After attachment (16 to 24 hours later), the cells were exposed to vehicle alone or to a range of concentrations of the test substance for 5 hours in 20 mL culture medium with reduced serum content (2 %). Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20 mL culture medium using $1.5x10^6$ cells per 75 cm^2 flask and in 5 mL culture medium using 200 cells per Petri dish (diameter of 60 mm). One flask and 3 Petri dishes were used per culture. These dishes were incubated for 6 to 8 days to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment (survival to treatment).

Cells in 75 cm^2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by reseeding $1.5x10^6$ cells into 20 mL of medium in 75 cm^2 flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (diameter of 100 mm) at $3x10^5$ cells per dish (8 dishes per culture) in 20 mL culture medium without hypoxanthine but containing 10 µg/mL 6-TG for selection of mutants. In addition, 200 cells per dish (diameter of 60 mm, 3 dishes per culture) were seeded in 5 mL culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes. Two trials were performed.

Treatment protocol with metabolic activation: The activation assay was performed independently. The procedure was identical to the non-activation assay except for the addition of S9 mix. In these experiments 19 mL instead of 20 mL culture medium and additionally 1 mL of S9 mix was added to the flasks for the treatment period, resulting in a concentration of 5 % S9 mix in the cultures. The number of 6-TG resistant mutants and viability were determined as in the non-activation assay. Two trials were performed.

Parameters assessed: The parameter “survival to treatment” in % was determined on the basis of the following calculation:

$$\frac{\text{Mean number of colonies (treated cultures) x 100}}{\text{Mean number of colonies (vehicle control cultures)}}$$

The “absolute population growth” was calculated using the following formula:

Absolute population growth (for each culture) = cell count 1 x cell count 2

The parameter “relative population growth” shows the cumulative growth of the treated cell populations, relative to the vehicle control.

$$\frac{\text{Absolute population growth treated culture x 100}}{\text{Absolute population growth of corresponding vehicle control culture}}$$

The ability of cells to form colonies at the time of mutant selection is measured by the parameter “absolute cloning efficiency”. It is expressed in %.

$$\frac{\text{Mean number of colonies per dish x 100}}{200}$$

The “mutant frequency” is calculated for each group by dividing the total number of mutant colonies by the number of cells seeded (usually 8-10 plates at $3x10^5$ cells per plate), corrected for the absolute cloning efficiency. The mutant frequency is expressed as 6-TG resistant mutants par 10^6 clonable cells.

$$\frac{\text{Total number of mutant colonies x 100}}{\text{Number of evaluated dishes x 3 x } 10^5 \text{ x C.E.}}$$

Acceptance criteria: The average cloning efficiency of the negative and vehicle controls should be at least 50 %. The average mutant frequency of the vehicle controls should not exceed 25 x 10^{-6} cells. The mutant frequency of the two cultures of the vehicle and /or the negative control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than 5 x 10^{-6}. The positive control should induce an average mutant frequency of at least three times that of the vehicle control. If not limited by the solubility of the test substance in the vehicle, the highest concentration should induce cytotoxicity of about 80 to 90 % or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the range of the negative control. For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency should be 10 % or greater. However, these criteria may be overruled by good scientific judgment.

Assessment criteria: Mutant frequencies were only used for assessment if at least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10 % or greater. A trial was considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies was observed. To be relevant, the increase in mutant frequencies should be at least two to three times that of the highest negative or vehicle control value observed in the respective trial. If this result could be reproduced in a second trial, the test substance was considered to be mutagenic.

Despite these criteria, a positive result was only considered relevant if no significant change in osmolality compared to the vehicle control was observed. Otherwise, unphysiological culture conditions might be the reason for the positive result. A test substance was judged as equivocal if there was no strictly concentration related increase in mutation frequencies but if one or more concentrations induced a reproducible and biologically relevant increase in mutant frequencies in all trials. An assay was considered negative if no reproducible and relevant increases of mutant frequencies were observed. However, these criteria may be overruled by good scientific judgment.

Statistical analysis: The statistical analysis relied on the mutant frequencies which were submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights. The two mutant frequency values obtained per group were, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay required at least two independent trials, the overall analysis without respectively with activation was the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups were included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of $\alpha = 0.05$ using the Dunnett test. The regression analysis part was performed on the basis of the actual concentrations thereby omitting the positive, negative and vehicle controls. If there was a significant concentration related increase of the mutant frequency ($\alpha = 0.05$) in the main analysis the highest concentration was dropped and the analysis repeated. This procedure was repeated until $p > 0.05$. Concentrations eliminated in that way were flagged correspondingly.

Findings

Cytotoxicity and precipitation: In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed to fluopyram at concentrations of up to and including 256 µg/mL. With or without S9 mix, fluopyram precipitation occurred in the medium at concentration of 128 µg/mL and above.

Good cloning conditions were demonstrated by the absolute cloning efficiency for the vehicle controls ranging from 52.6 % to 67.0 % and from 50.0 % to 64.8 % without and with metabolic activation, respectively.

Mutation assay: The test system proved to be sensitive on both experimental conditions (activation and non-activation) since treatment with the positive controls caused a biologically relevant increase in mutant frequencies as compared to the corresponding controls. There was no relevant increase in mutant frequencies after treatment with fluopyram at any concentration (up to the highest dose of 256 µg/mL) either with or without metabolic activation.

Conclusion

The aim of the study was to assess the point mutagenic potential of fluopyram at the HPRT locus in V79 cells. Fluopyram was tested up to its limit of solubility under culture conditions, as precipitation occurred in the culture medium at 128 µg/mL and above. With and without S9 mix there was no biologically relevant increase in mutant frequency above that of the vehicle controls. Adequate positive controls (ethyl methanesulfonate and dimethylbenzanthracene) were used for each experiment and induced clear mutagenic effects demonstrating the sensitivity of the test system and the activity of the S9 mix. Based on these results, fluopyram was considered to be non-mutagenic in the V79/HPRT forward mutation assay with and without metabolic activation.

4.9.1.2 In vivo data

***In vivo* genotoxicity (somatic cells) - Bone marrow or micronucleus**

Title:	Herbold, B. (2005): AE C656948 - Micronucleus-test on the male mouse, AT02753, M-263710-01, ASB2008-5557.
Guidelines:	OECD 474 (1997); Other guidelines claimed to be followed by the notifier: EEC 2000/32/EC Method B12 (2000).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Common name:		Fluopyram
Company code:		AE C656948
Description:		Light brown solid powder
Lot/Batch:		Mix-Batch:08528/0002
Purity:		94.7 %
Stability		Stable for the duration of the study
Test animals	Species	Mouse
	Strain	Hsd/Win: NMRI
	Age/Weight	6 to 12 weeks approximately; Weight at dosing: 36 to 43 g (males only)
	Source	Harlan, Netherlands
	No animals / dose	Range-finding test: 3 animals/sex Micronucleus assay: 5 males/group
	Animal husbandry	The animals were singly maintained in cages on bedding of soft wood granules. Husbandry was standardized with twelve hours of electrical lighting daily, 21.5-22°C room temperature and 45-

		57 % mean relative humidity
Control	Negative	None
	Solvent	0.5 % aqueous Cremophor emulsion
	Positive	Cyclophosphamide used in form of Endoxan 100 mg injection vials of dry substance (Baxter Oncology GmbH)

Test compound concentrations: Range-finding test: 2 intraperitoneal injections of 250, 500 and 1000 mg/kg bw separated by 24 hours; Micronucleus assay: 0, 250, 500 and 1000 mg/kg bw. The administered volume was 10 mL/kg in all of the dose groups.

Treatment and sampling times: Sampling took place 24 hours after the last intraperitoneal injection; the positive control was sampled 24 hours after the single intraperitoneal injection.

Tissues and cells examined: Bone marrow; 2000 polychromatic erythrocytes (PCEs) examined per animal; the number of normochromatic erythrocytes (NCEs, more mature RBCs) per 2000 PCEs was noted.

Details of slide preparation: At 24 hours after the second intraperitoneal injection of fluopyram or vehicle control, or 24 hours after the only one intraperitoneal injection of positive control, the appropriate groups of animals were sacrificed. Bone marrow smears were prepared from at least one intact femur for each animal. Cell smears were prepared and stained according to conventional cytological procedures. Coded slides were scored for the presence of micronuclei in 2000 PCEs per animal. The ratio of PCEs to NCEs was also recorded for each animal, as an indication of cytotoxicity to the target tissue. The number of normochromatic erythrocytes showing micronuclei was also established.

Evaluation Criteria: A statistically significant increase in micronucleated PCE frequency above the concurrent control should be interpreted against the background of historical vehicle and positive control data.

Statistical methods: The fluopyram group(s) with the highest mean (provided this superseded the negative control mean) and the positive control were checked by Wilcoxon’s non-parametric rank sum test with respect to the number of micronucleated (MN) polychromatic erythrocytes and the number of normochromatic erythrocytes. The rate of normochromatic erythrocytes containing micronuclei was examined if the micronucleus 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.

Findings

Toxicity: There was no mortality throughout the study. Clinical signs including apathy, semi-anesthetized state, roughened fur, weight loss, sternal recumbency, spasm, body stretching and difficulty in breathing were observed at all dose levels until the sacrifice. This demonstrated a relevant systemic exposure of the animals to the test substance.

Microscopic evaluation: The results of microscopic examination of the smears are summarized in Table 82.

Table 82: Number of normochromatic, micronucleated normochromatic and micronucleated polychromatic erythrocytes per 2000 polychromatic erythrocytes

Groups	Number of PCE evaluated	Number of NCE/2000 PCE	Number of MNNCE / 2000 PCE	Number of MNPCE/2000 PCE
Negative control		3775 ± 737	2.1 ± 2.0	4.0 ± 1.6
Fluopyram				
250 mg/kg	10000	5309 ± 1274	2.2 ± 1.5	4.4 ± 1.8
500 mg/kg	(5 x 2000)	6090 ± 852	1.5 ± 0.9	3.2 ± 2.3
1000 mg/kg		7866* ± 2018	1.3 ± 0.7	4.2 ± 1.5
Positive control		3243 ± 337	1.7 ± 0.8	28.6* ± 6.1

* $p < 0.01$ in non-parametric Wilcoxon ranking test

The positive control cyclophosphamide caused a significant increase in the number of micronucleated polychromatic erythrocytes compared to controls which demonstrated the sensitivity of the test system. On the other hand, fluopyram did not cause any increase in this parameter at any dose level used in the study. There was an increase in the number of normochromatic erythrocytes in all fluopyram treated groups compare to controls although the difference was only statistically significant at the highest dose level. This demonstrated a relevant and sufficient systemic exposure of animals to the test substance proving the reliability of the outcome of this assay.

Conclusion

The micronucleus test was conducted to investigate a possible clastogenic effect of fluopyram on bone-marrow erythroblasts. Results show an alteration of the polychromatic to normochromatic erythrocyte ratio demonstrating a relevant systemic exposure of bone marrow to the test substance. There were no significant variations in the incidence of micro-nucleated polychromatic erythrocytes between the control and the fluopyram treated groups. In contrast, the positive control animals showed a significant increase in the ratio of micronucleated polychromatic erythrocytes. In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered fluopyram in the micronucleus test on the male mouse.

4.9.2 Human information

No data submitted by the notifier.

4.9.3 Other relevant information

No data submitted by the notifier.

4.9.4 Summary and discussion of mutagenicity

Fluopyram (AE C656948) was tested in a minimum battery of standard genotoxicity and mutagenicity tests *in vitro* and *in vivo*. So far, these studies demonstrate that fluopyram has no genotoxic potential. There was no indication of gene mutation either in the presence or absence of metabolic activation in both the bacterial reverse mutation and mammalian gene mutation tests. The *in vitro* chromosome aberration test and the *in vivo* mouse micronucleus test were both negative and, thus, a clastogenic potential may be excluded. An overview on the currently available studies on mutagenicity is given in Table 83.

Table 83: Summary of genotoxicity tests with fluopyram

Study type	Metabolic activation	Concentrations that could be assessed	Results	Remarks
A. *In vitro* tests				
Ames Test with strains TA 1535, TA 100, TA 1537, TA 98 and TA 102 (Wirnitzer, 2006, ASB2008-5552) OECD 471	+/-	16, 50, 158, 500, 1581 and 5000 µg/plate	Negative	Studies with adequate positive controls were requested in the original DAR. Since an adequate demonstration of functionality of the S9 mix was provided, Ames tests are now regarded as acceptable
Ames Test with strains TA 1535, TA 100, TA 1537, TA 98 and TA 102 (Herbold, 2008, ASB2008-5554) OECD 471	+/-	16, 50, 158, 500, and 1581 µg/plate	Negative	
HPRT Test (V79 cells) (Herbold, 2006, ASB2008-5556) OECD 476	+/-	1.95 to 256 µg/mL	Negative	
Chromosome aberrations (V79 cells) (Nern, 2005, ASB2008-5555) OECD 473	+/-	30, 60, 120, 180 and 240 µg/mL	Negative	
B. *In vivo* tests				
Bone marrow micronucleus assay in mice – intraperitoneal administration (Herbold, 2005, ASB2008-5557) OECD 474	Not relevant; *in vivo* test	Dose levels = 250, 500 & 1000 mg/kg bw	Negative	

4.9.5 Comparison with criteria

Following criteria for classification for germ cell mutagens are given in DSD and CLP regulation:

Table 84: Comparison with criteria for DSD and CLP

DSD	CLP regulation
Category 1 To place a substance in Category 1, positive evidence from human mutation epidemiology studies will be needed. Examples of such substances are not known to date. It is recognised that it is extremely difficult to obtain reliable information from studies on the incidence of mutations in human populations, or on possible increases in their frequencies. Category 2 To place a substance in Category 2, positive results are needed from assays showing (a) mutagenic effects, or (b) other cellular interactions relevant to mutagenicity, in germ cells of mammals in vivo, or (c) mutagenic effects in somatic cells of mammals in vivo in combination with clear evidence that the substance or a relevant metabolite reaches the germ cells. With respect to placement in Category 2, at present the following methods are appropriate: 2 (a) in vivo germ cell mutagenicity assays: - specific locus mutation test, - heritable translocation test, - dominant lethal mutation test. These assays actually demonstrate the appearance of affected progeny or a defect in the developing embryo. 2 (b) in vivo assays showing relevant interaction with germ cells (usually DNA): - assays for chromosomal abnormalities, as detected by cytogenetic analysis, including aneuploidy, caused by	The classification in **Category 1A** is based on positive evidence from human epidemiological studies. Substances to be regarded as if they induce heritable mutations in the germ cells of humans. The classification in **Category 1B** is based on: — positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals; or — positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells; or — positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people. The classification in **Category 2** is based on: — positive evidence obtained from experiments in mammals and/or in some cases from in vitro experiments, obtained from: — somatic cell mutagenicity tests in vivo, in mammals; or — other in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays.

malsegregation of chromosomes, - test for sister chromatid exchanges (SCEs), - test for unscheduled DNA synthesis (UDS), - assay of (covalent) binding of mutagen to germ cell DNA, - assaying other kinds of DNA damage. These assays provide evidence of a more or less indirect nature. Positive results in these assays would normally be supported by positive results from in vivo somatic cell mutagenicity assays, in mammals or in mn (see under Category 3, preferably methods as under 3 (a)). 2 (c) in vivo assays showing mutagenic effects in somatic cells of mammals (see under 3 (a)), in combination with toxicokinetic methods, or other methodologies capable of demonstrating that the compound or a relevant metabolite reaches the germ cells. For 2 (b) and 2 (c), positive results from host-mediated assays or the demonstration of unequivocal effects in in vitro assays can be considered as supporting evidence. Category 3 To place a substance in Category 3, positive results are needed in assays showing (a) mutagenic effects or (b) other cellular interaction relevant to mutagenicity, in somatic cells in mammals in vivo. The latter especially would normally be supported by positive results from in vitro mutagenicity assays. For effects in somatic cells in vivo at present the following methods are appropriate: 3 (a) in vivo somatic cell mutagenicity assays: - bone marrow micronucleus test or metaphase analysis, - metaphase analysis of peripheral lymphocytes, - mouse coat colour spot test. 3 (b) in vivo somatic cell DNA interaction assays: - test for SCEs in somatic cells, - test for UDS in somatic cells, - assay for the (covalent) binding of mutagen to somatic cell DNA, - assay for DNA damage, e.g. by alkaline elution, in somatic cells. Substances showing positive results only in one or more in vitro mutagenicity assays should normally not be classified. Their further investigation using in vivo assays, however, is strongly indicated. In exceptional cases, e.g. for a substance showing pronounced responses in several in vitro assays, for which no relevant in vivo data are available, and which shows resemblance to known mutagens/carcinogens, classification in Category 3 could be considered.	Note: Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.

Comparison with criteria in DSD: No human data are available for fluopyram, hence a classification in category 1 is not possible. Neither in vivo heritable germ cell mutagenicity tests nor positive results from in vivo somatic cell mutagenicity tests in mammals are available, hence a classification in 2 is not necessary. In vitro and in vivo studies showed a negative outcome, hence a classification in category 3 is considered not necessary.

Comparision with criteria in CLP regulation: No human data are available for fluopyram, hence a classification as 1A is not possible. Neither vivo heritable germ cell mutagenicity tests nor positive results from in vivo somatic cell mutagenicity tests in mammals are available, hence a as 1B is not possible. In vitro and in vivo studies showed a negative outcome, hence a classification as 2 is considered not necessary.

4.9.6 Conclusions on classification and labelling

Based on the available studies and the comparison with the criteria a classification as germ cell mutagen is considered not necessary.

RAC evaluation of germ cell mutagenicity
Summary of the Dossier submitter's proposal Two bacterial mutation assays, a chromosomal aberration and mutation assay in mammalian cells *in vitro* and one micronucleus test *in vivo,* were summarised in the CLH report. All tests were negative. The DS thus argued that no classification for germ cell mutagenicity was warranted. **Comments received during public consultation** One Member State Competent Authority (MSCA) commented on mutagenicity, stating that a second *in vivo* test to investigate organ specific genotoxicity should have been conducted. **Assessment and comparison with the classification criteria** All mutagenicity studies presented were negative and of acceptable quality. RAC thus agrees with the DS that no classification for germ cell mutagenicity is warranted.

4.10 Carcinogenicity

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Long-term toxicity in rats

Title:	Kennel, P. (2008): AE C656948: Chronic toxicity and Carcinogenicity study of AE C656948 in the Wistar rat by dietary administration, SA 04312, M-298339-01, ASB2008-5439.
Guidelines:	OECD 453 (1981); EEC Directive 88/302/EEC Method B33 (1987).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.5 % a.i.
Vehicle or positive control:	None
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN)
Age/weight :	6 weeks approx.; at study start: 216 g to 219 g mean group weight for the males – 155 to 157 g mean group weight for the females
Source/breeder:	R. Janvier, Le Genest St Isle, France
Acclimation period:	13 days
Housing:	By sex in groups of 5, unless reduced by mortality or isolation. The cages were suspended, stainless steel wire mesh.
Environmental conditions :	Temperature: 22 ± 2°C
	Humidity: 55 ± 15 %
	Air changes: 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	A04CP1-10 (formerly referenced as A04C-10 P1) from S.A.F.E. (Scientific Animal Food and Engineering, Augy, *ad libitum* except at designated time periods
Water:	Filtered and softened tap water from the municipal water supplier, *ad libitum*

Animal assignment and treatment: Animals were weighed twice during the acclimatization phase. Their health status was checked on arrival and they were subjected to a detailed physical and ophthalmological examination once during the acclimatization phase. On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution among groups for each sex. Selected animals were in a weight range from 183 to 244 g for the males and 131 to 181 g for the females at the start of exposure to the test substance, i.e., within ± 20 % of the mean body weight on the day of randomization. Any animal deemed unsuitable for selection based on weight, ophthalmologic abnormalities or health status was not used for the study. On study Days 2 and 3 of the acclimatization phase, each animal was identified by a micro identification implant. Animals were assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 85: Study Groups for Carc.

Test group	Concentration in diet (ppm)	Dose per animal (week period 1 to 104)		Animals assigned/sex	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Main study 104 weeks	Interim sacrifice 52 weeks
1	0	0	0	60	10
2	30	1.20	1.68	60	10
3	150	6.0	8.6	60	10
4	750/375*	29	-	60 (males)	10 (males)
4	1500	-	89	60 (females)	10 (females)

*: concentration changed from 750 ppm to 375 ppm from week 85 onwards

The dose levels were selected based on the results from a previous 90-day dietary study in the rat (see 4.7.1.1) in which the NOAEL of 200 ppm was mainly based on effects observed in the liver, kidney and thyroid gland.

Diet preparation and analysis: The test substance was incorporated into the diet to provide the required dietary concentrations of 30, 150, 375, 750 or 1500 ppm. The test substance formulations were prepared to cover the dietary requirements over 6 to 8 weekly periods apart from the thirteenth formulation (F13) due to the decrease in dose level in the male high dose group and from the last formulation (F16) which covered the dietary needs until the end of the study. When not in use, the diet formulations were stored at ambient temperature. The homogeneity of the test substance in diet was verified at least from the first loads at all concentrations on the first formulation (F1) and on the first loads at 30 and 1500 ppm of formulations F2, F7 and F13, to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as the measured concentration. The concentration was checked at least for all loads at all dose levels for formulations F1, F2, F4, F7, F10, F13 and F16.

Statistics: Mean and standard deviation were calculated for each group and per time period. The Bartlett test was performed to compare the homogeneity of group variances. If the Bartlett test was not significant ($p>0.05$), means were compared using the analysis of variance (ANOVA), which was followed by Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant ($p\leq0.05$) (for body weight change parameters, terminal body weight, absolute and relative organ weight parameters, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, % neutrophils, % lymphocytes, prothrombin time), group means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance. If the Bartlett test was significant ($p\leq0.05$) (for body weight and average food consumption/day parameters), data were transformed using the log transformation. If the Bartlett test on log transformed data was not significant ($p>0.05$), means were compared using the ANOVA on log transformed data, which was followed by the Dunnett test (2-sided) on log transformed data if ANOVA indicated significance. If the Bartlett test was significant ($p\leq0.05$) even after log transformation, group

means were compared using the non-parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided), if the Kruskal-Wallis test indicated significance. If the Bartlett test was significant ($p \leq 0.05$) (for hematology parameters such as red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count), data were transformed using the square root transformation. If the Bartlett test on square root transformed data was not significant ($p > 0.05$), means were compared using the ANOVA on square root transformed data, which was followed by the Dunnett test (2-sided) on square root transformed data, if ANOVA indicated significance. If the Bartlett test was significant ($p \leq 0.05$) even after square root transformation, group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance.

For urine analysis (pH), group means were compared using the non-parametric Kruskal-Wallis which was followed by the Dunn test (2-sided), if Kruskal-Wallis test indicated significance. If one or more group variance(s) equaled 0, means were compared using non-parametric procedures. Group means were compared at the 5 % and 1 % levels of significance.

For survival analysis, adjusted mortality rates were estimated using Kaplan-Meier estimation procedures. Kaplan-Meier estimates were calculated separately for each sex and treatment group. Mortalities which were the result of animals dying following accidents (accidental trauma or died during anesthesia) or at scheduled sacrifice were considered to be censored observations. Statistical significance of differences in survival rates between treated and control groups and dose-related trend in survival were assessed using Cox's and Tarone’s tests on life table data. Probability values presented were two-sided for pairwise comparisons and trend test. Group mortality rates were compared at the 5 % and 1 % levels of significance. Survival analyses were performed on the carcinogenicity phase.

For neoplastic and non-neoplastic findings: When the incidences of the 30 and 150 ppm treated groups were equal to 0, only the high dose group was compared to the control group and no trend test was performed. When the number of lesion-bearing animals was equal to 1 in one group and was equal to 0 in the other groups, no statistical analysis was performed.

Not adjusted analyses: Selected lesions were analyzed by Cochran-Armitage method for trend (1-sided) and the Fisher’s exact test (1-sided) for control versus treatment comparisons. Cochran-Armitage trend significance is reported when there is existence of any monotone response in the incidence data (that is there is a relationship between the response and the dose represented by a regression line that is continually increasing (or decreasing), but perhaps not in a straight line).

Survival adjusted analyses: Further survival adjusted analyses, considering any possible intercurrent mortality differences due to the competing toxicity among the treated groups, were performed on lesions. For non-palpable tumors, each tumor was categorized as fatal if the tumor was a factor contributing towards the death of the animal, incidental otherwise. Incidental tumors and non-neoplastic lesions data were analyzed by logistic regression of tumor prevalence. Logistic regression analysis is based on the assumption that the diagnosed lesions were not directly responsible for the animal’s death. Treated and control group lesion rates and dose-related trends were compared using the corrected score test. Fatal tumors were analyzed by the life-table test. The life-table test is based on the assumption that all lesions were fatal. Statistical significance of differences in incidences between treated and control groups and dose-related trends were investigated using Cox’s and Tarone’s tests. Trend tests were conducted firstly including all groups. When both the trend test including all the dose levels and only the high dose group were significant, a second trend test excluding the high dose group was also done. The reported results reflect 1-sided testing. Group incidences were compared at the 5 % and 1 % levels of significance. All finding analyses were performed on the carcinogenicity phase.

Clinical signs and mortality: Animals were checked for morbidity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily. Detailed physical examinations including palpation for masses were performed weekly throughout the study.

Neurological examination: As the neurotoxicological potential of fluopyram was examined in specific studies (see 4.12.1.1), the respective parameters were not measured in this long-term study.

Body weight, food consumption and compound intake: Each animal was weighed at least weekly during the acclimatization period then weekly for the first 13 weeks of study and approximately every 4 weeks thereafter. Additionally, fasted animals were weighed prior to scheduled necropsy. These figures were referred to as terminal body weights.

Food consumption was recorded twice weekly during the first 6 weeks of treatment, then weekly up to Week 13, and once approximately every 4 weeks thereafter. The weekly mean achieved dosage intake in mg/kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as follows: Achieved dosage intake (mg/kg body weight/day) = x.

The monthly and overall mean achieved dosage intake for the 24 months of treatment were derived from the weekly data.

Ophthalmological examination: During the acclimatization phase, all animals were examined by indirect ophthalmoscopy. During the treatment period, funduscopic (indirect ophthalmoscopy) and biomicroscopic (slit lamp) examinations were performed on all surviving animals after approximately 12 and 24 months of treatment with fluopyram. Each eye was examined by direct ophthalmoscopy in the first instance, and then after instillation of an atropinic agent (Mydriaticum, Merck Sharp and Dohme), each eye was re-examined by means of a slit lamp and an indirect ophthalmoscope.

Hematology and clinical chemistry: Blood was sampled from Isoflurane anesthetized animals by puncture of the retro orbital venous plexus after overnight diet fasting. Blood was collected in tubes containing EDTA for hematology, lithium heparin (for plasma) and clot activator (for serum) for clinical chemistry and sodium citrate for coagulation. At terminal sacrifice, blood smears were prepared for all animals not sampled for hematology. When possible, a blood smear was prepared for the moribund animals, just before sacrifice.

Blood analyses were performed on all surviving animals of the interim sacrifice groups on Weeks 12 or 13, 25 or 26 and 51, and on the first ten suitable surviving rats of the terminal sacrifice groups on Weeks 12 to 13, 25 or 26, 51, 78 and 103 or 104.

Hematology: The following parameters were measured: hematocrit, hemoglobin, leukocyte count, erythrocyte count, platelet count, leukocyte differential count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, reticulocyte count, prothrombin time. A blood smear was prepared and stained with a Wright (Months 3, 6, 12 and 18) or May-Grünwald-Giemsa (Month 24) stains. It was examined only when the results were abnormal. For moribund and terminal sacrificed animals, the blood smears were stained with Wright stain (until October 05, 2006) or May-Grünwald-Giemsa stain (from October) for possible differential white blood cell determination (but were not examined).

Clinical chemistry: Any significant change in the general appearance of the plasma and the serum was recorded. Total bilirubin, glucose, urea, creatinine, total cholesterol, triglycerides, chloride, sodium, potassium, calcium and inorganic phosphorus concentrations and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on plasma samples, total protein and albumin concentrations were assayed on serum samples. Globulin and albumin/globulin ratio values were calculated.

Urinalysis: Urinalysis was performed on all the surviving animals of the interim sacrifice groups on Weeks 12 or 13, 24 or 25 and 52 or 53, and on the first ten suitable surviving rats of the terminal sacrifice groups during Weeks 12 or 13, 24 or 25, 52 or 53, 77 and 104. Diet and water were withdrawn during the overnight (approximately 16 hours) collection period. Any significant change in the general appearance of the urine was recorded. The urine volume was measured. pH was assayed using a Clinitek 200+ and Ames Multistix dipsticks. Urinary refractive index was measured using a RFM320 refractometer.

The following semi-quantitative parameters were assayed using a Clinitek 200+ and Ames Multistix dipsticks: glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen. Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

Necropsy procedure: On study Days 366 to 368 for the 12-month chronic phase, and on study Days 730 to 743 for the 24-month carcinogenicity phase, all surviving animals dedicated to chronic phase and carcinogenicity phase groups, respectively, were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day of sacrifice. Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of external surfaces, all orifices, all major organs, tissues and body cavities. All significant macroscopic abnormalities (including masses and their regional lymph nodes when possible) were recorded, sampled and examined microscopically.

Tissue collection: Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together. The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. Duplicate femoral bone marrow smears were prepared from sacrificed animals (except on weekends and public holidays), one of which was stained with May-Grünwald Giemsa, but not examined as no relevant changes were observed in hematology or bone marrow histology. The second smear was stored unstained for possible examination. Tissues 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.

Histopathology: All the above samples listed (except exorbital lachrymal gland, larynx/pharynx and nasal cavities) were embedded in paraffin wax. For the 12-month chronic and 24-month carcinogenicity phase, histological sections, stained with haematoxylin and eosin, were prepared from all organs and tissue samples.

12-month interim sacrifice (52 weeks of treatment): Histopathology examinations were performed as follows: all organs and tissue samples from animals sacrificed or dying during the treatment period, all organs and tissue samples from animals of control and high dose groups and liver, lung, kidney, thyroid gland and gross abnormalities from animals of the intermediate dose groups. For all unscheduled sacrificed or dead animals on study, the Study Pathologist made a determination of the factors having contributed to death. Initial examinations were performed by the Study Pathologist. Following the initial examination, an in-house review pathologist undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

Carcinogenicity phase (104 weeks of treatment): Histopathological examinations were performed on all organs and tissues embedded, including gross abnormalities, in all animals from all groups including

decedents. For all unscheduled sacrificed or dead animals on study, the Study Pathologist made a determination of the factors contributing to death. Initial examinations were performed by the Study Pathologist. Following the initial examination, an external review pathologist, undertook an independent « peer-review » of representative slides and diagnoses according to standardized operating procedures. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

Findings

Diet preparation analysis: Homogeneity and concentration results of fluopyram in the diet were within the in-house target range of 85 to 115 % of nominal concentration, except for 14 out of 242 results, which however were considered acceptable for use on the current study.

Clinical signs: During the first year of treatment there were no treatment-related clinical signs. During the second year of treatment, the following treatment-related clinical signs were observed in animals allocated to the carcinogenicity phase:

Table 86: Incidence of treatment related clinical signs during the second year of treatment (Days 367 to 742)

Sex	Males				Females			
Dose level (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Numer of animals examined	55	57	58	49	59	58	56	57
Hair loss	nc	nc	nc	nc	8 (13.6 %) [371]	10 (17.2 %) [371]	5 (8.9 %) [371]	17 (29.8 %) [371]
Prostration	0 (0 %) -	1 (1.8 %) [724]	0 (0 %) -	3 (6.1 %) [613]	nc	nc	nc	nc
General pallor	2 (3.6 %) [543]	2 (3.5 %) [624]	1 (1.7 %) [445]	4 (8.2 %) [445]	nc	nc	nc	nc
Wasted appearance	nc	nc	nc	Nc	10 (16.9 %) [464]	12 (20.7 %) [553]	11 (19.6 %) [483]	14 (24.6 %) [504]
Soiled anogenital region	2 (3.6 %) [704]	1 (1.8 %) [654]	1 (1.7 %) [641]	4 (8.2 %) [368]	nc	nc	nc	nc

nc: not concerned or no relevant change. (%): incidence. [ ]: first day of appearance during the second year of treatment.

In the female high dose group (1500 ppm), a higher incidence of hair loss and wasted appearance was noted, in comparison to the controls. In the male high dose group (750/375 ppm), a slightly higher incidence of the usual signs associated with morbidity (prostration, general pallor and soiled anogenital region) was noted, in comparison to the controls, reflecting the higher mortality observed in this group. No treatment-related clinical signs were noted at the mid and low dose levels in either sex.

Mortality: Within the first year in the male high dose group, 11/70 animals were found dead or were sacrificed prematurely for humane reasons compared to 6/70 in the control group. The main clinical signs in these early decedent males consisted of soiled fur or anogenital region (3/11) and focal swelling (2/11), together with usual signs associated with morbidity (limited use of hindlimbs, reduced motor activity, general pallor, wasted appearance). In view of the low mortality rate in the male control group and in other male treated groups during the first year of the study, the early deaths in the male high dose group were considered to be treatment-related. No clear factor contributing to the death of these animals could be established at the microscopic examination. No effect on mortality was noted in females.

Table 87: Mortality after 52 weeks of treatment

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Group size	70	70	70	70	70	70	70	70
Mortality (% mortality)	6 (8.6)	3 (4.3)	2 (2.9)	11 (15.7)	1 (1.4)	2 (2.9)	5 (7.1)	3 (4.3)

After 2 years of treatment, analysis of the survival rates showed that the mortality incidence was increased in the male high dose group and in the female low dose group ($p \leq 0.05$). Higher mortality in the male high dose group was considered to be treatment-related as the trend test was statistically significant in males, whereas in the female low dose group it was considered to be incidental and not related to treatment as the trend test was not statistically significant. No clear factor contributing to the death of these animals could be established.

Table 88: Mortality and survival rate after 104 weeks of treatment (animals allocated to the carcinogenicity phase)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Group size	60	60	60	60	60	60	60	60
Killed for humane reasons	17	15	6	20	12	24	19	23
Found dead	20	29	28	28	7	8	4	6
Dead during anesthesia	0	0	0	1	0	0	2	1
Total mortality (% mortality)	37 (61.7)	44 (73.3)	34 (56.7)	49 (81.7)	19 (31.7)	32 (53.3)	25 (41.7)	30 (50.0)
Adjusted survival rate (%) [a]	37.8 %	26.7 %	22.3 %	19.9 % *	68.3 %	46.7 % *	60.4 %	51.0 %

[a]: Kaplan-Meier estimated survival rates at the end of the study after adjusting for censored animals; *: $p \leq 0.05$

Body weight: In the female high dose group (1500 ppm), mean body weight or body weight gain parameters were essentially comparable to the controls throughout the first three months of treatment. Thereafter, mean cumulative body weight gain was lower than in the control group between weeks 14 to 26 (-29 %, $p \leq 0.01$), 26 to 54 (-15 %, not statistically significant) and 54 to 79 (-59 %, $p \leq 0.01$), whilst mean body weight was lower by 3, 5, 14 and 12 % at weeks 26, 54, 79 and 102 (statistically significant for most time points), when compared to the controls. In the male high dose group (750/375 ppm), mean body weight or body weight gain parameters were essentially comparable to the controls throughout the study. The few changes in mean body weight gain/day (reaching statistical significance) observed were considered to be incidental as they corresponded to both transient increases and decreases. At the mid and low dose levels (150 and 30 ppm), mean body weight or body weight gain parameters were unaffected by the treatment in both sexes over the two years of the study. The few changes (reaching statistical significance) observed were considered to be incidental as they were noted in isolation and with no dose-relationship.

Table 89: Mean body weights (BW) and cumulative body weight gains (BWG) (g) in male rats

Males				
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375** [#]
Initial BW (Day 1) (%C)	219 ± 11	219 ± 12 (100)	216 ± 12 (99)	218 ± 12 (100)
BW Week 2 (Day 8) (%C)	277 ± 14	278 ± 16 (100)	278 ± 14 (100)	279 ± 15 (101)
BW Week 14 (Day 92) (%C)	527 ± 37	526 ± 42 (100)	529 ± 41 (100)	526 ± 41 (100)
BW Week 26 (Day 176) (%C)	604 ± 45	600 ± 51 (99)	608 ± 49 (101)	609 47 (101)
BW Week 54 (Day 372) (%C)	697 ± 60	699 ± 74 (100)	704 ± 61 (101)	694 ± 61 (100)
BW Week 79 (Day 547) (%C)	719 ± 78	728 ± 86 (101)	733 ± 76 (102)	702 64 (98)
Final BW (Day 708) (%C)	672 ± 89	639 ± 86 (95)	686 ± 68 (102)	627 ± 64 (93)
BWG Weeks 1-2 (Days 1 to 8) (%C)	59 ± 5	59 ± 7 (100)	62 ± 9 (105)	61 * ± 6 (103)
BWG Weeks 1-14 (Days 1 to 92) (%C)	308 ± 34	307 ± 36 (100)	313 ± 40 (102)	308 ± 37 (100)
BWG Weeks 14-26 (Days 92 to 176) (%C)	77 ±16	74 ± 16 (96)	79 ± 17 (103)	82 17 (106)
BWG Weeks 26-54 (Days 176 to 372) (%C)	88 ±32	97 ± 35 (110)	93 ± 31 (106)	81 ± 34 (92)
BWG Weeks 54-79 (Days 372 to 547) (%C)	25 ± 34	31 ± 33 (124)	28 ± 47 (112)	23 ± 30 (92)
BWG Weeks 79-102 (Days 547 to 708) (%C)	-48 ± 57	-67 ± 49 (143)	-42 ± 55 (89)	-60 ± 45 (128)
Overall BWG (Days 1 to 708) (%C)	453 ± 91	421 ± 86 (93)	470 ± 69 (104)	410 ± 65 (91)

[#] 750 ppm up to study Day 588 (study Week 84), 375 ppm from study Day 589 onwards (study Week 85); C = control; nc = not calculated; * Statistically different (p≤0.05) from the control.

Table 90: Mean body weights (BW) and cumulative body weight gains (BWG) (g) in female rats

Females				
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**1500**
Initial BW (Day 1) (%C)	155 ± 10	156 ± 11 (101)	157 ± 10 (101)	157 ± 10 (101)
BW Week 2 (Day 8) (%C)	183 ± 13	186 ± 14 (102)	186 ± 13 (102)	183 ± 13 (100)
BW Week 14 (Day 92) (%C)	280 ± 22	287 ± 21 (103)	283 ± 23 (101)	278 ± 21 (99)
BW Week 26 (Day 176) (%C)	310 ± 25	316 ± 25 (102)	314 ± 26 (101)	300 * ± 25 (97)
BW Week 54 (Day 372) (%C)	350 ± 49	361 ± 40 (103)	351 ± 42 (100)	333 ± 36 (95)
BW Week 79 (Day 547) (%C)	412 ± 75	422 ± 67 (102)	408 ± 59 (99)	356 ** ± 45 (86)
Final BW (Day 708) (%C)	425 ±78	452 ± 92 (106)	425 ± 68 (100)	374 ** ± 56 (88)
BWG Weeks 1-2 (Days 1 to 8) (%C)	27 ±7	30 * ± 7 (111)	29 ± 7 (107)	27 ± 7 (100)
BWG Weeks 1-14 (Days 1 to 92) (%C)	124 ±17	131 * ± 16 (106)	126 ± 18 (102)	122 ± 16 (98)
BWG Weeks 14-26 (Days 92 to 176) (%C)	31 ± 10	30 ± 10 (97)	30 ± 9 (97)	22 ** ± 9 (71)
BWG Weeks 26-54 (Days 176 to 372) (%C)	39 ± 33	43 ± 24 (110)	37 ± 24 (95)	33 ± 20 (85)
BWG Weeks 54-79 (Days 372 to 547) (%C)	61 ± 42	60 ± 42 (98)	56 ± 32 (92)	25 ** ± 23 (41)
BWG Weeks 79-102 (Days 547 to 708) (%C)	21 ± 44	22 ± 52 (105)	25 ± 34 (119)	20 ± 39 (95)
Overall BWG (Days 1 to 708) (%C)	269 ± 73	296 ± 89 (110)	269 ± 64 (100)	216 ** ± 53 (80)

C = control; nc = not calculated; * Statistically different (p≤0.05) from the control. **Statistically different (p≤0.01) from the control.

Food consumption: Overall, mean food consumption was similar to the controls throughout the study in both sexes and at all dose levels, with the only exception of a slight reduction by up to 7 % (p≤0.01 or p≤0.05) in the male high dose group between study Days 18 to 39. The few other minor differences (reaching statistical significance) from controls were considered to reflect inter-individual variations but not treatment-related effects.

Table 91: Group mean food consumption (g/animal/day)

Fluopyram dosage level (ppm)	0	30	150	750/375	1500
Males					
Week period 1 to 13 (% C)	26.4	25.9 (98)	26.1 (99)	26.1 (99)	-
Week period 14 to 26 (% C)	25.3	25.2 (100)	25.8 (102)	25.7 (102)	-
Week period 27 to 52 (% C)	25.5	24.8 (97)	25.3 (99)	25.6 (101)	-
Week period 53 to 78 (% C)	24.8	24.2 (98)	24.9 (101)	25.7 (104)	-
Week period 79 to 104 (% C)	24.5	24.1 (98)	24.5 (100)	25.6 (105)	-
Females					
Week period 1 to 13 (% C)	19.0	19.5 (103)	19.6 (103)	-	18.7 (99)
Week period 14 to 26 (% C)	18.1	18.4 (102)	18.9 (104)	-	17.6 (97)
Week period 27 to 52 (% C)	18.2	18.8 (103)	18.6 (102)	-	18.0 (99)
Week period 53 to 78 (% C)	19.3	19.6 (102)	19.3 (100)	-	18.6 (96)
Week period 79 to 104 (% C)	20.5	21.8 (106)	20.8 (101)	-	20.9 (102)

Percentage from control in parentheses.

Achieved dosage: The mean achieved dietary intakes of fluopyram expressed in mg/kg body weight/day received by the animals during the study were as follows:

Table 92: Mean achieved dietary intake of fluopyram (mg/kg/day)

Sex	Males			Females		
Dosage level (ppm)	30	150	750/375*	30	150	1500
Week period 1 to 13	1.84	9.2	46	2.35	12	117
Week period 1 to 52	1.37	6.9	35	1.88	9.6	95
Week period 1 to 104	1.20	6.0	29	1.68	8.6	89

* 750 ppm up to study Day 588 (study Week 84), 375 ppm from study Day 589 onwards (study Week 85)

Ophthalmologic examination: At the end of the first year of treatment, the following treatment-related ophthalmologic findings were observed:

Table 93: Incidence of treatment related ophthalmologic findings noted at the first year examination

Sex	Males				Females			
Dose level of fluopyram (ppm)	0	30	150	750	0	30	150	1500
Number of animals examined	65	68	68	59	69	68	66	67
Retina fundus abnormal color: pale	0 (0 %)	0 (0 %)	1 (1.5 %)	0 (0 %)	0 (0 %)	1 (1.5 %)	0 (0 %)	4 (6 %)

(%): incidence.

In the female high dose group, abnormal color (pale) of the retinal fundus was observed in 4/67 animals, compared to no case in the controls. No treatment-related ophthalmological findings were noted at any dose level tested in males or at the mid and low dose levels in females at the end of the first year of

treatment. At the end of the second year of treatment, the following treatment-related ophthalmological findings were observed:

Table 94: Incidence of treatment related ophthalmological findings noted at the second year examination (animals allocated to the carcinogenicity phase)

Sex	Males				Females			
Dose level of fluopyram (ppm)	0	30	150	750/375	0	30	150	1500
Number of animals examined	25	21	31	14	43	31	37	32
Corneal opacity	2 (8.0 %)	2 (9.5 %)	7 (22.6 %)	3 (21.4 %)	2 (4.7 %)	0 (0 %)	1 (2.7 %)	0 (0 %)
Oedema of the cornea	1 (4.0 %)	1 (4.8 %)	4 (12.9 %)	3 (21.4 %)	2 (4.7 %)	0 (0 %)	1 (2.7 %)	0 (0 %)
Nuclear opacity of lens	12 (48 %)	11 (52.4 %)	27 (87.1 %)	12 (85.7 %)	35 (81.4 %)	26 (83.9 %)	31 (83.8 %)	30 (93.8 %)
Small retina vessels	1 (4.0 %)	1 (4.8 %)	3 (9.7 %)	3 (21.4 %)	2 (4.7 %)	2 (6.5 %)	2 (5.4 %)	14 (43.8 %)
Retina fundus abnormal color: pale	2 (8.0 %)	1 (4.8 %)	2 (6.5 %)	6 (42.9 %)	3 (7 %)	3 (9.7 %)	2 (5.4 %)	15 (46.9 %)
Hyperreflectivity in retina	1 (4.0 %)	0 (0 %)	0 (0 %)	1 (7.1 %)	1 (2.3 %)	1 (3.2 %)	1 (2.7 %)	3 (9.4 %)

(%): incidence.

In the female high dose group, a higher incidence of small retinal vessels, abnormal color (pale) of the retinal fundus and hyperreflectivity in retina was noted, in comparison to the controls. In the male high dose group, a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens, small retinal vessels and abnormal color (pale) of the retinal fundus was noted, in comparison to the controls. In the male mid dose group, a higher incidence of corneal opacity, oedema of the cornea, nuclear opacity of lens and small retinal vessels was noted, in comparison to the controls. No treatment-related ophthalmological findings were observed at the mid dose in females or at the low dose in either sex at the end of the second year of treatment.

Hematology: A significant tendency towards lower erythrocyte parameters (hemoglobin concentration, mean corpuscular volume, hematocrit and/or mean corpuscular hemoglobin) was observed in the female high dose group throughout the study. The same tendency was observed in high dose males at most time points however, without being significant. In addition prothrombin time was shorter in high dose females (significant at month 6 and 12) and the platelet count was significantly higher in high dose males and females at month six.

Clinical chemistry: Slightly higher mean total cholesterol concentrations were observed in the female high dose group throughout the study. In addition, a slightly higher mean total cholesterol concentration was observed in the female mid dose group at month 3, but as this change was observed in isolation and as its magnitude was low, it was considered not to be adverse.

Table 95: Total cholesterol concentrations (mean ± standard deviation) in females (mmol/L) with % change when compared to controls

Dose level of fluopyram	Control	30 ppm	150 ppm	1500 ppm
Month 3	1.76 ± 0.33	1.69 ± 0.37 (-4 %)	2.03 ± 0.28 * (+15 %)	2.38 ± 0.32 ** (+35 %)
Month 6	2.07 ± 0.37	2.01 ± 0.34 (-3 %)	2.32 ± 0.27 (+12 %)	2.72 ± 0.56 ** (+31 %)
Month 12	1.98 ± 0.33	2.01 ± 0.49 (+2 %)	2.19 ± 0.27 (+11 %)	2.74 ± 0.76 ** (+38 %)
Month 18	2.22 ± 0.31	2.78 ± 1.94 (+25 %)	2.21 ± 0.31 (0 %)	2.92 ± 0.76 (+32 %)
Month 24	2.44 ± 0.57	2.14 ± 0.68 (-12 %)	2.32 ± 0.51 (-5 %)	3.30 ± 0.46 ** (+35 %)

**: $p \leq 0.01$; *: $p \leq 0.05$.

In addition in the female high dose group, higher mean triglyceride concentrations were observed at Months 3 and 6 (+36 % at both time points, p≤0.01), and slightly lower mean glucose concentrations were noted at Months 6, 12 and 18 (-9 %, -13 % and -16 %, respectively, p≤0.01 or p≤0.05).

Slightly lower mean total bilirubin concentrations were seen in all female treated groups on one or more occasions. However, since the variations were not consistent throughout the sampling periods and were observed with no dose-relationship, they were considered not to be treatment-related. Activity of alkaline phosphatase (AP) was reduced in high dose males and females throughout the study being significant only at a few points but being consistent with observations in other studies and the observed changes in liver parameters in this study.

Urinalysis: In the high dose females, abnormal color of urine was noted at Month 6 (red color in 9/18 animals), Month 12 (orange to dark orange color in 14/20 animals) and Month 18 (orange color in 6/8 animals). In the male high and mid dose groups, a dose-related increase in incidence and severity of cellular casts was observed at Months 3 and 6, in comparison to the controls.

Table 96: Incidence and severity of cellular casts in urine in males

Dose level of fluopyram (ppm)		**control**	**30**	**150**	**750**
Month 3	slight	1/19	2/19	4/20	0/19
	moderate	0/19	0/19	4/20	6/19
	marked	0/19	0/19	2/20	5/19
	severe	0/19	0/19	0/20	6/19
	total	1/19	2/19	10/20	17/19
Month 6	slight	0/20	0/19	5/20	6/20
	moderate	0/20	0/19	1/20	9/20
	marked	0/20	0/19	1/20	4/20
	severe	0/20	0/19	0/20	0/20
	total	0/20	0/19	7/20	19/20

This effect was temporary (seen only after 3 and 6 months), since cellular casts (moderate severity) were observed in only 1/20 high dose males at Month 12, compared to no case in the controls and not observed anymore in this group at Months 18 and 24. No treatment-related findings were noted at the urinalysis at the mid dose level in females or at the low dose level in either sex.

Organ weights – 12-month chronic phase: In the female high dose group, mean terminal body weight was lower than the controls (-10 %, not statistically significant) but was unaffected at all dose levels tested in males and at the mid and low dose levels in females. In the high dose groups, mean absolute and relative liver weights were higher by between 39 to 54 % in females and by 17 to 18 % in males, when compared to the controls.

Table 97: Liver weight changes at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Mean absolute liver weight (g)	12.21 ± 1.33	12.70 ± 1.50 (+4 %)	12.43 ± 1.05 (+2 %)	14.31 * ± 1.92 (+17 %)	7.51 ± 0.99	7.37 ± 1.01 (-2 %)	7.67 ± 1.43 (+2 %)	10.44 ** ± 1.61 (+39 %)
Mean liver to body weight ratio	1.86 ± 0.12	1.93 ± 0.18 (+4 %)	1.88 ± 0.15 (+1 %)	2.19 ** ± 0.18 (+17 %)	2.17 ± 0.19	2.19 ± 0.22 (+1 %)	2.26 ± 0.20 (+4 %)	3.35 ** ± 0.33 (+54 %)

*: p≤0.05; **: p≤0.01

In the male high dose group, mean absolute and relative kidney weights were 28 % higher than the controls. In the female high dose group, mean kidney to body weight ratio was by 22 % higher than the controls.

Table 98: Kidney weight changes at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Mean absolute kidney weight (g)	3.51 ± 0.32	3.64 ± 0.33 (+4 %)	3.78 ± 0.23 (+8 %)	4.48 ** ± 0.70 (+28 %)	2.39 ± 0.24	2.40 ± 0.37 (0 %)	2.48 ± 0.37 (+4 %)	2.64 ± 0.36 (+10 %)
Mean kidney to body weight ratio	0.54 ± 0.03	0.55 ± 0.04 (+2 %)	0.58 ± 0.08 (+7 %)	0.69 ** ± 0.13 (+28 %)	0.70 ± 0.08	0.71 ± 0.09 (+1 %)	0.73 ± 0.08 (+5 %)	0.85 ** ± 0.08 (+22 %)

**: p≤0.01

In the female high dose group, mean absolute and relative thyroid gland weights were 23 to 38 % higher than the controls. This effect was associated with follicular cell hypertrophy at the microscopic examination.

Table 99: Thyroid gland weight changes at scheduled sacrifice of the chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Mean absolute thyroid gland weight (g)	0.0272 ± 0.0059	0.0280 ± 0.0036 (+3 %)	0.0244 ± 0.0066 (-10 %)	0.0295 ± 0.0059 (+8 %)	0.0201 ± 0.0052	0.0196 ± 0.0063 (-2 %)	0.0213 ± 0.0048 (+6 %)	0.0248 ± 0.0058 (+23 %)
Mean thyroid gland to body weight ratio	0. 0042 ± 0.0009	0.0043 ± 0.0006 (+3 %)	0.0038 ± 0.0012 (-10 %)	0.0045 ± 0.0008 (+9 %)	0.0058 ± 0.0013	0.0057 ± 0.0015 (-1 %)	0.0063 ± 0.0013 (+9 %)	0.0080 ** ± 0.0019 (+38 %)

**: p≤0.01

Organ weights – 24-month carcinogenicity phase: In the high dose groups, mean terminal body weight was 11 % lower in females and 7 % lower in males, compared to the controls (not statistically significant). Mean terminal body weight was unaffected at the mid and low doses in both sexes. In the female high dose group, mean absolute and relative liver weights were 39 to 56 % higher than the controls (statistically significant). This effect was associated with microscopic hepatocellular hypertrophy. In the male high and mid dose groups, mean absolute and/or relative liver weights were also slightly higher by between 5 to 12 %. This change was also considered to be treatment-related as it

was associated with hepatocellular hypertrophy at the microscopic examination. In the male low dose group, mean absolute and relative liver weights were 6 to 12 % lower, but these changes were judged not to be treatment-related in view of their low magnitude and in the absence of any associated change at the gross observation of microscopic examination. In high dose females kidney to body weight ratio and thyroid to body weight ratio were also significantly higher than in controls.

Table 100: Liver weight changes at scheduled sacrifice of the carcinogenicity phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Mean absolute liver weight (g)	12.56 ± 1.71	11.09 * ± 1.12 (-12 %)	14.02 * ± 2.06 (+12 %)	13.24 ± 2.25 (+5 %)	9.46 ± 2.19	9.69 ± 2.12 (+2 %)	9.89 ± 2.12 (+5 %)	13.16 ** ± 2.87 (+39 %)
Mean liver to body weight ratio	2.06 ± 0.33	1.94 ± 0.26 (-6 %)	2.22 ± 0.39 (+8 %)	2.32 ± 0.43 (+12 %)	2.37 ± 0.37	2.31 ± 0.30 (-3 %)	2.52 ± 0.35 (+6 %)	3.70 ** ± 0.59 (+56 %)

*: p≤0.05; **: p≤0.01

Gross pathology – 12-month chronic phase:

Unscheduled deaths: Two animals from the chronic phase were found dead before scheduled sacrifice: One control male (PT1M0678) was found dead on study Day 246, with blood at the brain surface and a mottled red thymus. One mid dose female (PT3F1032) died during anesthesia on study Day 81, with dark kidneys, small thymus, dark liver, white foci on spleen and a dilatation of uterine horns.

Terminal sacrifice: Treatment-related findings were found in liver and kidney. In the female high dose group, enlarged liver was found in 9/10 animals, dark liver in 8/10 animals and prominent lobulation on the liver in 4/10 animals, compared to no case in the controls. In the male high dose group, enlarged liver was found in 1/10 animals and prominent lobulation on the liver in 3/10 animals, compared to no case in the controls.

Table 101: Incidence of macroscopic changes in the liver, scheduled sacrifice of the chronic phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Obviously large	0/9	0/10	0/10	1/10	0/10	0/10	0/9	9/10
Dark	0/9	0/10	0/10	0/10	0/10	0/10	0/9	8/10
Prominent lobulation	0/9	0/10	0/10	3/10	0/10	0/10	0/9	4/10

In the female high dose group, dark kidneys were found in 8/10 animals, compared to no case in the controls. In the male high dose group, pale kidneys, enlarged kidneys or irregular surface on the kidneys were found in some animals, compared to no case in the controls.

Table 102: Incidence of macroscopic changes in the kidney, scheduled sacrifice of the chronic phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Dark	0/9	1/10	0/10	1/10	0/10	0/10	0/9	8/10
Pale	0/9	0/10	0/10	6/10	0/10	1/10	1/9	0/10
Obviously large	0/9	0/10	0/10	3/10	0/10	1/10	0/9	1/10
Irregular surface	0/9	0/10	1/10	3/10	0/10	0/10	0/9	0/10

Gross pathology – 24-month carcinogenicity phase: Treatment-related findings were noted in the liver and kidney.

Unscheduled deaths: Two hundred and seventy animals died before the end of the study. In the female high dose group, a higher incidence of enlarged liver, dark liver, white foci or red foci on the liver was observed, when compared to the controls. In addition, a liver nodule/mass was noted in one animal and correlated with a hepatocellular carcinoma noted at the microscopic examination. In the male high dose group, a higher incidence of enlarged liver and white foci on the liver was noted, when compared to the controls.

Table 103: Incidence of macroscopic changes in the liver, unscheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Nodule(s)/masse(s)	0/37	1/44	0/34	0/49	0/19	0/32	0/25	1/30
Obviously large	1/37	4/44	1/34	6/49	1/19	1/32	1/25	9/30
Dark	2/37	0/44	0/34	1/49	0/19	1/32	1/25	12/30
Focus (i), white	2/37	6/44	4/34	9/49	3/19	3/32	6/25	9/30
Focus (i), red	6/37	9/44	9/34	9/49	5/19	6/32	7/25	14/30

In the female high dose group, a higher incidence of dark kidneys, enlarged kidneys and irregular surface on the kidneys was observed, when compared to the controls. In the male high dose group, a higher incidence of enlarged kidneys and irregular surface on the kidneys was noted. In the male mid dose group, a higher incidence of enlarged kidneys was also observed.

Table 104: Incidence of macroscopic changes in the kidney, unscheduled sacrifices of the carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Dark	1/37	0/44	0/34	1/49	0/19	2/32	0/25	5/30
Obviously large	2/37	2/44	6/34	5/49	0/19	2/32	0/25	6/30
Irregular surface	3/37	2/44	4/34	8/49	1/19	2/32	1/25	7/30

Terminal sacrifice: In the female high dose group, a higher incidence of enlarged liver, dark liver, prominent lobulation and white foci on the liver was found when compared to the controls. In addition in this group, liver nodules/masses were noted and correlated with liver carcinoma or adenoma noted at the microscopic examination. The overall incidence (unscheduled deaths plus terminal sacrifice) of liver nodules/masses was 5/60 high dose females, compared to no case in the controls.

Table 105: Incidence of macroscopic changes in the liver, scheduled sacrifice of the carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Nodules/masses	0/23	0/16	0/26	1/11	0/41	1/28	2/35	4/30
Obviously large	1/23	0/16	2/26	0/11	6/41	1/28	7/35	28/30
Dark	0/23	0/16	1/26	2/11	0/41	1/28	1/35	19/30
Focus (i), white	4/23	1/16	4/26	3/11	14/41	7/28	9/35	22/30
Prominent lobulation	1/23	0/16	2/26	0/11	2/41	3/28	3/35	13/30

In the female high dose group, a higher incidence of dark kidneys, enlarged kidneys and irregular surface on the kidneys was observed, when compared to the controls. In the male high dose group, a higher rate of enlarged kidneys or irregular surface on the kidneys was noted.

Table 106: Incidence of macroscopic changes in the kidney, scheduled sacrifice of the carcinogenicity phase

Sex	**Males**				**Females**			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Dark	0/23	0/16	1/26	1/11	4/41	1/28	1/35	17/30
Obviously large	3/23	0/16	4/26	3/11	1/41	1/28	1/35	6/30
Irregular surface	5/23	0/16	7/26	8/11	1/41	2/28	3/35	6/30

Microscopic pathology – 12-month chronic phase:

Unscheduled deaths: One control male was found dead on Day 246; meningeal hemorrhage was considered to be the cause of death. One mid-dose female died during anesthesia on Day 81 without any significant microscopic findings explaining the cause of death.

Terminal sacrifice: Treatment-related non-neoplastic changes of fluopyram were found in the liver, kidney and thyroid gland.

Neoplastic findings: There was no evidence of a treatment-related effect on the incidence of neoplastic findings.

Non-neoplastic findings: In the liver of high dose females, a higher incidence of altered hepatocytes (eosinophilic foci), focal/multifocal hepatocellular vacuolation, increased number of mitoses, hepatocellular single cell necrosis and hepatocellular brown pigments was noted, when compared to the controls. In addition in this group, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed.

In the male high dose group, a higher incidence of altered hepatocytes (eosinophilic foci) was observed. In addition, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were observed. In the male mid dose group, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were noted. In the male low dose group, centrilobular to midzonal hepatocellular macrovacuolation was observed. Hepatocellular vacuolation or macrovacuolation noted in males at the three dose levels was also considered not to be adverse, as it was not associated with any degenerative change in the liver. No treatment-related changes were noted in the liver at the mid and low doses in females.

Table 107: Incidence and severity of microscopic changes in the liver, all animals of the chronic phase

Sex	**Males**				**Females**			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Number of animals examined	10	10	10	10	10	10	10	10
Focus(i) of hepatocellular alteration: eosinophilic: focal/multifocal								
Minimal	2	1	2	4	0	0	0	2
Slight	0	0	0	1	0	0	0	1
Total	2	1	2	5	0	0	0	3
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	0	0	2	5	0	0	0	0
Slight	0	0	1	5	0	0	0	2
Moderate	0	0	0	0	0	0	0	8
Total	0	0	3	10	0	0	0	10
Hepatocellular macrovacuolation: centrilobular to midzonal: diffuse								
Minimal	0	4	4	5	0	0	0	2
Slight	0	1	2	3	0	0	0	2
Moderate	0	0	0	1	0	0	0	3
Total	0	5	6	9	0	0	0	7
Hepatocellular vacuolation: focal/multifocal								
Minimal	0	4	0	2	2	2	2	3
Slight	0	1	0	0	0	0	0	1
Total	0	5	0	2	2	2	2	4
Increased number of mitoses								
Present	0	0	0	0	2	0	1	6
Total	0	0	0	0	2	0	1	6
Hepatocellular single cell necrosis: focal/multifocal								
Minimal	0	0	0	0	0	0	0	1
Slight	0	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	0	2
Hepatocellular brown pigment(s): focal/multifocal								
Minimal	0	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	0	1

In the kidney of high dose females, a higher incidence of tubular golden/brown pigments and of hyaline casts was noted, when compared to the controls. In the male high and mid dose groups, chronic progressive nephropathy was observed, together with a higher incidence of hyaline droplets. Chronic progressive nephropathy is a combination of thickened basement membranes (tubular and glomerular), basophilic tubules and hyaline casts with a variable inflammatory cell infiltrate. In addition in the male high dose group, a higher incidence of bilateral basophilic tubules was noted. No treatment-related changes were noted in the kidney at the mid dose in females or low dose in either sex.

Table 108: Incidence and severity of microscopic changes in the kidney, all animals of the chronic phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**1500**
Number of animals examined	10	10	10	10	10	10	10	10
Chronic progressive nephropathy: focal/multifocal								
Minimal	0	0	3	1	0	0	0	0
Slight	0	0	0	1	0	0	0	0
Total	0	0	3	2	0	0	0	0
Intratubular golden/brown pigments: focal/multifocal								
Minimal	0	3	1	0	1	2	1	4
Slight	0	0	0	0	0	0	0	1
Total	0	3	1	0	1	2	1	5
Hyaline droplets: proximal tubules								
Minimal	0	1	7	1	0	0	0	0
Slight	0	0	1	5	0	0	0	0
Moderate	0	0	0	4	0	0	0	0
Total	0	1	8	10	0	0	0	0
Basophilic tubules: bilateral: focal/multifocal								
Minimal	2	4	0	3	1	0	0	2
Slight	1	0	0	3	1	0	0	0
Total	3	4	0	6	2	0	0	2
Hyaline cast(s): focal/multifocal								
Minimal	2	1	0	1	1	0	1	4
Total	2	1	0	1	1	0	1	4

In the thyroid gland of high dose females and males, follicular cell hypertrophy was noted together with a higher incidence and severity of colloid alteration, when compared to the controls. In the male mid dose group, only follicular cell hypertrophy was observed. No treatment-related changes were noted in the thyroid gland at the mid dose in females or at the low dose in either sex.

Table 109: Incidence and severity of microscopic changes in the thyroid gland, all animals of the chronic phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	0	30	150	750	0	30	150	1500
Number of animals examined	10	10	10	10	10	10	10	10
Follicular cell hypertrophy: diffuse								
Minimal	0	0	2	2	0	0	0	4
Slight	0	0	0	2	0	0	0	1
Total	0	0	2	4	0	0	0	5
Colloid alteration								
Minimal	4	4	3	2	1	0	0	3
Slight	0	1	1	2	0	0	0	1
Moderate	0	0	0	3	0	0	0	0
Total	4	5	4	7	1	0	0	4

Microscopic pathology – 24-month carcinogenicity phase:

Unscheduled deaths: Increased mortality was observed in the male high dose group (28/49 animals found dead). No clear cause of death could be established for most of them.

Terminal sacrifice plus unscheduled deaths (i.e. all animals): Major treatment-related effects of fluopyram were found in the liver, kidney, thyroid gland and eye.

Neoplastic findings: At the end of the carcinogenicity phase, a higher incidence of tumors in the liver (carcinoma and adenoma) was noted in the female high dose group only, in comparison to the controls.

These findings were associated with non-neoplastic/preneoplastic changes and were seen at a dose causing marked hepatocellular toxicity. There was no evidence of a treatment-related increased incidence of tumors of any type in any other organ.

Table 110: Incidence of microscopic neoplastic changes in the liver, all animals of the carcinogenicity phase

Sex	**Males**				**Females**			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	58	60	60	60	59
M- Hepatocellular carcinoma								
Incidental	0	0	0	0	0	0	2	3
Total	0	0	0	0	0	0	2	3
B-Hepatocellular adenoma								
Incidental	2	1	2	1	2	2	0	9
Total	2	1	2	1	2	2	0	9*
Hepatocellular adenoma + carcinoma								
Total	2	1	2	1	2	2	2	11+**

+ One animal had both adenoma and carcinoma; *: $p \leq 0.05$, **: $p \leq 0.01$

Non-neoplastic findings: In the liver of high dose females, marked effects of the treatment indicative of toxicity were noted, including metabolic, degenerative and proliferative changes. They correspond to the exacerbation of microscopic findings observed after the chronic phase.

Proliferative changes like altered hepatocytes (eosinophilic foci), clear cell foci and multinucleated hepatocytes with anisocaryosis or increased number of mitoses were associated with metabolic morphological changes: centrilobular to panlobular hypertrophy, focal/multifocal hepatocellular vacuolation, centrilobular to midzonal hepatocellular macrovacuolation, brown pigments in Kupffer cells or hepatocellular brown pigments. Degenerative change (hepatocellular single cell necrosis) was also noted. In addition, minimal to slight extramedullary hematopoiesis was observed in the liver in this group.

In the male high and mid dose groups, treatment-related effects (seen also in the female high dose group) were noted. In a similar way, these changes result from exacerbation of findings noted at the end of the chronic phase. In the male high dose group, a higher incidence of altered hepatocytes (eosinophilic foci) was observed. In addition, centrilobular to panlobular hypertrophy and centrilobular to midzonal hepatocellular macrovacuolation were noted. In the male mid dose group, only eosinophilic foci and centrilobular to panlobular hypertrophy were observed. No treatment-related changes were noted in the liver at the mid dose in females or at the low dose in either sex.

Table 111: Incidence and severity of microscopic changes in the liver, all animals of the carcinogenicity phase

Sex	Males				Females			
Dose level fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	58	60	60	60	59
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	1	1	14	15	0	0	0	6
Slight	0	0	0	15	0	0	0	21
Moderate	0	0	0	0	0	0	0	21
Total	1	1	14**	30**	0	0	0	48**
Focus(i) of hepatocellular alteration: clear: focal/multifocal								
Minimal	8	6	6	11	0	3	3	8
Slight	2	2	1	5	1	1	1	3
Total	10	8	7	16	1	4	4	11**
Focus(i) of hepatocellular alteration: eosinophilic: focal/multifocal								
Minimal	12	20	18	15	23	17	14	25
Slight	4	3	10	12	6	7	15	13
Moderate	0	1	2	1	0	2	1	7
Marked	0	0	1	0	0	0	0	3
Total	16	24	31*	28**	29	26	30	48**
Hepatocellular vacuolation: focal/multifocal								
Minimal	10	4	13	7	5	10	7	13
Slight	0	2	3	0	1	4	2	9
Moderate	0	1	0	0	0	0	0	0
Total	10	7	16	7	6	14**	9	22**

*: p≤0.05, **: p≤0.01

Table 112: Incidence and severity of microscopic changes in the liver, all animals of the carcinogenicity phase (part 2/2)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	58	60	60	60	59
Increased number of mitoses								
Present	0	0	0	0	6	1	5	33
Total	0	0	0	0	6	1	5	33**
Multinucleated hepatocytes with anisocaryosis								
Present	1	0	1	0	4	2	6	38
Total	1	0	1	0	4	2	6	38**
Hepatocellular single cell necrosis: focal/multifocal								
Minimal	2	0	1	1	0	3	1	25
Slight	0	0	1	0	0	1	0	12
Total	2	0	2	1	0	4	1	37**
Hepatocellular brown pigment(s): focal/multifocal								
Minimal	0	0	0	0	1	1	2	22
Slight	0	0	0	0	0	0	0	2
Total	0	0	0	0	1	1	2	24**
Accumulation of brown pigments in Kupffer cells: focal/multifocal								
Minimal	6	2	9	5	8	7	9	27
Slight	1	0	1	2	4	3	2	4
Moderate	0	0	0	0	0	0	0	1
Total	7	2	10	7	12	10	11	32**
Hepatocellular macrovacuolation: centrilobular to midzonal: diffuse								
Minimal	0	0	0	9	0	0	0	5
Slight	0	0	2	1	0	0	0	4
Moderate	0	0	0	0	0	0	0	2
Total	0	0	2	10**	0	0	0	11**
Extramedullary hematopoiesis: multifocal								

Minimal	16	7	10	15	17	24	21	30
Slight	1	3	1	1	2	1	3	3
Moderate	1	0	0	0	0	0	0	0
Total	18	10	11	16	19	25	24	33**

**: p≤0.01

In the kidney, marked degenerative changes resulting from exacerbation of the microscopic findings noted at the end of the chronic phase were observed in both sexes at the high dose and in the male at the mid dose. These changes were mainly characterized by an increased incidence and severity of chronic progressive nephropathy. Specific findings (tubular hyperplasia, tubular dilatation or renal cysts) were judged to be associated with chronic nephropathy. In addition in the female high dose group, an increased incidence of tubular golden/brown pigments and collecting ducts hyperplasia was noted. Brown pigments were also previously noticed in the liver in this group. In the male high dose group, a higher incidence of tubular hypertrophy, collecting ducts hyperplasia and hyaline droplets was also noted. In the male mid dose group, a higher incidence of tubular hypertrophy was also noted. No treatment-related changes were noted in the kidney at the mid dose in females or at the low dose in either sex.

Table 113: Incidence and severity of microscopic changes in the kidney, all animals of the carcinogenicity phase

Sex	**Males**				**Females**			
Dose level fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	60	60	60	59	60
Chronic progressive nephropathy: focal/multifocal								
Minimal	17	24	16	11	17	12	11	19
Slight	7	8	10	18	3	7	11	13
Moderate	5	2	11	15	0	3	1	7
Marked	1	1	4	8	0	2	0	3
Severe	1	2	1	0	0	0	0	0
Total	31	37	42	52**	20	24	23	42**
Tubular hyperplasia: focal/multifocal								
Minimal	5	1	5	8	2	5	3	4
Slight	0	1	3	2	0	0	1	1
Moderate	0	0	0	1	0	0	0	0
Total	5	2	8	11*	2	5	4	5
Collecting ducts hyperplasia: unilateral: focal/multifocal								
Minimal	2	1	3	8	1	1	0	4
Slight	3	1	2	1	1	1	0	2
Moderate	0	0	0	0	0	0	0	2
Total	5	2	5	9	2	2	0	8
Tubular hypertrophy: focal/multifocal								
Minimal	5	8	18	17	7	13	11	8
Slight	4	3	6	2	2	3	2	4
Total	9	11	24**	19**	9	16	13	12

*: p≤0.05, **: p≤0.01

Table 114: Incidence and severity of microscopic changes in the kidney, all animals of the carcinogenicity phase (part 2/2)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	60	60	60	59	60
Intratubular golden/brown pigments: focal/multifocal								
Minimal	4	3	4	7	22	16	17	17
Slight	0	0	0	1	10	3	7	32
Moderate	0	0	0	0	0	0	0	8
Total	4	3	4	8	32	19	24	57**
Hyaline droplets: proximal tubules								
Minimal	0	1	1	5	1	1	0	1
Slight	2	0	0	4	0	1	1	1
Moderate	1	0	0	1	1	0	0	0
Marked	0	1	0	0	0	0	0	0
Total	3	2	1	10	2	2	1	2
Cortical tubular dilatation: focal/multifocal								
Minimal	6	2	9	17	4	0	6	7
Slight	3	3	5	11	0	4	1	6
Moderate	1	0	1	0	0	1	0	3
Total	10	5	15	28**	4	5	7	16**
Medullary tubular dilatation: focal/multifocal								
Minimal	5	12	8	21	11	4	10	9
Slight	4	2	13	17	2	3	2	11
Moderate	1	1	1	2	0	1	0	2
Total	10	15	22*	40**	13	8	12	22*
Renal cyst(s): focal/multifocal								
Present	7	3	6	13	2	2	3	3
Total	7	3	6	13*	2	2	3	3

*: $p \leq 0.05$, **: $p \leq 0.01$

In the thyroid gland, in the male and female high dose groups, exacerbation of the microscopic findings noted at the end of the chronic phase was observed: follicular cell hypertrophy together with a higher incidence of colloid alteration and a slightly higher number of follicular cell hyperplasia. At the mid dose, follicular cell hypertrophy was observed in males and a higher incidence of colloid alteration was noted in females. No treatment-related changes were noted in the thyroid gland at the low dose in either sex.

Table 115: Incidence and severity of microscopic changes in the thyroid gland, all animals of the carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	58	59	57	54	60	60	60	58
Follicular cell hyperplasia: focal/multifocal								
Minimal	0	0	0	2	1	1	0	2
Slight	0	0	0	1	1	1	1	2
Moderate	1	0	0	0	0	0	0	0
Marked	0	0	1	0	0	0	0	0
Total	1	0	1	3	2	2	1	4
Follicular cell hypertrophy: diffuse								
Minimal	1	0	3	5	0	0	1	7
Slight	0	0	1	2	0	0	0	4
Moderate	0	0	0	0	0	0	0	1
Total	1	0	4	7**	0	0	1	12**
Colloid alteration								
Minimal	18	5	12	10	5	6	10	17
Slight	8	9	16	20	2	1	5	15
Moderate	2	1	4	9	0	0	1	3
Marked	0	0	0	0	0	0	0	1
Total	28	15	32	39**	7	7	16*	36**

*: p≤0.05, **: p≤0.01

In the eye of high dose females, bilateral retinal atrophy was observed, together with a higher incidence of lens degeneration and peripheral bilateral retinal atrophy, when compared to the controls. Retinal atrophy was characterized by degeneration of the outer plexiform layer, outer nuclear layer and rod/cones lamina. No treatment-related changes were noted in the eye at all dose levels tested in males and at the mid and low doses in females.

Table 116: Incidence and severity of microscopic changes in the eye, all animals of the carcinogenicity phase

Sex	Males:				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750/375**	**0**	**30**	**150**	**1500**
Number of animals examined	60	60	60	59	60	60	60	60
Retinal atrophy: bilateral: diffuse								
Minimal	0	0	0	0	0	0	0	2
Slight	0	0	0	0	0	0	1	4
Moderate	0	0	1	0	0	0	0	19
Marked	0	0	0	0	0	0	0	2
Total	0	0	1	0	0	0	1	27**
Lenticular degeneration : focal								
Minimal	2	0	0	0	2	0	1	2
Slight	0	0	0	2	0	2	1	4
Moderate	0	0	1	0	0	1	2	3
Marked	0	1	0	0	1	0	0	0
Total	2	1	1	2	3	3	4	9*
Peripheral retinal atrophy: bilateral								
Minimal	1	0	0	1	3	0	1	3
Slight	0	0	0	0	0	1	0	2
Moderate	0	0	0	0	0	0	0	2
Total	1	0	0	1	3	1	1	7

*: p≤0.05, **: p≤0.01

Some indirect effects were noted in the testis and stomach: In the testis, a higher incidence of arteritis/periarteritis was noted in the male high and mid dose groups, when compared to the controls

(15/59 and 18/60, respectively, versus 8/60 in controls, p≤0.05). This vascular change was isolated (not found in sensitive tissues like aorta, mesenteric arteries) and is most likely explained by secondary hypertensive changes due to increased severity and incidence of chronic nephropathy. Therefore, this change was considered not to be a direct effect of the treatment.

In the stomach of high dose males, a higher incidence of regenerative non glandular hyperplasia: focal/multifocal (10/58 versus 6/58 in controls), non glandular erosion: focal/multifocal (7/58 versus 3/58 in controls) and submucosal edema (10/58 versus 4/58 in controls, (p≤0.05) was noted. These minor changes were mainly observed in animals found prematurely dead and were attributed to secondary stress due to morbidity. Therefore, their increased incidence was explained by the increased mortality rate in this male high dose group.

Conclusion

In a 2-year feeding study with fluopyram in Wistar rats, 30 ppm proved the No Observed Adverse Effect Level (NOAEL) in both males (equivalent to 1.2 mg/kg body weight/day) and females (equivalent to about 1.7 mg/kg body weight/day) due to non-neoplastic findings in liver, kidney and thyroid gland in the next higher dose level of 150 ppm. In addition, the eye was identified as a target organ but only at higher dose level. It was remarkable that, over the whole study period, male rats could be fed only about one third of the dose that could be administered to females. At higher dose level, a marked increase in mortality among the male animals clearly showed that the MTD was exceeded in this sex. At the top dose level of 1500 ppm, there was a treatment-related increase in the incidence of liver cell tumors (carcinoma plus adenoma) in females. In males, carcinogenicity was not obtained but testing was feasible until a dose level of 750 (first year) or 375 ppm (second year) only.

Carcinogenicity in mice

Title:	Wason, S. M. (2007): AE C656948, Carcinogenicity study of AE C656948 in the C57BL/6J mouse by dietary administration, SA 05094, M-295688-01, ASB2008-5440.
Guidelines:	OECD 451 (1981); EEC Directive 88/302/EEC – Annex V - Method B.32. (1987).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.5 % (Jan. 2005) to 94.7 % (May 2007)
Vehicle or positive control:	None
Stability of test compound:	Stable in rodent diet at 20 and 10000 ppm over a 105-day period at ambient temperature or over a 95-day freezing period followed by 10 days at ambient temperature

Species:	Mouse
Strain:	C57BL/6J
Age / weight:	6 weeks approx.; 20.7-21.1 g for male mean group weight 17.6-17.9 g for female mean group weight at dosing
Source/breeder:	Charles River Laboratories, St Germain-sur-l'Arbresle, France
Acclimation period:	13 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages. During the first week of acclimatization, the animals were housed by sex in groups of 3.
Environmental conditions :	Temperature: 22 ± 2°C Humidity: 55 ± 15 % Air changes: Approximately 10-15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum* except during designated time period
Water:	Tap water, *ad libitum* except during designated time period

Animal assignment and treatment: On the day of randomization, animals were allocated to dose groups using a computerized randomization procedure that ensured a similar body weight distribution for each sex. The acceptable body weight range for each sex was ± 20 % of the mean body weight on the day of randomization. Any animal deemed unsuitable for selection based on weight, clinical findings or health status was not used for the study. Animals were assigned to the test groups noted in the following table. Control animals received untreated diet. All other groups received the appropriate dietary concentrations at a constant (ppm) level.

Table 117: Test groups used in the study

Test group	Concentration in diet (ppm)	Dose per animal (week period 1 to 80)		Animals assigned/sex	
		Male (mg/kg bw/day)	Female (mg/kg bw/day)	Main study 78 weeks	Interim sacrifice 52 weeks
1	0	0	0	50	10
2	30	4.2	5.3	50	10
3	150	20.9	26.8	50	10
4	750	105	129	50	10

On Day 6 of the acclimatization phase, each animal was uniquely identified by an implant from Reseaumatique (Conches, France).

Dose selection: The dose levels had been selected based on the basis of the previous 28-day and 90-day dietary studies in the mouse (see 4.7.1.1) revealing an NOAEL of 150 ppm and a NOEL of 30 ppm.

Diet preparation and analysis: Fluopyram was incorporated into the diet to provide the required dietary concentrations. The test substance formulations were prepared approximately every 8 weeks. When not in use, the diet formulations were stored at ambient temperature. The stability of the test substance in the diet has been demonstrated in a previous 28-day rat study (see 4.7.1.1), where fluopyram was found to

be stable in the diet at 20 and 10000 ppm over a 105-day period at ambient temperature, and also for 95-days when stored frozen and then kept for 10 days at ambient temperature.

The homogeneity of the test substance in diet was verified at 30, 150 and 750 ppm on the first formulation and at 30 and 750 ppm on the sixth formulation, to demonstrate adequate formulation procedures. The mean value obtained from the homogeneity check was taken as measured concentration. In addition, the concentration at each dietary level was verified prior to administration to the animals for the following formulations: F1, F3, F6, F9 and F10 (at 30 and 750 ppm only).

Statistics: Statistical analysis was performed as described above (and at 4.7.1.1).

Clinical signs, mortality and body weight parameters: Clinical signs, mortality and body weight parameters were checked as described above (and at 4.7.1.1).

Food consumption and compound intake: Food consumption was recorded weekly during the first 13 weeks of treatment, and once approximately every 4 weeks thereafter. The weekly mean achieved dosage intake in mg/kg body weight/day for Weeks 1 to 13, then 1 week per month thereafter was calculated as described in the rat study described above. The monthly and overall mean achieved dosage intake for the 18 months of treatment were derived from the weekly data.

Hematology: Blood was sampled from Isoflurane anaesthetized animals by puncture of the retro-orbital venous plexus after overnight fasting. Blood was collected in tubes containing EDTA (0.5 mL). At terminal sacrifice, blood smears were prepared for all animals not sampled for hematology. When possible, a blood smear was prepared for the moribund animals, just before sacrifice.

Hematology was performed on all the surviving animals of the interim sacrifice groups and on the first ten surviving animals of the terminal sacrifice groups on Weeks 53 or 54. Hematology was performed on the first twenty surviving suitable mice of the terminal sacrifice groups on Weeks 53 or 54 and prior to necropsy on Week 79 or 80. The following parameters were measured: hematocrit, hemoglobin, leukocyte count, erythrocyte count, platelet count, leukocyte differential count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume. A blood smear was prepared and stained with Wright stain or May-Grünwald-Giemsa stain. It was examined only when the results of Advia 120 determinations were abnormal. For moribund and terminal sacrificed animals, the blood smears were stained with Wright stain or May-Grünwald-Giemsa stain for possible differential white blood cell determination.

Clinical chemistry, urinalysis, opthalmology: Not conducted in this study. These parameters are not required for carcinogenicity studies based on Guideline OECD 451.

Sacrifice and pathology: On study Days 366 to 368 for the 12-month interim kill, and on study Days 549 to 562 for the carcinogenicity phase, all surviving animals dedicated to the interim sacrifice group and carcinogenicity phase group, respectively, were sacrificed by exsanguination under deep anesthesia (Isoflurane). Animals were fasted overnight prior to sacrifice. All animals, including animals at scheduled sacrifice, found dead, or sacrificed during the course of the study, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. All significant macroscopic findings were recorded. Adrenal gland, brain, epididymides, heart, kidney, liver, ovary, spleen, testis, and uterus (including cervix) were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

The following organs or tissues were sampled: adrenal gland, aorta, articular surface (femoro-tibial), bone (sternum), bone marrow (sternum), brain, epididymis, esophagus, exorbital (lachrymal) gland, eye and optic nerve, gall bladder, Harderian gland, heart, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidney, larynx/pharynx, liver, lung, lymph nodes (submaxillary, mesenteric), mammary gland, nasal cavities, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord (cervical, thoracic, lumbar), spleen, stomach, submaxillary (salivary) gland, testis, thymus, thyroid gland (with parathyroid), tongue, trachea, urinary bladder, uterus (with cervix), vagina. Duplicate femoral bone marrow smears were prepared from sacrificed animals (except

on weekends and public holidays), one of which was stained with May-Gruenwald-Giemsa, but not examined as no relevant changes were observed in hematology or bone marrow histology. The second smear was stored unstained for possible examination. Tissues 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.

Histopathological examinations were performed on the thyroid gland in males only at the interim sacrifice. At the final sacrifice, histopathological examinations were performed on all organs and tissues embedded, including gross abnormalities, from all animals in all groups including decedents.

Findings

Diet preparation analysis: Homogeneity checks were between 85 to 107 % of nominal concentration and concentration checks were between 92 to 99 % of nominal concentration. Results were within the in-house target range of 85 to 115 % of nominal concentration and were therefore considered to be acceptable. Results are presented in the following table.

Table 118: Homogeneity and concentration of test material

	Nominal concentrations (ppm)	Analysed concentrations (ppm)	% of nominal concentration
Homogeneity (first preparation)	30	H1 27.2 H2 28.4 H3 27.9 M1 27.3 M2 27.6 M3 32.0 B1 29.4 B2 30.4 B3 31.9	91 95 93 91 92 107 98 101 106
	150	H1 156 H2 144 H3 140 M1 148 M2 145 M3 146 B1 151 B2 144 B3 144	104 96 93 99 97 97 101 96 96
	750	H1 731 H2 734 H3 735 M1 758 M2 742 M3 750 B1 737 B2 736 B3 725	97 98 98 101 99 100 98 98 97
Homogeneity (sixth preparation)	30	H1 197 H2 194 H3 193 M1 192 M2 193 M3 191 B1 194 B2 188 B3 190	93 90 105 100 85 96 107 93 94
	750	H1 753 H2 738 H3 736 M1 692 M2 729 M3 719 B1 696 B2 732 B3 732	100 98 98 92 97 96 93 98 98
Concentration (first formulation)	30 150 750	29.12 146.4 738.7	97 98 98
Concentration (third formulation)	30 150 750	27.6 143 693	92 95 92
Concentration (sixth formulation)	30 150 750	28.76 144 725.2	96 96 97
Concentration (ninth formulation)	30 150 750	29.8 145 725	99 97 97
Concentration (tenth formulation)	30 750	27.8 726	93 97

Mortality: There were no treatment-related mortalities at any dose level in either sex during the course of the study. During the first 53 weeks of the study, the mortality rate was low with no indication of a treatment-related effect.

Table 119: Mortality incidence of all animals during the first 53 weeks (Unscheduled deaths up to study Day 371)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	60	60	60	60	60	60	60	60
Killed for humane reasons	1 (1.7 %)	3 (5.0 %)	2 (3.3 %)	2 (3.3 %)	3 (5.0 %)	6 (10.0 %)	7 (11.7 %)	4 (6.7 %)
Found dead	-	-	-	-	-	1 (1.7 %)	-	1 (1.7 %)
Accidental trauma	1 (1.7 %)	-	-	-	-	-	-	-
Total number of unscheduled deaths	2 (3.3 %)	3 (5.0 %)	2 (3.3 %)	2 (3.3 %)	3 (5.0)	7 (11.7 %)	7 (11.7 %)	5 (8.3 %)

Percentage mortality in parentheses. No statistical analysis was done on this data

During the whole study period (at least 78 weeks), the mortality rate in animals allocated to the carcinogenicity phase of the study was very similar between the treated and control groups, with no indication of a statistically significant effect.

Table 120: Mortality incidence of animals of the carcinogenicity phase (Unscheduled deaths – whole study period)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	50	50	50	50	50	50	50	50
Killed for humane reasons	4 (8 %)	5 (10 %)	3 (6 %)	5 (10 %)	9 (18 %)	11 (22 %)	10 (20 %)	9 (18 %)
Found dead	2 (4 %)	4 (8 %)	6 (12 %)	3 (6 %)	2 (4 %)	2 (4 %)	-	3 (6 %)
Total number of unscheduled deaths	6 (12 %)	9 (18 %)	9 (18 %)	8 (16 %)	11 (22 %)	13 (26 %)	10 (20 %)	12 (24 %)

Percentage mortality in parentheses

Clinical signs of toxicity and neurotoxicity assessment: There were no treatment-related clinical signs observed at any dose level in either sex throughout the course of the study.

Body weight and body weight gain: At 750 ppm in males, mean body weight was comparable to controls from week 1 to 26. Between weeks 30 (study Day 204) to 58 (study Day 400) of the study mean body weight was reduced by up to 5 %, and mean cumulative body weight gain by up to 13 % over this period from the start of treatment. The effect was statistically significant at most time points during this period ($p \leq 0.05$ or 0.01). Thereafter, mean body weight and mean cumulative body weight gain were comparable to controls until the end of the study. Body weight parameters were affected in females at 750 ppm at only one occasion (reduced body weight gain at Weeks 14 to 26). At 150 or 30 ppm there were only minor effects (reduction and increase), that however became significant at some time points but were considered not to be of toxicological relevance.

Table 121: Mean body weights (BW) and body weight gains (BWG) (g)

Fluopyram dosage level (ppm)	0	30	150	750
Males				
Initial BW (Day 1) (%C)	20.8 ± 1.1	21.1 ± 1.0 (101)	20.8 ± 0.9 (100)	20.7 ± 1.1 (100)
BW Week 2 (Day 8) (%C)	21.9 ±1.1	21.9 ± 0.9 (100)	22.0 ± 1.1 (100)	21.7 ± 1.1 (99)
BW Week 14 (Day 92) (%C)	27.4 ± 1.5	27.9 ± 1.3 (102)	27.9 *± 1.1 (102)	27.5 ± 1.1 (100)
BW Week 26 (Day 176) (%C)	28.8 ± 1.8	29.4 ± 1.6 (102)	29.1 ± 1.5 (101)	28.4 ± 1.3 (99)
BW Week 54 (Day 372) (%C)	31.3 ±1.9	31.5 ± 1.4 (101)	30.8 ± 1.7 (98)	29.8 ** ± 1.3 (95)
Final BW Week 78 (Day 540) (%C)	31.8 ± 1.7	32.2 ± 1.6 (101)	31.6 ± 1.8 (99)	31.1 ± 1.8 (98)
BWG Weeks 1-2 (Days 1 to 8) (%C)	1.0 ± 0.6	0.8 ± 0.5 (80)	1.2 ± 0.7 (120)	1.0 ± 0.5 (100)
BWG Weeks 1-14 (Days 1 to 92) (%C)	6.5 ± 0.9	6.8 ± 0.8 (105)	7.1 **± 0.9 (109)	6.8 ± 0.9 (105)
BWG Weeks 14-26 (Days 92 to 176) (%C)	1.5 ± 1.1	1.5 ± 0.7 (100)	1.1 ± 1.0 (73)	0.8 **± 0.9 (53)
BWG Weeks 26-54 (Days 176 to 372) (%C)	2.5 ± 0.8	2.1 ± 0.8 (84)	1.8**± 0.8 (72)	1.3** ± 0.9 (52)
BWG Weeks 54-78 (Days 372 to 540) (%C)	0.3 ± 0.8	0.7 ± 1.1 (233)	0. 8*± 0.9 (267)	1.3 ** 0.9 (433)
Overall BWG Weeks 1-78 (Days 1 to 540) (%C)	10.9 ± 1.4	11.1 ± 1.4 (102)	10.8 ± 1.6 (99)	10.4 ± 1.5 (95)
Females				
Initial BW (Day 1) (%C)	17.9 ± 0.9	17.7 ± 0.8 (99)	17.7 ± 0.9 (99)	17.6 ± 0.9 (98)
BW Week 2 (Day 8) (%C)	18.3 ± 0.8	18.5 ± 0.8 (101)	18.1 ± 0.9 (99)	18.1 ± 0.9 (99)
BW Week 14 (Day 92) (%C)	22.5 ± 1.0	22.9 ± 1.1 (102)	22.7 ± 0.9 (101)	22.7 ± 0.9 (101)
BW Week 26 (Day 176) (%C)	24.2 ±1.3	24.5 ± 1.7 (101)	24.3 ± 1.1 (100)	23.7 ± 0.9 (98)
BW Week 54 (Day 372) (%C)	26.7 ± 2.0	26.8 ± 2.3 (100)	26.7 ± 1.9 (100)	25.8 ± 1.3 (97)
Final BW Week 78 (Day 540) (%C)	27.1 ± 1.8	27.8 ± 2.1 (103)	27.6 ± 1.7 (102)	27.0 ± 1.4 (100)
BWG Weeks 1- 2 (Days 1 to 8) (%C)	0.4 ± 0.4	0.8 **± 0.6 (200)	0.4 ± 0.7 (100)	0.5 ± 0.7 (125)
BWG Weeks 1-14 (Days 1 to 92) (%C)	4.6 ± 0.7	5.2 **± 1.0 (113)	5.0 * ± 1.0 (109)	5.1 ** ± 0.9 (111)
BWG Weeks 14-26 (Days 92 to 176) (%C)	1.7 ± 0.9	1.7 ± 1.1 (100)	1.6 ± 0.6 (94)	1.0 **± 0.6 (59)
BWG Weeks 26-54 (Days 176 to 372) (%C)	2.7 ± 1.3	2.3 ± 1.1 (85)	2.5 ± 1.2 (93)	2.1 ± 0.9 (78)
BWG Weeks 54-78 (Days 372 to 540) (%C)	0.6 ± 1.3	0.9 ± 1.4 (150)	1.1 ± 1.0 (183)	1.0 ± 1.1 (167)
Overall BWG Weeks (Days 1 to 540) (%C)	9.4 ± 1.6	10.0 ± 1.9 (106)	9.8 ± 1.6 (104)	9.4 ± 1.4 (100)

Food consumption: Food consumption was not affected by treatment at any dose level in either sex, throughout the course of the study.

Achieved dosage: The groups mean test material intakes are presented in Table 122 below.

Table 122: Mean achieved dietary intake of fluopyram (mg/kg/day)

Sex	Males			Females		
Dose level of fluopyram (ppm)	**30**	**150**	**750**	**30**	**150**	**750**
Weeks 1-13	5.1	25.5	128	6.4	32.0	156
Weeks 1-52	4.4	22.2	112	5.7	28.6	138
Weeks 1-80	4.2	20.9	105	5.3	26.8	129

Hematological findings: Slightly higher mean platelet counts were noted at 750 ppm in males, at Month 13 (+25 %, p≤0.01) and Month 19 (+22 %, p≤0.01).

Clinical chemistry findings: Not evaluated in this study.

Organ weight – 12-month interim sacrifice: There was no relevant change in terminal body weight of treated animals when compared to control animals. At 750 ppm and 150 ppm, mean absolute and relative liver weights were statistically significantly higher when compared to controls, in both sexes. Liver weight changes were found to be dose-related. No effect was seen on liver weight at 30 ppm.

Table 123: Liver weight changes at scheduled sacrifice, chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute liver weight (g)	1.26 ± 0.10	1.24 ± 0.09 (-2 %)	1.40* ± 0.15 (+11 %)	1.47** ± 0.13 (+17 %)	1.09 ± 0.13	1.07 ± 0.18 (-2 %)	1.31** ± 0.12 (+20 %)	1.40** ± 0.14 (+28 %)
Mean liver to body weight ratio (%)	4.53 ± 0.366	4.52 ± 0.232 (0 %)	5.20** 0.503 (+15 %)	5.68** 0.235 (+25 %)	4.70 ± 0.404	4.61 ± 0.688 (-2 %)	5.51** ± 0.365 (+17 %)	6.12** ± 0.325 (+30 %)

*: p≤0.05;**: p≤0.01

At 750 ppm, mean absolute and relative kidney weights were statistically significantly lower when compared to controls in both sexes. No effect on kidney weights was seen at 30 or 150 ppm in either sex.

Table 124: Kidney weight changes at scheduled sacrifice, chronic phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute kidney weight (g)	0.553 ± 0.064	0.538 ± 0.061 (-3 %)	0.554 ± 0.063 (0 %)	0.460* ± 0.077 (-17 %)	0.398 ± 0.056	0.385 ± 0.028 (-3 %)	0.388 ± 0.037 (-3 %)	0.342** ± 0.032 (-14 %)
Mean kidney to body weight ratio (%)	1.99 ± 0.173	1.96 ± 0.195 (-2 %)	2.06 ± 0.168 (+3 %)	1.78* ± 0.191 (-11 %)	1.72 ± 0.243	1.67 ± 0.134 (-3 %)	1.63 ± 0.131 (-5 %)	1.49** ± 0.083 (-13 %)

*: p≤0.05;**: p≤0.01

Organ weight – 18-month carcinogenicity phase: There was no relevant change in terminal body weights of treated males and females when compared to control animals. Mean absolute and relative liver weights were statistically significantly higher when compared to controls in both sexes at 750 ppm and 150 ppm. Mean absolute and relative liver weights were slightly higher when compared to controls in males at 30 ppm but no concomitant microscopic hepatocellular hypertrophy was noted. Therefore liver weight changes were considered to be adverse at 750 and 150 ppm only.

Table 125: Liver weight changes at terminal sacrifice, carcinogenicity phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute liver weight (g)	1.17 ± 0.12	1.26* ± 0.11 (+8 %)	1.34** ± 0.13 (+15 %)	1.49** ± 0.16 (+27 %)	1.26 ± 0.17	1.31 ± 0.16 (+4 %)	1.45** ± 0.21 (+15 %)	1.70** ± 0.23 (+35 %)
Mean liver to body weight ratio (%)	4.23 ± 0.358	4.50** ± 0.352 (+6 %)	4.83** ± 0.358 (+14 %)	5.53** ± 0.363 (+31 %)	5.24 ± 0.473	5.36 ± 0.387 (+2 %)	5.92** ± 0.602 (+13 %)	7.14** ± 0.957 (+36 %)

*: p≤0.05;**: p≤0.01

Mean absolute and relative kidney weights were statistically significantly lower when compared to controls in males at 750 ppm. These changes were not considered adverse since there were not accompanied by relevant microscopic findings. Mean kidney to body weight ratio was statistically significantly lower when compared to controls in females at 750 ppm. This change was considered to be treatment-related as it was associated with relevant microscopic findings in the kidney.

Table 126: Kidney weight changes at terminal sacrifice, carcinogenicity phase (% change when compared to controls)

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Mean absolute kidney weight (g)	0.542 ± 0.0501	0.557 ± 0.063 (+3 %)	0.556 ± 0.058 (+3 %)	0.491** ± 0.055 (-9 %)	0.432 ± 0.053	0.447 ± 0.050 (+3 %)	0.435 ± 0.038 (+1 %)	0.408 ± 0.043 (-6 %)
Mean kidney to body weight ratio (%)	1.95 ± 0.111	1.99 ± 0.181 (+2 %)	2.01 ± 0.155 (+3 %)	1.82** ± 0.156 (-7 %)	1.80 ± 0.131	1.83 ± 0.144 (+2 %)	1.78 ± 0.107 (-1 %)	1.71** ± 0.146 (-5 %)

**: p≤0.01

Mean absolute and relative heart weights were statistically significantly higher when compared to controls in females at 750 ppm (+ 17 to 19 %, p≤0.01). Mean heart to body weight ratio was statistically significantly higher when compared to controls in males at 750 ppm (+8 %, p≤0.05). In addition mean absolute and relative adrenal gland weights were statistically significantly higher when compared to controls in females at 750 ppm (+ 23 to 26 %, p≤0.01). Mean adrenal gland to body weight ratio was statistically significantly higher when compared to controls in males at 750 ppm (+24 %, p≤0.05). Unfortunately, no clinical chemistry data to support or disprove a causal connection between adrenal gland hypertrophy and cardial hypertrophy due to increased hormone synthesis by the adrenal gland are available in this study. Therefore at this stage no final judgment whether these changes were adverse effects or not could be made. In any case, they were confined to the top dose level. The results observed in the adrenal gland are to some extent consistent with results from the short term toxicity studies in mice, were zona fasciculate hypertrophy of the adrenal gland could be observed in the high dose groups.

Gross pathology – 12-month interim sacrifice:

Unscheduled deaths: One animal from the control group died prematurely before the end of the 12-month chronic phase, due to an accidental trauma on Day 301. No relevant macroscopic changes were observed except for a blood clot on the submaxillary glands.

Terminal sacrifice: At 750 ppm, enlarged liver was found in 1/10 males and 2/10 females. At 150 ppm, enlarged liver was found in 2/10 males.

Gross pathology – 18-month carcinogenicity phase:

Unscheduled deaths: Seventy-eight animals died prematurely before the end of the study. No treatment-related effect was established.

Terminal sacrifice: At 750 ppm, enlarged liver and dark liver was found in some males and females. At 150 ppm, enlarged liver was found in some females. These findings were correlated with relevant histopathological findings. At 750 ppm, prominent lobulation in liver was found in some males and females. Furthermore, enlarged thyroid gland was found in one high dose male and two high dose females as compared to no such effect at all other dose levels in both sexes.

Table 127: Incidence of macroscopic changes in the liver, terminal sacrifice of the carcinogenicity phase

Sex	**Males**				**Females**			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Obviously large	0/44	0/41	0/41	3/42	1/39	2/37	7/40	30/38
Dark	1/44	0/41	0/41	14/42	1/39	0/37	0/40	4/38
Prominent lobulation	1/44	0/41	1/41	5/42	0/39	0/37	0/40	8/38

Microscopic pathology – 12-month interim sacrifice

Due to an increased incidence of follicular cell adenomas observed in thyroid gland of the high dose group males (750 ppm), a microscopic examination was performed on the male thyroid gland, to establish in there were any pre-neoplastic changes in this tissue after 12 months of treatment. Follicular cell hyperplasia was noted in 2/10 and 2/9 males at 750 and 150 ppm, respectively.

Table 128: Incidence and severity of microscopic changes in the thyroid gland, all animals, chronic phase

Sex	**Males**			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**
Number of animals	9	10	9	10
Follicular cell hyperplasia: focal/multifocal				
Minimal	0	0	2	1
Slight	0	0	0	1
Total	0	0	2	2

Microscopic pathology – 18-month carcinogenicity phase

Non-neoplastic findings: Treatment-related effects of fluopyram were found in the liver, kidney and thyroid gland. In the liver, a higher incidence of eosinophilic foci of altered hepatocytes was observed in females at 750 ppm. Centrilobular to panlobular hypertrophy was observed in both sexes with a dose-related effect at 750 and 150 ppm. Hepatocellular cholestasis was noted in males at 750 ppm. Higher incidences of hepatocellular single cell degeneration/necrosis were noted in males at 750 and 150 ppm, together with interstitial mixed cell infiltrate, eosinophilic inclusion bodies and multinucleated hepatocytes in males at 750 ppm only. At 750 ppm, there was a markedly lower incidence of mainly centrilobular hepatocellular vacuolation with concomitant minimal to moderate hepatocellular hypertrophy in males. At 30 ppm, there was a higher incidence of mainly centrilobular hepatocellular

vacuolation in males. However, in the absence of an effect at the higher dose level of 150 ppm, this finding was considered to have occurred by chance at 30 ppm.

Table 129: Incidence and severity of microscopic changes in the liver, all animals, carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	49	49	49	50	48	50	50	50
Eosinophilic focus(i) of altered hepatocytes: focal/multifocal								
Minimal	1	0	0	0	0	1	2	0
Slight	0	0	0	2	0	0	0	2
Moderate	0	0	0	0	0	0	0	2
Marked	0	0	0	0	0	0	0	1
Total	1	0	0	2	0	1	2	5*
Centrilobular to panlobular hepatocellular hypertrophy: diffuse								
Minimal	0	0	16	3	0	0	18	24
Slight	0	0	22	11	0	0	0	2
Moderate	0	0	0	36	0	0	0	0
Total	0	0	38**	50**	0	0	18**	26**
Number of animals	49	49	49	50	48	50	50	50
Hepatocellular cholestasis: focal/multifocal								
Minimal	0	0	2	29	0	0	0	0
Slight	0	0	0	2	0	0	0	0
Total	0	0	2	31**	0	0	0	0
Hepatocellular single cell degeneration/necrosis: focal/multifocal								
Minimal	1	2	7	28	1	1	0	1
Slight	0	0	0	12	0	0	0	0
Moderate	0	0	0	0	0	0	0	1
Total	1	2	7*	40**	1	1	0	2
Interstitial mixed cell infiltrate: focal/multifocal								
Minimal	18	15	19	39	8	8	10	8
Slight	0	1	0	1	2	3	2	0
Total	18	16	19	40**	10	11	12	8
Eosinophilic inclusion bodies: focal/multifocal								
Minimal	2	3	5	18	0	0	0	0
Slight	0	0	0	1	0	0	0	0
Total	2	3	5	19**	0	0	0	0
Multinucleated hepatocytes: focal/multifocal								
Minimal	3	1	3	25	1	0	0	0
Slight	0	0	1	2	0	0	0	0
Total	3	1	4	27**	1	0	0	0
Hepatocellular vacuolation: mainly centrilobular: diffuse								
Minimal	21	15	13	0	3	4	1	0
Slight	6	19	11	2	2	5	0	1
Moderate	0	4	3	1	0	1	0	0
Total	27	38*	27	3**	5	10	1	1

*: $p \leq 0.05$, **: $p \leq 0.01$

In the kidney, higher incidences and/or severities of bilateral cortical basophilic tubules, hyaline casts(s) and interstitial mononuclear cell infiltrate were noted in females at 750 ppm. A higher incidence of glomerular congestion/hemorrhage(s), associated with higher severity of amyloid deposition (mainly observed within glomerular interstitium) was noted in females at 750 ppm.

Table 130: Incidence and severity of microscopic changes in the kidney, all animals, carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	50	50	50	50	48	50	50	50
Cortical basophilic tubules: bilateral								
Minimal	27	33	30	20	17	11	18	17
Slight	9	2	2	1	2	2	3	14
Moderate	0	0	1	1	0	1	0	3
Total	36	35	33	22**	19	14	21	34**
Glomerular congestion/hemorrhage(s): focal/multifocal								
Minimal	0	0	0	0	2	1	3	24
Slight	0	0	0	1	0	0	0	3
Moderate	0	0	0	0	0	1	0	0
Total	0	0	0	1	2	2	3	27**
Amyloid deposition: focal/multifocal								
Minimal	13	15	16	19	27	23	15	2
Slight	18	4	7	8	14	15	17	4
Moderate	1	1	0	2	2	2	8	27
Marked	0	0	0	0	0	0	0	8
Total	32	20	23	29	43	40	40	41
Hyaline cast(s): focal/multifocal								
Minimal	3	1	2	0	0	0	1	8
Slight	0	0	0	1	0	1	0	3
Moderate	0	0	1	0	0	1	0	0
Total	3	1	3	1	0	2	1	11**
Interstitial mononuclear cell infiltrate: focal/multifocal								
Minimal	27	27	25	25	33	27	26	16
Slight	5	2	3	2	9	11	12	25
Moderate	0	0	1	0	0	0	1	1
Total	32	29	29	27	42	38	39	42

**: p≤0.01

In the thyroid gland, a higher incidence of follicular cell hyperplasia was noted in both sexes at 750 ppm and as well in males at 150 ppm.

Table 131: Incidence and severity of microscopic changes in the thyroid gland, all animals, carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	50	50	50	50	48	50	50	50
Follicular cell hyperplasia: focal/multifocal								
Minimal	0	2	10	18	11	4	10	10
Slight	3	2	5	6	5	2	5	12
Moderate	1	2	2	5	1	2	3	6
Marked	0	0	3	3	0	0	1	4
Severe	0	0	1	0	0	0	0	1
Total	4	6	21**	32**	17	8*	19	33**

**: p≤0.01

Neoplastic findings: In the thyroid gland, a higher incidence of follicular cell adenoma was noted in males at 750 ppm, when compared to controls.

Table 132: Incidence of neoplastic microscopic changes in the thyroid gland, all animals, carcinogenicity phase

Sex	Males				Females			
Dose level of fluopyram (ppm)	**0**	**30**	**150**	**750**	**0**	**30**	**150**	**750**
Number of animals	50	50	50	50	48	50	50	50
Follicular cell adenoma								
Incidental	1	1	3	7	3	1	3	1
Total	1	1	3	7*	3	1	3	1

*: p≤0.05

Conclusion

Dietary administration of fluopyram over 18 months to the C57BL/6J mouse resulted in toxic effects on the liver, the kidneys and the thyroid at the top dose level of 750 ppm (equivalent to 105 mg/kg bw/day in males and 129 mg/kg bw/day in females). In male mice, higher incidence of follicular cell adenoma in the thyroid gland was observed at that dose level. There was no further evidence of carcinogenicity in this study but the occurrence of thyroid tumours in mice supports a need for classification and labeling. Non-neoplastic changes in the liver in both sexes and in the thyroid gland in males were also seen at the mid dose level of 150 ppm (equivalent to 20.9 mg/kg bw/day in males and 26.8 mg/kg bw/day in females). No treatment related changes were observed at 30 ppm and, thus, this lowest dose level is considered the NOAEL in this study. A dietary concentration of 30 ppm corresponded with a mean daily intake of 4.2 mg/kg bw by male mice and of 5.3 mg/kg bw by females.

4.10.1.2 Carcinogenicity: inhalation

No data submitted by the notifier.

4.10.1.3 Carcinogenicity: dermal

No data submitted by the notifier.

4.10.2 Human information

No data submitted by the notifier.

4.10.3 Other relevant information

Input from the joint global review

In the course of the joint global review process other, non-European authorities came to slightly different conlusions concerning the carcinogenic potential of fluopyram:

The global review partners, Germany, Canada, and US EPA all agree that the results of the carcinogenicity study in rats and mice show that fluopyram induced treatment-related increases in the incidences of liver tumors in female Wistar rats and thyroid follicular cell tumors in male C57BL/6J mice. Germany follows the EU cancer classification system in assessing the cancer risk, whereas PMRA of Canada and US EPA are employing the linear low-dose extrapolation method in assessing cancer risk for fluopyram.

For US EPA, the tumor incidence data and the related mechanistic results were evaluated according to the 2005 Guidelines for Carcinogen Risk Assessment by the Office of Pesticide Programs Cancer Assessment Review Committee. The Committee found the results of the mechanistic studies insufficient to support the proposed mode of action for the induction of liver tumors in female rats or

thyroid follicular cell tumors in male mice. The main deficiency included a lack of dose-response concordance with key precursor events and tumor incidence. The Committee classified fluopyram as "Likely to be Carcinogenic to Humans" based on tumors in two species and two sexes, and recommended the use of a linear low dose extrapolation model applied to the animal data (Q1*) for quantitative estimation of human risk. The unit risk, Q_1^* $(mg/kg/day)^{-1}$, of fluopyram based upon female rat liver combined adenoma and carcinoma tumor rates is 1.55×10^{-2} in human equivalents.

The PMRA came to the same general conclusions as the EPA regarding the mode of action and is using a q_1^* value of 1.72×10^{-2} for risk assessment, derived from the male mouse thyroid tumour data.

4.10.4 Summary and discussion of carcinogenicity

The long-term toxicity and the oncogenic potential of fluopyram were assessed in both mouse and rat. The studies were performed between 2005 and 2007, following the current OECD testing guidelines and in compliance with the GLP requirements. A summary of these studies and their results is given in table Table 133. In both species, the liver, the thyroid and the kidney were the main target organs of chronic toxicity. Carcinogenic effects comprised liver tumours in female rats and thyroid tumours in male mice but were confined to the highest dose levels in the respective studies.

Table 133: Summary of long-term toxicity/carcinogenicity with fluopyram

Species, type and duration of study, dose levels, references	**NOAEL**	**LOAEL**	**Major effects**
	mg/kg bw/day	**mg/kg bw/day**	
Rat – Combined chronic toxicity and carcinogencity, 104-weeks; 30, 150 & 750/375 (males) or 1500 ppm (females); Kennel, 2008, ASB2008-5439	1.2 / 1.7 (M / F)	6.0 / 8.6 (M / F) (Carc. 89)	Liver toxicity, nephropathy, follicular cell hypertrophy in the thyroid gland, eye lesions; Increased incidence of liver cell tumors (carcinoma and adenoma) in high dose females
Mouse – Combined chronic toxicity and carcinogencity, 78 weeks; 30, 150 & 750 ppm; Wason, 2007, ASB2008-5440	4.2 / 5.3 (M / F)	20.9 / 26.8 (M / F) (Carc. 105)	Nephropathy in high dose females, follicular cell hyperplasia in the thyroid gland, liver toxicity; Higher incidence of thyroid follicular cell adenomas in high dose males

Rat

In a combined chronic toxicity and carcinogenicity study, groups of 60 male and female Wistar rats were fed a diet containing 0, 30, 150 and 750 ppm (male animals) and 0, 30, 150 and 1500 ppm (females) fluopyram for 24 months. In males, the top dose level of 750 ppm had to be reduced to 375 ppm from week 85 onwards because of the high mortality in this group. Over the whole study period, these dietary concentrations corresponded to a mean daily intake of 0, 1.20, 6.0 and 29 mg/kg bw in male rats or 1.68, 8.6 and 89 mg/kg bw in females. Thus, for interpretation of results, it must be always taken into account that the top dose level tested in male rats was by three times lower than that one employed in females. In addition, satellite groups of 10 animals per sex and dose were subject to interim sacrifice after 1 year.

Overall, there was a statistically significant increase in mortality in males at 750/375 ppm during the study and after 24 months although no clear cause for these premature deaths could be established. At the highest dose levels of 750/375 or 1500 ppm, respectively, mean body weights were significantly reduced in both sexes at various times throughout the study. Main target organs were the liver, the kidneys and the thyroid gland but also the eyes.

Liver toxicity became apparent by an increase in organ weight at the two upper dose levels in male rats and in the highest dose group in females that was sometimes accompanied by gross pathological findings such as nodules/masses which correlated histologically with neoplastic changes. At the end of

the 2-year carcinogenicity phase, the incidence of liver cell tumors (carcinoma and adenoma) was significantly increased in females receiving 1500 ppm (equivalent to 89 mg/kg bw/day). The combined incidence of female rats with benign and malign liver tumours was 11 (including 3 animals with carcinoma) as compared to 2 in each of the control, low and mid dose groups. No such increment was seen in male rats but the very different actual compound intakes do not allow for a meaningful comparison.

Clinical chemistry findings suggesting hepatotoxicity were minor in nature and confined to the respective top dose level in both sexes. They comprised occasionally higher mean triglyceride concentrations and slightly lower mean glucose concentrations in the females. Activity of alkaline phosphatase was reduced in both sexes throughout the study but achieved statistical significance only occasionally. Histological changes included a higher incidence of altered hepatocytes (eosinophilic foci) and hepatocellular brown pigments, focal or multifocal hepatocellular vacuolation, increased number of mitoses, centrilobular to panlobular hypertrophy and hepatocellular single cell necrosis with females being much more affected. Again, this might be due to the higher dose that the female rats were fed. At the mid dose level of 150 ppm, however, histopathological lesions (hypertrophy) were confined to male rats and, thus, were in line with the increase in organ weight that was noted in the same sex only.

In the kidneys, marked degenerative changes such as chronic progressive nephropathy or focal/multifocal (medullar or cortical) tubular dilatation, together with an increased incidence of tubular golden/brown pigments (mainly in females) and collecting ducts hyperplasia, were observed at the high dose level. In addition, a higher incidence of hyaline droplets and of renal cysts was noted in male rats. At the mid dose level of 150 ppm, male rats still displayed a higher frequency of tubular hypertrophy or dilatation. During the first year of treatment, but not thereafter, urinalysis revealed higher incidences of abnormal color of urine (orange to red) in females and a higher incidence and severity of cellular casts in males. This latter finding was also confirmed in male rats receiving the intermediate dose.

Effects of fluopyram on the thyroid gland were demonstrated by an increased organ weight at the highest dose level in both sexes after 12 month. This finding was associated with histopathological changes (follicular cell hyperplasia and/or hypertrophy, colloid alteration) that were of less severity or frequency but yet to be noted at the mid dose level, too.

In addition, the eyes were affected by long-term treatment. Ophthalmologic examination revealed abnormal color of the retinal fundus in females after 12 months. At the 24-month examination, this condition was observed in females and males, together with small retinal vessels. In addition, hyperreflectivity in the retina was noted in females and corneal opacity, edema of the cornea and nuclear opacity in males. These effects were more severe at the top dose level after 2 years in females. Histologically, bilateral retinal atrophy was noted at the highest dose level, together with a higher incidence of lens degeneration.

A tendency towards lower erythrocyte parameters (hemoglobin concentration, mean corpuscular volume, hematocrit and/or mean corpuscular hemoglobin) was observed in the female high dose group throughout the study confirming evidence from short-term studies that the red blood cells might be an additional target. The same tendency was observed in high dose males at most time points, however, statistical significance was not achieved. The assumption of an effect on the blood was further substantiated by a more frequent occurrence of extramedullary hematopoiesis in the livers of high dose females. In contrast to the subchronic study, prothrombin time was this time shorter in the female high dose group with the difference being significant at months 6 and 12.

It may be concluded that, for both sexes, the lowest dose level of 30 ppm (equal to *ca* 1.2 or 1.7 mg/kg bw/d in males and females, respectively) was a clear NOAEL in this study, based on the occurrence mainly of histopathological changes at the next higher dose of 150 ppm. In females, this mid dose level (corresponding to a daily intake of about 8.6 mg/kg bw/d) was the NOAEL for the probably substance-related liver tumours that were confined to the highest dose of 1500 ppm (ca 89 mg/kg bw/d) and were

not seen up to the highest tested dose of 730/375 ppm (29 mg/kg bw/d) in males. The liver tumours occurring after long-term substance administration were assumed to result from liver toxicity that had become apparent after one year of treatment already and that could be noted also at the mid dose level. Mechanistic studies were performed to elucidate the mode of action behind tumour formation and are reported under section 4.12. Irrespective of these investigations, liver tumours in rats, in contrary to the mouse, are usually considered relevant to man and, thus, classification and labeling will be needed.

Mouse

Groups of 60 male and female C57BL/6J mice were fed a diet containing 0, 30, 150 or 750 ppm of fluopyram (corresponding to a mean compound intake of 0, 4.2, 20.9 and 105 mg/kg bw/day in males and 0, 5.3, 26.8 and 129 mg/kg bw/day in females, respectively) for up to 78 weeks. After 52 weeks, 10 males and 10 females from each group which had been allocated to the chronic phase of the study were killed and necropsied. The remaining 50 animals per sex and group (allocated to the carcinogenicity phase) continued treatment until scheduled sacrifice after at least 78 weeks of treatment.

There were no unscheduled deaths or clinical signs occurring during the study that could be attributed to treatment. The survival rate was not different among the control and dose groups. Body weight gain was decreased only in high and mid dose males and only during the second trimester of the study (weeks 26 to 54). Afterwards, some compensatory growth was observed resulting in a mean final body weight that was similar to the control group value.

A slightly higher mean platelet count was determined in top dose males but no other changes in hematological parameters were seen.

Pathological examination revealed that the liver, the thyroid gland and the kidneys were the main target organs also in mice.

Mean absolute and relative liver weights were markedly increased in high and mid dose males and females at interim sacrifice as well as at study termination. The increment exhibited a clear dose response. At these two upper dose levels, gross necropsy findings such as dark and enlarged livers were corroborated by an increase in non-neoplastic histopathological lesions such as centrilobular to panlobular hypertrophy, hepatocellular cholestasis, single cell degeneration/necrosis or eosinophilic foci. A few of these non-neoplastic effects on the liver were observed only in males pointing to a higher vulnerability of this sex with regard to hepatotoxicity.

Toxic effects on the thyroid were noted at the top dose level in both males and females and in the mid dose male group. The main non-neoplastic finding, *i.e.*, follicular cell hyperplasia became apparent in male mice at interim sacrifice already. In line with that, the only neoplastic change in this study consisted of a higher incidence of follicular cell adenoma in high dose males (7/50) as compared to the control group (1/50). This difference was statistically significant ($p \leq 0.05$).

Mean absolute and relative kidney weights were decreased at the 750 ppm dose level in both sexes. In addition, a higher incidence and/or severity of bilateral cortical basophilic tubules, hyaline casts(s) and interstitial mononuclear cell infiltrates, glomerular congestion/hemorrhage(s), and more pronounced amyloid deposition (mainly in the glomerular interstitium) was noted at this dose but only in females.

No adverse treatment-related effects were observed at the lowest dose level. In male mice, mean absolute and relative liver weight was higher than in the control group but by less than 10 % although the difference was statistically significant. More important, this organ weight increase was not accompanied by liver cell hypertrophy. The only histopathological finding at this dose level was diffuse centrilobular vacuolation that, in the lack of other lesions, is not considered adverse. Thus, the No Observed Adverse Effect Level (NOAEL) in this study was 30 ppm in males (equivalent to 4.2 mg/kg/day) and females (equivalent to 5.3 mg/kg/day). The thyroid tumours, although benign in

nature and occurring at the highest dose and in one sex only, confirm a need for classification and labeling.

Mechanistic evaluation

Mechanistic studies are presented in detail in section 4.12. Here, the mode of action analysis according to the IPCS MoA framework is presented, which is based on these mechanistic data. The evaluation has been performed by the Joint Meeting on pesticide residues of the FAO and WHO (Pfeil and Boobis 2010) and has been summarised in tabular form (Table 134). This mode of action analysis is important for the decision on classification and labeling of fluopyram for carcinogenicity since non-relevance to humans of thyroid tumors in mice and liver tumors in rat was postulated by the applicant. In agreement with earlier evaluations of the BfR and the JMPR it is suggested to follow this argumentation with respect to the murine thyroid tumors but not for the hepatic tumors in rats for the following reasons: Strength, consistency and specificity of the suggested MoA is only partially convincing (e.g. gene-expression analysis has shown that fluopyram partially induces induction of other genes than phenobarbital). Other MoA have not convincingly been excluded. Hence there are remaining uncertainties that do not allow concluding on non-relevance of these tumors to humans.

Table 134: Mode of action analysis (Pfeil and Boobis, 2010).

IPCS MoA	**Thyroid tumors in mice**	**Liver tumors in rats**
Theory of the case	HPT disturbance by induction of T4 excretion, subsequent TSH increase	CAR mediated enzyme induction, cell prolifer.& subsequent hyper-trophy, hyperplasia and foci devel.
Key events	1. induction of hepatic enzymes 2. increase metabolism of T4 3. decrease in T4 half-life & conc. 4. increase in serum TSH 5. thyroid follicular cell hypertrophy and hyperplasia	1. activation of nuclear receptors 2. induction of hepatic CYP 3. liver cell prolifer. ↑, apoptosis↓ 4. hepatocellular hypertrophie and hyperplasia 5. develop. of altered hep. foci
Concordance of dose-response	Good correlation	Partially good correlation
Temporal association	Yes	Yes
Strength, consistency and specificity	Yes	Partially
Biological plausibility and coherence	Yes	Yes
Other MoA?	Possible, TPO inhibition excluded	Possible, genotox. excluded, no other pot. mech excluded (ER, GJIC, AhR)
Uncertainties, incon-sistencies and data gaps	No major inconsistencies and data gaps	Yes; CAR activation not shown, gene expression different from PB (known CAR activator), other MoA possible
Overall assessment and conclusion	MoA supported	MoA not supported, relevance of liver tumors in rats to humans possible

4.10.5 Comparison with criteria

For comparision of carcinogenic effects with DSD and CLP criteria see Table 135 below.

Table 135: Criteria for classification as carcinogen according to DSD and CLP.

DSD	CLP regulation
The placing of a substance into Category 1 is done on the basis of epidemiological data; placing into Categories 2 and 3 is based primarily on animal experiments. For classification as a Category 2 carcinogen either positive results in two animal species should be available or clear positive evidence in one species, together with supporting evidence such as genotoxicity data, metabolic or biochemical studies, induction of benign tumours, structural relationship with other known carcinogens, or data from epidemiological studies suggesting an association. Category 3 actually comprises 2 sub-categories: (a) substances which are well investigated but for which the evidence of a tumour-inducing effect is insufficient for classification in Category 2. Additional experiments would not be expected to yield further relevant information with respect to classification; (b) substances which are insufficiently investigated. The available date are inadequate, but they raise concern for man. This classification is provisional; further experiments are necessary before a final decision can be made. For a distinction between Categories 2 and 3 the arguments listed below are relevant which reduce the significance of experimental tumour induction in view of possible human exposure. These arguments, especially in combination, would lead in most cases to classification in Category 3, even though tumours have been induced in animals: - carcinogenic effects only at very high dose levels exceeding the 'maximal tolerated dose'. The maximal tolerated dose is characterised by toxic effects which, although not yet reducing lifespan, go along with physical changes such as about 10 % retardation in weight gain, - appearance of tumours, especially at high dose levels, only in particular organs of certain species known to be susceptible to a high spontaneous tumour formation, - appearance of tumours, only at the site of application, in very sensitive test systems (e.g., i.p. or s.c. application of certain locally active compounds), if the particular target is not relevant to man, - lack of genotoxicity in short-term tests in vivo and in vitro, - existence of a secondary mechanism of action with the implication of a practical threshold above a certain dose level (e.g., hormonal effects on target organs or on mechanisms of physiological regulation, chronic stimulation of cell proliferation), - existence of a species - specific mechanism of tumour formation (e.g. by specific metabolic pathways) irrelevant for man. For a distinction between Category 3 and no classification arguments are relevant which exclude a concern for man: - a substance should not be classified in any of the categories if the mechanism of experimental tumour formation is clearly identified, with good evidence that this process cannot be extrapolated to man, - if the only available tumour data are liver tumours in certain sensitive strains of mice, without any other supplementary evidence, the substance may not be classified in any of the categories,	A substance is classified in Category 1 (known or presumed human carcinogens) for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as: 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 (see section 3.6.2.2). 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 (1) 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 (suspected human carcinogens) 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 (see section 3.6.2.2). Such evidence may be derived either from limited (1) evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies. […] 3.6.2.2.3. Strength of evidence involves the enumeration of tumours in human and animal studies and determination of their level of statistical significance. Sufficient human evidence demonstrates causality between human exposure and the development of cancer, whereas sufficient evidence in animals shows a causal relationship between the substance and an increased incidence of tumours. Limited evidence in humans is demonstrated by a positive association between exposure and cancer, but a causal relationship cannot be stated. Limited evidence in animals is provided when data suggest a carcinogenic effect, but are less than sufficient. The terms ‘sufficient’ and ‘limited’ have been used here as they have been defined by the International Agency for Research on Cancer (IARC) and read as follows: (a) Carcinogenicity in humans The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between exposure to the agent and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence;

- particular attention should be paid to cases where the only available tumour data are the occurrence of neoplasms at sites and in strains where they are well known to occur spontaneously with a high incidence.	— limited evidence of carcinogenicity: a positive association has been observed between exposure to the agent and cancer for which a causal interpretation is considered to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence. (b) Carcinogenicity in experimental animals Carcinogenicity in experimental animals can be evaluated using conventional bioassays, bioassays that employ genetically modified animals, and other in-vivo bioassays that focus on one or more of the critical stages of carcinogenesis. In the absence of data from conventional long-term bioassays or from assays with neoplasia as the end-point, consistently positive results in several models that address several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals. The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories: — sufficient evidence of carcinogenicity: a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites; — limited evidence of carcinogenicity: the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs. 3.6.2.2.4. Additional considerations (as part of the weight of evidence approach (see 1.1.1)). Beyond the determination of the strength of evidence for carcinogenicity, a number of other factors need to be considered that influence the overall likelihood that a substance poses a carcinogenic hazard in humans. The full list of factors that influence this determination would be very lengthy, but some of the more important ones are considered here. 3.6.2.2.5. The factors can be viewed as either increasing or decreasing the level of concern for human carcinogenicity. The relative emphasis accorded to each factor depends upon the amount and coherence of evidence bearing on each. Generally there is a requirement for more complete information to decrease than to increase the level of concern. Additional considerations should be used in evaluating the tumour findings and the other factors in a case-by-case manner. 3.6.2.2.6. Some important factors which may be taken into consideration, when assessing the overall level of concern are:

	(a) tumour type and background incidence; (b) multi-site responses; (c) progression of lesions to malignancy; (d) reduced tumour latency; (e) whether responses are in single or both sexes; (f) whether responses are in a single species or several species; (g) structural similarity to a substance(s) for which there is good evidence of carcinogenicity; (h) routes of exposure; (i) comparison of absorption, distribution, metabolism and excretion between test animals and humans; (j) the possibility of a confounding effect of excessive toxicity at test doses; (k) mode of action and its relevance for humans, such as cytotoxicity with growth stimulation, mitogenesis, immunosuppression, mutagenicity. Mutagenicity: it is recognised that genetic events are central in the overall process of cancer development. Therefore evidence of mutagenic activity in vivo may indicate that a substance has a potential for carcinogenic effects.

There are no relevant data from epidemiological studies submitted by the notifier, hence no classification with cat. 1 according to DSD regulation or with 1A according to CLP regulation is required.

There are two different types of tumors (liver and thyroid) in two different species (rat and mouse), which have been detected in two different experiments occurring at relatively high doses. Thus, classification with cat. 2 or 3 according to DSD or with 1B or 2 according to CLP regulation could be considered according to the afformentioned criteria. Since for the thyroid tumors mechanistic data suggest that they are not relevant to humans, they are not further considered for classification. Only low incidences of liver carcinomas were observed in one species and one sex. Considering that only one tumor type was regarded of relevance we propose to classify with cat 3 according to DSD (R40) or Carc. 2 according to CLP (H351).

Conclusions on classification and labelling

The carcinogenicity of fluopyram does meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the carcinogenicity studies and respective mechanistic studies fluopyram should be considered for classification and labelling as carcinogenig cat. 3 (R40 – limited evidence of a carcinogenic effect).

According to the criteria in Reg. 1272/2008, based on the results of the carcinogenicity studies and respective mechanistic studies fluopyram should be considered for classification and labelling as Carc. 2 (H351 – suspected of causing cancer).

RAC evaluation of carcinogenicity[1]

Summary of the Dossier submitter's proposal

Two carcinogenicity studies were summarised in the CLH report, one in C57BL/6J mice with doses 0, 30

[1] Note that the numbering of Tables for the RAC evaluation on the carcinogenicity part is in a separate order comparing to the rest of the background document due to the insertion of additional key information section.

150 or 750 ppm (0, 4.2/5.3, 20.9/26.8, 105/129 mg/kg bw m/f) in the diet and one in Wistar rats with doses 0, 30, 150, 750(concentration changed from 750 ppm to 375 ppm from week 85 onwards) and 1500 ppm (0, 1.2/1.68, 6.9/8.6, 29/-, -/89 mg/kg bw in m/f) in the diet . Both studies were performed under GLP. Tumours were induced in the liver in female rats (adenomas and carcinomas) but not in male rats. It should be noted that male rats were considerably more sensitive to the substance than female rats and only one half to one quarter of the highest dose given to females could be given to the males. In this study mortality was high with a survival rate for males of 37.8 % in controls decreasing to 19.9% at the high dose and 68.3 % in control females decreasing to 51.0% at the high dose. At the high dose there was also a reduction in body weight in both sexes. In mice, follicular cell adenomas were seen in the thyroid in males but not in females. No difference in sensitivity between the sexes was seen in this study and there were no treatment-related deaths or clinical signs.

The DS argued that the mode of action (MoA) for the formation of the thyroid tumours was not relevant to humans. The DS stated, however, that there was no convincing evidence that the liver tumours observed were caused by a MoA not relevant to humans. The DS noted that Constitutive Androstane Receptor (CAR) activation, indicative of a MoA potentially not relevant to humans, is not shown and gene expression is different from phenobarbital (PB; known CAR activator). Further, although genotoxicity is excluded, no other potential mechanisms have been excluded (estrogen receptor (ER), gap junction intercellular communication (GJIC), aryl hydrocarbon receptor (AhR)). Since there are tumours only in one sex and one species in one experiment and the tumours where mainly benign, the DS considered classification as Carc. 2 – H351 as appropriate.

Comments received during public consultation

Two MSCAs supported the proposed classification. One industry representative and one individual argued that classification for carcinogenicity was not warranted. A substantial amount of new information was also submitted by Industry.

The new data included studies on CAR activation and some studies and argumentation on other MoAs and is summarised in the section "additional key elements" in the background document. Further details can be found in the RCOM.

Additional key elements

A number of mechanistic studies were submitted during public consultation. Since the studies were claimed confidential, only study summaries are reported here:

Confidential study (2011): Fluopyram, Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations), non-guideline, non-GLP

Fluopyram (94.7% purity), was administered by oral gavage to 2 groups of 15 male C57BL/6J mice for 3 days at 100 and 300 mg/kg bw/d. Phenobarbital (99.6% purity) was administered by gavage to one group of 15 male C57BL/6J mice for 3 days at 80 mg/kg bw/d. A control group of 15 males received the vehicle alone (0.5% methylcellulose).

During the whole study period no mortalities and treatment-related clinical signs and body weight effects were seen.

After 3 days of fluopyram exposure, at 100 and 300 mg/kg bw/d, mean T4 levels in the plasma were decreased (-26% and -34%, $p<0.01$) whilst mean TSH levels were unchanged compared to controls. In the PB treated animals, mean T4 levels in the plasma were decreased (-38%, $p<0.01$), whilst mean TSH levels in the plasma were unchanged.

Table 1

Mean plasma hormone values ± SD (% change compared to mean control values)				
Group	Control	Fluopyram 100 mg/kg bw/d	Fluopyram 300 mg/kg bw/d	PB 80 mg/kg bw/d
T4 (nmol/L)	34.2 ± 8.7	25.4* ± 6.1 (-26%)	22.6* ± 4.6 (-34%)	21.3* ± 3.1 (-38%)

TSH (ng/mL)	3.45 ± 0.40	3.37 ± 0.24	3.53 ± 0.29	3.55 ± 0.37

*: The group mean is significantly different from the control at p≤0.01

In the bile, no statistically significant change in mean T4 levels were seen after 3 days of fluopyram exposure. According to the author the decrease in mean T4 levels (-19%, p≤0.01) observed in the bile of PB treated animals was most likely due to technical reasons and was not considered to be a treatment-related effect.

Table 2

Mean bile hormone values ± SD (% change compared to mean control values)				
Group	Control	Fluopyram 100 mg/kg bw/d	Fluopyram 300 mg/kg bw/d	PB 80 mg/kg bw/d
T4 (nmol/L)	79.2 ± 7.9	79.0 ± 6.1	83.9 ± 8.5	64.5* ± 4.4 (-19%)

*: The group mean is significantly different from the control at p≤0.01

An increase in the levels of Tsh transcript (+21% (not statistically significant; n.s.); +49%, p<0.01) was observed in the pituitary gland after fluopyram (+21% n.s.; +49%, p<0.01) and PB (+46%, p<0.01) exposure.

Table 3

Mean Relative Quantity ± SD (% change compared to mean control values)				
Gene transcript	Control	Fluopyram 100 mg/kg bw/d	Fluopyram 300 mg/kg bw/d	PB 80 mg/kg bw/d
Tsh b *	0.994 ± 0.124	1.138 ± 0.230 (+21%)	1.404* ± 0.253 (+49%)	1.381* ± 0.198 (+46%)

*: beta subunit Tsh

In conclusion, fluopyram administration by oral gavage at nominal concentrations of 100 and 300 mg/kg bw/d, in the C57BL/6J mouse for 3 days, induced a decrease in plasma T4 levels associated with an increase in the levels of Tsh transcript (beta subunit) in the pituitary gland, whereas no increase of plasma TSH levels were detected.

Confidential study (2012a): Fluopyram, Mechanistic 3-day toxicity study in the mouse by oral gavage (thyroid hormone investigations), non-guideline, non-GLP,
Fluopyram, (94.7% purity), was administered daily by oral gavage for 3 days to groups of 15 male C57BL/6J mice, at dose levels of 100 or 300 mg/kg bw/d. T4 and TSH blood hormone levels were examined at four time points 2h, 8h, 14h and 48h after the last gavage administration. Four control groups of each 15 males received the vehicle alone (0.5% methylcellulose). On the day of sacrifice, bood samples were taken for hormone level measurements (T4 and TSH).
During the whole study period no mortalities or treatment-related clinical signs were seen.
In the fluopyram treated animals, after 3 days of exposure, mean T4 levels in the plasma were statistically significantly (p≤0.01) decreased by between -18% and -41%. The magnitude of the response occurred in a dose-related manner at all time-points apart from the 2h time point. No statistically significant change in mean TSH levels was observed at any time point.

Table 4

Time point	**Blood hormone (nmol/L)**	**Control**	**Fluopyram (100 mg/kg bw/d)**	**Fluopyram (300 mg/kg bw/d)**
2h	T4	31.5 ± 9.1	22.8**± 6.1 (-28%)	24.0*± 6.3 (-24%)

	TSH	2.7 ± 0.5	2.6 ± 0.5 (-1%)	2.7 ± 0.5 (+1%)
8h	T4	38.2 ± 7.0	28.8** ± 5.4 (-25%)	22.4** ± 4.1 (-41%)
	TSH	2.8 ± 0.6	3.0 ± 0.5 (+7%)	3.1 ± 0.7 (+13%)
14h	T4	25.5 ± 5.3	20.9** ± 4.5 (-18%)	18.6** ± 4.3 (-27%)
	TSH	3.1 ± 0.4	3.1 ± 0.7 (+2%)	3.1 ± 0.5 (+2%)
48h	T4	34.5 ± 8.5	25.4** ± 6.1 (-26%)	24.1** ± 4.4 (-30%)
	TSH	3.1 ± 0.6	3.2 ± 0.5 (+3%)	3.2 ± 0.6 (+2%)

*: The group mean is significantly different from the control at p≤0.05
**: The group mean is significantly different from the control at p≤0.01

In conclusion, fluopyram administration to C57BL/6J male mice by oral gavage for three days at concentrations similar to or above the top dose level administered in the mouse cancer bioassay induced a statistically significant decrease in plasma T4 levels at all the time points examined. However, no change in plasma TSH level was detected in this short term assay.

Confidential study (2012b, amended 2013): Fluopyram, Mechanistic 28-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations), non-guideline, GLP
Fluopyram (94.7% purity), was administered daily via the diet for at least 28 days to male C57BL/6J mice at dose levels of 30, 75, 150, 600 and 750 ppm (5, 13, 25, 102 and 128 mg/kg bw/d). Additionally, one group of male C57BL/6J mice received 80 mg/kg bw/d PB (100% purity) by gavage and the negative control group were kept on unchanged diet only for 28 days. Each group consisted of 15 male mice with the exception of the untreated group, the 750 ppm fluopyram and the PB group, where 15 additional males each were allocated for a one month recovery period. The top dose level of 750 ppm represented the dose level at which thyroid tumors were detected in the mouse cancer bioassay.
In the liver, hepatic UDP-glucuronosyltransferases (UDPGT) activity was measured using either bilirubin or T4 as substrate.

During dosing, neither mortalities and treatment-related clinical signs nor changes in body weight and food consumption were seen with fluopyram exposure.

Fluopyram and PB induced a statistically significant decrease in mean T4 levels in plasma in all dose levels, but no statistically significant change in mean TSH levels was observed.

Table 5

Mean plasma hormone values ± SD (% change compared to mean control values) after 28 days exposure							
	Fluopyram (ppm)						**PB** mg/kg bw/d
Group	0	30	75	150	600	750	80
T4 (nmol/L)	26.1 ± 6.9	18.9* ± 3.1 (-28%)	17.9* ± 3.4 (-31%)	19.5* ± 4.6 (-25%)	16.5* ± 2.3 (-37%)	16.3* ± 2.9 (-38%)	20.1* ± 2.7 (-23%)
TSH (ng/mL)	1.4 ± 0.6	2.1 ± 0.9 (+50%)	1.6 ± 0.5 (+14%)	1.2 ± 0.3 (-14%)	1.6 ± 0.7 (+14%)	1.6 ± 0.6 (+14%)	1.6 ± 0.4 (+14%)

*: The group mean is significantly different from the control at p≤0.01

After one month recovery no statistically significant changes in mean T4 and TSH plasma levels were observed when compared to the control animals either in the fluopyram or in PB-exposed animals.

Table 6

Mean plasma hormone values ± SD (% change compared to control mean values) after 1 month recovery			
	Fluopyram (ppm)		**PB** mg/kg bw/d
Group	0	750	80
T4 (nmol/l)	28.5 ± 5.4	27.1 ± 5.6 (-5%)	28.4 ± 7.5 (-0.4%)
TSH (ng/ml)	1.5 ± 0.3	1.4 ± 0.4 (-7%)	1.6 ± 0.5 (+7%)

A dose-related increase in the level of Tsh transcript in the pituitary gland was recorded at 600 and 750 ppm. An increase in the level of Tsh transcript in the pituitary gland was also recorded in PB treated animals.

Table 7

Mean relative quantity ± SD of Tsh b transcript (% change compared to control mean values) after 28 days exposure							
	Fluopyram (ppm)						**PB** mg/kg bw/d
Group	0	30	75	150	600	750	80
Tsh b	1.156 ±0.334	1.223±0.354 (+6%)	1.303±0.344 (+13%)	1.297±0.451 (+12%)	1.655*±0.461 (+43%)	1.783**±0.725 (+54%)	1.764**±0.586 (+53%)

*: The group mean is significantly different from the control at p≤0.05
**: The group mean is significantly different from the control at p≤0.01

After one month recovery in the pituitary gland a slight increase in the level of Tsh transcript was recorded in fluopyram treated animals. No statistically significant changes were observed in PB treated animals after the recovery phase.

Table 8

Mean relative quantity ± SD of Tsh b transcript (% change compared to control mean values) after 1 month recovery			
	Fluopyram (ppm)		**PB** mg/kg bw/d
Group	0	750	80
Tsh b	1.046 ±0.087	1.169*±0.184 (+12%)	1.090±0.139 (+4%)

*: The group mean is significantly different from the control at p≤0.05

Fluopyram and PB induced mean absolute and relative liver weight increase in the mice after 28-day exposure.

Table 9

Mean liver weight ± SD (% change compared to control) after 28 days exposure		
	Fluopyram (ppm)	**PB** mg/kg bw/d

Group	0	30	75	150	600	750	80
Absolut liver weight (g)	1.31 ± 0.08	1.37 ± 0.13 (-5%)	1.4 ± 0.09 (+7%)	1.45±0.13 (+11%)	1.67* ±0.12 (+27%)	1.78*±0.12 (+36%)	1.44*±0.12 (+10%)
Liver to body weight ratio (%)	5.338 ± 0.31	5.57±0.306 (+4%)	5.667±0.278 (+6%)	5.825*±0.275 (+9%)	6.786*±0.468 (+27%)	7.088*±0.341 (+33%)	6.19*±0.291 (+16%)

*: The group mean is significantly different from the control at p≤0.01

After recovery phase there was no significant change in liver weight parameters in treated animals when compared to the controls.

Table 10

Mean liver weight ± SD (% change compared to control) after 1 month recovery			
	Fluopyram (ppm)		**PB** mg/kg bw/d
Group	0	750	80
Absolut liver weight (g)	1.32 ± 0.1	1.37 ± 0.18 (+4%)	1.33 ± 0.09 (+1%)
Liver to body weight ratio (%)	4.984 ± 0.296	5.19 ± 0.5 (+4%)	5.085 ± 0.282 (+2%)

A dose-related statistically significant increase in the UDPGT enzymatic activity using bilirubin and thyroxine as substrates was recorded after 28 days fluopyram exposure.

Table 11

Mean enzymatic UDPGT activities ± SD (% change compared to control) after 28 days exposure							
	Fluopyram (ppm)						**PB** mg/kg bw/d
Group	0	30	75	150	600	750	80
UDPGT (Bilirubin)	1.985 ± 0.577	2.217 ± 0.375 (+12%)	2.394 ± 0.327 (+21%)	2.624 ±0.198 (+32%)	2.758* ± 0.324 (+39%)	2.947** ± 0.422 (+48%)	2.821 ±0.676 (+42%)
UDPGT (Thyroxine)	0.77 ± 0.144	0.768± 0.19 (0%)	0.854 ± 0.198 (+11%)	1.168 ± 0.36 (+52%)	1.412** ± 0.253 (+83%)	1.026 ± 0.271 (+33%)	1.02 ±0.21 (+32%)

*: The group mean is significantly different from the control at p≤0.05
**: The group mean is significantly different from the control at p≤0.01

There was no significant change in the hepatic UDPGT enzymatic activitiy in the fluopyram and PB treated animals after the recovery phase.

Table 12

Mean enzymatic UDPGT activities ± SD (% change compared to control) after 1 month recovery			
	Fluopyram (ppm)		**PB** mg/kg bw/d
Group	0	750	80
UDPGT (Bilirubin)	1.795 ± 0.313	1.908 ± 0237 (+6%)	1.992 ± 0.293 (+11%)

UDPGT (Thyroxine)	0.822 ± 0.115	0.774 ± 0.327 (-6%)	0.808 ± 0.096 (-2%)

In conclusion, fluopyram administration to C57BL/6J male mice via the diet for 28 days induced a decrease in plasma T4 levels correlated with an increase in the levels of Tsh transcript (beta subunit) in the pituitary gland, together with an increase in the UDPGT-bilirubin and UDPGT-T4 enzymatic activities. In addition, absolute and relative liver weights were significantly increased. These effects were not detected after a four-week recovery phase except for a marginal increase in Tsh transcript level.
All these effects occurred in a dose-related manner, apart from the decrease in plasma T4 for which there was no dose-related concordance in terms of the magnitude of the response observed. At the low dose of 30 ppm, the only finding was a decrease in plasma T4 levels. According to the author, the relevance of this is doubtful based on the lack of clear dose response concordance.

Confidential study (2012c): Fluopyram, 28-day toxicity study for proliferation assessment in the C57BL/6J male mouse, non-guideline, GLP

Fluopyram, (94.7% w/w), was administered daily in the diet to groups of adult male C57BL/6J mice (15 mice/dose level) for 28 days at a dose level of 750 ppm (127 mg/kg bw/d). A group of animals dosed by oral gavage with 80 mg/kg bw/d PB (100% purity) acted as a positive control for thyroid effects. A control group received untreated diet. All animals were exposed to BrdU in their drinking water during the last week of the study.
The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations.

There were no deaths, clinical signs of toxicity and no effects on food consumption or body weight parameters for the group treated with fluopyram. Mean water containing BrdU consumption in the fluopyram or PB animals was decreased by 7.9% (not statistically significant for fluopyram and statistically significant ($p<0.05$) for PB) compared to the control values.
Enlarged (14/15 animals) and dark (5/15 animals) livers were found in the fluopyram treated group and in PB group (3/15 and 4/15 animals, respectively). No microscopic changes were noted in the thyroid gland. Dark thyroid glands were noted in 2/15 animals treated with PB but not associated with any microscopic findings.
Assessment of cell proliferation in the thyroid revealed a 1.7 fold increase ($p<0.01$) in BrdU labelling index for mice treated with fluopyram when compared to the controls. Investigation of cell proliferation in the thyroid of PB treated mice revealed no change in the BrdU labeling index.

Table 13

Mean mean BrdU labelling after 28 days of treatment			
	Fluopyram (ppm)		**PB** mg/kg bw/d
Group	0	750	80
Mean	12.45	21.09**	12.68
STD	6.17	5.78	3.71

** The group mean is significantly different from the control at $p \leq 0.01$

In conclusion, a clear increase in thyroid cell proliferation, as evidenced by a 1.7 fold increase ($p<0.01$) in BrdU labelling index, was observed following dietary administration of fluopyram at 750 ppm for 28 days, to the male C57BL/6J mouse. This proliferation increase was not seen in thyroid cells of PB treated mice. According to the author, this result was consistent with the literature, which shows that PB induces thyroid cell proliferation in mice at a dose level of 1800 ppm (approx. 200 mg/kg bw/d).

Confidential study (2013a, amended 2013): Fluopyram, 28-day toxicity study for thyroid cell proliferation in the C57BL/6J male mouse, non-guideline, GLP

Fluopyram (94.7% purity), was administered daily in the diet for at least 28 days to male C57BL/6J mice at dose levels of 0, 30, 75, 150, 600, 750 and 1500 ppm (0, 5, 13, 25, 99, 124 and 247 mg/kg bw/d). Each group consisted of 15 male mice with the exception of the untreated group and the 1500 ppm group, where 15 additional males were allocated for a one month recovery period. All animals were exposed to BrdU in their drinking water during the last week of the study. The duodenum was used as a positive control for the cell proliferation investigations.
No treatment-related clinical signs, effects on body weight parameters, food or water consumption were observed at any dose level tested. One animal from the 1500 ppm group was found dead on day 28 of the recovery phase.
Assessment of follicular cell proliferation in the thyroid revealed a 1.21, 1.40, 1.61 and 2.33 fold increase in BrdU labeling index at 150, 600, 750 and 1500 ppm, respectively, when compared to the controls. The effects are statistically significant at the three highest dose levels.

Table 14

Mean BrdU labelling after 28 days of treatment (Proliferation rate of 1000 cells)							
	Fluopyram (ppm)						
N	14	15	15	15	15	15	15
Group	0	30	75	150	600	750	150
Mean	21.55	17.81	19.51	26.09	30.11**	34.78**	50.21***
STD	4.75	7.37	5.64	8.62	8.53	7.61	10.24

**: Statistically higher than the control group ($p<0.01$)
***: Statistically higher than the control group ($p<0.001$)

In the recovery group cell proliferation in the thyroid revealed no change in BrdU labeling index in the 1500 ppm dose group when compared to the controls.

Table 15

Mean BrdU labelling after one month recovery (Proliferation rate of 1000 cells)		
	Fluopyram (ppm)	
N	14	14
Group	0	1500
Mean	17.57	11.56
STD	5.18	4.78

** The group mean is significantly different from the control at $p \leq 0.01$

In conclusion, a clear increase in thyroid cell proliferation, was observed following dietary administration of fluopyram at 750 and 1500 ppm for at least 28 days to the male C57BL/6J mouse. This effect showed complete reversibility (no cell proliferation observed) after a one month recovery period.

Confidential study (2013b): 28-day dietary study to determine potential role of the nuclear pregnane X receptor (Pxr) and the CAR on the thyroid changes following the administration of Fluopyram to male mice (C57BL/6J and Pxr KO/Car KO), non-guideline, GLP

Fluopyram (94.7% purity), was administered daily in the diet for 28 days to the mice at dose levels of 750 and 1500 ppm, corresponding to 125 and 256 mg/kg bw/d in C57BL/6J male mice and to 130 and 247 mg/kg bw/d in Pxr KO/Car KO male mice. Each group consisted of 15 male mice. All animals were exposed to BrdU (80 mg/100 ml) in their drinking water during the last week of the study.
In liver microsomes, P450 enzyme activity (PROD for CYP2B and benzyloxyquinoline-O- debenzylation

(BQ) for CYP3A) and bilirubin glucuronidation activity were measured. Liver microsomes were incubated with 125I-thyroxine and the formation of T4- glucuronide was determined. Additional thyroid and duodenum (positive control) slides were stained for BrdU cell proliferation determinations. Gene transcript analyses of Thyroid Stimulating Hormone beta subunit (Tshb) by quantitative Polymerase Chain Reaction (q-PCR) were undertaken in pituitary gland. Beta-actin (Actb) was selected as reference gene.

No treatment-related clinical signs, mortalities, effects on body weight parameters, food or water consumption were observed at any dose level tested.
In wild type animals mean absolute and relative liver weights were statistically significantly and dose-dependently increased. Dose-related enlarged livers were noted at both doses associated with hepatocellular hypertrophy, single cell necrosis, increased number of mitoses (only 1500 ppm) and interstitial mixed cell infiltrate.
In knock-out animals, at 1500 and 750 ppm, mean absolute and relative liver weights were dose-related increased, but in a lower incidence that in wild type animals. No macroscopic and microscopic changes were noted.

Table 16

Mean liver weight ± SD (mean % control) and incidence of macroscopic changes in the liver			
	Males wild-type (C57BL/6J) mice		
Fluopyram dose level (ppm)	0	750	1500
Mean absolute liver weight (g)	1.277 ± 0.06859	1.8035** ± 0.15861 (+41%)	2.115*** ± 0.19106 (+66%)
Mean liver to body weight ratio (%)	4.9285 ± 0.18549	6.8482** ± 0.44559 (+39%)	7.9883*** ± 0.48285 (+62%)
Mean liver to brain weight ratio (%)	288.9877 ± 17.75730	409.6459** ± 35.06164 (+42%)	480.1812*** ± 43.73024 (+66%)
enlarged	0/15	7/15	14/15
	Males Pxr KO/Car KO mice		
Mean absolute liver weight (g)	1.2945 ± 0.08045	1.4008** ± 0.07952 (+8%)	1.4307** ± 0.07729 (+11%)
Mean liver to body weight ratio (%)	5.3119 ± 0.24617	5.7075** ± 0.27193 (+7%)	5.7970** ± 0.23142 (+9%)
Mean liver to brain weight ratio (%)	297.0051 ± 15.48663	315.5953* ± 26.98886 (+6%)	336.8503** ± 18.1761 (+13%)

*P≤0.05; **P≤0.01; ***P≤0.001

In the thyroid gland proliferation test in wild-type mice higher proliferative indexes were noted (2.6-fold at 1500 ppm and 1.8-fold at 750 ppm) when compared to controls. In knock-out mice no change was noted between control and treated groups.

Table 17

Thyroid gland proliferation index			
	Males wild-type (C57BL/6J) mice (N=15)		
Fluopyram dose level (ppm)	0	750	1500

Mean	14.28	26.08***	36.61***
STD	3.96	7.16	10.27
	Males Pxr KO/Car KO mice (N=15)		
Mean	10.05	9.91	8.27
STD	3.88	4.06	3.38

***P≤0.001

The total cytochrome P450 content of the liver microsomal fractions from male C57BL/6J mice exposed to 1500 ppm and 750 ppm fluopyram in the food was increased 3.7- and 3.6-fold, respectively. In detail, hepatic microsomal PROD was increased by 151- and 70-fold, BQ activity by 7.9- and 5.5-fold, thyroxine glucuronosyl transferase (T4-GT) activity by 1.9- and 1.8-fold and bilirubin glucuronidation was increased by 2.0-, and 1.8-fold respectively, over the concurrent control.
Fluopyram had no effect on the total microsomal P450 content in the Pxr KO/Car KO mice.
Both at 1500 and 750 ppm, Pxr KO/Car KO mice showed little induction of PROD (1.4-fold) when compared to wild type animals. For BQ the activity was decreased to 1.7-fold and 1.5-fold, respectively. No effect on T4-GT at 750 ppm, and decreased T4-GT activity to 1.3-fold at 1500 ppm were observed.

Table 18

Mean ± SD (mean % control ± SD)			
Fluopyram dose level (ppm)	0	750	1500
	Males wild-type (C57BL/6J) mice (N=15)		
Total P450 nmol/mg protein	0.34 ± 0.22 (100.0 ± 63.5)	1.24 ± 0.16*** [a] (363.3 ± 47.2)	1.25 ± 0.18*** (367.3 ± 51.3)
PROD pmols resorufin formed/min/mg protein	2.01 ± 0.20 (100.0 ± 10.0)	140.21 ± 15.11*** [a] (6991.0 ± 753.5)	302.14 ± 84.76*** (15065.3 ± 4226.4)
BQ nmols 7-OH quinolone formed /min/mg protein	2.77 ± 0.34 (100.0 ± 12.3)	15.20 ± 1.89*** [a] (549.3 68.3)	21.94 ± 1.83*** (792.8 ± 66.1)
T4-GT pmol T4-glucoronide formed/min/mg protein	0.58 ± 0.17 (100.0 ± 29.9)	1.06 ± 0.17*** [a] (183.8 ± 29.2)	1.09 ± 0.25*** (189.5 ± 43.6)
BIL-GT nmol bilirubin-glucuronide formed/min/mg protein	0.73 ± 0.11 (100.0 ± 14.4)	1.30 ± 0.22*** [a] (177.4 ± 30.0)	1.43 ± 0.42*** (195.6 ± 57.6)
	Males Pxr KO/Car KO mice (N=15)		
Total P450 nmol/mg protein	0.24 ± 0.09 (100.0 ± 36.5)	0.27 ± 0.12 (112.3 ± 49.7)	0.27 ± 0.12 (111.0 ± 47.5)
PROD pmols resorufin formed/min/mg protein	2.27 ± 0.30 (100.0 ± 13.1)	3.20 ± 0.81*** (141.3 ± 35.6)	3.24 ± 1.16** (142.9 ± 51.0)
BQ nmols 7-OH quinolone formed /min/mg protein	3.51 ± 0.39 (100.0 ± 11.0)	2.40 ± 0.40*** (68.5 ± 11.3)	2.09 ± 0.35*** (59.4 ± 10.1)
T4-GT pmol T4-glucoronide formed/min/mg protein	0.57 ± 0.19 (100.0 ± 34.0)	0.66 ± 0.17 (115.9 ± 29.9)	0.43 ± 0.15* (75.3 ± 26.7)

BIL-GT nmol bilirubin-glucuronide formed/min/mg protein	0.70 ± 0.24 [a] (100.0 ± 34.1)	0.66 ± 0.24 [a] (94.5 ± 34.1)	0.61 ± 0.28 (86.5 ± 39.4)

[a] n = 14
*statistically different from control p<0.05; **p<0.01; *** p<0.001.

In male C57BL/6J mice exposed to fluopyram Tshb gene transcription in the pituitary gland was up-regulated in a dose related manner (1.7-fold, and 1.6-fold, at 1500 and 750 ppm, respectively) when compared with controls.
In Pxr KO/Car KO mice Tshb gene transcription in the pituitary gland was slightly down-regulated (1.2-fold) at 1500 ppm and very slightly down-regulated (1.1-fold) at 750 ppm fluopyram in the food.

Table 19

Mean Relative Quantity ± SD of gene transcripts in pituitary glands (mean % control)			
Fluopyram dose level (ppm)	0	750	1500
	Males wild-type (C57BL/6J) mice (N=15)		
Tshb	1.23 ± 0.289	1.92** ± 0.306 (+56%)	2.05*** ± 0.586 (+67%)
	Males Pxr KO/Car KO mice (N=15)		
Tshb	1.25 ± 0.264	1.14 ± 0.177	1.04* ± 0.165 (-17%)

*statistically different from control p<0.05; **p<0.01; *** p<0.001.

In conclusion, clear increases in PROD activity seen in WT C57BL/6J mice after exposure to fluopyram, with minimal PROD induction in Pxr KO/Car KO mice, indicates that fluopyram is an inducer of CYP2b in WT mice. The induction of BQ activity in WT mice and decrease in BQ activity in mice not expressing Car and Pxr receptors indicates that fluopyram is also a CYP3a inducer. Also liver weight increase was more prominent in WT mice and macroscopic and microscopic liver changes were not found in Pxr KO/Car KO mice. In the pituitary gland, Tshb gene transcription was up-regulated in WT mice after Fluopyram exposure but not in knock out mice. In the thyroid gland proliferation test in WT mice increase of cell proliferation was observed but not in Car and Pxr depleted mice, suggesting that Car activation seems be involved also in thyroid gland effects.

Confidential study (2013c): Fluopyram: Enzyme and DNA-Synthesis induction in cultured rat hepatocytes, main study; non-guideline, non-GLP

Primary rat hepatocytes were isolated by in situ perfusion from approximately 7 weeks old female Han Wistar rats.
The potential of fluopyram to induce characteristic effects of CAR/PXR activation in cultured rat hepatocytes was investigated. The hepatocytes were exposed to fluopyram (purity 98.7% w/w) at 6 concentrations (1, 3, 10, 30, 100 and 300 µM) for 96 hours. Then CYP2B, CYP2B/3A and CYP3A activities were measured. Additionally, replicative DNA synthesis (S-phase) determined by the incorporation of BrdU followed by immunostaining and cellular adenosine 5'-triphosphate (ATP) concentration determined by luminometry indicative of cytotoxicity was tested. Phenobarbital (10, 100 and 1000 µM) and epidermal growth factor (EGF; 25 ng/mL) were included as positive control for induction of CYP2B/3A activities (PB only) and cell proliferation. 0.1% v/v DMSO was used as vehicle control.
In hepatocytes treated with fluopyram a dose-dependent increase in PROD, BROD and BQ activity was observed. PROD and BROD activity was increased to 2.8-fold and 4.2-fold of control value at 30 µM, respectively. The degree of induction decreased as the concentration of fluopyram was increased to 100 and 300 µM. BQ showed a maximum of activity of 18–fold at 100 µM.
Culturing primary human hepatocytes, for 96 hours with PB resulted also in an increase in PROD and

BROD of up to 4.6-fold and 5.8-fold at 100 µM, and BQ activity of up to 12-fold at 1000 µM, respectively.

Table 20

Treatment	**Enzyme activity measurement** Mean ± SD (mean % control ± SD)		
n = 3 per group	**PROD** (pmol resorufin formed/min/mg protein)	**BROD** (pmol resorufin formed/min/mg protein)	**BQ** (nmol 7-Hydroxyquinoline formed/min/mg protein)
Vehicle Control	0.247 ± 0.046 (100.0 ± 18.7)	1.734 ± 0.255 (100.0 ± 14.7)	0.045 ± 0.009 (100.0 ± 20.5)
PB 10 µM	0.529 ± 0.123* (213.9 ± 49.7)	3.835 ± 0.309*** (221.1 ± 17.8)	0.077 ± 0.006** (170.8 ± 13.4)
PB 100 µM	1.145 ± 0.222** (462.9 ± 89.9)	10.142 ± 0.704*** (584.8 ± 40.6)	0.119 ± 0.004*** (265.5 ± 8.4)
PB 1000 µM	1.110 ± 0.196** (448.9 ± 79.1)	9.560 ± 1.419*** (551.3 ± 81.8)	0.538 ± 0.019*** (1198.8 ± 43.2)
Fluopyram 1 µM	0.456 ± 0.079* (184.5 ± 32.0)	4.661 ± 1.087* (268.7 ± 62.7)	0.121 ± 0.011*** (268.7 ± 24.3)
Fluopyram 3 µM	0.437 ± 0.146 (176.7 ± 59.1)	5.343 ± 0.639*** (308.1 ± 36.8)	0.206 ± 0.015*** (457.9 ± 34.1)
Fluopyram 10 µM	0.675 ± 0.027*** (272.9 ± 11.0)	7.223 ± 0.844*** (416.5 ± 48.7)	0.373 ± 0.033*** (831.1 ± 73.8)
Fluopyram 30 µM	0.688 ± 0.148** (278.2 ± 59.9)	7.299 ± 2.398* (420.9 ± 138.3)	0.701 ± 0.048*** (1561.5 ± 107.8)
Fluopyram 100 µM	0.465 ± 0.119* (187.9 ± 48.2)	6.480 ± 0.662*** (373.7 ± 38.1)	0.808 ± 0.121*** (1800.9 ± 269.2)
Fluopyram 300 µM	0.456 ± 0.019** (184.4 ± 7.8)	1.693 ± 0.217 (97.7 ± 15.7)	0.066 ± 0.011 (147.2 ± 25.5)

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

In the replicative DNA synthesis test dose-dependent increases following treatment with PB (up to 2.9-fold) and fluopyram (maximal stimulation at 30 µM fluopyram, 3.9-fold) could be observed. Exposure of hepatocytes to higher fluopyram concentrations resulted in less stimulation of S-phase. Treatment with EGF resulted in an increase in replicative DNA synthesis to 4.2-fold control values.

Table 21

Treatment n = 5 per group	**Labelling Index (%)** Mean ± SD; (mean % control ± SD)
Vehicle Control	10.97 ± 2.32 (100.0 ± 21.1)
PB 10 µM	24.59 ± 3.00*** (224.3 ± 27.4)
PB 100 µM	32.34 ± 2.07*** (294.9 ± 18.8)
PB 1000 µM	29.57 ± 2.99*** (269.7 ± 27.3)
Fluopyram 1 µM	30.82 ± 4.04*** (281.0 ± 36.8)
Fluopyram 3 µM	33.31 ± 3.66*** (303.8 ± 33.4)
Fluopyram 10 µM	37.79 ± 2.386*** (344.6 ± 26.0)
Fluopyram 30 µM	42.56 ± 2.09*** (388.1 ± 19.1)

Fluopyram 100 µM	28.67 ± 2.80*** (261.4 ± 25.5)
Fluopyram 300 µM	[a]
EGF 25ng/mL	46.07 ± 3.29*** (420.1 ± 30.0)

[a] Analysis unable to be performed due to excessive cytotoxicity.
*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Fluopyram (1 - 100 µM) had little, or no, effect on cellular ATP concentrations after 96 hours exposure. At a concentration of 300 µM a marked decrease in ATP concentration, demonstrating cytotoxicity, was observed.

Table 22

Treatment n = 6 per group	**ATP (% Control)** Mean ± SD
Vehicle Control	100.0 ± 8.7
PB 10 µM	80.5 ± 14.6
PB 100 µM	90.8 ± 14.6
PB 1000 µM	108.9 ± 14.5
Fluopyram 1 µM	89.0 ± 8.2
Fluopyram 3 µM	104.4 ± 5.0
Fluopyram 10 µM	106.4 ± 10.0
Fluopyram 30 µM	119.3 ± 7.4*
Fluopyram 100 µM	110.5 ± 10.0
Fluopyram 300 µM	31.3 ± 8.6***

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

In conclusion, fluopyram and Pb exhibited similar effects in rat hepatocytes, including cytochromes P450 activation as demonstrated by dose-dependent increase in PROD, BROD and BQ activity, representing induction of CYP2B and CYP3A, respectively. In common with PB, fluopyram stimulated replicative DNA synthesis (S-phase) in the primary rat hepatocytes. These data suggest that fluopyram is an activator of both CAR and PXR.

Confidential study (2013d): Fluopyram: Enzyme and DNA-Synthesis induction in cultured human hepatocytes, main study; non-guideline, non-GLP
The potential of fluopyram to induce characteristic effects of CAR/PXR activation in cultured human hepatocytes was investigated. Primary human hepatocytes were exposed to fluopyram (batch code AE C656948-01-06, purity 98.7% w/w) at 6 concentrations (1, 3, 10, 30, 100 and 300 µM) for 96 hours.
In hepatocytes treated with fluopyram a dose-dependent increase in PROD, BROD and BQ activity could be observed. PROD activity was increased to 1.9-fold of control value at 100 µM. Treatment with 300 µM fluopyram reduced PROD activity to 5% of the control, reflecting the cytotoxicity shown by the ATP and S-phase. BROD activity was increased up to 2-fold at 300 µM. BQ showed a maximum of activity of 1.8–fold at 10 µM, which could indicates that fluopyram is an inducer of CYP3A at low concentrations.
Culturing primary human hepatocytes with PB for 96 hours, resulted also in an increase in PROD, BROD and BQ activity of up to 3.1-fold, 4-fold and 5.2-fold at 1000 µM, respectively.

Table 23

Treatment	**Enzyme activity measurement** Mean ± SD (mean % control ± SD)		
n = 3 per group	**PROD** (pmol resorufin formed/min/mg protein)	**BROD** (pmol resorufin formed/min/mg protein)	**BQ** (nmol 7-Hydroxyquinoline formed/min/mg protein)

Vehicle Control	0.132 ± 0.005 (100.0 ± 3.5)	0.612 ± 0.107 (100.0 ± 17.5)	0.066 ± 0.016 (100.0 ± 24.2)
PB 10 µM	0.306 ± 0.026*** (231.1 ± 19.8)	0.865 ± 0.105* (141.2 ± 17.1)	0.079 ± 0.002 (119.6 ± 2.5)
PB 100 µM	0.224 ± 0.021** (169.6 ± 16.0)	0.741 ± 0.033 (121.0 ± 5.5)	0.156 ± 0.004*** (236.8 ± 6.5)
PB 1000 µM	0.413 ± 0.045*** (312.0 ± 34.2)	2.473 ± 0.089*** (403.8 ± 14.5)	0.344 ± 0.045*** (523.3 ± 67.9)
Fluopyram 1 µM	0.206 ± 0.035* (155.6 ± 26.2)	0.550 ± 0.011 (89.9 ± 1.8)	0.105 ± 0.027 (159.4 ± 41.1)
Fluopyram 3 µM	0.236 ± 0.018*** (178.6 ± 13.9)	0.515 ± 0.102 (84.1 ± 16.6)	0.110 ± 0.011* (167.5 ± 16.9)
Fluopyram 10 µM	0.200 ± 0.116 (151.4 ± 87.7)	0.671 ± 0.100 (109.5±)16.4	0.120 ± 0.011** (182.2 ± 16.1)
Fluopyram 30 µM	0.179 ± 0.053 (135.5 ± 40.0)	0.840 ± 0.040* (137.1 ± 6.5)	0.088 ± 0.008 (134.4 ± 11.6)
Fluopyram 100 µM	0.246 ± 0.037** (186.1 ± 27.8)	1.182 ± 0.206* (193.0 ± 33.6)	0.037 ± 0.005* (56.2 ± 7.2)
Fluopyram 300 µM	0.007 ± 0.012*** (5.4 ± 9.4)	1.222 ± 0.269* (199.5 ± 43.9)	0.008 ± 0.003** (12.7 ± 5.3)

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

In replicative DNA synthesis there were no changes observed following treatment with either PB or fluopyram. At a concentration of 100 µM fluopyram a decrease in hepatocyte confluency was noted. At 300 µM no cells could be analysed due to extensive cytotoxicity. Treatment with EGF resulted in an increase in replicative DNA synthesis to 15-fold control values, indicating that the hepatocytes could proliferate following exposure to proliferative stimuli.

Table 24

Treatment n = 5 per group	**Labelling Index (%)** Mean ± SD; (mean % control ± SD)
Vehicle Control	0.49 ± 0.09 (100.0 ± 18.5)
PB 10 µM	0.43 ± 0.16 (88.4 ± 32.4)
PB 100 µM	0.31 ± 0.11* (63.8 ± 22.2)
PB 1000 µM	0.43 ± 0.15 (87.4 ± 30.3)
Fluopyram 1 µM	0.54 ± 0.13 (109.9 ± 27.2)
Fluopyram 3 µM	0.45 ± 0.03 (91.7 ± 7.1)
Fluopyram 10 µM	0.51 ± 0.16 (103.7 ± 33.2)
Fluopyram 30 µM	0.43 ± 0.04 (88.7 ± 7.9)
Fluopyram 100 µM	0.32 ± 0.04** (66.1 ± 9.1) [a]
Fluopyram 300 µM	[b]
EGF 25ng/mL	7.13 ± 0.30*** (1461.4 ± 60.7)

[a] Decrease in hepatocyte confluency, [b]Analysis unable to be performed due to excessive cytotoxicity.
*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

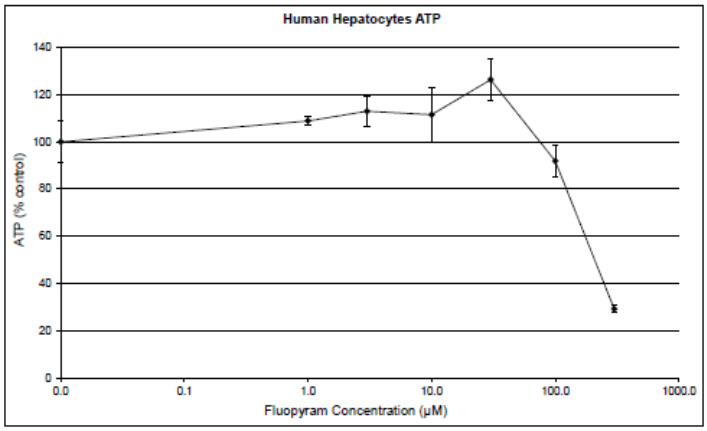
A slight decrease in ATP concentration was observed at fluopyram exposure of 100 µM, which was more severe at 300 µM, indicating the start of cytotoxicity at 100 µM, which was clearly evident at 300 µM.

Table 25

Treatment n = 6 per group	**ATP (% Control)** Mean ± SD
Vehicle Control	100.0 ± 8.7
PB 10 µM	110.0 ± 5.2*
PB 100 µM	103.4 ± 7.1
PB 1000 µM	110.8 ± 9.5
Fluopyram 1 µM	108.9 ± 1.6*
Fluopyram 3 µM	113.0 ± 6.3*
Fluopyram 10 µM	111.5 ± 11.5
Fluopyram 30 µM	126.3 ± 8.6***
Fluopyram 100 µM	91.9 ± 6.9
Fluopyram 300 µM	29.1 ± 1.4***

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

Figure 1



In conclusion, Fluopyram and PB exhibited similar effects in human hepatocytes, including cytochromes P450 activation as presumed via the Pregnane X Receptor (PXR) and the CAR. This is demonstrated by dose-dependent increase in PROD, BROD and BQ activity, representing induction of CYP2B and CYP3A, respectively.
Treatment with fluopyram and PB had no effect on replicative DNA synthesis in the primary cells indicating that human CAR/PXR did not mediate compound-stimulated DNA synthesis (S-phase) in human hepatocytes, whilst the positive control EGF elicited a 15-fold increase in S-phase.

Confidential study (2013e): Fluopyram: Assessment of Pentoxyresorufin-O-depentylation and Benzyloxyresorufin-O-debenzylation in 50 liver microsomal samples; non-guideline, non-GLP
PROD and BQ activity in microsomal samples prepared from liver of control, fluopyram or PB treated male C57BL/6J mice were tested. Microsomal samples were originally from rat liver taken in confidential study (2012b*, amended 2013*). In this study fluopyram (batch number: 94.7% purity), was administered daily via the diet for at least 28 days to the mice at dose levels of 30, 75, 150, 600 and 750 ppm (5, 13, 25, 102 and 128 mg/kg bw/d). Additionally, one group of mice received 80 mg/kg bw/d PB (100% purity) by gavage and the negative control group were kept on unchanged diet only for 28 days. The 28-day exposure was followed by a one month recovery period for control, PB and fluopyram high dose groups.
28 days exposure to fluopyram and PB resulted in a dose-dependent increase in PROD activity to a maximum 47.2-fold (at 750ppm) and 32.7-fold, respectively.
Following a 28-day recovery period on control diet, PROD activity in both fluopyram and PB treated mice returned to control levels.

Table 26

Treatment, 28 days exposure n = 5 per group	**PROD (pmol resorufin formed/min/mg protein)** Mean ± SD; (mean % control ± SD)
Control	4.65 ± 1.12 (100.0 ± 24.2)
Fluopyram 30ppm	67.22 ± 6.95*** (1447.1 ± 149.5)
Fluopyram 75ppm	156.89 ± 46.37*** (3377.5 ± 998.3) a
Fluopyram 150ppm	170.99 ± 29.09*** (3680.8 ± 626.2)

Fluopyram 600ppm	201.51 ± 45.02*** (4338.0 ± 969.1)
Fluopyram 750ppm	219.06 ± 31.31*** (4715.8 ± 674.0)
PB 80 mg/kg bw/d	151.87 ± 48.84*** (3269.3 ± 1051.4)
Treatment, 28-day recovery n = 5 per group	
Control	4.63 ± 0.82 (100.0 ± 17.7)
Fluopyram 750ppm	5.27 ± 0.75 (113.9 ± 16.1)
PB 80 mg/kg bw/d	5.33 ± 0.31 (115.1 ± 6.6)

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

28 days dietary administration of fluopyram resulted in a dose-dependent increase in BQ activity to a maximum 6.2-fold at 750ppm. 28 days administration of PB at 80 mg/kg bw/d resulted in a 3.0-fold increase in BQ activity.
Following a 28-day recovery period on control diet, BQ activity in both fluopyram and PB treated mice returned to control levels.

Table 27

Treatment, 28-day exposure n = 5 per group	**BQ (nmol 7-Hydroxyquinoline formed/min/mg protein)** Mean ± SD; (mean % control ± SD)
Control	7.59 ± 0.67 (100.0 ± 8.8)
Fluopyram 30ppm	10.68 ± 1.92* (140.7 ± 25.3)
Fluopyram 75ppm	16.71 ± 2.61*** (220.2 ± 34.4)
Fluopyram 150ppm	21.98 ± 1.76*** (289.7 ± 23.2)
Fluopyram 600ppm	39.20 ± 7.49*** (516.6 ± 98.6)
Fluopyram 750ppm	47.24 ± 3.85*** (622.6 ± 50.8)
PB 80 mg/kg bw/d	23.00 ± 5.24*** (303.1 ± 69.1)
Treatment, 28-day recovery n = 5 per group	
Control	6.93 ± 1.01 (100.0 ± 14.5)
Fluopyram 750ppm	6.68 ± 1.27 (96.4 ± 18.4)
PB 80 mg/kg bw/d	6.45 ± 1.22 (93.1 ± 17.7)

*statistically different from control p<0.05; ** p<0.01; ***p<0.001.

In conclusion, fluopyram or PB resulted in PROD and BQ activity increase after a 28-day exposure to male C57BL/6J mice with signs of complete reversibility after recovery period.

Assessment and comparison with the classification criteria

Liver tumours

In the rat carcinogenicity study, liver cell carcinomas and adenomas were observed in female rats at the

highest dose (1500 ppm, 89 mg/kg bw).

The mortality rate in control males after 24 months was twice as high as in control females (male control: 61.7%, high dose: 81.7%; female control: 31.7%, high dose: 50%). The reason is unclear. It can be argued that the toxicity is well above the MTD, as the mortality is between 15 and 32 % relative to the control, but given the high mortality also in the controls, this is less certain. No historical control data were provided for this study. However, data on Wistar rats from the same period of time is available from Charles River (Giknis and Clifford, 2011), indicating that 1 case of liver carcinoma was found in 40 studies, while adenomas were seen in 9 out of 40 studies with a frequenzy of between 1 and 11 (median 1).

One of the DS arguments for the Cat 2 classification proposal is the lack of CAR activation data. Data supporting CAR activation, including studies with knock out mice, were provided during PC. These studies reported the following:

- Activation of the CAR
  as shown in knock out mice (*Confidential study, 2013b)*, see Table 28 below;
- Specific CYP enzyme induction (CYP 2B family) including hypertrophy of liver
  shown in different oral repeated dose studies with female rats as gene transcription activation of phase I enzymes and as Cyp 450 isoenzyme profile as PROD activity and others;
- Increased hepatocellular proliferation in the rat
  shown in different oral studies with female rats treated for 3 to 28 days and as replicative DNA synthesis (S-phase) stimulation in *in vitro* study with primary rat hepatocytes (*Confidential study, 2013c);*
- Lack of hepatocellular proliferation (S-phase) in human
  shown in *in vitro* study with primary human hepatocyte (*Confidential study (2013d);*
- Reversibility of effects,
  28-day rat study (only with females) plus one month recovery showed recovery, although not complete;

Key event K/O mice study

(28-day oral male mouse study with WT (C5/BL/6J) and Pxr KO/Car KO strain)

Table 28[2]

Effects	WT mouse 1500 ppm Fluopyram	Pxr KO/Car KO mouse 1500 ppm Fluopyram
liver weight, - mean absolute - mean liver to BW ratio - mean liver to brain weight ratio	 + 66% +62% +66%	 +11% +9% +13%
Liver, enlarged in mice	14/15	-

[2] Note that the numbering of Tables for the RAC evaluation on the carcinogenicity part is in a separate order comparing to the opinion document due to the insertion of the additional key information section.

Hepatocellular hypertrophy	15/15	-
Hepatocellular necrosis, min-slight	10/15	-
Hepatocytes, increased number of mitosis	3/15	-
Interstitial cell infiltration, focal	11/15	-
Increased PROD	151x	1.4x
Increased BQ	7.9x	1.7x
Increased Bil-GT	2.0x	-

Based on the data provided RAC considers that it has been demonstrated that a CAR mediated MoA contributes to the formation of liver tumours.

The possibility that other MoAs could be operating was also considered. Industry provided some experimental evidence against one of the MoAs and argumentation against several others (such as no structural similarities with estrogen and that the other MoAs would be seen in the K/O mice). RAC considers these arguments reasonable.

- Genetic toxicity can be ruled out based on the mutagenicity studies submitted.
- PPARα activation seems not likely due to lack of induction of Cyp4a1.
- Significant induction of the Cyp1a1 gene was reported in the CLH report. An assessment of microsomal proteins revealed a slight increase in mean EROD measurements in several repeated dose toxicity studies in the rat. This can be indicative of activation of AhR. However, for an AhR agonist the increase in EROD is normally considerably larger than found in the studies described in the CLH report. It can also be argued that activation of the AhR would be expected in both species and sexes, whereas liver tumours are seen only in one species and one sex. Also in the cells *in vitro* it would be expected to see proliferation if AhR activation would occur. It has to our knowledge not been shown that this applies to human hepatocytes, such as used in *Confidential study* (2013d) but it would be a reasonable assumption.
- There is no histopathological evidence for estrogens, statins, metals and infection mechanisms, and no structural dissimilarity to estrogens.

Table 29 Gene expression given as mean fold change relative to controls for female Wistar rats exposed to fluopyram for 3, 7, or 28 days.

28 days Dose (ppm)		**30**	**75**	**150**	**600**	**1500**
Associated receptor	Rat Genes	**3 days**				
Ahr	*Cyp1a1*	-1.2	1.1	1.7	**7.3**	**62.7**
Car	*Cyp2b1*	-1.6	1.1	**3.3**	**49.6**	**244.1**
Pxr	*Cyp3a3*	1.1	**1.5**	**2.6**	**8.2**	**21.5**
Pparα	*Cyp4a1*	-1.1	NC	-1.1	NC	-1.3
Associated receptor	Rat Genes	**7 days**				
Ahr	*Cyp1a1*	1.4	1.8	**4.6**	**63.6**	**222.9**

Car	*Cyp2b1*	2.6	3.1	**14.4**	**326.5**	**1434.0**
Pxr	*Cyp3a3*	1.5	**1.9**	**3.6**	**12.4**	**28.6**
Pparα	*Cyp4a1*	-2.1	-2.4	-2.3	-2.3	**-3.2**
Associated receptor	Rat Genes	**28 days**				
Ahr	*Cyp1a1*	1.8	2.3	**8.1**	**100.9**	**354.7**
Car	*Cyp2b1*	2.7	1.7	**10.9**	**212.5**	**1543.8**
Pxr	*Cyp3a3*	**1.8**	**3.7**	**5.3**	**17.1**	**50.4**
Pparα	*Cyp4a1*	-1.2	-1.1	NC	-1.2	-1.4

Taken together, RAC considers the possible contribution of other MoAs sufficiently excluded.

Thyroid tumours:

In the carcinogenicity study in male mice, follicular cell adenoma was observed (7/50 investigated male mice) in the thyroid gland at the highest dose (750 ppm, 105 mg/kg bw/d). However, in female control mice 3 adenomas in 48 investigated thyroid glands were found. Historical control data were not reported.

For the discussion of the MoA for follicular cell adenomas, an extensive database was submitted during PC. The evidence seems to point to a CAR mediated (phenobarbital-like; PB) MoA. The key events documented by Industry during PC are:

- Activation of the CAR
  Shown in a study conducted with Pxr KO/Car KO mice (see Table 30 below).

- Induction of Phase II liver enzymes (e.g. UDPGT) including hypertrophy of liver. This was presented in different oral repeated dose studies with male mice as gene transcription activation of phase II enzymes and as enzyme activation such as UDPGT-Bil and UDGPT-T4. RAC notes, however, that the concentration of T4 was greatly reduced already 2 hours after administration of the substance (100 mg/kg bw/d), a time-point too early to be affected by induction (an increase in the amount) of UDPGT. Furthermore, when the activity of UDPGT was measured after 28 daily administrations of fluopyram, a statistically significant increase was only observed at the two top dose levels (>102 mg/kg bw/d), whereas the concentration of T4 was decreased already from 5 mg/kg bw/d. It is thus not certain that UDPGT is a key event.

- Decrease of T4 (fast clearance), increase of TSH, T3 unaffected
  Shown in different oral repeated dose studies with male mice and i.v 125I-thyroxine clearance study.

- Increased thyroid cell proliferation
  Shown in a 28-day oral study with male mice.

Table 30 *Key event K/O mice study*

*(*28-day oral male mouse study with WT (C5/BL/6J) and Pxr KO/Car KO strain)

Effects	WT mouse 1500 ppm Fluopyram	Pxr KO/Car KO mouse 1500 ppm Fluopyram
Thyroid gland proliferation index (mean)	+ 2.6	-

Increased T4-GT	1.9x	-1.3x
Increased Bil-GT	2.0x	-
Tshb gene transcription in pituitary gland	+67%	-17%

Humans are often less sensitive to induction of thyroid tumours compared to rodents. It can, however, not be concluded that thyroid tumours in rodents are never relevant to humans. During PC, data were provided, including studies on knock-out mice, supporting CAR activation. Industry argued that:

"The main reasons for the difference in response between rodents and humans are as follows:

I. Rodents are more sensitive to thyroid hormone changes
II. Rodents have enhanced thyroid hormone elimination
III. Thyroxine binding globulin is major plasma protein in humans (which acts as a buffer), but not in rodents
IV. Consequence, the concentration of unbound T4 is greater in rodents than humans, resulting in greater susceptibility to metabolism and excretion and compensatory increase in thyroid follicular cell turnover, which over time can result in thyroid tumors."

This, together with the fact that the DS did not consider the thyroid tumours suitable for classification could lead to no classification. However, while the DS did not consider the thyroid tumours relevant for classification, their argumentation as to why not, was not extensive. Concerning non-relevance to humans humans, CLP guidance states for example that "*certain thyroid tumours in rodents mediated by UDP glucuronyltransferase (UGT) induction (IARC, 1999; EU Specialised Experts, 1999)*". The MoA, it was argued by Industry, is indeed UGT mediated. However, the specialised experts excluded "*liver enzyme inducing agents such as PB*" from their recommendation. The specialised experts were not convinced that substances acting as liver enzyme inductors could significantly alter the levels of thyroid hormones. However, subsequent data shows that liver enzyme inductors may indeed alther the levels of thyroid hormones.

Although there is a plausible MoA suggested for induction of thyroid tumours via a CAR mediated MoA, the evidence of such tumours is scarce. There are studies where PB enhances the tumorigenic potency of genotoxic carcinogens (Hiasa *et al*, 1982, 1983: McClain *et al*, 1988). In these studies no induction of thyroid tumours were seen after treatment with PB alone, but the treatment period was short. Dellarco *et al.* (2006) reported on a substance inducing similar effects on the thyroid as fluopyram and inducing tumours but although it was possibly via CAR activation, this was not shown.

Based on the data provided, RAC considers that in this case, it can be demonstrated that a CAR mediated MoA contributes to the formation of thyroid tumours.

- Other MoA have partly been excluded. As for liver tumours, genetic toxicity can be ruled out based on the mutagenicity studies submitted.
- Damage to Follicular cells - No histopathological evidence of overt cytotoxicity was observed in the thyroid in rodent studies
- Inhibition of thyroid peroxidase - Mechanistic studies using hog thyroid microsomes showed that fluopyram did not affect thyroid peroxidase
- Inhibition of T4 to T3 via indirect MoA is unlikely as serum levels of T3 were unchanged in rodent studies

Taken together, RAC considers the possible contribution of other MoAs in this case to be sufficiently excluded.

RAC concludes that it has beensufficiently demonstrated that the thyroid tumours induced by fluopyram

are caused by a CAR mediated MoA. This MoA might give rise to thyroid tumours in rodents.

The relevance of this MoA based on enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UGT enzymes, **is in the case of Fluopyram considered by RAC not to be relevant to humans.**

Conclusion on classification:

Thyroid tumours: RAC concludes that it is sufficiently well shown that the thyroid tumours induced by fluopyram were caused by a CAR mediated MoA. This MoA might give rise to thyroid tumours in rodents. The relevance of such an MoA based on enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UGT enzymes, is consider by RAC not to be relevant to humans. RAC considers the possible contribution of other MoAs sufficiently excluded.

Liver tumours: Based on the data provided, RAC concludes that a CAR mediated MoA was contributing to the formation of liver tumours. This MoA gives rise to liver tumours in rodents, but there is evidence that the effects in human cells differ from rodent cells. RAC considers the possible contribution of other MoAs sufficiently excluded. RAC concludes that the CAR mediated MoA is assumed to be of no relevance to humans.

RAC concludes that no classification for carcinogenicity is warranted.

4.11 Toxicity for reproduction

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Multi-generation study

Title:	Milius, A. D., Bommegowda, S. (2008): Technical Grade AE C656948: A Two Generation Reproductive Toxicity Study in the Wistar Rat, 201855, M-299334-01, ASB2008-5478.
Guidelines:	OECD 416 (2001).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered acceptable.

Materials and methods

Test Material:	AE C656948
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle or positive control:	Acetone
Stability of test compound:	Stable in the diet at concentrations of 5 ppm and 5000 ppm when stored for 7 days at room temperature followed by 28 days of storage in the freezer
Species:	Rat
Strain:	Wistar Han Crl: WI(HAN)
Age / weight:	(P) 8 weeks; (P) Males: 224.9 – 279.1 g; Females: 145.6 – 203.3 g
Source/breeder:	Charles River Laboratory, Raleigh, NC, US
Acclimation period:	Six days
Housing:	Animals were housed individually (except during the mating phase and as noted below for the F_1 and F_2 pups) in suspended stainless steel cages and deotized cage board in the bedding trays. During gestation and lactation, individual dams (and litters) were housed in polycarbonate cages with corn-cob bedding
Environmental conditions:	Temperature: 18 – 26 °C
	Humidity: 30 – 70 %
	Air changes: At least 10 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles
Diet:	Purina Mills Rodent Lab Chow 5002 meal a*d libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: Following a minimum of six days of quarantine/acclimation, animals were examined by a veterinarian and released for study use. The animals were randomly assigned to either a control or one of three chemically-treated groups using a weight stratification-based computer program (INSTEM Computer Systems, Stone, Staffordshire, UK). Only those animals falling within +/- 20 % of the mean for all animals (per sex) were placed on study. Once animals were assigned to their dose groups, each rat on study had a microchip (Biomedic Data Systems, Inc. Seaford, DE) subcutaneously implanted on its' dorsal surface in the region between the scapulae. At a minimum, the chip was encoded with a unique number, specifying the animal's sex, dose group, cage number, and study affiliation. In addition, a laminated cage card, essentially duplicating the information present on the chip, was attached to the outside of each animal's cage. Pups born alive were identified by tattoo and pups found dead were identified with an indelible marking pen.

Study schedule: One hundred and twenty female and one hundred and twenty male rats were assigned to one of four treatment groups (30 animals/sex/group): nominal doses of 0, 40, 220 and 1200 ppm fluopyram in the diet (see Table 136). Animals were exposed to the treated feed throughout the entire in-life phase of the study. In-life phases included: Premating: 10 weeks; Mating: 14 days; Gestation: approximately 22 days; and Lactation: weaning on Day 21. F_1-pups were maintained after weaning for approximately six weeks prior to initiation of the second generation.

Table 136: Animal Assignment

Test group	Dose in Diet[a] (ppm)	Animals/group			
		P Males	P Females	F_1 Males	F_1 Females
Control	0	30	30	30	30
Low (LDT)	40	30	30	30	30
Mid (MDT)	220	30	30	30	30
High (HDT)	1200	30	30	30	30

[a] = Diets were administered from beginning of the study until sacrifice. LDT – Low dose tested, MDT – Mid dose tested, HDT – High dose tested

Mating procedure: Males and females were exposed to the test substance for ten weeks prior to mating. Mating was accomplished by co-housing one female with one male for up to 14 consecutive days. During the mating phase, vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination was observed in the vaginal smear was designated Day 0 of gestation for that female. In order to evaluate those females which might be inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in polycarbonate nesting cages, following the 14-day mating period.

Dose selection rationale: Doses were selected based upon the preliminary results which emerged in the rat over the course of a pilot reproductive toxicity testing study conducted with the test chemical at doses of 0, 30, 150, 750 and 1500 ppm fluopyram (Milius, 2008, ASB2008-5480). In that study, there were no compound-related effects observed on body weight, food consumption or clinical observations at any dietary level tested. Liver and kidney weight changes were observed in the males of the 750- and 1500-ppm dose groups. Females also exhibited liver weight changes in both the 750- and 1500-ppm dose group. Changes in clinical chemistry and hematology parameters were also noted at these same dose levels in either the males or females. Based on these results, the doses selected for the two-generation reproduction toxicity study were 0, 40, 220, and 1200 ppm fluopyram. This dose range was intended to produce evidence of toxicity at the highest dietary concentration and no parental or reproductive effects at the lowest dietary concentration.

Dosage preparation and analysis: The test substance was dissolved in acetone and then mixed with the feed. The control test diet was prepared in the same manner as the chemically-treated test diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained in the freezer until the study was completed and the analytical data deemed satisfactory. Replacement

admixtures for each treatment group were prepared weekly (or at greater intervals, if within freezer stability limits) during the entire study and stored at freezer conditions until presented to the animals. Additionally, the entire batch of replacement admixture for each treatment group was used for subsequent weekly feeding if within freezer stability limits. The concentration of the test substance in the feed for the females was adjusted down by 50 % during the lactation period (Days 0-21) as follows, to avoid the large increase in dosage (mg/kg bw/day) that is otherwise associated with increased feed consumption that occurs during lactation. Thus, during lactation days 0-21, the dietary concentrations were reduced from 40, 220 and 1200 ppm to 20, 110 and 600 ppm, respectively.

Calculation for test substance intake is: [Mean analytical concentration (ppm) specific for each phase / 1000 X mean weekly food consumption (g/kg/body weight/day) for each phase]. Exceptions are that weeks 18 and 22 were analyzed but not included in substance intake calculations due to this being the developmental landmark phase in which food consumption is not measured. The concentration of fluopyram in the various test diets was analytically verified for batches intended for weeks 1, 2, 3, and at monthly intervals thereafter (Bayer CropScience LP, Environmental Research, 17745 S. Metcalf, Stilwell, KS).

Statistics and calculation of reproductive and offspring indices: The data were analyzed using applications provided by DATATOX (Instem Computer Systems), SAS (SAS Institute, Inc.), or TASC (Toxicology Analysis Systems Customized, 1993). Parametric data (including body weight gain and food consumption) were analyzed using a univariate Analysis of Variance (ANOVA), and if significant differences were observed, a Dunnett's Test was performed. Nonparametric data (e.g., number of estrous cycles, litter size, and number of implantation sites) was first analyzed by the Kruskal-Wallis test and then subjected to Dunn's Test if significant differences were identified. Nonparametric dichotomous data (e.g. fertility and gestation indices) were initially analyzed by the Chi-Square Test and if significance was observed between groups then by the Fisher's Exact Test with the Bonferroni adjustment. To the extent possible, the frequency of gross lesions was first examined visually, then, in the event of questionable distribution, by statistical analysis using the Chi-Square and Fisher's Exact tests. Differences between the control and test substance-treated groups were considered statistically significant when $p \leq 0.05$ or $p \leq 0.01$.

The following reproductive indices were calculated from breeding and parturition records of animals in the study:

$$\text{Mating Index (\%)} = \frac{\text{\# inseminated females}^{a}}{\text{\# of females co-housed}} \times 100$$

$$\text{Fertility Index (\%)} = \frac{\text{\# of pregnant females}^{b}}{\text{\# of inseminated females}} \times 100$$

$$\text{Gestation Index (\%)} = \frac{\text{\# of females with live pups}}{\text{\# of pregnant females}} \times 100$$

[a] Includes pregnant females not observed sperm positive or with an internal vaginal plug.

[b] Includes females which did not deliver but had implantation sites.

The following viability indices were calculated from lactation records of litters in the study:

$$\text{Birth Index (\%)} = \frac{\text{total \# of pups born/litter}}{\text{total \# of implantation sites/litter}} \times 100$$

$$\text{Livebirth Index (\%)} = \frac{\text{\# of live pups born/litter}}{\text{total \# of pups/litter}} \times 100$$

$$\text{Viability Index (\%)} = \frac{\text{\# of live pups/litter on day 4 (pre-culling)}}{\text{\# of live pups born/litter}} \times 100$$

$$\text{Lactation Index (\%)} = \frac{\text{\# of live pups/litter on day 21}}{\text{\# of live pups/litter on day 4 (post-culling)}} \times 100$$

Mortality and clinical observations: Mortality checks (cage side observations) were performed twice daily (AM and PM) during the workweek and once daily on weekends and holidays. Cage side observations characterized mortality, morbidity, behavioral changes, signs of difficult or prolonged delivery and overt toxicity by viewing the animal in the cage. In the event a possible clinical sign was observed during the cage side evaluation, the animal was removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs included both observing the animal in the cage and removing the animal to perform a physical examination and was conducted at least once per week throughout the entire in-life phase of the study.

Body weights and food consumption: Body weight and food consumption was measured and fresh feed provided once per week for both males and females during the 10-week premating period. During the mating period and until sacrifice, body weight for the males and unmated females were measured once per week. Also during the mating period, fresh feed was provided for both males and unmated females once each week without measuring food consumption. During gestation, dam body weight was measured on Days 0, 6, 13, and 20, and fresh feed was provided and food consumption measured once each week. During lactation, dam body weight was measured on Days 0, 4, 7, 14, and 21. Fresh feed was provided and food consumption measured once per week, with the exception of week one when food consumption was measured twice (Days 0-4 and 4-7).

Estrous cyclicity: The estrous cycle (determined by examining daily vaginal smears) was characterized for all P- and F_1-generation females, over a three-week period prior to mating. Additionally, the estrous cycle stage was determined for all females just prior to termination.

Sperm parameters: For all P- and F_1-generation males at termination, sperm was collected from one testis and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, an evaluation of the morphology and motility was performed on sperm sampled from the distal portion (closest to the urethra) of the vas deferens. Sperm motility and counts was conducted using IVOS (Integrated Visual Operating Systems, 2005). Morphology and counts were conducted on the control and highest dose group.

Hematology and clinical chemistry: Prior to sacrifice, 10 adult rats/sex/group were fasted overnight with water available prior to blood sample collection. Each adult rat was anesthetized with inhaled Isoflurane and blood samples were collected from the orbital sinus (~500 µL for hematology: in tubes containing EDTA and ~1000 µL for serum chemistry) using a capillary tube. Samples were transferred to the clinical pathology department as soon as possible after collection.

Hematologic parameters: Hematocrit (HCT); Hemoglobin (HGB); Total erythrocyte count (RBC); Erythrocyte indices (MCV, MCH, MCHC); Red cell distribution width (RDW); Differential leukocyte

count; Total leukocyte count (WBC); RBC morphology; Total platelet count (PLT); Hemoglobin Distribution Width (HDW)

Serum Chemistry parameters: Albumin (ALB); Globulin (GLOB); A/G ratio (A/G); Alanine aminotransferase (ALT); Aspartate aminotransferase (AST); Alkaline phosphatase (ALK); Total protein (TP); Total bilirubin (T.BIL); Glucose (GLUC); Cholesterol (CHOL); Triglyceride (TRIG); Urea nitrogen (BUN); Creatinine (CREAT); Sodium (Na); Potassium (K); Chloride (Cl); Calcium (Ca); Phosphorus (PO_4); Creatine Phosphokinase (CPK)

Litter observations: The following litter observations (X) were made (see Table 137).

Table 137: F1 / F2 Litter Observations [a]

Observation	Time of Observation (lactation day)						
	Day 0	Day 4[a]	Day 4[b]	Day 7	Day 14	Day 21	Days (0-21)
Number of live pups							X
Pup weight	X	X		X	X	X	
External alterations	X	X		X	X	X	
Number of dead pups							X
Sex of each pup (M/F)	X						
Preputial Separation	Performed post weaning						
Vaginal Patency	Performed post weaning						

[a] Before standardization (culling) [b] After standardization (culling)

The size of each litter was adjusted on lactation Day 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (e.g., three females and five males). No adjustments were made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS. Culled pups were sacrificed by decapitation. Grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded. The F_1- and F_2-pups not culled on lactation Day 4 were maintained with the dam until weaning on lactation Day 21. On lactation Day 21, a sufficient number of F_1-pups/sex/litter was maintained to produce the next generation. F_1-pups not selected to become parents of the next generation and all F_2-pups were sacrificed, examined macroscopically and had organs weighed. One pup/sex/litter for each generation had tissues collected and evaluated for any structural abnormalities or pathological changes, particularly as they related to the organs of the reproductive system. Dead pups were examined grossly for external and internal abnormalities, and a possible cause of death was determined for pups stillborn or found dead.

Postmortem observations: Parental animals: All surviving parental males were sacrificed as soon as possible after the last litters were produced. Maternal animals were sacrificed following the weaning of their respective litters (lactation Day 21). The animals were subjected to postmortem examinations as follows.

Male rats were euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all males. For all males at termination, sperm was collected from one testes and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, the morphology and motility of sperm samples from the distal portion (closest to the urethra) of the vas deferens was evaluated. Sperm motility and counts were conducted using IVOS (Integrated Visual Operating System, 2005).

Each dam (both P- and F_1-generations) was euthanized by carbon dioxide asphyxiation and a gross external examination was performed. Terminal body weights (fasted terminal body weights from those animals bled) were measured and the recording of all gross pathologic alterations, weighing designated organs, and saving all gross lesions was conducted on all females. The uterus was excised and the

implantation sites, if present, were counted. Females which were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation Day 24. Females that were never observed as being inseminated and/or with an internal vaginal plug and did not deliver at least 24 days after the completion of the mating phase, were sacrificed and necropsied. A gross necropsy was performed on these animals as described above. In addition, patency of the cervical/uterine *os* in these females was examined via flushing of the uterine horns with 10 % buffered formalin.

Animals found moribund while on study were sacrificed and a gross necropsy performed. Animals found dead were necropsied as soon as possible. Necropsy examinations included those parameters previously described. Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or to determine the cause of death. The F_1-offspring not selected as parental animals and all F_2-offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination) as follows. As summarized in Table 138, the following tissues were collected (X) or collected and weighed (XX). Micropathology was performed on those tissues designated with (XXX). Tissues designated with 'O' were also collected in the F generations (from 21-day weanlings).

Table 138: Tissue list

XX/O	Brain	XXX/O	Epididymis
XXX	Pituitary	XXX/O	Coagulating Gland
XXX	Liver	XXX/O	Ovary
XXX	Kidney	XXX/O	Oviduct
XXX/O	Spleen	XXX/O	Prostate
XX	Thyroid	XXX/O	Seminal Vesicle
XX/O	Thymus	XXX/O	Testis
XXX	Adrenal	XXX/O	Uterus
X/O	Gross Lesions	XXX/O	Vagina
XXX/O	Cervix		

Findings

Dose preparation analysis

Homogeneity Analysis: The mean concentrations of fluopyram in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 5- or 5000-ppm, were determined to be 5.18 ppm (range 5.00-5.39 ppm; %RSD = 2.66) and 4975 ppm (range 4866-5115 ppm; %RSD = 1.79), respectively. Based on a %RSD $\leq$ to10 %, fluopyram was judged to be homogeneously distributed in the feed over a concentration range of 5-5000 ppm.

Stability analysis: Following 7 days of room temperature storage, the analytically-determined concentration of the a.i. of the test substance in the 5- and 5000-ppm admixture was determined to be 5.36 ppm (5.18 ppm on Day 0) and 5229 ppm (5,153 ppm on Day 0), respectively. Following 28 days of freezer storage, the concentration of the test substance in the 5- and 5000-ppm admixtures was determined to be 5.02 ppm (5.18 on Day 0) and 5014 ppm (4975 on Day 0), respectively. Fluopyram mixed in rodent ration was judged to be stable at room temperature for at least seven days and following freezer storage for a minimum of 28 days, over a concentration range of 5-5000 ppm.

Concentration analysis: Mean analytical concentrations for each dose group were 39.6, 220 and 1198 ppm, ranging from 99–100 % of the corresponding nominal concentrations of 40, 220 and 1200 ppm, respectively. Mean analytical concentrations for each dose group during lactation were 20.0, 108 and 593 ppm, ranging from 98-100 % of the corresponding reduced nominal concentrations of 20, 110 and 600 ppm, respectively. Mean recovery was 101 % and ranged from 94-109 % for rodent ration spiked with 20, 40, 220 or 1200 ppm of fluopyram.

Parental animals

Mortality and clinical signs: There were no test substance-related mortalities or clinical observations observed during the course of this study at any dietary level tested in either generation.

Body weight and food consumption:

Males: The P-generation males did not exhibit any test substance-related effects on body weight or body weight gain at any dietary level tested after 15 weeks of exposure. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested.

The F_1-generation males did not exhibit any test substance-related effects on body weight or body weight gain at any dietary level tested. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested. Sporadic statistical significant effects on food consumption, considered not to be test substance-related, were observed in both the 220 and 1200 ppm dose groups.

Females (premating): The F_1-generation females of the 1200 ppm dose group exhibited declines in body weight gain (declined 9.8 %) when compared to the controls. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested. A slight decline in food consumption from Day 56 to 63 on a g/animal/day was observed in the 1200 ppm dose group and was considered to be incidental.

The *P*-generation females showed slight declines in body weight in the 1200 ppm dose group with statistical significance observed on Day 63 (declined 5.5 %) and body weight gain declined 20 % when compared to controls. No test substance-related findings were observed on food consumption during the 10-week premating period at any dietary level tested. A slight decline in food consumption from Day 56 to 63 on a g/animal/day was observed in the 1200 ppm dose group and is considered to be incidental. Results are summarized in Table 139.

Table 139: Mean (S.E.) Body Weight and Food Consumption

Observations/study week	Dose Group			
	0 ppm	40 ppm	220 ppm	1200 ppm
P Generation Males				
Mean body weight (g) - Week 15 S.E.	444.5 7.47	456.2 5.99	458.6 7.69	448.4 4.61
Mean weight gain (g) Weeks 1-15	196.3	210.9	207.1	195.8
Mean food consumption (g/animal/day) Weeks 1-10	23.3	23.8	23.5	23.5
Mean food consumption (g/kg/day) Weeks 1-10	68.8	70.2	68.4	69.6
P Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	244.9 2.81	237.6 3.52	241.0 2.72	234.2 2.69
Mean weight gain (g) Weeks 1-10	72.9	64.1	66.8	58.3
Mean food consumption (g/animal/day) Weeks 1-10	17.2	16.9	16.9	16.8
Mean food consumption (g/kg/day) Weeks 1-10	80.8	80.5	80.0	80.7
F_1 Generation Males				
Mean body weight (g) - Week 14 S.E.	462.0 7.09	451.9 8.21	465.6 6.78	456.5 5.74
Mean weight gain (g) Weeks 1-14	193.7	186.5	191.5	189.2
Mean food consumption (g/animal/day) Weeks 1-10	23.1	22.7	22.8	23.8
Mean food consumption (g/kg/day) Weeks 1-10	64.2	64.4	62.2	66.2
F_1 Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	237.5 3.51	244.1 3.97	244.5 3.63	230.6 3.14
Mean weight gain (g) Weeks 1-10	62.4	66.7	61.3	56.3
Mean food consumption (g/animal/day) Weeks 1-10	16.2	16.8	16.4	15.8
Mean food consumption (g/kg/day) Weeks 1-10	77.1	78.6	75.4	76.8

Females (gestation): Statistically significant body weight declines, relative to control, were observed in the P generation females of the 1200 ppm dose group from gestation Day 0-13 (mean decline of 5.5 %). The lower dose groups did not exhibit any effects on body weight. There was no effect on body weight gain during gestation at any dietary level tested. There were no effects on food consumption observed at any dietary level tested. In the 1200 ppm F_1generation dose group, a significant increase in body weight gain (increased 13.2 % relative to control) was observed and correlates with the increased food consumption on both a g/animal/day and g/kg/day basis observed in this same dose group. No effect on body weight, body weight gain or food consumption was observed at any other dietary level tested. Results are summarized in Table 140 and Table 141 for P and F_1 generation females, respectively.

Table 140: Mean (S.E.) Body Weight and Food Consumption for P Generation Females During Gestation

Observations/study week	Dose Group			
	0 ppm	40 ppm	220 ppm	1200 ppm
P Generation Females - Gestation				
Mean body weight (g) - Day 0 S.E.	247.4 3.28	239.3 3.95	243.1 2.46	232.7** 2.50
Mean body weight (g) - Day 6 S.E.	263.8 3.12	254.8 4.18	261.5 2.54	248.5** 3.51
Mean body weight (g) - Day 13 S.E.	286.0 3.20	276.9 4.61	283.8 2.87	272.6** 3.01
Mean body weight (g) - Day 20 S.E.	349.7 3.94	333.6 6.21	343.7 4.35	336.1 3.83
Mean weight gain (g) - Days 0-20 S.E.	102.3 2.13	94.4 4.28	100.6 3.20	103.4 2.90
Mean food consumption (g/animal/day) Days 0-20	20.0	20.1	20.0	20.0
Mean food consumption (g/kg/day) Days 0-20	75.3	78.0	75.8	79.6

** Statistically different from control, $p \leq 0.01$.

Table 141: Mean (S.E.) Body Weight and Food Consumption F1 Generation Females During Gestation

Observations/study week	Dose Group			
	0 ppm	40 ppm	220 ppm	1200 ppm
F_1 Generation Females - Gestation				
Mean body weight (g) - Day 0 S.E.	240.2 3.74	247.5 4.17	241.5 3.54	231.2 3.51
Mean body weight (g) - Day 6 S.E.	253.3 3.78	261.3 4.02	256.2 3.29	249.4 3.54
Mean body weight (g) - Day 13 S.E.	270.8 3.98	279.6 4.14	273.4 3.89	269.7 4.06
Mean body weight (g) - Day 20 S.E.	329.3 4.87	338.3 5.51	326.8 5.75	332.1 4.92
Mean weight gain (g) - Days 0-20 S.E.	89.1 2.84	90.8 3.04	85.3 4.16	100.9* 2.71
Mean food consumption (g/animal/day) Days 0-20	18.6	18.4	17.2	19.6
Mean food consumption (g/kg/day) Days 0-20	73.0	70.2	66.9	78.4

* Statistically different from control, $p \leq 0.05$.

Females (lactation): In the 1200 ppm P generation dose group, a statistically significant decline in body weight was observed on Day 0. No other effect on body weight in this dose group during lactation was observed. Body weight was not affected at any other dietary level tested. There was no test substance-related effects on food consumption observed at any dietary level tested. Body weight of F_1 generation animals was unaffected at any dietary level tested during lactation. Reported body weight and selected food consumption results for lactation are summarized in Table 142.

Table 142: Mean (S.E.) Body Weight and Food Consumption - Lactation

Observations/study week	Dose Group			
	0 ppm	40 ppm	220 ppm	1200 ppm
P Generation Females - Lactation				
Mean body weight (g) - Day 0 S.E.	267.9 3.34	264.2 3.94	270.3 2.79	255.8* 2.65
Mean body weight (g) - Day 4 S.E.	276.5 3.50	266.2 4.66	276.8 2.80	267.2 3.69
Mean body weight (g) - Day 7 S.E.	282.7 3.63	273.9 4.31	284.7 3.00	273.1 3.50
Mean body weight (g) - Day 14 S.E.	299.3 4.26	290.7 4.10	299.9 3.06	289.0 3.10
Mean body weight (g) - Day 21 S.E.	287.5 3.67	280.4 4.06	286.7 2.85	277.9 2.89
Mean food consumption (g/animal/day) Days 0-21	45.6	42.8	43.5	44.1
Mean food consumption (g/kg/day) Days 0-21	161.1	155.5	152.6	161.4
F_1 Generation Females - Lactation				
Mean body weight (g) - Day 0 S.E.	257.7 3.25	266.8 4.37	260.6 4.56	258.5 3.95
Mean body weight (g) - Day 4 S.E.	263.0 4.31	274.4 4.45	271.4 4.63	263.0 3.70
Mean body weight (g) - Day 7 S.E.	273.3 4.14	282.6 4.62	279.9 4.63	271.3 4.18
Mean body weight (g) - Day 14 S.E.	293.5 3.85	297.5 5.19	296.8 4.14	291.3 3.91
Mean body weight (g) - Day 21 S.E.	286.4 4.24	290.6 4.45	293.3 3.75	284.5 4.59
Mean food consumption (g/animal/day) Days 0-21	45.8	46.3	45.1	46.2
Mean food consumption (g/kg/day) Days 0-21	166.5	163.4	161.5	168.7

* Statistically different from control, $p \leq 0.05$.

Test Substance Intake: Based on food consumption, body weight and dietary analyses results, the doses expressed as mean daily mg test substance/kg body weight during the pre-mating period (10 weeks) are presented in Table 143. Calculation for test substance intake is: Mean analytical concentration (ppm) specific for premating / 1000 X mean weekly food consumption (g/kg/body weight/day) during premating.

Table 143: Mean test substance intake (mg/kg body weight/day)

Phase of Study	40 ppm in mg/kg bw/day[a]	220 ppm in mg/kg bw/day[a]	1200 ppm in mg/kg bw/day[a]
Premating (P-gen) - Male	2.7	15.1	83.1
Premating (P-gen) – Female	3.2	17.6	96.3
Gestation (P-gen) – Female	3.0	15.5	90.3
Lactation (P-gen) – Female	3.1	15.9	92.5
Premating (F_1-gen) - Male	2.6	13.9	82.4
Premating (F_1-gen) – Female	3.1	16.8	95.6
Gestation (F_1-gen) – Female	2.8	14.4	95.9
Lactation (F_1-gen) – Female	3.3	18.1	103.2

Reproductive function

There were no test substance-related effects observed on the estrous cycle number or length in either generation at any dietary level tested. There were no test substance-related effects observed on any sperm parameter evaluated at any dietary level tested for either generation. These data are summarized in Table 144.

Table 144: Sperm Measures

Sperm Analysis		Dose Group (ppm)			
		0 ppm	40 ppm	220 ppm	1200 ppm
P Generation Males					
Sperm Motility	% Motile	89.8	89.5	89.8	89.9
	% Progressive	64.0	62.7	64.6	63.3
Sperm Counts (sperm/gram)	Testis	38.2	N/A	N/A	34.92
	Epididymis	240.5	N/A	N/A	219.7
Sperm Morphology (mean total number)	Normal	198.7	N/A	N/A	197.3
	Abnormal	0.9	N/A	N/A	2.0
	Detached Head	0.3	N/A	N/A	0.8
F_1 Generation Males					
Sperm Motility	% Motile	87.8	86.4	87.0	87.2
	% Progressive	60.4	61.1	61.8	61.9
Sperm Counts (sperm/gram)	Testis	28.3	N/A	N/A	29.1
	Epididymis	189.4	N/A	N/A	161.9
Sperm Morphology (mean total number)	Normal	197.0	N/A	N/A	195.3
	Abnormal	2.0	N/A	N/A	4.2
	Detached Head	1.0	N/A	N/A	0.5

N/A – Indicates evaluation deemed unnecessary.

Reproductive performance: Overall reproductive performance was not affected for any parameter (e.g., mating, fertility or gestation indices, days to insemination, gestation length, or the median number of implants) in either generation at any dietary level tested. Results for both the P- and F_1-generation animals are summarized in Table 145.

Table 145: Reproductive Performance

Observation	Dose Group (ppm)			
	0 ppm	40 ppm	220 ppm	1200 ppm
P Generation – F_1 Offspring				
Number Cohoused	30	30	30	30
Number Mated	30	29	30	28
Number of Animals Delivered	30	25	28	26
Number of Animals with Implants	30	25	29	26
Mating Index	100.0	96.7	100.0	93.3
Fertility Index	100.0	86.2	96.7	92.9
Gestation Index	100.0	100.0	96.6	100.0
Days to Insemination\Mean (S.E.)	3.0 (0.43)	2.4 (0.22)	2.7 (0.43)	2.3 (0.20)
Gestation Length (days)\Mean (S.E.)	21.9 (0.12)	21.8 (0.10)	21.8 (0.09)	21.8 (0.09)
F_1 Generation – F_2 Offspring				
Number Cohoused	30	30	30	30
Number Mated	29	30	30	30
Number of Animals Delivered	27	27	27	28
Number of Animals with Implants	27	27	27	29
Mating Index	96.7	100.0	100.0	100.0
Fertility Index	93.1	90.0	90.0	96.7
Gestation Index	100.0	100.0	100.0	96.6
Days to Insemination\Mean (S.E.)	2.9 (0.41)	3.0 (0.43)	2.3 (0.23)	2.8 (0.32)
Gestation Length (days)\Mean (S.E.)	21.8 (0.11)	21.7 (0.09)	21.6 (0.11)	21.5 (0.10)

Clinical Chemistry

P-generation: Test substance-related clinical chemistry changes were limited to creatinine, total protein, and albumin that were increased in 1200-ppm males.

F_1-generation: Test substance-related clinical chemistry changes were limited to urea nitrogen (UN) and total protein that were increased in 1200-ppm males, and cholesterol that was increased in 1200-ppm females.

Hematology

P-generation: Test substance-related hematology changes were limited to decreased hemoglobin and hematocrit in the 1200 ppm females.

F_1-generation: Test substance-related hematology changes were limited to increased white blood cell and monocyte Ab counts and decreased hemoglobin in 1200-ppm females.

Parental post-mortem results

P-generation: There were no test substance-related effects on adult terminal body weights for the males or females. Test substance-related organ weight changes for males and/or females were limited to the kidneys, liver, and spleen. Kidney (right and left) weights were increased in 1200-ppm males (absolute and relative), spleen weights were decreased in 1200-ppm females (absolute), and liver weights were increased in 1200-ppm males and females (absolute and relative). The decrease in the absolute spleen weights in the females was considered not to be an adverse effect since no corresponding micropathology or hematology findings were observed to support this finding.

F_1-generation: There were no test substance-related effects on adult terminal body weights for the males or females. Test substance-related organ weight changes for males and/or females were limited to the kidneys, liver and spleen. Kidney (right and left) weights were increased in 1200-ppm males (absolute and relative), spleen weights were decreased in 1200 ppm females (absolute and relative), and liver weights were increased in 1200-ppm males and females (absolute and relative). Mean relative spleen weight was also decreased in 220-ppm females (as compared to the controls).

The decrease in absolute spleen weights (absolute and/or relative) in 1200- and 220-ppm females was not associated with corresponding micropathology findings. However, as in F_1 animals white blood cell parameters were changed and spleen weights were later found to be affected in F1 and F2 pups as well, this finding may be treatment related and further studies may be required to clarify reasons for this observation.

Pathology:

Macroscopic examination: No test substance-related gross necropsy findings were observed in either the males or females at any dietary level tested.

Microscopic examination: Test substance-related micropathology findings for P-generation males and/or females that were statistically significantly different from controls included kidneys - increased incidence of protein droplet nephropathy and lymphocytic infiltration in 1200-ppm males, and liver - increased incidence of centrilobular hypertrophy in 1200-ppm males and females. There was no evidence of relevant changes in the kidneys and liver of the 220-ppm males and females. These findings are in accordance with findings of other studies over a similar duration.

Ovarian follicle counts from F_1-generation females: None of the mean primordial (preantral) follicular, antral follicular, or corpora luteal counts for F_1-generation females were statistically different from controls. Ovarian follicular counts were not affected.

Offspring

Viability and clinical signs: There was no test substance-related effects observed on the viability of the pups at any dietary level tested. There were no test-substance-related clinical observations observed in either generation at any dietary level tested. Mean litter size and pup viability (survival) during lactation are summarized in Table 146.

Table 146: Litter parameters for F1 and F2 generations

Observation	Dose Group (ppm)			
	0 ppm	**40 ppm**	**220 ppm**	**1200 ppm**
F_1 Generation				
Total Number of Implantation Sites (Mean)	377 (12.6)	274 (11.0)	323 (11.1)	304 (11.7)
Total Number born	354	257	303	295
Number stillborn	1	1	0	1
Sex Ratio Day 0 (% male)	53.2	54.0	46.0	44.6
Mean litter size	11.8	10.3	10.8	11.3
Birth index	93.9	91.8	89.6	96.8
Live birth index	99.7	99.7	100.0	99.7
Viability index	99.7	96.0	99.3	97.8
Lactation index	98.8	99.5	99.6	99.5
F_2 Generation				
Total Number of Implantation Sites (Mean)	301 (11.1)	311 (11.5)	297 (11.0)	323 (11.1)
Total Number born	287	299	289	303
Number stillborn	4	0	1	3
Sex Ratio Day 0 (% male)	47.4	49.7	49.1	44.1
Mean litter size	10.6	11.1	10.7	10.8
Birth index	95.0	96.3	97.1	91.0
Live birth index	98.7	100.0	99.7	99.2
Viability index	99.6	98.2	98.1	98.3
Lactation index	98.6	94.9	98.8	99.6

[b] Before standardization (culling); [c] After standardization (culling); [d] Does not include number stillborn

Pup body weight (combined male and female)

F_1-Pups: Pup body weights at birth for all three treated groups were comparable to the control group. In the 1200 ppm dose group, pup weight gain was declined for both males (statistically declined 7.8 %) and females (non-statistically declined 6.2 %) from Days 7-14 of lactation. In addition a small and non-significant decline in thymus and spleen weights was observed. No test substance-related effects were observed on body weight or body weight gain at any other dietary level tested.

F_2-Pups: Pup body weights at birth for all three treated groups were comparable to the control group. In the 1200 ppm dose group, non-statistical declines by Day 4 (6.1 % less than control) were observed with significant body weight declines observed by Day 21 (decline of 8.1 %). Overall body weight gain throughout lactation was declined by 8.6 %, relative to control. There were no test substance-related effects on pup body weight observed at any other dietary level. Selected mean pup body weight data are presented in Table 147, Table 148 and Table 149 for combined sexes, males and females, respectively.

Table 147: Mean (S.E.) Male/Female Combined Pup Weights (g)

F_1 Generation						F_2 Generation				
Lactation Day	0	40	220	1200		Lactation Day	0	40	220	1200
0	5.9	5.9	6.0	5.9		0	6.0	5.8	5.8	5.7
S.E	0.09	0.10	0.09	0.07		S.E	0.09	0.07	0.13	0.08
4[b]	9.7	9.3	9.8	9.5		4[b]	9.8	9.6	9.4	9.2
S.E.	0.21	0.22	0.21	0.22		S.E.	0.28	0.19	0.27	0.19
4[c]	9.7	9.3	9.8	9.5		4[c]	9.8	9.6	9.4	9.2
S.E.	0.21	0.22	0.20	0.22		S.E.	0.28	0.19	0.26	0.19
7	15.6	14.9	15.5	15.2		7	15.6	15.2	15.0	14.5
S.E.	0.29	0.39	0.29	0.34		S.E.	0.41	0.30	0.46	0.31
14	32.1	30.9	31.8	30.6		14	31.8	31.0	30.8	29.6
S.E.	0.46	0.72	0.50	0.53		S.E.	0.72	0.87	0.76	0.49
21	49.1	47.2	48.8	46.8		21	49.2	48.7	46.9	45.2*
S.E.	0.74	0.96	0.75	0.80		S.E.	1.09	0.81	1.10	0.80
GAIN	43.2	41.4	42.9	40.9		GAIN	43.2	42.9	41.1	39.5*

[b] Before standardization (culling); [c] After standardization (culling); Statistically different from control, $p \leq 0.05$

Table 148: Mean (S.E.) Male Pup Weights (g)

F_1 Generation						F_2 Generation				
Lactation Day	0	40	220	1200		Lactation Day	0	40	220	1200
0	6.1	6.0	6.1	6.0		0	6.2	6.0	5.9	5.9
S.E	0.10	0.10	0.10	0.07		S.E	0.11	0.08	0.13	0.09
4[b]	9.9	9.6	10.0	9.7		4[b]	10.0	9.8	9.6	9.4
S.E.	0.23	0.23	0.22	0.23		S.E.	0.28	0.20	0.28	0.18
4[c]	10.0	9.6	10.1	9.7		4[c]	10.0	9.8	9.6	9.4
S.E.	0.22	0.23	0.22	0.23		S.E.	0.28	0.21	0.28	0.19
7	16.0	15.4	15.9	15.5		7	16.0	15.5	15.2	14.8
S.E.	0.29	0.37	0.30	0.37		S.E.	0.42	0.32	0.47	0.33
14	32.7	32.0	32.3	31.0		14	32.3	31.4	31.1	30.1
S.E.	0.48	0.60	0.53	0.53		S.E.	0.69	0.88	0.79	0.51
21	50.2	49.0	49.7	47.6		21	50.3	49.6	47.8	46.1**
S.E.	0.75	0.88	0.82	0.83		S.E.	1.09	0.88	1.17	0.81

[b] Before standardization (culling); [c] After standardization (culling); ** Statistically different from control, $p \leq 0.01$

Table 149: Mean (S.E.) Female Pup Weights (g)

F_1 Generation						F_2 Generation				
Lactation Day	0	40	220	1200		Lactation Day	0	40	220	1200
0	5.8	5.6	5.8	5.7		0	5.8	5.6	5.6	5.6
S.E	0.10	0.08	0.09	0.08		S.E	0.09	0.06	0.12	0.08
4[b]	9.5	9.0	9.7	9.4		4[b]	9.6	9.3	9.0	9.1
S.E.	0.21	0.19	0.20	0.23		S.E.	0.29	0.18	0.30	0.21
4[c]	9.5	9.0	9.7	9.3		4[c]	9.6	9.3	9.0	9.1
S.E.	0.22	0.19	0.21	0.23		S.E.	0.29	0.19	0.30	0.20
7	15.3	14.4	15.2	14.9		7	15.4	14.9	14.7	14.3
S.E.	0.30	0.36	0.30	0.35		S.E.	0.42	0.30	0.48	0.31
14	31.5	30.2	31.4	30.2		14	31.4	30.6	30.3	29.4
S.E.	0.46	0.66	0.49	0.54		S.E.	0.75	0.86	0.77	0.51
21	48.0	46.0	48.2	45.9		21	48.2	47.8	45.6	44.6*
S.E.	0.77	0.84	0.75	0.86		S.E.	1.12	0.77	1.09	0.84

[b] Before standardization (culling); [c] After standardization (culling); * Statistically different from control, $p \leq 0.05$

Sexual maturation

A slight delay in preputial separation in the F_1-males of the 1200 ppm dose group (mean = 42.5 days) was observed, relative to control. Although statistically significant, the number of days to passing was well within this laboratory's historical control range (42.4 – 43.8) and is considered to be secondary to the decline in male body weight gain observed during lactation. There were no findings on preputial separation in any other dietary level tested. There was no effect observed on vaginal patency at any dietary level tested. Anogenital distance was examined on lactation Day 0 for the F_2-pups with no effects on this measurement noted.

Organ weights

There were no statistically significant differences in the mean organ weights for F_1-male and female pups, relative to controls. Test substance-related organ weight changes for F_2-pups that were statistically significantly different from controls included spleen and thymus which were decreased in 1200-ppm males, females and combined pups (absolute and relative). Other mean organ weights for F_2-pups that were statistically significantly different from controls included brain – increased in 1200-ppm male pups (relative). This change was not considered to be test substance-related for one or more of the following reasons: the organ weight difference was associated with the statistically significant decrease in day 21 mean body weights, the change was not dose-related, and/or the change was relatively small.

Pathology

Macroscopic examination: All gross lesions for F_1 and F_2 pups were considered to be incidental and not test substance-related.

Microscopic examination: All micropathology lesions in the 21-day F_1 and F_2 pups were considered to be incidental and/or background and were not considered to be test substance-related.

Conclusion

The parental male systemic LOAEL was 1200 ppm (82.8 mg/kg bw/day in males) based on increased clinical chemistry parameters (creatinine, total protein, albumin and urea nitrogen), increased kidney weight associated with an increased incidence of protein droplet nephropathy and lymphocytic infiltration, and increased liver weights associated with an increased incidence of centrilobular hypertrophy. The parental female systemic LOAEL was 1200 ppm (96.0 mg/kg bw/day in females) based on decline in body weight and/or body weight gain during premating, decreased body weight during gestation in the P-generation, increased body weight and food consumption during gestation in the F_1-generation, increased cholesterol and increased white blood cell and monocyte Ab counts in the F_1-generation, decreased hemoglobin and/or hematocrit in the P- and/or F_1-generation, increased liver weights associated with an increased incidence of centrilobular hypertrophy and minimal to slight,

alveolar macrophages in the P- and/or F_1-generation. The parental systemic NOAEL was 220 ppm (14.5 mg/kg bw/day in males, 17.2 mg/kg bw/day in females).

The reproductive NOAEL was 1200 ppm in both males and females (82.8 mg/kg bw/day in males and 93.1 mg/kg bw/day females), based on no reproductive findings observed up to the highest dose tested.

The offspring LOAEL was 1200 ppm (82.8 mg/kg bw/day). The LOAEL was based on maternal effects leading to secondarily-mediated effects on pup weight and pup weight gain. Also noted was a slight delay in preputial separation and decrease in spleen and thymus weight. As these organs represent important parts of the immune system and the effect was worse in the F_2 generation than in the F_1 generation, the necessity of a developmental immuntox study should was considered in the first DAR. However, since no other immune parameters were affected and these effects were small in extent a study on developmental immunotoxicity was finally not regarded necessary (van Goethem, D. 2009; ASB2009-3177). However, the offspring NOAEL was 220 ppm (14.5 mg/kg bw/day).

4.11.1.2 Human information

No data submitted by the notifier.

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

Developmental toxicity study in rats

Title:	Wason, S. (2008): AE C656948 - Developmental toxicity study in the rat by gavage SA 05276 M-299438-01, ASB2008-5481.
Guidelines:	OECD 414 (2001).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered acceptable.

Materials and methods

Test Material:	AE C656948
Description:	Beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.6 %
Vehicle or positive control:	Aqueous solution of methylcellulose 400 at 0.5 %
Stability of test compound:	Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5 %) at concentrations of 0.0868 and 250 g/L for a period of 33 days under similar conditions to those of the current study.

Species:	Rat
Strain:	Sprague-Dawley Crl:CD(SD)
Age / weight:	11 to 13 weeks approx. (at mating); 246 to 301 g for the females
Source/breeder:	Charles River laboratories, St Germain-sur-l'Arbresle, France
Acclimation period:	5 days prior to mating
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions :	Temperature: 22 ± 2°C
	Humidity: 55 ± 15 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: Adult virgin female rats were mated on a one-to-one basis with stock males of the same strain and supplier for each group. Each morning following pairing, rats showing spermatozoa in a vaginal smear or sperm plug *in situ* were considered as pregnant. The day where evidence of mating was found, was designated as GD 0. The females were assigned to control and treated groups each day of pairing using a body weight dependent randomization procedure. Body weight means were checked after the mating period to ensure similar means among all groups.

Test substance dosage formulations and analysis: The appropriate amount of fluopyram was suspended (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5 % and stored at approximately 5°C (± 3°C). Formulations were prepared twice (F_1 and F_2) during the study. Homogeneity of the suspensions was checked on the first formulation (F_1) for the lowest and the highest concentrations (3 and 45 g/L). In addition, the intermediate concentration (15 g/L) of the first formulation (F_1) and all concentrations of the second formulation (F_2) were checked. Homogeneity and concentration checks were between 99 and 102 % of nominal values. Stability of the test substance in suspension in the vehicle at concentrations of 0.0868 and 250 g/L was determined in a previous study and was found to be stable for 33 days under similar conditions to those of the current study.

Table 150: Study design and animal assignment

Test group	Test substance	Dose levels mg/kg bw/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	0	0	0	10	23
2		30	3	10	23
3	fluopyram	150	15	10	23
4		450	45	10	23

Doses were administered daily by gavage to each female from GD 6 to 20, based on the animal's most recent body weight, and at a volume of 10 mL/kg. Control animals received an equivalent volume of vehicle alone (0.5 % aqueous methylcellulose).

Observations: All rats were observed daily for clinical signs and twice daily for mortality (except at weekends and public holidays when checking was carried out once daily).

Body weight and food consumption: Body weights were measured on GD: 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was measured at the following intervals: full feeder on GD: 1, 6, 8, 10, 12, 14, 16 and 18 and empty feeder weights were measured on GD: 6, 8, 10, 12, 14, 16, 18, and 21.

Cesarean sections: On GD 21, all females were sacrificed by inhalation of carbon dioxide, for examination of uterine content. Autopsies were performed blind with regard to the animal study identification. Each female was first subjected to macroscopic examination of the visceral organs. The liver was weighed for pregnant females, retained from all females and subjected to a microscopic examination.

The reproductive tract was weighed (gravid uterine weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and late), number of live and dead fetuses, sex and individual weights of live fetuses. Dead fetuses were defined as fetuses showings distinct digits visible on fore and hind-paws. Runt fetuses were defined as live fetuses weighing less than 4 g at Cesarean section of the dam (=small fetuses). Uterine horn(s) without visible implantations were immersed in a 10 % solution of ammonium sulfide to visualise any sites which were not apparent. Intra-uterine death was classified as early resorptions when macroscopic discrimination between fetal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between fetal residues and placental material was possible. Tissues and carcasses of dams were then discarded.

Fetal examination: All data were recorded without knowledge of treatment group. All live fetuses were subjected to external examination and then sacrificed by subcutaneous injection (0.02 mL/fetus) of Dolethal (18.22 g/100 ml, sodium pentobarbital). Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, fixed in absolute ethanol and stained according to a modification of the Tyl and Marr technique for skeletal examination of bone and cartilage.

Classification of structural deviations:

Malformations: A permanent structural change that is likely to adversely affect the survival or health.

Variations: A change that occurs within the normal population under investigation and is unlikely to adversely affect survival or health (this might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development).

Statistics: Means and standard deviations for all maternal and litter parameters were calculated for each group. Statistical analyses were performed on the following variables using TERATEST Phase 1, Version 12, TERATEST Phase 4, Version 4 or SAS programs (Version 8.2). Maternal endpoints were body weight change calculated according to time periods, calculated corrected body weight change, average food consumption calculated according to time periods, liver weight. Litter based and fetal endpoints were number of corpora lutea, number of implantation sites, number of resorption (early and late), pre- and post-implantation loss percentages, fetal sex, fetal death status, fetal body weight. Homogeneity of variances between control and treated groups was evaluated using the Bartlett test. If not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance.

If the Bartlett test was significant (for body weight change, corrected body weight change, number of corpora lutea, number of implantation sites or number of resorptions parameters), group means were compared using the non parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance. If the Bartlett test was significant, a log transformation (for food consumption, liver weight or fetal body weight) or an arcsine root transformation (for pre- or post-implantation loss percentages) was performed. If the Bartlett test on transformed data was not significant, means were compared using the analysis of variance (ANOVA), which was followed by the Dunnett test (2-sided) if ANOVA indicated significance. If the Bartlett test on transformed data was significant, group means were compared using the non parametric Kruskal-Wallis test, which was followed by the Dunn test (2-sided) if the Kruskal-Wallis test indicated significance. If the Bartlett test

was significant for fetal sex (male vs. female fetuses) and fetal death status (live vs. dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test (for fetal sex parameter) or the Fisher Exact test (2-sided) (for fetal death status parameter). Death status was analyzed both using the fetus as the statistical unit and using the litter as the statistical unit. If one or more group variance(s) equaled 0, means were compared using non parametric procedures. The homogeneity of group variances, results of the ANOVA or the Kruskal-Wallis tests were evaluated at the 5 % level of significance. Group means were compared at the 5 % and 1 % levels of significance.

Findings

Maternal observations

Mortality and clinical signs: There were no mortalities or treatment related clinical signs during the course of the study.

Pregnancy rate: There was no effect on pregnancy rates, which were 96 % in all treated group and in the controls.

Body weight: At 450 mg/kg bw/day, mean maternal body weight remained static between GD 6 to 8, compared with a mean maternal body weight gain of 6.8 g over the corresponding period in the control group, the effect being statistically significant ($p \leq 0.01$). Between GD 8 to 10 and 10 to 14, mean maternal body weight gain was 22 % (not statistically significant) and 54 % ($p \leq 0.01$) lower than in the controls, respectively. At other intervals maternal weight gain was similar to the controls. The overall effect between GD 6 to 21 was a 16 % lower mean maternal body weight gain ($p \leq 0.01$) compared with the controls.

At 150 mg/kg bw/day, mean maternal body weight essentially remained static between GD 6 to 8, compared with a mean maternal body weight gain of 6.8 g in the controls over this period. Between GD 10 to 14, mean maternal body weight gain was 26 % lower than in the controls. The effect was statistically significant ($p \leq 0.05$ or $p \leq 0.01$) at both these intervals. At other intervals maternal weight gain was similar to the controls. The overall effect between GD 6 to 21 was a 6 % lower mean maternal body weight gain compared with the controls, though the effect was not statistically significant.

At 30 mg/kg bw/day, mean maternal body weight gain was 31 % lower than in the control group between GD 6 to 8, though the effect was not statistically significant. Thereafter, body weight gain was similar to the controls. Mean maternal corrected body weight change (maternal body weight change independent of the uterine weight) was less pronounced at 450 mg/kg bw/day (37.6 g) and 150 mg/kg bw/day (45.8 g) than in the control group (59.4 g), the effect being statistically significant ($p \leq 0.01$) at 450 mg/kg bw/day. At 30 mg/kg bw/day, mean maternal corrected body weight change was similar to the controls.

Food consumption: Mean maternal food consumption was between 13 % to 15 % lower than the controls at 450 mg/kg bw/day and between 10 % to 18 % lower than the controls at 150 mg/kg bw/day, for each interval between GD 6 and 14, the effect being statistically significant at each interval ($p \leq 0.01$ at 450 mg/kg bw/day and $p \leq 0.05$ or $p \leq 0.01$ at 150 mg/kg bw/day). Thereafter, food consumption was comparable to the controls at both dose levels. At 30 mg/kg bw/day, mean maternal food consumption was 10 % lower than the controls between GD 6 to 8, the effect being statistically significant ($p \leq 0.01$), thereafter, food consumption at this dosage was similar to the controls.

Maternal liver weights, necropsies and microscopic findings: Mean maternal liver weights were 40 % higher at 450 and 15 % higher at 150 mg/kg bw/day, compared with the controls. The effect was statistically significant ($p \leq 0.01$) at both dose levels. Mean maternal liver weights were similar to the controls at 30 mg/kg bw/day.

Table 151: Mean liver weight of pregnant females

Dose levels of fluopyram (mg/kg bw/day)	**0**	**30**	**150**	**450**
Number	22	22	22	21
Mean liver weight (g) ± SD (% of controls)	13.95 ± 1.69 (-)	14.06 ± 1.61 (101)	16.05 ** ± 2.11 (115)	19.47 ** ± 2.33 (140)

** p≤0.01; SD: Standard deviation

At autopsy of the dams, enlarged liver was observed in 4/23 females at 450 mg/kg bw/day compared with 0/23 cases in the control group. Histopathological changes were observed in the liver at 450 and 150 mg/kg bw/day and consisted of diffuse centrilobular hepatocellular hypertrophy.

Table 152: Incidence of changes in the liver

Sex	**Females**			
Dose levels of fluopyram (mg/kg bw/day)	0	30	150	450
Number of animals examined	23	23	23	23
Centrilobular hepatocellular hypertrophy, diffuse				
minimal	0	1	8	0
slight	0	0	10	1
moderate	0	0	2	21
marked	0	0	0	1
Total	0	1	20	23

Litter data

At 450 mg/kg bw/day, mean fetal body weight was 5 % (p≤0.05) lower for both the combined and separate sexes. At 150 and 30 mg/kg bw/day, mean fetal body weight for the combined sexes and the separate sexes was not statistically significantly different when compared to the control group. Other litter parameters including number of live fetuses, early or late resorptions and dead fetuses, were unaffected by treatment.

Table 153: Mean fetal body weights

Dose levels of fluopyram (mg/kg bw/day)	**0**	**30**	**150**	**450**
Number of litters	22	22	22	22
Mean litter body weight (g) for combined sexes ± SD (% of controls)	5.51 ± 0.39 (100)	5.48 ± 0.20 (100)	5.39 ± 0.35 (98)	5.26 * ± 0.36 (95)
Mean litter body weight (g) for males ± SD (% of controls)	5.67 ± 0.34 (100)	5.59 ± 0.26 (99)	5.54 ± 0.38 (98)	5.37 * ± 0.36 (95)
Mean litter body weight (g) for females ± SD (% of controls)	5.40 ± 0.42 (100)	5.38 ± 0.19 (100)	5.25 ± 0.37 (97)	5.13 * ± 0.37 (95)

* p≤0.05; SD: Standard Deviation

Fetal necropsy findings

External observations: There were no malformations or treatment-related increase in variations observed at the external fetal observation.

Visceral observations: There were two cases at 450 and 150 mg/kg bw/day and one case at 30 mg/kg bw/day of the malformation ‘renal pelvis; dilated severe’ compared with no cases in the control group.

However, the incidence at both the individual and litter level was within in-house variability at all three dose levels, when data from the low dose from a recent in-house study, where there was clearly no treatment-related effect on this parameter, was taken into consideration. Therefore, at such a low incidence with no clear indication of a dose response, this finding was considered to have occurred by chance.

There were two other malformations observed at the visceral examination, 'eventration of the diaphragm and caudate lung lobe absent' in one fetus at 450 mg/kg bw/day, and 'situs inversus (total)' in one control fetus. In isolation, these findings were considered to have occurred spontaneously.

At 450 mg/kg bw/day, the incidence of the variations 'thymic remnant present (unilateral/bilateral)' and 'ureter (unilateral/bilateral); convoluted and /or dilated' was higher at the fetal and/or litter level than in the control group, and was outside the in-house historical control range for both parameters. The higher incidence of these two variations was considered to be treatment-related at this dose level. At 150 mg/kg bw/day, the occurrence of these two variations was slightly higher than in the control group, but was considered to be incidental in view of the in-house historical control range.

Table 154: Visceral findings

Group	**Fluopyram (mg/kg bw/day)**				**Historical Control Range**	**Fluopyram (mg/kg bw/day)**				**Historical Control Range**
	0	**30**	**150**	**450**		**0**	**30**	**150**	**450**	
Observations	Fetal incidence (mean % of fetuses affected)*					Litter incidence (% of litters affected)				
Thymus remnant present (unilateral/ bilateral)	6/146 (3.9)	7/147 (4.6)	14/155 (9.2)	21/149 (14.5)	2/153-11/175 (1.3-6.0)	5/22 (22.7)	5/22 (22.7)	8/22 (36.4)	10/22 (45.5)	1/19-8/24 (5.3-33.3)
Ureter (unilateral /bilateral) convoluted and/or dilated	46/146 (33.2)	57/147 (36.9)	72/155 (46.2)	88/149 (58.6)	22/153-78/175 (20.5-45.1)	17/22 (77.3)	17/22 (77.3)	20/22 (90.9)	20/22 (90.9)	17/25-23/24 (68.0-95.8)

* mean % of litters affected defined as: sum of % of live fetuses affected per litter/no. of litters with live fetuses examined

Skeletal observations: The only malformation observed was one case of the finding 'two (except atlas and axis) cervical centrum (unilateral) cartilage fused', at 450 mg/kg bw/day. In isolation, this finding was considered to be fortuitous.

At 450 mg/kg bw/day, there was a higher incidence of the variations 'at least one thoracic centrum split/split cartilage' and 'at least one thoracic centrum: dumbbell and/or bipartite / normal cartilage', compared with the control group. The incidence was outside the in-house historical control range at both the fetal and litter level for both findings. The increased incidence of these two variations was therefore considered to be treatment-related at this dose level. At 150 mg/kg bw/day, the incidence of the variation 'at least one thoracic centrum: dumbbell and/or bipartite / normal cartilage' was slightly elevated, but was considered to be incidental at this dose level, when compared with the in-house historical control range.

Table 155: Skeletal findings

Group	Fluopyram (mg/kg bw/day)				Historical Control Range	Fluopyram (mg/kg bw/day)				Historical Control Range
	0	30	150	450		0	30	150	450	
Observations	Fetal incidence (mean % of fetuses affected)*					Litter incidence (% of litters affected))				
At least one thoracic centrum: split/split cartilage	0/157 (0.0)	1/160 (0.6)	0/167 (0.0)	4/159 (2.6)	0/189-1/155 (0.0-0.6)	0/22 (0.0)	1/22 (4.5)	0/22 (0.0)	4/22 (18.2)	0/25-1/21 (0.0-4.8)
At least one thoracic centrum : dumbbell *or incomplete ossification* and/or bipartite/normal cartilage	3/157 (1.8)	9/160 (5.3)	12/167 (7.1)	29/159 (20.8)	1/144-12/176 (0.7-7.2)	2/22 (9.1)	7/22 (31.8)	9/22 (40.9)	14/22 (63.6)	1/19-9/23 (5.3-39.1)

* mean % of litters affected defined as: sum of % of live fetuses affected per litter/no. of litters with live fetuses examined; or incomplete ossification : additional term included in the in-house historical control range.

Conclusion

In conclusion, fluopyram administered by oral gavage to the Sprague-Dawley rat caused significant maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centrilobular hepatocellular hypertrophy at 450 and 150 mg/kg bw/day. Developmental toxicity was observed at the high dose level only in terms of slightly lower fetal body weight, and a slightly increased incidence of two visceral and two skeletal minor variations. Therefore, the maternal No Observed Adverse Effect Level (NOAEL) was considered to be 30 mg/kg bw/day and the fetal No Observed Effect Level (NOEL) was considered to be 150 mg/kg bw/day.

Developmental toxicity study in rabbits

Title: Kennel, P. (2006): AE C656948 - Developmental toxicity study in the rabbit by gavage, SA 05014, M-279773-01, ASB2008-5483.

Guidelines: OECD 414.

Deviations: None.

GLP: Yes.

Acceptability: The study is considered acceptable.

Materials and methods

Test Material: AE C656948

Description: Beige powder

Lot/Batch: Mix-Batch:08528/0002

Purity: 94.6 %

Vehicle or pos. control: Aqueous solution of methylcellulose 400 at 0.5 %

Stability of test compound: Stable in suspension in the vehicle (aqueous solution of methylcellulose 400 at 0.5 %) at concentrations of 0.0868 and 250 g/L for a period of 33 days under similar conditions to those of the current study.

Species:	Rabbit
Strain:	New Zealand White Crl:KBL (NZW)
Age / weight:	18 weeks approx. at arrival; 3.17 to 3.90 kg for the females
Source/breeder:	Charles River laboratories, Châtillon-sur-Chalaronne, France
Acclimation period:	At least 5 days prior to dosing
Housing:	Animals were caged individually in stainless steel wire mesh cages
Environmental conditions:	Temperature: 19 ± 2°C Humidity: 55 ± 15 % Air changes: Approximately 10 to 15 changes per hour Photoperiod: Alternating 16-hour light and 8-hour dark cycles (5 am- 9 pm)
Diet:	Laboratory animal pellets 110C-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: Time-mated female New Zealand White rabbits were used in this study. Stock males from the same strain were used by the supplier to naturally mate nulliparous females. The day of insemination was designated as GD 0. The animals were approximately 18 weeks of age on arrival and were received on GD 1 or 2. On each day of mating, the females were allocated to control and treated groups using a computerized randomization procedure. Body weight means were checked to ensure similar means among all groups. The doses were administered daily by gavage at a volume of 4 mL/kg to each female from GD 6 to GD 28 inclusive, based on the animal's most recent body weight. Control animals received an equivalent volume of vehicle alone (methylcellulose). The experimental groups were as follows:

Table 156: Study design and animal assignment

Test group	Test substance	Dose levels mg/kg bw/day	Concentrations g/L	Volume (mL/kg)	Number of animals
1	0	0	0	4	23
2	Fluopyram	10	2.50	4	23
3		25	6.25	4	23
4		75	18.75	4	23

Test substance dosage formulations and analysis: The appropriate amount of test substance was periodically (six formulations for each dose level) suspended (w/v) in an aqueous solution of methylcellulose 400 (Fluka, Mulhouse, France) at 0.5 % and stored at approximately 5°C (± 3°C). Homogeneity of the suspensions was checked during the first formulation for the lowest and highest concentrations. All concentrations were checked. Homogeneity and concentration checks were between 97 and 106 % of nominal values and were therefore inside the in-house target range of 90 to 110 % of nominal concentration. Stability of the compound in suspension in the vehicle was determined at 0.0868 and 250 g/L in 0.5 % aqueous methylcellulose in a previous study and was found to be stable for 33 days under similar conditions to those of the current study.

Observations: All rabbits were observed daily for clinical signs and twice daily for mortality (except once daily on weekends and public holidays).

Body weight and food consumption:

Body weights were measured on GD: 3, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 29. Full feeder weights were measured on GD: 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28. Empty feeder weights were measured on GD: 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 29.

Cesarean sections: Animals killed *in extremis* by intravenous injection of Dolethal® (Sanofi, Libourne, France) or found dead, were autopsied. A macroscopic examination of the visceral organs was performed and the number of ribs counted. The number and type of implantations and corpora lutea were noted when present. In the case of no visible uterine implants, but with corpora lutea, uterine horn(s) were immersed in a 20 % solution of ammonium sulfide according to the Salewski method (1964). The liver was taken and preserved in 10 % neutral buffered formalin. Tissues and carcasses were then discarded.

On GD 29, surviving females were killed by intravenous injection of Dolethal® for examination of their uterine content. Each female was first subjected to macroscopic examination of the visceral organs and the number of ribs was recorded. In the case of no visible uterine implants, but with corpora lutea, uterine horn(s) were immersed in a 20 % solution of ammonium sulfide according to the Salewski method. The liver was weighed for pregnant females and was taken from all females and preserved in 10 % neutral buffered formalin.

The reproductive tract was weighed (gravid uterine weight), dissected out and the following parameters recorded: number of corpora lutea, number of implantation sites, number of resorption sites (classified as early and late), number of live and dead fetuses, individual weights of live fetuses. Dead fetuses were defined as dead conceptuses showings distinct digits visible on fore and hind-paws. All the live fetuses were and subjected to an external examination. Resorption death was classified as early resorptions when macroscopic discrimination between fetal residues and placental material was not possible and late resorption when distinct macroscopic discrimination between fetal residues and placental material was possible. Tissues and carcasses of dams were then discarded.

Fetal examination: All data were recorded without knowledge of treatment group. After internal examination of the neck, the head of fetuses from approximately half of each litter was immersed in Bouin's fluid and the internal structures examined after fixation. The bodies of all fetuses were dissected for soft tissue abnormalities and sexed. Then the fetuses were fixed in absolute ethanol before staining. A modification of the Staples and Schnell staining technique was used and a subsequent skeletal examination was performed.

Statistics: Statistics were performed as described above (4.7.1.1).

Findings

Maternal observations

Mortality: On GD 21 one female at 75 mg/kg bw/day died and one female was sacrificed due to accidental trauma on GD 15 in the control group. Both deaths were attributable to a gavage error. The macroscopic observation showed hemorrhaging in the lung of both females together with hemorrhaging and foam in the trachea of one female and a trachea filled with fluid for the other female. In addition, one female was killed for humane reasons on GD 23 at 25 mg/kg bw/day, following a slight loss in body weight and a reduction in food consumption between GD 20 and 22. Clinical signs in this female consisted of a limited use of the right hindlimb on GD 22 and 23. The macroscopic observation showed a severe fracture of the right hindlimb, in association with massive subcutaneous hemorrhaging and a distal epiphysal femoral disjunction. The condition of this animal was considered to be due to accidental trauma.

Abortions: There were no abortions throughout the study.

Clinical signs: There were no treatment-related clinical signs in any group.

Pregnancy rate: The pregnancy rate was 96 % in all groups without treatment-related effects.

Body weight: At 75 mg/kg bw/day, mean body weight gain was reduced between GD 14 and 18 (0.02 kg vs. 0.09 kg for controls, $p \leq 0.01$) and between GD 18 and 22 (0.02 kg vs. 0.07 kg for controls, $p \leq 0.01$), in comparison to controls. Thereafter, mean body weight gain was similar to the controls, resulting in an overall body weight gain between GD 6 and 29 of 0.20 kg compared to 0.31 kg for the controls, though the effect was not statistically significant. Mean body weight change at 25 and 10 mg/kg bw/day was comparable with the controls. Maternal corrected body weight change (i.e., maternal body weight change independent of the uterine weight) was more pronounced at 75 mg/kg bw/day (-0.25 kg) compared with the controls (-0.17 kg), though the effect was not statistically significant. Maternal corrected body weight change at 25 and 10 mg/kg bw/day was comparable with the controls.

Food consumption: At 75 mg/kg bw/day, mean maternal food consumption was reduced by between 22 to 34 % ($p \leq 0.01$) for all intervals between GD 14 to 26, in comparison to controls. Mean maternal food consumption at 25 and 10 mg/kg bw/day was similar to the controls.

Maternal necropsy findings and liver weights: There were no treatment-related macroscopic findings or liver weight effects.

Litter data

At 75 mg/kg bw/day, mean fetal body weight for the combined sexes and for the individual sexes were 11 % lower than the controls ($p \leq 0.01$ for combined sexes and males, $p \leq 0.05$ for females). There was no effect on mean fetal body weight at 25 and 10 mg/kg bw/day. Other litter parameters, including number of live fetuses, early or late resorptions, fetal death status and percentage of male fetuses were unaffected by treatment at all dose levels tested.

Fetal necropsy findings

External observations: There were two fetuses noted with malformations, but as they occurred as isolated incidences at 10 mg/kg bw/day and in the control group, they were considered to be fortuitous. The number of 'runt' fetuses (BW<28.0g) also called small fetuses, classed as a variation, was increased at 75 mg/kg bw/day, where the mean percentage of fetuses classified as small was 12.5 % and the percentage of litters affected was 47.6 %, compared with 3.0 % and 23.8 %, respectively, in the control group. At 25 and 10 mg/kg bw/day, the mean percentage of small fetuses and percentage of litter affected were very similar to the control values.

Visceral observations: At 75 mg/kg bw/day, there were two fetuses from separate litters with the malformation 'gall bladder absent', compared to no instance in the current control group. However considering the low incidence of this finding and that it has already been observed at similar incidence in previous studies conducted in the same strain, it was considered not to be treatment-related. All other malformations occurred as isolated findings or at a similar frequency across the dose groups including the controls and were considered to have occurred by chance. There was no treatment-related effect on variations at the visceral examination.

Skeletal observations: The few malformations which were observed at the skeletal examination both occurred as isolated incidences or at a higher incidence in the controls and were therefore considered to be fortuitous. There was no treatment-related effect on variations at the skeletal examination.

Conclusion

In conclusion, a dose level of 75 mg/kg bw/day of fluopyram administered to the pregnant female New Zealand White rabbit caused maternal toxicity, as evidenced by reduced mean body weight gains and food consumption. Fetal toxicity at this dose level consisted of a reduced body weight in both sexes. A dose level of 25 mg/kg bw/day of fluopyram was considered to be a No Observed Effect Level (NOEL) both in the dam and in terms of fetal development in the New Zealand White rabbit.

4.11.2.2 Human information

No data submitted by the notifier.

4.11.3 Other relevant information

No data submitted by the notifier.

4.11.4 Summary and discussion of reproductive toxicity

Three studies were performed to assess the reproductive toxicity of fluopyram: one rat two generation reproduction study, one developmental toxicity study in the rat and one developmental toxicity study in the rabbit.

In the two-generation reproduction study in rats, systemic effects in male rats were confined to the top dose level of 1200 ppm (*ca* 82.8 mg/kg bw/day). Liver and kidney toxicity at this dose became apparent by altered clinical chemistry parameters (increase in creatinine, total protein, albumin and urea nitrogen), higher kidney weights associated with a more frequent occurrence of protein droplet nephropathy and lymphocytic infiltration, and increased liver weights associated with centrilobular hypertrophy. In females, at the same dietary dose level of 1200 ppm (~ 96.0 mg/kg bw/day), a decline in body weight and/or body weight gain during the premating period and during gestation in the P-generation was noted. In contrast, body weight and food consumption were increased during gestation in the F_1-generation. Further findings comprised an increase in cholesterol level and in white blood cell and monocyte absolute cell counts in the F_1-generation, lower hemoglobin and/or hematocrit values in the P- and/or F_1-generation, higher liver weights associated with centrilobular hypertrophy and minimal to slight lung alveolar macrophages in the P- and/or F_1-generation. Based on these observations, the parental systemic NOAEL was 220 ppm (corresponding to 14.5 mg/kg bw/day in males and 17.2 mg/kg bw/day in females).

The reproductive NOAEL was 1200 ppm in both males and females (82.8 mg/kg bw/day in males and 93.1 mg/kg bw/day females) because no reproductive findings were observed up to the highest dose tested.

In the offspring, effects on pup body weight and body weight gain at the highest dose level of 1200 ppm (82.8 mg/kg bw/day) might be secondary to maternal toxicity. In line with that, a slight delay in preputial separation was observed. A decrease in spleen and thymus weights in pups might indicate an adverse effect on the immune system. However, since no other immune parameters were affected and these effects were small in extent a study on developmental immunotoxicity was not regarded necessary. Nonetheless, the clear offspring NOAEL was 220 ppm (14.5 mg/kg bw/day).

In the rat developmental toxicity study, there were no unscheduled mortalities or treatment-related clinical signs in the dams. At the highest and mid dose levels of 450 and 150 mg/kg bw/day, dams did not gain weight between GD 6-8. Thereafter, body weight and body weight gain remained lower throughout the study and food consumption was reduced. At necropsy, a dose-related significant increase in liver weight was noted and diffuse centrilobular hepatocellular hypertrophy was observed in a majority of dams. At the top dose level, hepatomegaly was noted in 4 females. The NOAEL for maternal toxicity was 30 mg/kg bw/day in this study, in spite of a transient reduction in maternal body weight gain and food consumption during the first three days of treatment (GD 6-8).

Fluopyram was not teratogenic and signs of fetotoxicity were confined to the highest dose level of 450 mg/kg bw/day. In the group receiving this dose, mean fetal body weights were by 5 % lower than in the controls. Litter parameters were not affected but there was an increase in the incidence of a few visceral (‘thymic remnant present’ and ‘ureter convoluted and/or dilated’), and skeletal variations (‘at least one thoracic centrum split/split cartilage’ and ‘at least one thoracic centrum dumbbell and/or bipartite/normal cartilage’). The mid dose level of 150 mg/kg bw/day was considered the fetal NOAEL.

In the developmental toxicity study in rabbits, there were no treatment-related maternal deaths or clinical signs. At the high dose level of 75 mg/kg bw/day, mean body weight gain and food

consumption were reduced in comparison to controls. At necropsy, no treatment-related macroscopic findings were noted. Mean fetal body weight was 11 % lower at this dose. In line with that, individual and litter incidence of very small fetuses (classified as 'runts') was higher. There were no treatment-related visceral or skeletal findings at any dose level. Thus, the mid dose level of 25 mg/kg bw/day was considered the NOAEL for both maternal and developmental toxicity.

Table 157: Summary of reproductive toxicity with fluopyram

Type of study **Doses**	**NO(A)EL (mg/kg bw/d)**	**LOAEL (mg/kg bw/d)**	**Adverse effects at LOAEL**	
Reproductive toxicity studies				
Two-generation rat (Milius, A. D.; Bommegowda, S., 2008, ASB2008-5478) 0, 40, 220, 1200 ppm	14.5/17.2 (M/F)	82.8/93.1 (M/F)	Parents	Liver and kidney toxicity
	82.8/93.1 (M/F)	>93.1 (M/F)	Reproduction	None
	14.5 (M/F)	82.8 (M/F)	Offspring	Decreased body weight and body weight gain, slight delay in preputial separation, decreases in spleen and thymus weights,
Developmental toxicity studies				
Developmental toxicity rat, (Wason, 2008, ASB2008-5481) 0, 30, 150 450 mg/kg bw/day	30	150	dams	Reduced body weight gain and food consumption, liver toxicity
	150	450	fetus	Decreased fetal body weight, increased incidence of certain visceral and skeletal variations
Developmental toxicity rabbit, (Kennel, 2006, ASB2008-5483) 0, 10, 25, 75 mg/kg bw/day	25	75	dams	Reduced body weight gain and food consumption.
	25	75	fetus	Decreased fetal body weight (-11 %).

4.11.5 Comparison with criteria

Table 158: Toxiclogical results concerning adverse effects on sexual function and fertility

Toxicological result	DSD criteria	CLP criteria
No evidence for impairment of fertility and reproduction	Category 1: Substances known to impair fertility in humans Category 2 Substances which should be regarded as if they impair fertility in humans - clear evidence in animal studies of impaired fertility in the absence of toxic effects, or, - evidence of impaired fertility occurring at around the same dose levels as other toxic effects but which is not a secondary nonspecific consequence of the other toxic effects - other relevant information Category 3 Substances which cause concern for human fertility - results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of impaired fertility in the absence of toxic effects, or evidence of impaired fertility occuring at around the same dose levels as other toxic effects, but which is not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2, - other relevant information	Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on sexual function and fertility in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility and - and where the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

Table 159: Toxiclogical results concerning adverse effects on development

Toxicological result	DSD criteria	CLP criteria
Maternal: Rat: bw gain and food consumption ↓, liver (enlarged, hepatocelluar hypertrophy) Rabbit: bw gain and food consumption ↓ Maternal NOAEL: Rat: 30 mg/kg bw/d Rabbit: 25 mg/kg bw/d Developmental: Rat: foetal wt ↓, few skeletal and visceral (thymus, ureter) variations ↑ Rabbit: foetal wt ↓, incidence of small foetuses ↑ Developmental NOAEL: Rat: 150 mg/kg bw/d Rabbit: 25 mg/kg bw/d	Category 1: Substances known to cause developmental toxicity in humans Category 2 Substances which should be regarded as if they cause developmental toxicity to humans - clear results in appropriate animal studies in the absence of marked maternal toxicity, or, - at around the same dose levels as other toxic effects but which is not a secondary nonspecific consequence of the other toxic effects - other relevant information Category 3 Substances which cause concern for humans owing to possible developmental toxic effects - results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of developmental toxicity in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects, but which are not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in Category 2, - other relevant information	Category 1A: Known human reproductive toxicant Category 1B: Presumed human reproductive toxicant largely based on data from animal studies - clear evidence of an adverse effect on development in the absence of other toxic effects, or - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects Category 2: Suspected human reproductive toxicant - some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on development and - the evidence is not sufficiently convincing to place the substance in Category 1 (deficiencies in the study). - the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects

There are no appropriate epidemiological studies demonstrating reproductive effects in humans. Thus, classification as toxic to reproduction category 1A according Regulation (EC) No 1272/2008 is not warranted. Likewise, category 1 according to DSD is not warranted.

Appropriate animal studies show no effects of the substance on reproduction or fertility as summarised above. Thus, classification as toxic to reproduction category 1B or 2 according to CLP is not warranted. Likewise, category 2 or 3 according to DSD is not warranted either.

There are no appropriate epidemiological studies demonstrating developmental effects in humans. Thus, classification as toxic to development 1A according to CLP is not warranted. Likewise, category 1 according to DSD is not warranted.

Appropriate animal studies show only minor effects of the substance on development. These effects consist in slight changes in fetal body weights and visceral variations occurring at maternaly toxic doses as summarised above. Thus, classification as toxic to reproduction 1B or 2 according to CLP is not warranted. Likewise, category 2 or 3 according to DSD is not warranted either.

4.11.6 Conclusions on classification and labelling

The reproductive toxicity of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the reproductive toxicity studies fluopyram does not warrant classification and labelling for reproductive toxicity.

According to the criteria in Reg. 1272/2008, based on the results of the reproductive toxicity studies fluopyram does not warrant classification and labelling for reproductive toxicity.

The developmental toxicity of fluopyram does not meet the DSD and CLP criteria.

According to the criteria in Dir. 67/548, based on the results of the developmental toxicity studies fluopyram does not warrant classification and labelling for developmental toxicity.

According to the criteria in Reg. 1272/2008, based on the results of the developmental toxicity studies fluopyram does not warrant classification and labelling for developmental toxicity.

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

One two-generation reproduction study in rats with the doses 0, 40, 220 or 1200 ppm in the diet and two teratogenicity studies, one performed in rats with the doses 0, 30, 150 or 450 mg/kg bw/d and one in rabbits with the doses 0, 10, 25 or 75 mg/kg bw/d, were summarised in the dossier.

Two-generation reproduction study

In the P- and F1-generations, increased liver weights associated with an increased incidence of centrilobular hypertrophy were observed. In parental males only, an increase in some clinical chemistry parameters, increases in kidney weight (associated with an increased incidence of protein droplet nephropathy) and lymphocytic infiltration were found. In parental females (P and F1) some effects on body weight and blood parameters were observed. The parental systemic NOAEL was 220 ppm (14.5 mg/kg bw/d in males, 17.2 mg/kg bw/d in females).

The offspring NOAEL was 220 ppm (14.5 mg/kg bw/d) based on maternal effects leading to secondary effects on pup weight and pup weight gain. Also, a slight delay in preputial separation and decreases in spleen and thymus weights were noted.

No reproductive findings were observed up to the highest dose tested resulting in a reproductive NOAEL of 1200 ppm in both males and females (82.8/93.1 mg/kg bw/d).

Teratogenicity studies

Rat:

In the rat study, maternal toxicity in terms of lower maternal body weight gain and food consumption, higher liver weight and diffuse centrilobular hepatocellular hypertrophy was seen at mid and high dose levels. Developmental toxicity was observed at the high dose only in terms of slightly lower fetal body weight, and a slightly increased incidence of two visceral variations and malformations (thymic remnant present, ureter convoluted and/or dilated) and two skeletal variations and malformations (incomplete ossification of thoracic vertebrae and split thoracic vertebrae). Split cartilage in the thoracic centrum was found in 4/159 foetuses in the higest dose group (450 mg/kg bw/d) and in 1/160 foetuses in the lowest dose group (30 mg/kg bw/d) but these were within the historical control range. No findings were seen in the mid dose group. The split cartilage is identified as a malformation according to Solecki *et al.* (2001). However, the findings of all incidences were observed only in one single thoracic centrum and only in the highest dose group in presence of maternal toxicity. The maternal NOAEL was considered to be 30 mg/kg bw/d and the fetal NOAEL was considered to be 150 mg/kg bw/d.

Rabbit:
Maternal toxicity was observed in the form of reduced mean body weight gains and food consumption. Foetal toxicity was found at the same dose level, consisting of reduced body weight in both sexes. For both, the maternal and the foetal developmental toxicity, the NOEL was set at 25 mg/kg bw/d.

Overall conclusion
The appropriate animal studies showed no effects of the substance on reproduction or fertility. Additionally, in the summarised teratogenicity studies, only minor effects on development were seen. These effects consisted of slight changes in foetal body weights and visceral variations occurring at maternally toxic doses. Thus, classification as toxic to reproduction was not considered warranted by the DS.

Comments received during public consultation

One MSCA commented, suggesting that classification as Repr. 2 - H361d would be warranted based on the increased incidences of certain visceral and skeletal malformations/variations in rats and the malformation 'gall bladder absent' in rabbits.

The DS responded that the malformation 'gall bladder absent' occurring in rabbits was within the historical control range. Furthermore, there were no treatment-related skeletal malformations in the rat. The reported effects (incomplete ossification of thoracic vertebrae and split thoracic vertebrae) are considered to be variations and do not require classification for developmental toxicity.

Assessment and comparison with the classification criteria

The findings on reproductive toxicity were limited to absent gall bladder and skeletal variations (incomplete ossification). The gall bladder findings (two cases) were within the range of historical controls. This effect is thus not considered to be treatment related. Variants may, according to the CLP regulation, not lead to classification if considered to be of low toxicological significance. RAC considers the effects not severe enough to warrant classification. RAC therefore agrees with the DS that no classification for reproductive toxicity is warranted. RAC regards the findings of split cartilage in thoracic centrum to be a malformation. Split cartilage occured in the highest dose group in association with maternal toxicity. No effects were seen in the middle dose group. One single incidence of split cartilage in the lowest dose group was within the historical control range. In all incidences the findings were observed only in one single thoracic centrum per animal.

RAC agrees that the effects were not severe enough to be a basis for classification and therefore agrees with the DS that classification as toxic to reproduction is not warranted.

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

In an acute neurotoxicity study, a single dose of fluopyram was administered by gavage to young adult Wistar rats at nominal doses of 0 (vehicle control), 125, 500 or 2000 mg/kg bw. Effects in males and/or females consisted of decreased motor and locomotor activity on the day of treatment, urine stain, and decreased body temperature. A clear NOAEL of 125 mg/kg bw could be established for male rats

whereas in females motor and locomotor activity was still impaired at this lowest dose level. Therefore, a follow-up study was conducted under the same conditions but only in females at nominal doses of 0 (vehicle), 25, 50 or 100 mg/kg bw to establish an overall No-Observed-Adverse-Effect Level (NOAEL). Because slight decreases in (loco)motor activity became apparent at 100 mg/kg bw, the next lower dose of 50 mg/kg bw was considered the NOAEL for females. There were no gross or microscopic lesions in the nervous system up to the highest dose of 2000 mg/kg bw.

In a 90-day neurotoxicity study, fluopyram was administered in the diet for 13 weeks to Wistar rats at nominal concentrations of 0, 100, 500 and 2500 ppm. No evidence of neurotoxicity was observed at any treatment level and, accordingly, the highest dose (equivalent to a mean daily intake of 164 mg/kg bw by male rats and of 197 mg/kg bw by females) was considered the NOAEL for this endpoint. Treatment-related findings of general toxicty at the high dose consisted of decreases in body weight, total body weight gain and food consumption in males and females, some alterations in clinical chemistry and hematological parameters and an increase in the organ weights of liver, thyroid and kidneys. A few of these findings such as lower food consumption, hematological changes and an increase in relative liver weight were also observed at the next lower dose level but were rather minor in nature. Thus, this mid dose of 500 ppm (corresponding to a mean daily intake of about 33 mg/kg bw in males and 41 mg/kg bw in females) was considered the overall NOAEL in this study. An overview on the available studies on neurotoxicity is given in Table 160.

Table 160: Summary of neurotoxicity studies with fluopyram

Type of study; dose levels	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Adverse effects / target organs
Acute neurotoxicity in the rat (initial study in both sexes); 0, 125, 500 and 2000 mg/kg bw Gilmore, R. G. and Hoss, H. E., 2007 ASB2008-5365	125 in males and < 125 in females	500 in males and < 125 in females	↓ motor and locomotor activity, clinical signs, ↓ body temperature
Acute neurotoxicity in the rat (follow-up study in females); 0, 25, 50 and 100 mg/kg bw Gilmore, R. G. and Hoss, H. E., 2007 ASB2008-5365	50	100	↓ motor and locomotor activity
Subchronic (90-day) neurotoxicity in the rat; 0, 100, 500 and 2500 ppm Gilmore, 2008, ASB2008-5366	164/197 (M/F) for neurotoxicity; 33.2/41.2 (M/F) for systemic effects	164/197 (M/F) for systemic effects	No neurotoxic potential; systemic effects: bw (gain) and food consumption ↓, changes in hematological and clinical chemistry parameters; organ weights of liver, thyroid, and kidneys ↑

Acute neurotoxicity – rat

Report: Gilmore, R. G. and Hoss, H. E. (2007): An acute Oral Neurotoxicity Screening Study with Technical Grade AE C656948 in Wistar Rats, 201656, M-289073-01, ASB2008-5365.

(Unusually, this report included two independent studies, i.e., the so-called "initial study" employing animals of both sexes and a "follow-up study" in females using lower dose levels.)

Guidelines: OECD 424 (1997).

Deviations: None, with regard to the initial study. In contrast, the follow up study was not in compliance with OECD 424 because the range of parameters was quite limited and the requirement of a 14-day post-observation period was not fulfilled.

GLP: Yes.

Acceptability: The initial study is considered to be acceptable whereas the follow-up study in females is only supplementary. However, because a full study in both sexes is available, the information obtained from both experiments, when taken together, is considered complete and sufficient to address this annex point.

Materials and methods

Test material: AE C656948 (Fluopyram)

Description: Beige powder

Lot/Batch Mix-Batch:08528/0002 (used in both studies)

Purity 94.7 % (May, 2005, certified through May, 2007)

Dose preparation and analysis: Dosing solutions were prepared by suspending the test substance in 2 % (v/v) Cremophor EL in deionized water to give nominal concentrations of 2.5 – 200 mg/mL for the different dose groups. In each dosing suspension, the concentration of fluopyram in the vehicle was

measured using high- performance liquid chromatographic/ultra violet (HPLC/UV) analysis. The homogeneity and stability of the test compound in the vehicle were established using samples at nominal concentrations of 2.5, 5 and 200 mg/mL that either bracketed or included the dose levels employed in the present study. Homogeneity was considered sufficient if the percent relative standard deviation (% RSD) was <5 %.

Species	Rat
Strain	Wistar HAN CRL: WI (HAN)
Age / weight	At least 9 weeks; Initial Study: 252.2 to 315.3 g for the males; 167.0 – 206.9 g for the females; Follow-Up Study: 166.6 – 214.7 g for females
Source/breeder	Charles River Laboratories, Inc. (Raleigh, NC)
Acclimation period	Initial Study – 8 days; Follow-Up Study – 7 days
Housing	Animals were caged individually in suspended stainless steel wire-mesh cages.
Environmental conditions	Temperature: 22 □ 4°C
	Humidity: 50 □ 20 %
	Air changes: Minimum daily average of 10.13 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles
Diet	Purina Mills Rodent Lab Chow 5002 in meal form provided for ad libitum consumption during the acclimation period and throughout the study except during neurobehavioral testing
Water	Tap water, ad libitum except during neurobehavioral testing

Study design, treatment and dose selection: In the initial experiment, groups of 12 male and female rats were given a single dose of fluopyram by oral gavage at nominal doses of 0 (vehicle) control, 125, 500 or 2000 mg/kg bw. The test substance was administered to non-fasted animals at a dosing volume of 10 mL/kg bw. Treatment was followed by a 14-day post-observation period.

The oral route of exposure was chosen in accordance with the test guideline requirements and because of the expected route of human exposure. The rat was selected due to its general acceptance and suitability as a rodent species for toxicological testing of this type. A large historical database is available for the Wistar strain that was employed in this study. For dose selection, the results of an acute oral toxicity study in young-adult female Wistar rats were primarily considered (see 4.2.1.1). The outcome of this study supported the use of a limit dose of 2000 mg/kg bw that might be expected to produce weak systemic toxicity. Taking into account that additional parameters are investigated in an acute neurotoxicity study, the middle dose of 500 mg/kg bw was selected to produce minimal or no effects whereas the low dose of 125 mg/kg bw was considered to be the probable NOAEL.

Neurobehavioral testing for loco(motor) activity and by means of a functional observation battery (FOB) was performed on all animals. Dosing and neurobehavioral testing was staggered over two days for each sex to accommodate the schedule. In addition, the following observations and measurements were included: clinical observations, mortality and body weight. Subsequent to sacrifice and gross necropsy at day 14 or 15 after exposure, half of the rats from the control and high-dose groups became subject of histopathology on selected tissues of the nervous system. In addition, brain weights were determined in these animals.

Because motor and locomotor activity were impaired in females even at the lowest dose level of 125 mg/kg bw, a follow-up study only in that sex was initiated in order to establish a clear NOAEL. For this purpose, 12 females/group were treated in the same manner as in the initial study but were sacrificed

two or three days after dosing already. This time, intended dose levels were 0, 25, 50 and 100 mg/kg bw. In-life observations were confined to FOB and activity measurements. The animals were neither subjected to gross necropsy nor to micropathology. An overview on the study design is given in Table 161.

Table 161: Experimental design of the acute neurotoxicity study

	Initial Study – Dose Groups (mg/kg bw)				**Follow-Up Study - Dose Groups (mg/kg bw), Females Only**			
	Control	125	500	2000	Control	25	50	100
Total number of animals/sex/	12/sex	12/sex	12/sex	12/sex	11-12b	12	12	12
Behavioral Testing (FOB, Motor Activity)	12/sex	12/sex	12/sex	12/sex	11-12b	12	12	12
Neuropathology	6/sex	- [a]	- [a]	6/sex	-	-	-	-

[a] Tissues from the mid- and low-dose groups were not examined, as no treatment-related neuropathology was noted at the highest dose level. [b] One control female was found dead after dosing.

Animal assignment: Following acclimation, the animals were weighed and those with individual body weights of more or less than 20 % of the mean value for each sex were rejected. The remaining animals were randomly assigned to the control and different dose groups by means of software from INSTEM Computer Systems. Each rat was identified by cage card and tail mark but this number did not reveal the respective dose group. In addition, animals were assigned an identification number that specified the rat's sex, treatment group, and cage number and by which each individual was uniquely identified.

The study design for the initial study required a total of 96 rats (48 males and 48 females) and an additional 48 females were utilized for the follow-up study to establish a NOAEL for motor and locomotor activity. An additional 24 animals (8 males and 8 females, each for the initial study and 8 females for the follow-up study) were tested (FOB and motor activity) during the pre-treatment week but not treated in case they were needed to replace animals (e.g. if mis-dosing would have occurred).

Mortality and clinical observations: Cage-side observations were performed at least once daily for mortality or clinical signs of a moribund status. In addition, detailed physical examinations for clinical signs of toxicity were carried out and recorded each day for the whole study period.

Body weight and food consumption: Animals were weighed weekly as a component of the FOB. Additionally, all animals were weighed prior to dosing and, in the initial study, at scheduled termination. Animals from the follow-up study were not further weighed after dosing since a NOAEL for body weight effects had been established in the initial study yet. Food consumption was not measured in this study.

Neurobehavioral assessment: In the initial study, FOB and motor activity measurements were performed on all animals on four occasions: one week prior to treatment, approximately 1 hour (minimum) after administration of the dose, and again seven and 14 days following treatment. In the follow-up study, females were tested on two occasions: one week prior to treatment and approximately 1 hour (minimum) after treatment. The time schedule for post-dosing examinations was based on toxicokinetic considerations. Since the acute oral toxicity study did not provide information on the time to peak effect, data from an ADME study (see 4.1) were used. In the respective portion of this study, adult male and female Wistar rats (4/sex/dose) received a single oral (gavage) dose of 5 mg fluopyram/kg bw in 0.5 % aqueous tragacanth at a dosing volume of 10 mL/kg. The t_{max} in plasma occurred 40-60 minutes after treatment in males and 2-3 hours after treatment in females. Because of this experience, in the neurotoxicity study, the FOB began approximately one hour (minimum) following dose administration, with the automated test of activity concluding at about 4 hours after treatment.

Sets of eight animals (maximum) were evaluated individually using the FOB and then, approximately 30 minutes after the last animal in the set had finished being tested in the FOB, were placed individually into the mazes to measure activity. The order of testing and assignment of animals to mazes were done in a semi-random manner, such that groups were balanced across test times and test devices, and no

animal would be tested more than once in the same maze. Testing was performed without knowledge of the group assignment by the technicians.

The FOB closely followed the battery of tests described by Moser (J. Am. Coll. Toxicol., 1989, 8, pp. 85-93) with each animal tested individually. Scoring criteria and explicitly-defined scales were used to rank the severity of observations that do not readily lend themselves to quantitation. When applicable, observations were scored for intensity as follows: 1) slight (barely perceptible or infrequent) or 2) moderate to severe. Data were collected while the rats were in their home cage, during handling, and in an open field for 2 minutes (in the center of a flat surface with a perimeter barrier, such as a cart). In addition, reflex and physiologic observations and measurements were made while the animals were sitting on the cart surface following open field observations. The functional parameters evaluated in this study are summarized in Table 162.

For determination of motor and locomotor activities, rats were placed individually into figure-eight mazes and activity was measured for a total of 60 minutes. This whole observation period was further partitioned into six ten-minute intervals. The figure-eight maze was selected as an established and widely-used automated activity-measuring device that can be used to detect both increases and decreases in activity. A Columbus Instruments (Columbus, Ohio, U.S.A.) Universal Maze Monitoring System and a personal computer were used for automated data collection. Each maze consisted of a series of inter-connected alleys, converging on a central arena and was covered by transparent plastic. Eight infrared emitter / detector pairs (three in each of the figure-eight alleys and one in each of the blind alleys) measured activity; i.e., when a beam was interrupted, an activity count was registered. Motor activity was measured as the total number of beam interruptions that occurred during the test session and its intervals. In contrast, locomotor activity was measured by eliminating consecutive counts for a given beam. Thus, for the latter parameter, only one interruption of a given beam was counted until the rat relocated in the maze and interrupted one of the other beams. Habituation was evaluated as a decrement in activity during the test session.

Table 162: Parameters of the FOB

HOME CAGE OBSERVATIONS	HANDLING OBSERVATIONS	OPEN FIELD OBSERVATIONS
Posture*	Reactivity*	Rearing+
Piloerection	Muscle tone*	Piloerection*
Involuntary motor movements e.g.:	Palpebral closure*	Respiratory abnormalities
Repetitive chewing	Lacrimation* / chromodacryorrhea	Posture*
Convulsions*	Salivation*	Involuntary motor movements e.g.:
Tremors*	Nasal discharge	Repetitive chewing
Abnormal movements*	Red/crusty deposits (stains)*	Convulsions*
Gate abnormalities	Fur appearance	Tremors*
Vocalizations	Emaciation	Stereotypic behavior*
Decreased activity	Bite marks	Bizarre behavior*
Repetitive head bobbing	Eye prominence*	Abnormal movements *
Increased reactivity	Broken teeth/malocclusion	Gait abnormalities* / Gait score*
	Missing Toe Nail(s)	Vocalizations
SENSORY OBSERVATONS	Dehydration	Arousal/ general activity level*
Approach response+	Cool-to-Touch	Urination / defecation*
Touch response+		
Auditory response*	PHYSIOLOGICAL	NEUROMUSCULAR
Pain response*	OBSERVATIONS	OBSERVATIONS
Pupil response*	Body weight*	Forelimb grip strength*
Pupil size	Body temperature+	Hindlimb grip strength*
Air righting reflex+		Landing foot splay*

*Required parameters; +Recommended additional parameters

Sacrifice and pathology: In the initial study, all animals were subjected to a complete gross necropsy at scheduled termination involving an examination of all organs, body cavities, cut surfaces, external

orifices and body surfaces. A minimum of six males and six females at each dose level were selected for perfusion and collection of tissues, with replacement, as necessary, if the perfusion was considered inadequate. These animals were deeply anesthetized using an intraperitoneal dose (50 mg/kg bw) of pentobarbital and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by universal fixative (1 % (w/v) glutaraldehyde and 4 % (w/v) EM-grade formaldehyde) in phosphate buffer. The entire brain and spinal cord, both eyes (with optic nerves) and selected (bilateral) peripheral nerves (sciatic, tibial and sural), the gasserian ganglion, gastrocnemius muscle, both forelimbs, gross lesions in neural tissues or skeletal muscle and physical identifier were dissected from each animal and post-fixed in 10 % buffered formalin. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio was calculated.

Histopathological examinations were performed on a comprehensive battery of neural tissues from perfusion-fixed control and high-dose rats of both sexes. For this purpose, eight coronal sections of the brain and sections from three levels of the spinal cord (cervical, thoracic, lumbar) and the Cauda equina were embedded in paraffin and stained with hematoxylin and eosin (H&E), as well as the eyes, optic nerves and gastrocnemius muscle. Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings, gasserian ganglion and peripheral nerve tissues from the sciatic, tibial and sural nerves were embedded in glycol methacrylate (GMA). After sectioning, these samples were stained using a modified Lee’s stain. In addition, histopathology was performed on any gross lesions collected at necropsy. In the low and mid dose groups, histopathology of the nervous system was not performed because there were no remarkable findings at the top dose level that could be attributed to treatment.

Statistics: All data with equal variances were analyzed further using different Analysis of Variance (ANOVA) procedures. This was followed by a Dunnett's test if a significant F-value had been found to determine which groups, if any, were significantly different from the control group.

Continuous pathology data (e.g. brain weight) was initially evaluated using Bartlett's test to analyze for homogeneity of variance among groups. Groups with homogeneous variances were analyzed further using an ANOVA followed by Dunnett's test for pair-wise comparisons. In cases of non-homogeneous distribution, continuous data were analyzed using the nonparametric Kruskal-Wallis test followed by a Mann-Whitney U test for pair-wise comparisons. Neurohistological findings were evaluated using a Chi-Square procedure, followed by a one-tailed Fischer's Exact Test in cases of significant variation by the Chi-Square analysis. The level of statistical significance was generally set at $p \leq 0.05$, with the exception of Bartlett’s test which was tested at $p \leq 0.001$.

Findings

Dose analysis: For the initial study, actual dose levels ranged from 92 % to 101 % of the nominal concentrations of 125, 500 and 2000 mg/kg bw. In fact, the analytically-confirmed doses were 126, 498 and 1840 mg/kg bw for both males and females. In the follow-up study, the female rats received actual doses of 25, 51 and 100 mg/kg bw that were nearly identical to the nominal concentrations (100 % to 102 %). Homogeneity of the test substance in the vehicle was acceptable as the 2.5, 5 and 200 mg/mL concentrations had percent relative standard deviations of no more than 1.2 %, 1.9 % and 1.7 %, respectively. The stability of fluopyram in the vehicle at room temperature storage was confirmed at nominal concentrations of 2.5, 5 or 200 mg/mL with no appreciable decreases in concentration within eight days.

Mortality: There were no compound-related deaths at any dose level in either sex. However, one control female from the follow-up study was found dead on day 1 of the study. The cause of death could not be determined but was clearly not due to the test substance. Necropsy findings for this animal included bilateral mild dilatation of the renal pelvis, multiple discolored zones on both kidneys and abnormal (thickened) contents in the urinary bladder.

Clinical signs: In the initial study, compound-related clinical signs were limited to urine stain in four high-dose males with none in the control, low- or mid-dose groups. This finding became first evident on day 0 and generally resolved within two to five days after treatment. There were no compound-related clinical signs in males at lower dose levels or in females at any dose level. Incidental observations occurring at a low frequency and without dose response comprised areas of hair loss described as alopecia on various occasions in one control male and in one or two control or mid-dose females, each. One mid-dose female had a dermal lesion described as a scab that was observed during the pretreatment week already. In the follow-up study, there were no compound-related signs at any dose level.

Body weight: In the initial study, body weight was not affected by treatment at any dose level in either sex. In the follow-up study, this parameter was not recorded after dosing.

FOB: In the initial study, the only notable and possibly treatment-related findings were limited to the groups of females receiving the high and intermediate doses. There was a statistically significant decrease in mean body temperature in mid- and high-dose females (i.e. 37.4°C and 36.9°C, respectively vs. 37.9°C for controls). Also there were statistical differences from control with regard to the ease of removal from the home cage in high-dose females with a similar (non-statistical) trend in mid-dose females. This latter observation became apparent by a higher number of rats that resisted when they were taken out of their cages but, on the other hand, a lower incidence of animals that vocalized at this occasion. In the control group, vocalization was mentioned in 6 female rats whereas this sign of reluctance was noted in three mid-dose rats and in only one animal from the high-dose group. In males at any dose level or in low-dose females, there were no findings that could be attributed to treatment.

In addition, there were a few findings that were statistically different from the control group but were not considered substance-related because the difference was apparent prior to treatment already or because they occurred only occasionally and were not consistently seen throughout the study. This included an increased number of urine pools on the open field in males assigned to the high-dose and a statistical decrease in mean foot splay in mid-dose females. The results obtained by FOB in the initial study are summarized in Table 163.

Table 163: Functional observational battery results – Initial study

Males		Dose Level (mg/kg bw)			
	Observation	**Control**	**125**	**500**	**2000**
Pre-treatment week	Urination - Number of Pools Mean ± S.D.	1.0±1.0	1.3±1.4	1.6±1.6	3.1±2.3*
Day 0	No Findings	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Day 7	No Findings	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)	12(100) 0(0)
Day 14	Handling - Other: Not Observed Alopecia, Present	 11(92) 1(8)	 12(100) 0(0)	 12(100) 0(0)	 12(100) 0(0)
Females		**Dose Level (mg/kg bw)**			
	Observation	**Control**	**125**	**500**	**2000**
Pre-treatment week	Handling - Other: Not Observed Alopecia, Present	12(100) 0(0)	12(100) 0(0)	10(83) 2(17)	12(100) 0(0)
	Handling - Other: Not Observed Scab, Present	12(100) 0(0)	12(100) 0(0)	11(92) 1(8)	12(100) 0(0)
	Reflex/Physiologic Observations – Foot Splay Mean ± S.D.	84±17	84±16	66±14*	81±15
Day 0	Handling – Ease of Removal: Minimal Resistance Minimal Resistance with Vocalizations	 6(50) 6(50)	 5(42) 7(58)	 9(75) 3(25)	 11(92) * 1(8)
	Handling - Other: Not Observed Alopecia, Present	 11(92) 1(8)	 12(100) 0(0)	 10(83) 2(17)	 12(100) 0(0)
	Reflex/Physiologic Observations - Body Temperature (°C) Mean ± S.D.	37.9 ±0.4	37.8 ±0.3	37.4 ±0.5*	36.9 ±0.5*
Day 7	Handling - Other: Not Observed Alopecia, Present	 10(83) 2(17)	 12(100) 0(0)	 10(83) 2(17)	 12(100) 0(0)
	Reflex/Physiologic Observations – Foot Splay Mean ± S.D.	79±16	71±15	61±11*	69±14
Day 14	Handling - Other: Not Observed Alopecia, Present	 10(83) 2(17)	 12(100) 0(0)	 10(83) 2(17)	 12(100) 0(0)

For discrete data, values represent the number of affected animals and % incidence (in parentheses)

* Statistically different from control ($p \leq 0.05$)

In the follow-up study, there were no compound-related findings prior to treatment or in the Day 0 FOB at any dose level.

Motor activity: A comparison based on the inherent variability of the average pretreatment values in the initial study for motor and locomotor activity among the four groups of males and females provides a measure of the magnitude of the difference that should be considered biologically significant. For motor activity, the pretreatment values for groups that later received the test substance averaged from 12 % lower to 17 % higher than controls for males and from 7 % lower to 22 % higher than controls for females (see Table 164). For locomotor activity, the pretreatment values for groups that later received the test substance averaged from 10 % lower to 28 % higher than controls for males and from 12 % lower to 22 % higher than controls for females (see Table 165). In the follow-up study, for motor and locomotor activity the pretreatment values for groups that later received the test substance averaged from 9 % to 15 % lower than controls and from 7 % to 20 % lower than controls, respectively (see the following table). As a general guide, these results confirm that differences of approximately +20 % are within the range of normal variability in this laboratory for groups of 10-12 rats/sex/dose level and, therefore, should not be considered biologically significant.

For the overall 60-minute test session in the initial study, dose-related and statistically significant decreases in motor and locomotor activity were evident on the day of treatment in both sexes at the mid- and high-dose levels and in low-dose females (see Table 164 and Table 165). These changes were considered compound-related effects. In contrast, measures of motor and locomotor activity were not affected by treatment in low-dose males on day 0. After day 0, there were no consistent effects, neither in males nor in females, at any dose. The few differences did not gain statistical significance and there was no clear dose response.

Motor and locomotor activity data from the initial study were further analyzed for the individual 10-minutes intervals of each session. This more detailed analysis confirmed the outcome when the whole session was considered. On day 0, high-dose males and females exhibited statistically lower levels of motor and locomotor activity during intervals 1 through 6. For mid-dose males, motor and locomotor activity was statistically lower than in the control group during intervals 2 through 5 or 6. Similarly, in mid-dose females, interval motor and locomotor activity was statistically lower during intervals 1 or 2 through 6. In low-dose females, motor and locomotor activity was significantly reduced during intervals 3, 5 and 6 and during intervals 3 through 6, respectively whereas there were no differences in low-dose males at the lowest dose level during any interval.

Table 164: Summary session motor activity – Initial study (percent difference from control)

Test Day	Dose Level (mg/kg bw)		
	125	500	2000
Males			
Pretreatment	17	-12	-6
Day 0	-5	-51*	-71*
Day 7	4	-14	-20
Day 14	6	-9	-9
Females			
Pretreatment	13	-7	22
Day 0	-26*	-53*	-72*
Day 7	1	-11	19
Day 14	6	17	8

* statistically different from control ($p \leq 0.05$; ANOVA)

Table 165: Summary session locomotor activity – Initial study (percent difference from control)

Test Day	Dose Level (mg/kg bw)		
	125	500	2000
Males			
Pretreatment	28	-8	-10
Day 0	-4	-49*	-73*
Day 7	12	-9	-22
Day 14	13	-3	-15
Females			
Pretreatment	16	-12	22
Day 0	-31*	-58*	-77*
Day 7	7	-16	12
Day 14	7	7	11

* statistically different from control ($p \leq 0.05$; ANOVA)

At later test sessions, statistical differences from control were occasionally observed for measures of motor and locomotor activity in few intervals. Thus, in high-dose males, motor and locomotor activity was decreased on day 7 during interval 3 and motor activity was statistically increased on day 7 during interval 6 in high-dose females. These isolated findings were not attributed to treatment because a consistent pattern was lacking. Thus, habituation was apparently not affected by treatment with fluopyram at any dose level in either sex.

In the follow-up study in females, measures of motor and locomotor activity are difficult to interpret. Upon pretreatment testing already, motor and locomotor activity counts were reduced in all three dose groups as compared to the control. At all three dose levels, this difference became more pronounced after substance administration (see Table 166 and

Table 167). Nonetheless, the mean values were not statistically significantly different from the control. However, in the high dose group, a decrease by 38 % was observed for both parameters that is clearly outside the ± 20 % range of normal biological variation. Therefore, it cannot be excluded that this change was treatment-related.

Table 166: Summary session motor activity – Follow-up study (percent difference from control)

Test Day	Dose Level (mg/kg bw)		
	25	50	100
Females			
Pretreatment	-11	-9	-15
Day 0	-22	-20	-38

Table 167: Summary session locomotor activity – Follow-up study (percent difference from control)

Test Day	Dose Level (mg/kg bw)		
	25	50	100
Females			
Pretreatment	-11	-7	-20
Day 0	-14	-18	-38

This assumption is further supported by analysis of the individual 10-minutes intervals. Differences in motor and locomotor activity from the control groups were evident in females that received 100 mg/kg bw dose level and were considered to be related to treatment. These differences from control in females treated with 100 mg/kg during intervals 1 or 2 through 6.

Pathology: There were no gross lesions at terminal sacrifice in males or females in the initial study at any dose level that might be compound-related. In addition, there were no effects on terminal body weight in any group or on absolute or relative brain weight in perfused animals at any level of exposure. Body and brain weight data from the initial study is summarized in Table 168. Microscopic evaluation did not reveal compound-related histological lesions in the nervous system in high-dose males or females in the initial study. Therefore, it was not necessary to perform necropsy or histopathology on animals in the follow-up study.

Table 168: Body weights, absolute and relative brain weights – Initial study

Weights (g)	Dose Level (mg/kg bw)			
	Control	125	500	2000
Males				
Body wt [a] (g)	312.6±23.5	328.4±19.8	316.1±17.4	307.3±21.1
Brain wt [b] (g)	1.826±0.078	1.833±0.057	1.806±0.038	1.866±0.052
Brain/body wt [b] (%)	0.577±0.037	0.547±0.019	0.565±0.023	0.619±0.046
Female				
Body wt [a] (g)	209.1±15.2	207.7±10.5	204.7±7.6	209.2±7.9
Brain wt [b] (g)	1.812±0.081	1.739±0.083	1.750±0.038	1.721±0.025
Brain/body wt [b] (%)	0.878±.045	0.842±0.056	0.841±0.012	0.828±0.016

[a] n=12, [b] n=6

Conclusion

Compound-related effects with possible involvement of the nervous system following a single oral dose of the test substance were observed in mid- and high-dose males and females and in low-dose females in the initial study. These findings consisted of decreased motor and locomotor activity on the day of treatment, urine stain, decreased body temperature and a higher reluctance to removal from the home cage although the number of animals that vocalized during removal was lower. There were no compound-related gross or microscopic lesions at the highest dose of 2000 mg/kg bw. Thus, a specific neurotoxic potential of fluopyram is not likely and the weak effects are rather due to systemic toxicity. The NOAEL for male rats was 125 mg/kg bw whereas no clear NOAEL could be established for females. Therefore, a follow-up study in females was initiated in which the NOAEL was established at 50 mg/kg bw that is based on a decrease in motor and locomotor activity at the next higher dose level of 100 mg/kg bw although the differences from the controls did not gain statistical significance.

Subchronic neurotoxicity - rat

Report:	Gilmore, R. G. (2008): A Subchronic Neurotoxicity Screening Study with Technical Grade AE C656948 in Wistar Rats. 201833, M-299110-01, ASB2008-5366.
Guidelines:	OECD 424 (1997).
Deviations:	None.
GLP:	Yes.
Acceptability:	The study is considered to be acceptable.

Materials and methods

Test material:	AE C656948 (Fluopyram)	
Description:	Beige powder	
Lot/Batch	Mix-Batch:08528/0002	
Purity	94.7 % (May, 2005, certified through May, 2007)	
Species	Rat	
Strain	Wistar HAN CRL: WI (HAN)	
Age / weight	8 weeks approx.; 219.6 – 285.9 g range for males and 142.9 – 174.9 g range for females	
Source/breeder	Charles River Laboratories, Inc. (Raleigh, NC, U.S.A.)	
Acclimation period	10 days	
Housing	Animals were caged individually in suspended stainless steel wire-mesh cages.	
Environmental conditions	Temperature:	22 □ 4°C
	Humidity:	50 □ 20 %
	Air changes:	Approximately 10 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles
Diet	Purina Mills Rodent Lab Chow 5002 in meal form provided for ad libitum consumption during the acclimation period and throughout the study except during neurobehavioral testing	
Water	Tap water, ad libitum	

Study design, dose selection and treatment: A 90-day feeding study in rats was run to investigate a neurotoxic potential that might become apparent upon repeated administration. The in-life phase of the study was performed between February 26, 2007 and June 1, 2007 at the test facility of Bayer CropScience LP, Stilwell, Kansas, United States. The oral application route was employed in accordance with the test guideline requirements and because of the expected exposure of consumers. Four dose groups of 12 male and 12 (only 11 from week 4 onwards) female rats each were administered the test substance via their diet at nominal concentrations of 0, 100, 500 and 2500 ppm over 13 weeks. These dose levels had been selected on the basis of the experiences obtained in the subchronic feeding study in rats (see 4.7.1.1) and in the pre-mating phase of the two-generation reproduction study (see 0). An overview on the study design is given in Table 169.

Table 169: Experimental design of the 90-day oral neurotoxicity study in rats

	Dose levels in ppm (mg/kg bw/day)			
Experimental parameter	Control	100 (♂ 6.69) (♀ 8.05)	500 (♂ 33.2) (♀ 41.2)	2500 (♂ 164.2) (♀ 197.1)
Total number of animals/sex/group	12	12	12	12
Mortality, clinical signs, body weights, food consumption	10-12/sex	11-12/sex	10-12/sex	10-12/sex
Behavioral testing (FOB, motor activity)	10-12/sex	11-12/sex	10-12/sex	10-12/sex
Clinical chemistry, hematology, organ weights and tissue collection	4-6/sex	5-6/sex	5-6/sex	5-6/sex
Neuropathology	6/sex	-	-	6/sex
Ophthalmic examination	11-12/sex	11-12/sex	11-12/sex	11-12/sex

The parameters that were examined in this study allowed evaluation of both systemic and neurotoxicity and, in addition, were appropriate to prove that a sufficiently high toxic dose was tested.

The study design did not include concurrent positive controls but previous studies conducted at this laboratory had demonstrated the sensitivity of the test system and the methods used as well as the adequacy of training of technical personnel. Thus, sensitivity, reliability, and validity of the Functional Observational Battery (FOB) had been demonstrated in studies with acrylamide, carbaryl and in untreated rats. To assess motor activity, studies on untreated animals and with reference substances that increase (triadimefon) or decrease (chlorpromazine) motor activity had established that the test procedures were valid. Studies performed at this laboratory with trimethyltin and acrylamide proved the suitability of histopathological procedures to detect lesions in peripheral nerves and the central nervous system.

Animal assignment: Following acclimation, the animals were weighed and those with body weights that were more or less than 20 % of the mean weight for each sex were rejected. The remaining animals were randomly assigned to a control group or one of three dietary levels in order that, for each sex, groups had comparable body weights when treatment was initiated. Randomization procedures utilized software from INSTEM Computer Systems.

Each rat was identified by cage card and tail tattoo with a number that did not reveal the animal's treatment group. In addition, animals were assigned an identification number that specified the rat's sex, treatment group, and cage number and identified it uniquely.

Diet preparation and analysis: The diet was prepared every other week. Acetone served as a solvent in the diet preparation process and was allowed to evaporate. The control diet was prepared the same way but, of course, without the test substance. A sample of each batch of mixed feed was taken and retained in the freezer (daily average temperatures -22.62 to -24.74°C) until the study was complete and the analytical data deemed satisfactory. Feed was available for ad libitum consumption for a period of one week prior to changing, at which time any uneaten feed was collected and disposed of by incineration. The concentration of fluopyram in the diet was weakly measured by LC-MS/MS analysis. The stability [following both room temperature (~ 22°C) and freezer (~ -23°C) exposure] and homogeneity of the test substance in the feed were established by analysis of samples at nominal concentrations of 5 and 5000 ppm.

Mortality and clinical observations: Cage-side observations were conducted twice daily on work days and once daily on holidays and weekends for mortality or clinical signs of morbidity. Detailed physical examinations for clinical signs of toxicity were carried out and recorded weakly.

Body weight, food consumption and test substance intake: Individual body weights were determined weekly. Additionally, all animals were weighed on the day of sacrifice for terminal body weight measurement. Individual food consumption was measured weekly. The mean daily intake of the active ingredient (mg a.i./kg bw/day) was calculated using weekly body weight and food consumption data.

Ophthalmology: Pre-exposure and pre-terminal (week 12) ophthalmic examinations were conducted on all animals in a semi-darkened room. The pupillary reflex was tested using a penlight or transilluminator with Finnoff (Welch Allyn, Inc., Skaneateles Falls, NY), and then a mydriatic agent was applied to each eye to dilate the pupil. After mydriasis, the conjunctiva, cornea and lens were examined with a slit lamp microscope (Kowa SL-15, Kowa Company, Ltd., Tollerance, CA 90502, USA), and the vitreous humor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope (HEINE OMEGA, Heine USA, Ltd., Dover, NH, USA).

Hematology and clinical chemistry: Evaluation of hematological and blood clinical chemistry standard parameters was performed on all surviving non-perfused animals (i.e., 4 – 6 per sex and dietary level) on the day of sacrifice after 13-weeks of treatment. Blood samples were obtained from fasted (overnight) animals via the retroorbital sinus while under light anesthesia (IsoFlo7; Isoflurane).

Neurobehavioral assessment: All animals on study were tested using the FOB and motor activity on five occasions - once during the week prior to initiating the exposure and again during weeks 2, 4, 8 and 13. The order of testing and assignment of animals to mazes were done in a semi-random manner, such that groups were balanced across test times and test devices, and no animal would be tested more than once in the same maze. On the day prior to each test day, the appropriate animals were placed in the correct sequence that had been established for testing on that day. Animals were then transferred to the room where testing took place and allowed to acclimate with minimal disturbance until testing on the following day. The dose group identification was concealed prior to testing to ensure that testing would be conducted without knowledge of the group assignment. The test room was a standard animal room that was maintained on the same light-dark cycle and settings for temperature and relative humidity as the animal room, with tests conducted during the light phase. Sets of eight animals (maximum) were evaluated individually using the FOB and then, approximately 30 minutes after the last animal in the set had finished being tested in the FOB, all eight rats were placed individually into the mazes to measure activity. Each week, testing was staggered over two days for each sex to accommodate the schedule for behavioral testing. Males and females were tested on separate days, with the open field and mazes cleaned during the ensuing interval to reduce the residual scent from the other sex. For description of the FOB and of the methods for (loco)motor activities measurement, see the details given in the sub-section on acute neurotoxicity testing above.

Sacrifice and gross pathology: At termination, all rats were weighed, killed and subjected to a complete gross necropsy involving an examination of all body surfaces, external orifices, organs, body cavities, and cut surfaces. For specific neuropathology, the first six males and six females at each dietary level were selected for perfusion and collection of tissues, with replacement, as necessary, if the perfusion was considered inadequate. These animals were deeply anesthetized using an intraperitoneal dose (50 mg/kg) of pentobarbital and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by Universal fixative (1 % (w/v) glutaraldehyde and 4 % (w/v) EM-grade formaldehyde in phosphate buffer. The entire brain and spinal cord, both eyes (with optic nerves) and selected (bilateral) peripheral nerves (sciatic, tibial and sural), the gasserian ganglion, gastrocnemius muscle, both forelimbs, gross lesions in neural tissues or skeletal muscle were dissected from each animal and post-fixed in 10 % buffered formalin. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio was calculated.

The remaining animals (4 – 6 per sex and dietary level) were sacrificed by carbon dioxide asphyxiation after blood samples for hematological and clinical chemistry evaluations had been taken. Following necropsy, the liver, kidneys and thyroid of these non-perfused animals were collected, weighed and preserved in 10 % buffered formalin to allow, if necessary, subsequent histopathological evaluation. Organ to body weight ratios were calculated.

Histopathology: Microscopic examinations were conducted on a comprehensive selection of neural tissues from perfusion-fixed control and high-dose rats of both sexes. Eight coronal sections of the brain and sections from three levels of the spinal cord (cervical, thoracic, lumbar) and the Cauda equina were embedded in paraffin and stained with hematoxylin and eosin (H&E). Dorsal root ganglia (including

dorsal and ventral root fibers) from the cervical and lumbar swellings and gasserian ganglion were embedded in glycol methacrylate (GMA). Eyes, optic nerves and gastrocnemius muscle were embedded in paraffin and stained using H&E. Peripheral nerves (sciatic, tibial and sural) were embedded in GMA and cut in cross/transverse-section, as well as longitudinal section. GMA-embedded tissues were sectioned at 2-3 μm and stained using a modified Lee's stain. In addition, histopathology was performed on any gross lesion collected at necropsy. Tissues from perfusion-fixed animals at the low- and mid-dose levels were not subjected to micropathology because no compound-related lesions were present in the high-dose group.

The liver, kidneys and thyroid of the non-perfused animals from all dietary levels were trimmed, processed and sectioned according to standard procedures for light microscopy (paraffin with H&E stain), if deemed necessary by the study director.

Statistics: Statistical evaluations were performed using software from either INSTEM Computer Systems or SAS. With the exception of Bartlett's test for which it was $p \leq 0.001$, the level used to establish statistical significance was $p \leq 0.05$.

Continuous data were analyzed using an Analysis of Variance (ANOVA), followed by a Dunnett's test if a significant F-value was determined in the ANOVA for the whole study period or certain weeks or intervals.

For the FOB, continuous data were first analyzed using a Repeated-Measures ANOVA, followed by a one-way ANOVA if there was a significant interaction between dose group and test week. Categorical data collected in the FOB were analyzed in a similar manner, using General Linear Modeling (GLM) and Categorical Modeling (CATMOD) Procedures, with post-hoc comparisons using Dunnett's test and an Analysis of Contrasts, respectively. Motor and locomotor activity (activity for the entire session and activity for each 10-minute interval) were analyzed using ANOVA procedures. Session activity data were first analyzed using a Repeated-Measures ANOVA, followed by a one-way ANOVA if there was a significant interaction with test occasion. Interval data were subjected to a two-way Repeated-Measures ANOVA, using both test interval and test occasion as the repeated measures, followed by a Repeated Measures ANOVA to determine on which weeks there was a significant treatment by interval interaction.

For pathology, continuous data were evaluated initially using Bartlett's Test to analyze for homogeneity of variances among groups. Homogeneous data were further analyzed using an Analysis of Variance (ANOVA) followed by Dunnett's Test for pair-wise comparisons. In the event of non-homogeneous data, statistical analysis was performed using the non-parametric Kruskal-Wallis Test followed by a Mann-Whitney U Test for pair-wise comparisons. Micropathology frequency data were analyzed using a Chi-Square Test followed by a one-tailed Fisher's Exact Test in cases of significant variation by the Chi-Square analysis.

Findings

Diet analysis: Analytically determined concentrations of the active ingredient in the three dose groups averaged at 98 % to 102 % of the nominal concentrations. Based on these results, the actual mean analytically-confirmed dietary levels for this study were 100, 512 and 2458 ppm.

Homogeneity of the test substance was within the acceptable range for concentrations that bracketed those used in this study. These concentrations of 5 and 5000 ppm had percent relative standard deviations (%RSD) of 2.7 % and 1.8 %, respectively.

The stability of fluopyram in the feed was established at room temperature at dietary concentrations of 5 and 5000 ppm, with no appreciable decrease in concentration within seven days of storage. In addition, fluopyram was stable at freezer conditions for 28 days, with no appreciable decrease in concentration at 5 and 5000 ppm.

Actual daily test substance intake: The mean daily consumption of fluopyram for males and females that received diets with nominal concentrations of 0, 100, 500 and 2500 ppm fluopyram was calculated as follows: 0, 6.69, 33.2 and 164.2 mg/kg bw/day, respectively, for males and 0, 8.05, 41.2 and 197.1 mg/kg bw/day, respectively, for females.

Mortality: There were no unscheduled deaths in males or females at any dietary level. However, according to the original study report, during week 4 FOB one female from the control, low-dose, mid-dose and high-dose group each were found to be in the wrong cages. Apparently, these rats had been exposed to a wrong dietary level for some time. Therefore, these animals were killed without further examinations and the individual data obtained so far removed from analysis because they were deemed invalid. Accordingly, the number of animals in all female groups was only 11 from week 4 onwards.

Clinical signs: Occasional clinical signs such as malocclusion of the upper incisors, red lacrimal or nasal stain or areas of hair loss on the forelimbs or the thorax could not be attributed to treatment because only very few animals were affected and because these findings did not show a dose response and were not consistently seen throughout the study.

Body weight (gain): Body weight and body weight gain tended to be lower at the upper dose level in both sexes. In females, this decrease was statistically significant over the most part of the study (see Table 170 and

Table 171). This decrement in body weight (gain) is considered a weak systemic effect of fluopyram administration but was clearly confined to a rather high dose of 2500 ppm.

Table 170: Body weight and body weight gain (mean (g) ± s.d.) in male rats (n=12/group)

Treatment day	**Dose level (ppm)**			
	Control	**100**	**500**	**2500**
Day 0	244.3±13.5	244.4±19.0	245.6±9.2	247.2±8.7
Day 21	325.7±23.6	325.3±34.8	327.3±12.9	309.8±13.5
Day 28	347.8±24.8	344.5±36.9	347.3±10.8	331.1±13.8
Day 35	365.5±25.9	360.7±38.1	366.8±14.8	344.9±14.7
Day 42	377.2±29.3	369.8±37.9	375.0±12.3	353.6±17.2
Day 49	388.0±30.4	380.6±40.1	387.6±16.3	360.9±17.3
Day 56	398.5±29.2	391.4±41.6	400.0±17.2	373.5±18.2
Day 63	405.9±30.1	399.9±43.7	410.8±18.3	384.2±20.0
Day 70	414.6±32.5	408.8±42.9	420.4±19.2	394.7±19.9
Day 77	420.3±31.8	413.0±44.8	426.3±18.0	399.5±21.7
Day 84	426.6±32.2	421.9±47.1	431.1±19.0	408.0±21.0
Day 91	432.9±34.5	428.7±44.8	437.8±22.2	416.2±22.7
Total body weight gain	188.6±26.3	184.3±31.1	192.3±18.3	169.0±19.3

Table 171: Body weight and body weight gain (Mean (g) ± s.d.) in female rats

Treatment day	Dose levels (ppm) Number of animals			
	Control	100	500	2500
Day 0	156.0±9.0 12	158.3±9.4 12	152.4±5.8 12	155.8±7.7 12
Day 21	196.7±14.7 11	196.4±16.4 11	189.8±7.6 11	182.4±7.6* 11
Day 28	205.6±16.2 11	206.7±18.5 11	199.9±8.7 11	194.7±7.6* 11
Day 35	213.7±18.2 11	213.2±16.7 11	207.9±7.8 11	201.4±10.3* 11
Day 42	217.5±17.3 11	216.1±17.0 11	209.1±8.0 11	197.9±12.3* 11
Day 49	222.9±15.9 11	219.4±17.4 11	214.1±9.5 11	199.3±15.3* 11
Day 56	226.4±15.5 11	224.9±19.8 11	219.9±10.2 11	208.9±10.0* 11
Day 63	231.1±19.5 11	229.5±18.4 11	226.1±9.7 11	211.3±10.4* 11
Day 70	236.2±18.8 11	233.1±20.3 11	226.9±9.3 11	212.1±12.1* 11
Day 77	236.4±17.7 11	236.4±22.2 11	229.0±11.9 11	208.4±22.6* 11
Day 84	240.8±18.1 11	239.9±21.8 11	234.1±12.8 11	216.7±13.6* 11
Day 91	245.8±20.3 11	243.2±22.1 11	239.1±11.4 11	223.2±11.6* 11
Total body weight gain	89.3±13.4 11	85.1±12.7 11	86.8±8.5 11	66.5±10.2* 11

*=p≤0.05, compared to control.

Food consumption: In high-dose males, food consumption was statistically significantly decreased (15 %) on day 21. Also, food consumption was non-statistically significantly decreased (6-8 %) in high-dose males on day 35 through day 49 but, this time, the difference did not gain statistical significance. In high-dose females, food consumption was statistically significantly decreased (13-24 %) beginning on day 21 and continuing for all remaining weeks measured and, thus, paralleled the decrement in body weight and body weight gain. In addition, food consumption was statistically significantly reduced by 7-12 % in mid-dose females beginning on day 21 through day 42, day 63 through day 70 and again on day 91. In contrast, food consumption was not affected by treatment in mid-dose males or in low-dose males or females. Since there seems to be a dose response, impaired food consumption is regarded as a treatment-related effect with females being more affected.

Ophthalmology: No treatment-related ophthalmologic findings were seen in either sex at any dose level.

Clinical chemistry: Probably treatment-related changes in clinical chemistry parameters were rather minor and were confined to the top dose level. Cholesterol and triglyceride levels were increased in high-dose males and/or females. Furthermore, total protein was significantly increased in high dose animals of both sexes. This finding was associated with an increase in albumin in high dose males and an increase in globulin in high dose females. Glucose concentration was significantly reduced in high dose animals of both sexes. In addition, in high dose females, the chloride concentration was significantly reduced and the urea nitrogen was significantly elevated. Most of these alterations that are summarized in Table 172 might be due to changes in liver function that are induced by fluopyram as well known from other studies with that compound at comparable dose levels.

Hematology: Clear effects on red blood cell parameters such as reductions in total and mean corpuscular hemoglobin (MCH), hemoglobin distribution width (HDW), hematocrit or mean corpuscular volume (MCV) were seen in high dose females also suggesting systemic toxicity. A clear dose response was

seen because significant decreases in MCH and MCV were apparent in the low and mid dose groups, too. However, since the differences to the control group were rather small (less than 10 %) and because total hemoglobin was not affected at doses other than the highest, these latter findings were not considered to be adverse. In males, similar changes occurred but were less pronounced and are more difficult to interpret since the dose response was not that clear. The hematological findings are summarized in Table 173.

Table 172: Clinical chemistry findings in the 90-day neurotoxicity study on rats (means ± standard deviations)

Parameters	**Dose levels (ppm)**			
	Control	**100**	**500**	**2500**
Males[a]				
Total protein (g/dL)	6.7±0.1	6.8±0.2	6.9±0.2	7.2±0.2*
Albumin (g/dL)	4.0±0.1	4.1±0.1	4.0±0.3	4.4±0.1*
Glucose (mg/dL)	110±9	117±9	109±7	94±5*
Cholesterol (mg/dL)	57±14	58±11	110±127	82±14$
Females[b]				
Chloride (mmol/L)	106±1	106±1	105±2	103±1*
Urea nitrogen (mg/dL)	15±1	16±2	17±1	19±2*
Total protein (g/dL)	6.6±0.1	7.0±0.3	7.0±0.4	7.2±0.1*
Globulin (g/dL)	2.5±0.2	2.7±0.2	2.7±0.1	2.9±0.1*
Glucose (mg/dL)	103±6	104±8	99±11	88±5*
Cholesterol (mg/dL)	51±3	50±6	65±16	86±19*
Trigyceride (mg/dL)	30±8	32±11	31±5	57±26*

[a]n = 6; [b]n = 4-5; * Statistically different from the control ($p \leq 0.05$ Anova + Dunnett's test). $ Statistically different from the control ($p \leq 0.05$ Kruskal-Wallis + Mann-Whitney U-tests).

Table 173: Haematological findings in the subchronic neurotoxicity study in rats (means ± standard deviations)

Parameters	**Dose levels (ppm)**			
	Control	**100**	**500**	**2500**
Malesa				
Haemoglobin (g/dL)	17.5±0.5	17.2±0.4	16.8±1.0	16.6±0.5
Haematocrit (%)	49.0±1.8	48.5±0.6	45.9±2.8*	46.2±1.7
MCV (µm3)	52.7±1.1	50.4±1.5*	50.7±0.7*	49.5±1.1*
HDW (g/dL)	2.52±0.28	2.62±0.27	3.17±0.26*	3.00±0.34*
Females[b]				
Haemoglobin (g/dL)	17.0±0.3	16.8±0.6	16.6±0.7	15.3±0.3*
Haematocrit (%)	46.2±0.8	46.8±1.2	45.3±2.1	42.0±0.9*
MCV (µm3)	56.3±1.3	54.2±1.2*	52.8±1.0*	49.0±1.1*
MCH (pg)	20.7±0.8	19.4±0.4*	19.3±0.3*	17.9±0.7*
HDW (g/dL)	2.26±0.14	2.18±0.15	2.37±0.15	2.90±0.18*

[a] n = 6; [b] n = 4-5; * $p \leq 0.05$ (Anova + Dunnett's test)

FOB findings: The data obtained by means of the Functional Observational Battery did not reveal evidence of treatment-related neurobehavioral changes.

Motor and locomotor activity: Summary session (60-minute) motor and locomotor activity measurements are presented in Tables 170 and 171, respectively. An examination of inherent variability, using the average pretreatment values among the four groups of males and females, provides a measure of the magnitude of the difference that should be considered biologically significant. For motor activity, the pretreatment values for groups that later received the test substance averaged from 12 % to 18 % higher than animals assigned to the control group for males and from 13 % lower to 8 % higher than controls for females. For locomotor activity, the pretreatment values for groups that later received the test substance averaged from 5 % to 14 % higher than controls for males and from 12 % lower to 10 % higher than controls for females. As a general guide, these results confirm that differences of approximately ± 20 % are within the range of normal variability in this laboratory for groups of 10-12 rats/sex/dietary level and, therefore, are not biologically significant. A large variation must be taken into account. Thus, it may be concluded that, for the overall 60-minute test session, motor and locomotor activity was not affected by treatment at any dietary level in either sex. There were inter-group differences but these were either small (i.e., in the pre-treatment range), were not dose-related and/or not consistently seen throughout the study. Furthermore, these differences did not achieve statistical significance.

Table 174: Motor activity in rats, summary session results (Percent differences from control)[a]

Week No.	Dose levels in ppm (mg/kg bw/day)		
	100 (♂ 6.69)	500 (♂ 33.2)	2500 (♂ 164.2)
Males[b]			
Pretreatment	+12	+18	+15
Week 2	+23	+19	+28
Week 4	+20	+5	-1
Week 8	+16	-1	+3
Week 13	+8	+9	+19
Females[c]			
Pretreatment[b]	+1	-13	+8
Week 2	-19	-8	+4
Week 4	-7	-9	+3
Week 8	0	-14	-13
Week 13	+0.5	+5	+3

[a]Percent greater (+) or less (-) than concurrent control. [b]N=12 for pretreatment week, week 2, week 8 and week 13; N=10 for week 4. [c]N=12 for pretreatment week and week 2; N=11 for week 4 and week 8; N=10 for week 13 control animals and N=11 for remaining dietary levels.

Table 175: Locomotor activity in rats, summary session results (Percent differences from control)[a]

Week No.	Dose levels in ppm (mg/kg bw/day)		
	100 (♂ 6.69)	**500 (♂ 33.2)**	**2500 (♂ 164.2)**
Males[b]			
Pretreatment	+5	+14	+14
Week 2	+28	+21	+25
Week 4	+25	+13	-1
Week 8	+35	+11	+13
Week 13	+11	+20	+29
Females[c]			
Week No.	100 (♀ 8.05)	500 (♀ 41.2)	2500 (♀ 197.1)
Pretreatment[b]	+10	-12	+10
Week 2	-12	-8	-4
Week 4	-9	-17	-7
Week 8	+4	-16	-24
Week 13	+3	+5	-9

[a] Percent greater (+) or less (-) than concurrent control. [b] N=12 for pretreatment week, week 2, week 8 and week 13; N=10 for week 4. [c] N=12 for pretreatment week and week 2; N=11 for week 4 and week 8; N=10 for week 13 control animals and N=11 for remaining dietary levels.

Motor and locomotor activity data were also analyzed for differences at each 10-minute interval of each test session. For males and females, interval motor and locomotor activity were not affected by treatment at any dietary level in either sex. Habituation was not affected by treatment with fluopyram in males or females at any dietary level.

Gross pathology: There were no compound-related gross lesions at terminal sacrifice in males or females at any dietary level.

Organ weights: Mean body weight data and mean organ weight (absolute and relative) data for perfused and non-perfused animals are summarized in Table 176. Compound-related organ weight changes, relative to control, included increased absolute and relative liver weights in high- and mid-dose animals of both sexes and increased kidney weights (absolute and relative) in high-dose males. Other mean organ weights for males and females that were statistically different from controls included an increase in relative liver weights in mid-dose males and females. Thyroid weight (absolute and relative) was increased in a dose dependent manner in male rats (+ 32 % in high dose males, + 24 % in mid-dose males). This effect was in agreement with findings in other studies. However, in this study it was not statistically significant but the low number of animals must be taken into account. There were no significant differences in brain weights between control and treated perfused rats.

Table 176: Absolute and relative organ weights in rats (means with standard deviations)

Weights (g)	**Dose levels in ppm (mg/kg bw/day)**			
	Control	**100 (♂ 6.69)**	**500 (♂ 33.2)**	**2500 (♂ 164.2)**
Perfused males				
Body Wt.a	419.7±39.2	413.4±49.3	440.3±20.9	418.1±23.2
Brain Wt.b	1.916±0.107	1.906±0.075	1.918±0.069	1.825±0.139
Brain/Body Wt.b	0.436±0.027	0.439±0.035	0.445±0.021	0.427±0.041
Non-perfused males				
Body Wt. b	399.2±34.1	391.1±56.0	417.4±18.1	375.1±8.3
Liver Wt. b	14.937±0.755	15.971±2.618	18.183±3.361	21.357±1.080*
Liver/Body Wt b.	3.752±0.162	4.079±0.203	4.351±0.722*	5.695±0.265*
Kidney Wt. b	2.817±0.170	2.841±0.427	3.573±1.171	3.362±0.289$
Kidney/Body Wt. b	0.711±0.085	0.727±0.032	0.853±0.258	0.897±0.085$
Thyroid Wt. b	0.025±0.004	0.026±0.006	0.031±0.008	0.033±0.006
Thyroid/Body Wt. b	0.0063±0.0011	0.0067±0.0016	0.0074±0.0019	0.0087±0.0015
Weights (g)	Control	100 (♀ 8.05)	500 (♀ 41.2)	2500 (♀ 197.1)
Perfused females				
Body Wt.c	248.4±19.3	244.4±22.6	238.2±10.3	222.6±11.5*
Brain Wt.b	1.894±0.101	1.781±0.075	1.632±0.379	1.769±0.124
Brain/Body Wt.b	0.762±0.046	0.726±0.067	0.674±0.159	0.775±0.063
Non-perfused females				
Body Wt. d	225.8±19.0	220.1±18.6	210.0±12.1	193.3±15.9*
Liver Wt. d	7.612±1.033	7.595±0.457	8.497±0.992	10.729±0.487*
Liver/Body Wt. d	3.371±0.358	3.460±0.202	4.045±0.402*	5.569±0.359*
Kidney Wt. d	1.736±0.152	1.781±0.116	1.686±0.157	1.656±0.066
Kidney/Body Wt. d	0.769±0.042	0.811±0.035	0.802±0.052	0.860±0.055
Thyroid Wt. d	0.020±0.005	0.020±0.005	0.019±0.003	0.023±0.004
Thyroid/Body Wt. d	0.0091±0.0029	0.0091±0.0015	0.0090±0.0011	0.0120±0.0023

[a] N= 12; [b] N= 6; [c]N=10-11 [d] N= 4-5; * Statistically different from the control ($p \leq 0.05$ Anova + Dunnett's test). $ Statistically different from the control ($p \leq 0.05$ Kruskal-Wallis, Anova + Mann-Whitney U-tests).

Histopathology: There were no treatment-related findings in neural and/or non-neural tissues from perfusion-fixed high-dose males or females that were related to administration of the test substance. Tissues from animals at lower dose levels were, therefore, not examined. Liver, kidney and thyroid tissues were not examined microscopically, because the microscopic findings that are associated with the measured changes in tissue weight have been sufficiently established in other studies.

Conclusion

In this 90-day feeding study in rats, no evidence of neurotoxicity was observed up to the highest dose level of 2500 ppm that was equivalent to a mean daily intake of about 164 mg/kg bw in males and of 197 mg/kg bw in females. Thus, a relevant neurotoxic potential of fluopyram, if administered via the diet, can be excluded.

Systemic toxicity became apparent at the top dose level and comprised decreases in body weight, total body weight gain and food consumption, increases in relative and absolute liver, thyroid and kidney weights and changes in various clinical chemistry and haematology parameters in high-dose males and females. A few of these findings such as lower food consumption, hematological changes and an increase in relative liver weight were noted at the mid dose level already but were rather minor in nature. Accordingly, this intermediate dose level of 500 ppm (corresponding to 33 mg/kg bw/day in males and 41 mg/kg bw/day in females) is considered the overall NOAEL in this study. The same or very similar effects have been observed in other short-term studies in rats.

4.12.1.2 Immunotoxicity

A study on immunotoxicity was announced in the process of the joint global review but has not yet been submitted.

4.12.1.3 Specific investigations: other studies

Studies on metabolites

A data package including an acute oral toxicity study, *in vitro* genotoxicity tests and a 28-day short term toxicity study was provided for each of two plant metabolites of fluopyram (fluopyram-pyridyl-carboxylic acid, AE C657188, and fluopyram-methyl-sulfoxide, AE 1344122) that had not been found in the rat. Since these metabolites occur in plants only they are not considered relevant for classification of fluopyram in the context of Reg 1272/2008. Hence, no detailed report on these metabolites is presented in this CLH dossier but only an overview. The first metabolite occurred in grapes, potatoes, beans, red bell peppers, and in rotational crops whereas the second was only detected in low amounts in a rotational crop study so far. Fluopyram-pyridyl-carboxylic acid and -methyl-sulfoxide are also known to be a metabolites of the fungicide fluopicolide.

A brief overview on the toxicological studies and their results is given in Table 177 suggesting that these metabolites were of no toxicological concern and that further investigations were not warranted. Both substances exhibited a very low acute oral toxicity and proved negative in the genotoxicity test battery. The subacute feeding studies revealed a lower toxicity of these metabolites when compared to fluopyram active ingredient.

Table 177: Summary of the toxicity studies with two plant metabolites of fluopyram

Metabolite	Acute oral toxicity	Genotoxicity	28-day oral study
Fluopyram-pyridyl-carboxylic-acid	> 2000 mg/kg bw, no mortality or bw effects up to this dose clinical signs confined to piloerection; mortality observed at 4000 mg/kg bw Coleman, D.G. , 2007, ASB2008-5367	Ames-test (+/- S9): Negative Chromosome-aberration (+/- S9): Negative HPRT/V79-forward-mut. Assay(+/- S9): Negative Kitching, J., 2000, ASB2008-5368 Lloyd, M., 2003, ASB2008-5369 Herbold, B., 2003, ASB2008-5370	NOAEL: 20000 ppm (equiv. to 1570 mg/kg bw/d); i.e., the highest dose level tested Kennel, P., 2001, TOX2005-950
Fluopyram-methyl-sulfoxide	> 2000 mg/kg bw, no mortality, clinical signs or bw effects up to this dose Schuengel, M., 2003, ASB2008-5372	Ames-test (- S9*): Negative Chromosome-aberration (+/- S9): Negative HPRT/V79-forward-mut. Assay(+/- S9): Negative Herbold, B., 2003, ASB2008-5373 Lloyd, M., 2003, ASB2008-5374 Herbold, B., 2003, ASB2008-5375	NOAEL: 2000 ppm (equiv. to 152 and 167 mg/kg bw/day in males and females, respectively); clinical signs, bw ↓, food consumption ↓, renal toxicity (clinical chemistry, histopatho-logy) at 20000 ppm McElligott, A., 2003, ASB2008-5376

* limited study since a non-adequate positive control substance for testing under activation conditions was used

Mechanistic studies on the active substance

Title:	Blanck, M. (2008a): Fluopyram (AE C 656948): 7-day mechanistic study in the female Wistar rat by dietary administration, SA 07323, M-299274-01, ASB2008-5441.
Guidelines:	None.
Deviations:	Not applicable.
GLP:	Yes.
Acceptability:	The study is considered to be supplementary as it was a non-standard study with a limited range of parameters and was performed to provide mechanistic information.

Materials and methods

Test Material:	Fluopyram
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle:	None
Stability of test compound:	Stable for a period covering the study duration
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – females only
Age / weight:	11 weeks approx.; at study start: 228 g to 254 g
Source/breeder:	R. Janvier, Le Genest St Isle, France
Acclimation period:	12 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 – 24°C Humidity: 40 – 70 % Air changes: Approximately 10-15 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: Fluopyram was administered in the diet for 7 days to a group of 15 female rats at the dose of 3000 ppm (*ca.* 193 mg/kg/day). A negative control group with the same number of animals received plain diet. Animal housing and husbandry were in accordance with current regulations. A solution of BrdU at 80 mg of BrdU/100 mL of drinking water was administered to all animals over the treatment period to allow liver cell proliferation evaluation.

Investigations: The animals were observed twice daily for mortality and clinical signs. Body weight, food and water consumption were monitored.

On the day of necropsy, blood samples were taken from fasted animals in all groups by puncture of the abdominal aorta under isofluran anesthesia. The animals were sacrificed by exsanguination and necropsied. Brain and liver were weighed fresh at scheduled sacrifice only. Duodenum and two central sections of the liver taken in the left and medial lobes were fixed by immersion in neutral buffered 10 % formalin. The remaining portions of the liver from ten females from each group were kept for microsomal preparations and determination of total cytochrome P-450 content and isoenzyme activities.

For histopathology, duodenum and the two central sections of the liver were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and examined from all the animals. For cell proliferation assessment, an immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all study animals. The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin.

The zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an automated image analysis system. The mean labeling indexes (periportal, centrilobular and combined) and standard deviation were calculated for each zone and each group. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

The remaining portions of the liver from all surviving animals were homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate. Results were compared to those generated with well known reference compounds.

The results were analysed by appropriate statistical methods.

Findings

There was no mortality and no treatment-related clinical signs occurring in any group during the course of the study. There were no relevant changes in mean bodyweight and in water consumption in treated animals compared to controls. There was a slight reduction in food consumption in the treated group, however, this was essentially due to a single rat.

At necropsy, higher liver weights (+ 40 % approximately) were observed in animals treated with fluopyram compared to controls. This was associated with enlarged livers observed at the macroscopic examination in nearly all animals and with hepatocellular hypertrophy in all animals at the microscopic examination. In addition, dark livers were observed in 13/15 females as compared to 1/15 animals in the control group.

Table 178: Liver weight changes and microscopic findings after 7 days of treatment with fluopyram at 3000 ppm

Dosage level (ppm)	0	3000
Absolute liver weight (g) Relative liver weight (%)	5.61 ± 0.49 2.53 ± 0.13	7.86 ± 0.93** (+40 %) 3.63 ± 0.320** (+43 %)
Diffuse centrilobular to panlobular hepatocellular hypertrophy	0/15	15/15
Diffuse mainly periportal hepatocellular vacuolation	11/15	1/15

**: p≤0.01

Cell proliferation was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean BrdU labeling indexes were found to be approximately 4 times higher in treated animals (p≤0.01), when compared to controls.

Table 179: Mean BrdU labeling index in the liver after 7 days of treatment with fluopyram at 3000 ppm

Dosage level (ppm)	0	3000
Number of animals	14	14
BrdU positive cells in the centrilobular zone	44.54 ± 22.31	179.68 ± 95.18**
BrdU positive cells in the periportal zone	28.55 ± 16.80	112.94 ± 58.19**
Overall BrdU positive cells	36.54 ± 18.70	146.31 ± 70.26**

**: p≤0.01

Assessment of total cytochrome P-450 content and microsomal proteins revealed a slight increase in total P-450 content and in mean EROD activity, a moderate increase in mean PROD and UDPGT activities and a marked increase in mean BROD activity.

Table 180: Liver enzyme activties after 7 day of treatment with fluopyram at 3000 ppm

Dosage level (ppm)	0	3000
Number of animals	10	10
Total P-450 (nmol/mg prot.)	0.91 ± 0.17	1.23 ± 0.20 ** (+35 %)
EROD (pmol/min/mg prot.)	47.99 ± 3.73	103.18 ± 13.74 ** (+115 %)
PROD (pmol/min prot.)	6.65 ± 0.70	28.55 ± 14.12** (+329 %)
BROD (pmol/min/mg prot.)	6.39± 1.12	74.51 ± 50.89 ** (+1066 %)
UDPGT (nmol/min /mg prot.)	6.42 ± 0.61	30.69 ± 1.94 ** (+378 %)

**: p≤0.01; (%) as compared to control

Conclusion

This data showed that fluopyram, when administered for 7 days at a dose level of 193 mg/kg bw/day, has the ability to induce xenobiotic metabolising enzymes in the rat liver. For the individual enzymes, the extent of induction was very different ranging from minimal (total cytochrome P-450) to marked (BROD). Pathological examination confirmed hypertrophy and cell proliferation in the liver.

Title: Blanck, M. (2008b): Phenobarbital 7-day mechanistic study in the female Wistar rat by gavage, SA 07325, M-299491-01, ASB2008-5442.

Guidelines: No specific guideline available.

Deviations: Not applicable.

GLP:	Yes.
Acceptability:	The study is considered to be supplementary.

Materials and methods

Test Material:	Phenobarbital
Description:	White crystalline powder
Lot/Batch:	06100228
Purity:	99,6 %
Vehicle:	Methylcellulose 400
Stability of test compound:	Stable for a period covering the study duration
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – females only
Age / weight:	11 weeks approx.; at study start 226 g to 263 g
Source/breeder:	R. Janvier, Le Genest St Isle, France

Acclimation period:	12 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*
Water:	Tap water, *ad libitum*

Animal assignment and treatment: Phenobarbital was administered once daily by oral gavage for 7 days to a group of 15 females at a dose of 80 mg/kg/day in 0.5 % aqueous solution of methylcellulose 400. A negative control group with the same number of animals received the vehicle only (0.5 % aqueous solution of methycellulose 400). Animal housing and husbandry were in accordance with current regulations. A solution of BrdU at 80 mg of BrdU/100 mL of drinking water was administered to all animals for 7 days to allow evaluation of liver cell proliferation.

The same parameters as in the previous study with fluopyram (Blanck, 2008, ASB2008-5441) were investigated.

Findings

One treated animal was found dead on day 5 of the study but no cause of death could be established, perhaps because this rat was discarded without necropsy. All animal receiving phenobarbital showed a reduced motor activity.

Overall, there was no mean body weight gain in the phenobarbital group compared to 7 g in the control group. In contrast, food and water consumption were not affected by the treatment.

At necropsy, higher liver weights (+20 % approximately) were observed in animals treated with phenobarbital compared to controls. This was associated with enlarged (3/14) and dark (5/14) livers observed at the macroscopic examination and with hepatocellular hypertrophy in all animals at the microscopic examination.

Table 181: Liver weight changes and microscopic findings after 7 days of treatment with phenobarbital at 80 mg/kg bw/d

Dosage level (mg/kg bw/d)	0	80
Absolute liver weight (g) Relative liver weight (%)	5.55±0.40 2.47±0.16	6.63±0.98** (+19 %) 3.02±0.36** (+22 %)
Diffuse centrilobular to panlobular hepatocellular hypertrophy	0/15	14/14
Hepatocellular necrotic foci	0/15	4/14
Diffuse mainly periportal hepatocellular vacuolation	7/15	3/14

**: p≤0.01

Cell proliferation was assessed separately in the centrilobular and the periportal zones of the hepatic lobules. In the centrilobular and periportal areas, the mean BrdU labeling indexes were found to be 2 fold higher in treated animals, when compared to controls (p≤0.01). Centrilobular index was higher than periportal index in treated animals.

Table 182: Mean BrdU labeling index in the liver after 7 days of treatment with phenobarbital at 80 mg/kg bw/d

Dosage level (mg/kg bw/d)	0	80
Number of animals	15	14
BrdU positive cells in the centrilobular zone	21.73 ± 16.34	55.21 ± 43.31**
BrdU positive cells in the periportal zone	16.70 ± 10.02	33.19 ± 18.22**
Overall BrdU positive cells	19.22 ± 12.52	44.20 ± 27.39**

**: p≤0.01

Changes in the activities of microsomal liver enzymes are described in Table 183. Assessment of total cytochrome P-450 content and microsomal proteins revealed a slight increase in total P-450 content and UDPGT activities, a moderate increase in mean PROD activity and a marked increase in BROD activity (all statistically different from the control group p≤0.01).

Table 183: Results of the hepatotoxicity testing after 7 day of treatment with phenobarbital at 80 mg/kg bw/d

Dosage level (mg/kg bw/d)	0	80
Number of animals	10	10
Total P-450 (nmol/mg prot.)	0.95 ± 0.20	1.49 ± 0.38 ** (+ 57 %)
EROD (pmol/min/mg prot)	38.25 ± 6.42	47.56 ± 9.75 * (+ 24 %)
PROD (pmol/min prot.)	4.89 ± 0.61	26.36 ± 17.55 ** (+ 439 %)
BROD (pmol/min/mg prot.)	4.91 ± 0.70	94.43 ± 62.94 ** (+ 1823 %)
UDPGT (nmol/min /mg Prot)	6.99 ± 0.52	13.47 ± 1.66 ** (+ 93 %)

* : p≤0.05; **: p≤0.01; (%) as compared to control

Conclusion

The well known ability of phenobarbital to induce enhanced activity of hepatic enzymes like total cytochrome P-450, PROD, BROD and UDPGT and to cause liver cell hypertrophy and proliferation was confirmed in this oral 7-day study in rats at a daily dose level of 80 mg/kg bw. However, the magnitude of effects was different from that seen with fluopyram (Blanck, 2008, ASB2008-5441), especially when EROD activity was concerned. Furthermore, the effect of PB on hepatic cell proliferation was much more prominent in the centrilobular region where proliferation by fluopyram equally affected the centrilobular and the periportal parts of the liver.

Title:	Rouquie, D. (2008a): AE C656948 Mechanistic 14-day toxicity study in the mouse by dietary administration (hepatotoxicity and thyroid hormone investigations), SA 07215, M-299522-01, ASB2008-5444.
Guidelines:	No specific guideline available.
Deviations:	Not applicable.
GLP:	Yes.
Acceptability:	The study is considered to be supplementary.
Materials and methods:	
Test Material:	Fluopyram
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle or positive control	None
Stability of test compound	Stable for a period covering the study duration
Species:	Mouse – Male only
Strain:	C57BL/6J
Age / weight :	8 weeks approx.; 21.2 g to 25.1 g at start of treatment
Source/breeder:	Charles River Laboratories, St Germain-sur-l'Arbresle, France
Acclimation period	5 to 6 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions :	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*, (except prior to sacrifice)
Water:	Tap water, filtered and softened, *ad libitum* (except prior to sacrifice)

Animal assignment and treatment: Groups of 15 male mice were fed fluopyram at a dietary concentration of 2000 ppm for either 3 or 14 days. The mean daily intake of the test compound accounted for 308 or 314 mg/kg bw. The dose level was choosen because it was below the MTD in a subacute study in mice (see 4.7.1.1) but well above the dietary level of 750 ppm at which a carcinogenic effect (thyroid follicular cell adenoma in male mice) had been observed in the long-term study (see 4.10.1.1). Two concurrent control groups of 15 males each were also employed. Animal housing and husbandry were in accordance with current regulations.

The animals were monitored for mortality and the occurrence of clinical signs, body weight development and food consumption. Special parameters were investigated to allow evaluation of liver and thyroid function. For data analysis, appropriate statistical methods were applied.

Hormone measurements: On study day 4 or study day 15, i.e., at the end of the exposure periods, blood samples were taken under isoflurane anesthesia from all animals by puncture of the retro-orbital venous plexus. Plasma was prepared from each blood sample and kept frozen at approximately -80°C until the determination of TSH, T3 and T4 hormone levels with specific radio-immunoassay kits (supplied by Amersham for TSH and by DIASORIN for T3 and T4).

Pathology: At scheduled termination, mice were sacrificed by exsanguination under deep anesthesia and necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh. A piece of median and left liver lobes of 5 animals per groups as well as the thyroid gland (with parathyroid glands) of all animals were sampled and fixed in 10 % neutral buffered formalin for subsequent histological examination (hematoxylin-eosin staining). The remaining portions of livers from those animals and the entire liver of the remaining animals of each group were used for microsome preparation.

Measurement of microsomal liver enzymes: For this purpose, the livers of 3 animals were pooled (each time 2 entire livers and the remaining portion of 1 liver used for histological examination) and homogenized. Accordingly, in total, 5 samples per group were prepared in which total cytochrome P-450 content and a specific cytochrome P-450 isozyme profile (including EROD, BROD and PROD activities) were measured. In addition, Phase II enzymatic activities were determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate.

Findings

There was no mortality in any groups throughout the study and no clinical signs occurred. Body weights or body weight gains were not affected throughout the study although there was a slight reduction in food consumption at the beginning of treatment with fluopyram (-12.5 % at the end of the 3-day exposure period and -5.1 % after 1 week in the 14-day exposure period). Thereafter, food consumption was comparable to controls.

Hormone measurements: The TSH, T3 and T4 levels are summarized in Table 184. The figures obtained after 3 or 14 days in the control and treatment groups were very similar.

Table 184: Mean levels of thyroid/pituitary hormones after 3 and 14 days of exposure to fluopyram

	3-day exposure		14-day exposure	
Group	**Control**	**2000 ppm (308 mg/kg bw/d)**	**Control**	**2000 ppm (314 mg/kg bw/d)**
T3 (nmol/L)	1.62 ± 0.15	1.64 ± 0.25 (+1 %)	1.45 ± 0.18	1.52 ± 0.38 (+5 %)
T4 (nmol/L)	43.7 ± 8.1	30.7** ± 6.0 (-30 %)	38.1 ± 9.1	27.7** ± 8.7 (-27 %)
TSH (ng/mL)	3.81 ± 0.23	4.48** ± 0.31 (+18 %)	3.81 ± 0.28	4.09* ± 0.44 (+7 %)

*: p≤0.05; **: p≤0.01

While T3 levels were apparently not affected by treatment, a significant decrease in T4 levels and a significant increase in TSH levels were seen in treated groups as compared to the controls. These results were consistent with the well known feedback regulation mechanism of thyroid hormones. When thyroid hormones levels get lower, the pituitary gland will produce more TSH to stimulate the thyroid gland in order to restore the normal level of thyroid hormones.

Pathology: Liver weights were markedly increased in the treated group compared to control groups after only 3 days of exposure and after 14 days of exposure (approximately +60 % in all cases). This was associated with enlarged and/or dark livers seen at the macroscopic examination and with hepatocellular hypertrophy in all examined animals and single cell necrosis on many occasions especially after 14 day of exposure. In addition, an increased number of mitoses were observed in all animals after 3 days of exposure.

Table 185: Pathological liver effects after 3 and 14 days of exposure to fluopyram

	3-day exposure		14-day exposure	
Group	**Control**	**2000 ppm (308 mg/kg bw/d)**	**Control**	**2000 ppm (314 mg/kg bw/d)**
Absolute liver weight (g)	1.24±0.12	1.97±0.17** (+59 %)	1.25±0.10	1.99±0.22** (+59 %)
Relative liver weight (%)	5.40±0.57	8.71±0.54** (+61 %)	5.23±0.33	8.42±0.94** (+61 %)
Centrilobular to panlobular hepatocellular hypertrophy	0/5	5/5	0/5	5/5
Increase mitosis	0/5	5/5	0/5	0/5
Single cell necrosis	0/5	1/5	0/5	4/5

*: p≤0.05; **: p≤0.01

No relevant changes were observed in the thyroid gland.

Measurement of microsomal liver enzymes: Treatment with fluopyram over 3 or 14 days induced a clear increase in total cytochrome P-450 content and a marked increase in BROD and PROD activities. In contrast, EROD activity was only slightly increased. These results were quite similar to the changes observed in the rat with the exception of the 4-nitrophenol UDPGT activity which was induced in the rat but not affected or even decreased in the mouse.

Table 186: Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of exposure to fluopyram

	3-day exposure		14-day exposure	
Group	**Control**	**2000 ppm (308 mg/kg bw/d)**	**Control**	**2000 ppm (314 mg/kg bw/d)**
P-450 (nmol/mg Prot.)	1.08 ± 0.16	2.33 ± 0.19 (+116 %) **	1.26 ± 0.49	2.15 ± 0.06 (+71 %) *
EROD (pmol/min/ mg Prot.)	90.25 ± 11.11	302.52 ± 43.49 (+235 %) **	99.05 ± 8.98	262.24 ± 72.87 (+165 %) **
PROD (pmol/min/ mg Prot.)	4.93 ± 0.83	143.42 ± 57.05 (+2890 %) **	4.19 ± 0.49	94.80 ± 44.77 (+2163 %) **
BROD (pmol/min/ mg Prot.)	12.99 ± 2.34	1145.28 ± 262.93 (+8717 %) **	12.83 ± 2.11	1175.30 ± 163.99 (+9061 %)**
UDPGT (nmol/min/ mg Prot.)	16.04 ± 1.42	15.36 ± 0.53 (difference from control not calculated)	17.09 ± 0.90	14.32 ± 0.63 (-16 %)**

*: p≤0.05; **: p≤0.01

Conclusion

As in rats, fluopyram demonstrated the ability to induce total cytochrome P-450, PROD, BROD and EROD activities in mouse liver after 3 and/or 14 days of dietary treatment at a high dose level of 2000 ppm (> 300 mg/kg bw/day) but, in contrast to the rat, might have caused a minimal repression of UDPGT activity. Hepatotoxicity became further apparent by an organ weight increase and concomitant histological lesions. Rapid onset of these changes in the 3-day experiment is remarkable. Furthermore, fluopyram administration resulted in a disturbance of thyroid hormone balance in male C57BL/6J mice by causing a decrease in T4 levels and a concomitant increase in TSH suggesting efficacy of a feed-back mechanism. However, there was no histological evidence of toxic effects on the thyroid. This might be due to the relatively short exposure period.

Title: Rouquie, D. (2008b): Phenobarbital mechanistic 14-day toxicity study in the mouse by oral gavage (hepatotoxicity and thyroid hormone investigations), SA 07326, M-299521-01, ASB2008-5445.

Guidelines:	No specific guideline available.
Deviations:	Not applicable.
GLP:	Yes.
Acceptability:	The study is considered to be supplementary.

Materials and methods

Test Material:	Phenobarbital
Description:	White crystalline powder
Lot/Batch:	06100228
Purity:	99,6 %
Vehicle or positive control:	Methylcellulose 400
Stability of test compound:	Stable for a period covering the study duration
Species:	Mouse – Male only
Strain:	C57BL/6J
Age / weight:	8 weeks approx.; 19.9 g to 24.2 g at start of treatment
Source/breeder:	Charles River Laboratories, St Germain-sur-L'Arbresle, France
Acclimation period:	6 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum,* (except prior to sacrifice)
Water:	Tap water, filtered and softened, *ad libitum,* (except prior to sacrifice)

Animal assignment and treatment: Groups of 15 male mice were administered phenobarbital in 0.5 % aqueous solution of methycellulose 400 by oral gavage once daily for 3 or 14 days at a dose level of 80 mg/kg bw. This dosage was selected on the basis of previous studies conducted with phenobarbital. Two concurrent control groups of 15 males each receiving only the vehicle were also employed. Animal housing conditions and the whole study design including the range of parameters investigated were the same as in the abovementioned study with fluopyram (Rouquie, 2008, ASB2008-5444).

Findings

There was no unscheduled mortality in this study and no treatment-related clinical signs were observed. The first days of exposure were associated with body weight effects in treated groups. For the 3-day exposure group, an overall mean body weight loss of 0.6 g was observed at the end of treatment compared to a gain of 0.3 g in the control group. In the 14-day exposure group, there was a mean body

weight loss of 0.3 g ($p \leq 0.05$) in the treated group compared with a gain of 0.4 g in the control group on study Day 7 although overall mean bodyweight was not affected at the end of treatment period. There was a slight reduction in food consumption at the beginning of treatment but not during the second week of treatment.

Hormone measurements: The TSH, T3 and T4 levels are summarized in Table 187. After 3 days of treatment, the T3 level in the phenobarbital group was significantly lower than in the concurrent control group. This difference had disappeared after a 14-day exposure period but assessment is difficult because the mean value for the control group was much lower now. Thus, an impact of the test substance is not very likely.

In contrast, there is much stronger and more consistent evidence of a decrease in T4 concentrations after 3 or 14 days of treatment and a (most likely compensatory) increase of the TSH level after 14 days.

Table 187: Mean levels of thyroid/pituitary hormones after 3 and 14 days of exposure to phenobarbital

	3-day exposure		14-day exposure	
Group	**1 Control**	**2 Phenobarbital**	**3 Control**	**4 Phenobarbital**
T3 (nmol/L)	1.72 ± 0.25	1.54* ± 0.17 (-10 %)	1.61 ± 0.20	1.57 ± 0.19 (-2 %)
T4 (nmol/L)	36.7 ± 6.2	26.8** ± 3.5 (-27 %)	32.4 ± 6.5	26.1* ± 6.7 (-19 %)
TSH (ng/mL)	4.44 ± 0.27	4.41 ± 0.54 (-1 %)	4.47 ± 0.37	4.89* ± 0.58 (+9 %)

*: $p \leq 0.05$; **: $p \leq 0.01$

Pathology: Higher liver weights were observed in animals treated with Phenobarbital compared to controls after both 3 and 14 days of exposure. This was associated in several occasions with enlarged and/or dark livers and frequently with hepatocellular hypertrophy.

No relevant changes were observed in the thyroid gland.

Table 188: Pathological liver effects after 3 and 14 days of exposure to phenobarbital

	3-day exposure		14-day exposure	
Group	**1 Control**	**2 Phenobarbital**	**3 Control**	**4 Phenobarbital**
Absolute liver weight (g)	1.30±0.12	1.36±0.09 (+5 %)	1.31±0.16	1.60**±0.16 (+22 %)
Relative liver weight (%)	5.70±0.33	6.32**±0.21 (+11 %)	5.38±0.50	6.65**±0.47 (+23 %)
Centrilobular to panlobular hepatocellular hypertrophy	0/5	4/5	0/5	5/5
Increased mitosis	0/5	3/5	0/5	0/5

*: $p \leq 0.05$; **: $p \leq 0.01$

Measurement of microsomal liver enzymes: Treatment with Phenobarbital induced the expected clear increase in total cytochrome P-450 content and a marked increase in BROD and PROD activities. EROD activity was only slightly increased whereas 4-nitrophenol UDPGT activity was unaffected. Those results are in line with the changes observed in the rat with the exception of the 4-nitrophenol UDPGT activity which was also induced in that species.

Table 189: Cytochrome P-450 content and enzymatic activities in the liver after 3 and 14 days of exposure to phenobarbital

	3-day exposure		14-day exposure	
Group	**1 Control**	**2 Phenobarbital**	**3 Control**	**4 Phenobarbital**
P-450 (nmol/mg Prot.)	0.94 ± 0.06	2.31**± 0.20 (+ 146 %)	0.98 ± 0.12	1.33* ± 0.29 (+ 36 %)
EROD (pmol/min/ mg Prot.)	48.08 ± 6.87	190.65** ± 97.94 (+ 297 %)	35.34 ± 10.48	167.88** ± 96.47 (+ 375 %)
PROD (pmol/min/ mg Prot.)	6.01 ± 1.99	88.99** ± 29.89 (+ 1381 %)	4.98 ± 0.59	71.97** ± 21.55 (+ 1345 %)
BROD (pmol/min/ mg Prot.)	17.33 ± 1.58	871.66** ± 148.84 (+ 4930 %)	18.82 ± 3.30	554.00** ± 119.35 (+ 2844 %)
UDPGT (nmol/min/ mg Prot.)	16.24 ± 0.56	17.23 ± 0.82 N.S.	15.18 ± 1.28	12.96 ± 2.14 N.S.

*: p≤0.05; **: p≤0.01

Conclusion

In this study in mice, it was confirmed that phenobarbital exerted liver toxicity in male C57BL/6J mice by induction of microsomal liver enzymes and causing of gross and histopathological changes in this organ. Furthermore, it has the potential to modify the thyroid hormone balance by causing a decrease at least in T4 and a concomitant increase in TSH concentration.

Title:	Rouquie, D. (2008 c); Mechanistic 3-day toxicity study in the male mouse (QPCR investigations of gene transcripts in the liver), SA 08151, M-308073-01-2, ASB2008-8219.
Guidelines:	No specific guideline available.
Deviations:	Not applicable.
GLP:	Yes.
Acceptability:	The study is considered to be supplementary.

Materials and methods

Test Material:	Fluopyram
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Reference compound	Phenobarbital (batch number: 06100228: white powder, 99.6% purity)
Stability of test compound	Stable for a period covering the study duration

Species:	Mouse (males only)
Strain:	C57BL/6J
Age / weight:	8 weeks approx.; 19.9 g to 23.4 g at start of treatment
Source/breeder:	Charles River Laboratories, St Germain-sur-L'Arbresle, France
Acclimation period:	6 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, filtered and softened, *ad libitum*

Groups of 10 male C57BL/6J mice received either fluopyram at a dietary level of 2000 ppm or phenobarbital by oral gavage once a day at a dose level of 80 mg/kg bw for three days. An untreated control group of the same size was also employed. It was the aim of the study to get insight into the molecular regulation of sulfotransferase and UDP glucoronosyltransferase gene transcripts which are known to encode enzymes that inactivate T3 and T4. In addition, transcripts encoding for three enzymes from the Cytochrome P450 family were investigated. A list of the genes analysed is given in Table 190 below.

Animals were sacrificed on study day 4 by exanguination under deep anesthesia. All animals were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded but not sampled except upon decision of the Study Pathologist. The liver was weighed fresh and sacrificed.

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

10 µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H_2O MQ was used as template instead of first strand cDNA.

Table 190: List of genes analyzed

Gene family	Isoform	Refset ID	Taqman assay ID (Applied Biosystems)
Cytochrome P450	Cyplal	NM 009992.3	Mm00487218 ml
Cytochrome P450	Cyp2b9	NM 010000.2	Mm00657910 ml
Cytochrome P450	CypSal 1	NM 007818.3	Mm00731567 ml
Sulfotransferase	Sultal	NM 133670.1	Mm00467072 ml
Sulfotransferase	Sulta2	NM 009286.1	Mm02394381 gl
Sulfotransferase	Sultn	NM 016771.2	Mm00502030 ml
UGT	Ugtlal	NM 201645.2	Mm02603337 ml
UGT	Ugt2bl	NM 152811.1	Mm00514184 ml
UGT	Ugt2b5	NM 009467.1	MmO 1623253 si
Beta-2 microglobulin	B2m	NM 009735.3	Mm00437762 ml

Beta-2 microglobulin (B2m) was selected as reference gene for the quantitative calculations of transcripts.

Findings

There were no unscheduled deaths or body weight effects in any group, however, animals treated with phenobarbital showed reduced motor activity throughout the treatment period. At necropsy, mean absolute and relative liver weights were increased by 60 % to 61 % for fluopyram treated animals and by 17 % to 19 % for phenobarbital treated animals, when compared to the control animals. Results are presented in Table 191 below.

Table 191: Liver weights

Test substance	Control Diet	Fluopyram (2000 ppm)	PB (80 mg/kg/day)
Mean absolute liver weight (g)	1.20±0.10	1.93**±0.12 (+61 %)	1.40**±0.13 (+17 %)
Mean liver to body weight ratio (%)	5.34±0.36	8.55**±0.40 (+60 %)	6.34**±0.29 (+19 %)

** $p<0.01$

Quantitative PCR analyses in fluopyram treated animals revealed an up-regulation of sulfotransferase transcripts (from +92 % to +463 %, $p<0.01$) and UDP glucoronosyltransferase transcripts (from+173 % to +273 %, $p<0.01$). Similarly, an up-regulation of sulfotransferase transcripts Sultlal and Sultn (+62 % and +96 %, respectively, $p<0.01$) and UDP glucoronosyltransferase transcripts (from +82 % to +119 %, $p<0.01$) was observed in phenobarbital treated animals except for Sult2a2. In contrast, an equivalent effect of both substances could not be shown for Cyp 1a1 that was induced by fluopyram but repressed by PB. For most genes, effects were more pronounced with fluopyram than with phenobarbital.

Table 192: qPCR Analysis

	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)		
Gene transcripts	Control	Fluopyram (2000 ppm)	Phenobarbital (80 mg/kg/day)
Cyplal	1.29 + 0.30	4.81**+ 1.19 (+272 %)	1.20 + 0.24NS (-7 %)
Cyp2b9	14.71 + 30.17	48.61*+ 91.21 (+230 %)	21. 11 ±30.19 NS (+43 %)
Cyp3al 1	1.51 + 0.56	43.59** ±34.38 (+2783 %)	7.76** ±4.11 (+413 %)
Sultlal	1.19 + 0.39	2.29** ±1.20 (+92 %)	1.92* ±0.46 (+62 %)
Sult2a2	0.51+0.22	2.90** ±2.08 (+463 %)	0.63 ± 0.35 NS (+22 %)
Sultn	1.41+0.56	5.93**+ 3.90 (+321 %)	2.76** ±1.18 (+96 %)
Ugtlal	1.08+ 0.16	4.03** ±0.80 (+273 %)	2.36** ±0.26 (+119 %)
Ugt2bl	1.04 + 0.25	2.84** ±0.71 (+173 %)	1.97** ±0.53 (+90 %)
Ugt2b5	1.30 + 0.23	4.30** ±2.16 (+231 %)	2.36** ±0.87 (+82 %)

NS : Not statistically significant; * : Statistically different from the control group ($p<0.05$)

** : Statistically different from the control group ($p<0.01$)

Conclusion

It can be assumed that several enzymes responsible for conjugation of T4 were induced on the mRNA level. Since enzyme activity was not measured in this assay, it is only possible to conclude that transcription of these genes was increased. This observation does not necessarily mean that T4 catabolism or excretion were increased. However, as several differences between fluopyram and Phenobarbital in terms of gene expression were detected, these results strongly suggest that substantial equivalence of PB and fluopyram is doubtful.

Title: Rouquie, D. (2008 d): Mechanistic 3-day toxicity study in the male mouse (Pharmacokinetic investigations of the clearance of intravenously administered 125I-Thyroxine), SA 08159, M-308369-01-2; ASB2008-8220.

Guidelines: No specific guideline available.

Deviations: Not applicable.

GLP: Yes.

Acceptability: The study is considered to be supplementary.

Materials and methods

Test Material: Fluopyram

Description: Light beige powder

Lot/Batch: Mix-Batch:08528/0002

Purity: 94.7 %

Reference compound Phenobarbital (batch number: 06100228: white powder, 99.6%

	purity)
Stability of test compound	Stable for a period covering the study duration
Species:	Mouse (males only)
Strain:	C57BL/6J
Age / weight:	8 weeks approx.; 19.8 g to 25.3 g at start of treatment
Source/breeder:	Charles River Laboratories, St Germain-sur-L'Arbresle, France
Acclimation period:	6 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 – 24°C
	Humidity: 40 – 70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*
Water:	Tap water, filtered and softened, *ad libitum*

Fluopyram was administered continuously via the diet at a dose level of 2000 ppm to a group of 5 male C57BLl6J mice for 3 days. A similarly constituted group of 5 males received untreated diet and acted as a control group. In addition, a further group of 5 males received 80 mg/kg/day Phenobarbital by oral gavage for a 3 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucoronidation. On Study Day 4 each animal received by intravenous injection via the tail 250 µL of diluted ^{125}I-Thyroxinesolution corresponding to a dose 1 µCi of ^{125}I-thyroxin per animal.. Approximately 3 hours post-administration with ^{125}I-Thyroxine each animal received 0.1 mg of NaI in 250 µL of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal after 1 hour 20 minutes, 2, 4, 6 and 24 hours post ^{125}I-Thyroxine administration. The level of ^{125}I-radioactivity in each sample was measured using a Cobra gamma scintillation counter. The rate of ^{125}I-radioactivity was indicative of the rate of Thyroxine (T4) clearance from the blood.

Animals were checked daily for mortality and clinical signs. Body weights were recorded on Study Days 1 and 4. Due to technical difficulties encountered with the intravenous injection of ^{125}I-Thyroxine, Thyroxine (T4) clearance data for 9 animals only (5 control animals, 1 fluopyram treated animal and 3 Phenobarbital treated animals) was obtained from this first group of animals (subgroup I). Consequently, 5 additional animals were incorporated onto the study (subgroup 2). One animal acted as a control and received untreated diet, whilst the remaining 4 animals were treated with 2000 ppm fluopyram. The results obtained in the main and the complementary experiments were combined.

Findings

There were no mortalities or clinical signs during the course of the study. There was no statistically significant effect on body weight.

The results show that following an intravenous injection of ^{125}I-Thyroxine the radioactivity level in the blood of fluopyram treated animals was lower than that in the blood of the corresponding control animals. This decrease in the level of radioactivity in the blood of fluopyram treated animals was observed at all time points examined and reflects a more rapid clearance of Thyroxine in these animals over a 24 hour period, compared with the controls. A partly similar response was seen in animals treated with the reference compound Phenobarbital. The results are summarised in Table Table 193.

Table 193: Thyroxin (T4) clearance

	Whole blood radioactivity after a single administration of ^{125}I-Thyroxin Mean ± Standard deviation (cpm)		
	Control	**Fluopyram (2000 ppm)**	**Phenobarbital (80 mglkglday)**
Number of animals	6	5	3
Time point 1 h20min	11434 ± 1624	4767 ± 1953	5775 ± 2615
Time point 2h	11025 ± 1415	4686 ± 1999	5905 ± 2095
Time point 4h	9811 ± 1756	4984 ± 1491	5651 ± 995
Time point 6h	8692 ± 1397	4566 ± 1342	6021 ± 1046
Time point 24h	2686 ± 454	1955 ± 199	2309 ± 446

Conclusion

The results suggest a more rapid T4 clearance after treatment with fluopyram or phenobarbital than in untreated mice. However, after 24 hours, the values were quite similar. Phenobarbital is known to induce an increase in thyroxin clearance in the mouse through induction of glucoronidation of this hormone. A similar mechanism may be assumed for fluopyram.

Title: Rouquie, D. (2009): Definitive mechanistic 4-day toxicity study in the male mouse (Pharmacokinetic investigations of the clearance of intravenously administered 125I-Thyroxine), SA 08288, ASB2009-3240.

Guidelines: No specific guideline available.

Deviations: Not applicable.

GLP: Yes.

Acceptability: The study is considered to be supplementary.

Materials and methods

Test Material:	Fluopyram
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Reference compound	Phenobarbital (batch number: 06100228: white powder, 99.6% purity)
Stability of test compound	Stable for a period covering the study duration

Species:	Mouse (males only)	
Strain:	C57BL/6J	
Age / weight:	8 weeks approx.; 20.3 g to 23.5 g at start of treatment	
Source/breeder:	Charles River Laboratories, St Germain-sur-L'Arbresle, France	
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.	
Environmental conditions:	Temperature:	20 – 24°C
	Humidity:	40 – 70 %
	Air changes:	Approximately 10-15 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles (7 am- 7 pm)
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from S.A.F.E. (Scientific Animal Food and Engineering, Augy, France), *ad libitum*,	
Water:	Tap water, filtered and softened, *ad libitum*	

The objective of this mechanistic study was to determine the potential effects of fluopyram in the male mouse on the clearance of Thyroxine (T4) in the blood following continuous dietary administration for 4 days.

Fluopyram was administered continuously via the diet at a dose level of 2000 ppm to a group of 8 male C57BL/6J mice for 4 days, a similarly constituted group of 8 males received untreated diet and acted as a control group. In addition, a further group of 8 males received 80 mg/kg/day Phenobarbital by oral gavage for a 4 day period. Phenobarbital acted as a reference compound known to induce an increase in T4 clearance in the mouse through induction of T4 glucuronidation and sulfonation. On Study Day 5 each animal received 250 µl of diluted ^{125}I-Thyroxine solution in 0.9% sterile saline by intravenous injection via the tail vein. Approximately 3 hours after administration of ^{125}I-Thyroxine, each animal received 0.1 mg of NaI in 250 µl of 0.9% sterile saline by intraperitoneal injection. A blood sample was collected from the retro-orbital venous plexus of each animal 40 minutes, 1.5, 4, and 24 hours post ^{125}I-Thyroxine administration. The level of ^{125}I radioactivity in each sample was measured using a gamma scintillation counter. The level of ^{125}I radioactivity was indicative of the rate of Thyroxine (T4) clearance from the blood. Animals were checked daily for mortality and clinical signs. Body weights were recorded on Study Days 1 and 5.

Findings

There were no clinical signs during the course of the study. One animal was sacrificed after intravenous injection of the radiolabeled Thyroxine due to a technical problem during the injection. There was no statistically significant effect on body weight.

The results show that following an intravenous injection of ^{125}I-Thyroxine, the radioactivity level in the blood of the fluopyram treated animals was lower than the blood radioactivity level of the concurrent control animals. This decrease in the level of radioactivity in the blood of fluopyram treated animals was observed at all time points examined and is considered to reflect a more rapid clearance of Thyroxine in these animals over a 24 hour period, compared with the controls. A partly similar response was seen in animals treated with the reference compound Phenobarbital.

Table 194: Whole blood radioactivity after a single administration of 125I-Thyroxin Mean

	Whole blood radioactivity after a single administration of ^{125}I-Thyroxin Mean ± Standard deviation (cpm)		
	Control	**Fluopyram (2000 ppm)**	**Phenobarbital (80 mglkglday)**
Number of animals	8	8	7
Time point 40min	19726 ± 1468	6163** ±2025	10691** ±1197
Time point 1h 30min	16930 ± 1001	6388** ±1982	10592** ±1245
Time point 4h	13781 ±1099	6111** ±1304	9312** ± 1330
Time point 24h	3889 ± 561	2562** ± 482	2653** ± 547

** : Statistically different from the control group ($p<0.01$)

Conclusion

In conclusion, these results indicate that the clearance of Thyroxine from the blood of animals treated with 2000 ppm fluopyram was increased when compared to control animals over a 24 hour period.

Title: Freyberger, A. (2008): AE C656948 (Fluopyram) In vitro studies on the potential interactions with thyroid peroxidase-catalyzed reactions, AT04481, M-299276-01, ASB2008-5443.

Guidelines: No specific guideline available.

Deviations: Not applicable.

GLP: Yes.

Acceptability: The study is considered to be supplementary.

Materials and methods

Test Material:	AE C656948
Description:	Light beige powder
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle:	Dimethylsulfoxide
Positive controls:	Amitrole (3-amino-1,2,4-triazole) from Sigma (Lot number 083K0649) ETU (ethylenethiourea, 2-imidazolidinethione) from Riedel-de-Haën (Lot number 3223X)
Metabolic system:	No metabolic system was used
Biological raw material:	Hog thyroid glands from domestic pigs were obtained from Bayer CropScience, Monheim. They were trimmed free of excess fat and connective tissue and stored at -80°C until use.
Stability of test compound:	Stable for a period covering the study duration

Microsome preparation: Interactions of fluopyram with TPO-catalyzed reactions were studied *in vitro* using solubilized hog thyroid microsomes as an enzyme source. Amitrole and Ethylenethiourea (ETU) served as positive control substances. Solubilized hog thyroid microsomes were prepared according to a standard procedure as described in Neary *et al*., 1984. No statistical evaluation was performed.

Determination of TPO-catalyzed guaiacol oxidation: Guaiacol oxidation was used as a measure for peroxidase activity. Incubations were carried out at room temperature in 0.1 M potassium phosphate buffer, pH 7.4 in a total volume of 1.0 mL. Guaiacol (125 µL of 40 mM solution in water, final concentration 5 mM), TPO (approximately 0.1 ΔE/min, corresponds to 3.5 µL of microsomal preparation) and test compound were preincubated for 1 minute, then the reaction was initiated by addition of hydrogen peroxide (20 µL of 12.5 mM solution in water, final concentration 250 µM). Test compounds were added in 20 µL DMSO, likewise control incubations lacking test compounds contained the same amount of solvent. The following final concentrations were used: Fluopyram: 3.0 – 30 – 300 µM, Amitrole: 1.0 µM. The initial linear increase (ΔE/min) of the absorption at 470 nm was used to calculate the peroxidase activity.

Determination of TPO-catalyzed iodine formation: Incubations were carried out as described above, however, guaiacol was replaced by potassium iodide (100 µL of 100 mM solution in water, final concentration 10 mM). The following final concentrations were used: Fluopyram: 3.0 – 30 – 300 µM, Amitrole: 0.1 µM, ETU: 5 µM. The initial linear increase (ΔE/min) of the absorption at 350 nm was used to calculate the enzymatic activity.

Findings

The results of the TPO-catalyzed oxidation of guaiacol are summarized in Table 195. Amitrole, the positive control, at a concentration of 1 µM inhibited the initial rate of thyroid peroxidase (TPO)-catalyzed oxidation of the model substrate guaiacol by 44.6 %. Fluopyram, at concentrations up to 300 µM did not affect this reaction.

Table 195: Effect of fluopyram on TPO-catalyzed guaiacol reaction

Compound	Concentration (µM)	ΔE/min. ± SD	% of control
Vehicle	-	0.121 ± 0.006	100
Fluopyram	3	0.122 ± 0.002	100.8
	30	0.123 ± 0.005	101.6
	300	0.124 ± 0.001	102.5
Amitrole	1	0.054 ± 0.003	44.6

The results of the TPO-catalyzed iodine formation are summarized in Table 196. Up to 300 µM fluopyram did not affect TPO-catalyzed iodine formation. Neither the initial rate of the reaction was affected, nor was a temporary suppression of iodine formation observed. In contrast, Ethylenethiourea (ETU), a trap of the iodinating intermediate, temporarily suppressed iodine formation, whilst Amitrole at a concentration of 0.1 µM inhibited the initial rate of this reaction by 50 %.

Table 196: Effect of fluopyram on TPO-catalyzed iodine formation

Compound	Concentration (µM):	ΔE/min. ± SD	% of control
Vehicle	-	0.259 ± 0.012	100
Fluopyram	3	0.269 ± 0.012	103.9
	30	0.246 ± 0.005	95.0
	300	0.260 ± 0.012	100.4
Amitrole	0.1	0.131 ± 0.013	50.6

Conclusion

Fluopyram at concentrations of up to 300 µM (the highest concentration tested) had no impact of TPO-catalyzed guaiacol oxidation or on iodine formation. These findings suggest that fluopyram does not affect thyroid hormone synthesis at the level of TPO. However, potential effects of fluopyram metabolites have not been studied.

Reference:	KIIA 5.5.4 /23;Tinwell, H.;2011
Report:	Tinwell, H. (2011): Fluopyram (AE C 656948): Mechanistic investigation in the female rat by dietary administration for up to 7 days, M-408029-01 ASB2012-7372
Guidelines:	No specific guideline available
Deviations:	Not applicable
GLP:	Yes
Acceptability:	The study is considered supplementary as it was a non-standard study with a limited range of parameters and was performed to provide mechanistic information.

Fluopyram was administered daily in the diet to groups of adult female Wistar rats (30 rats/dose level) for 3 days (Subgroup 1; n = 15/group) or for 7 days (Subgroup 2; n = 15/group) at dose levels of 30, 75, 150, 600 and 1500 ppm. These doses equated to 2.4, 6.2, 12.0, 46.1 and 117.6 mg/kg/day, respectively for those animals sacrificed after 3 days of treatment. For those animals sacrificed following 7 days of treatment the doses corresponded to 2.3, 5.6, 11.6, 44.1 and 118.5 mg/kg/day, respectively. A group of animals dosed by oral gavage with 80 mg/kg/day phenobarbital (a CAR/PXR nuclear receptor activator) acted as a positive control for the parameters investigated in this present study. A control group received untreated diet. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. Body weight and food consumption were recorded at the start and end of the treatment period for each subgroup. All animals were necropsied in the morning following three or seven days of treatment, the liver was weighed and sampled for investigation of several parameters. Specifically, samples were fixed and examined microscopically.

Additional slides were stained for Ki67 for cell proliferation determinations. The duodenum was also sampled and used as a positive control tissue for the cell proliferation investigations. Small portions of the liver were frozen in liquid nitrogen and used for gene expression investigations. The remaining portions of the liver from 5 randomly chosen females/group that were sacrificed after 7 days treatment were homogenized for microsomal preparations in order to determine cytochrome P-450 and UDPGT isoenzyme profiles.

There were no clinical signs and no effects on food consumption or body weight parameters for any of the groups treated with fluopyram (both sacrifice times). Clinical signs consisting of reduced motor activity were, however, recorded for all females dosed with phenobarbital and some of these females had ocular discharge in one or both eyes. In addition, mean absolute body weight gain was reduced by 23% (not statistically significant) for those females dosed with phenobarbital for 7 days.

Fluopyram induced treatment-related changes in all liver parameters investigated. The number of parameters affected and also the magnitude of the responses were dose related as described below.

At 30 ppm, there were no treatment-related changes in any of the parameters measured at either of the sacrifice times.

At 75 ppm, a marginal but statistically significant increase in the expression of Cyp3a3 was recorded both after three (+48.4%; $p \leq 0.05$) and seven days (+95%; $p \leq 0.01$) of treatment. This increase in gene expression was, however not associated with any increase in enzyme (BROD) activity following seven days of treatment.

At 150 ppm, a slight, though significant, increase in hepatic cell proliferation (centrilobular and perilobular) was observed. This effect was similar in magnitude at both sacrifice times. In addition, increased gene expression of Cyp2b1 as well as Cyp3a3 was recorded at this dose level and for both sacrifice times. Following 7 day treatment the gene expression of Cyp1a1 was statistically significantly

increased. These increases in gene expression for the Phase I enzymes were not associated with increased PROD, BROD or EROD enzyme activity following seven days of treatment.

At 600 ppm, the increased cell proliferation was more marked than that already observed at 150 ppm. For example, the % change (compared to controls) in global cell proliferation at 3 days was 266.6% compared to 86.0% at 150 ppm and at 7 days it was 191.7% compared to 106% at 150 ppm. A slight but statistically significant increase in liver weight (absolute and relative to body weight) was recorded following 3 days treatment and in one female minimal centrilobular to panlobular hepatocellular hypertrophy was observed following 7 days treatment. Statistically significant increases in enzyme activity (BROD, PROD and both isoforms of UDPGT) were also recorded following 7 days treatment. Statistically significant increases were recorded at both sacrifice times for the gene expression of Phase I (Cyp2b1; Cyp3a3) and Phase II enzymes (Udpgtr2; Gstm4 and Ephx1). Gsta2 was significantly increased following 7 days treatment. Cyp1a1 gene expression was statistically significantly increased at both sacrifice times but was not associated with any enzyme activity at this dose level.

At 1500 ppm, the effects on cell proliferation were even more marked than those recorded at 600 ppm. Specifically, the % change (compared to controls) in global cell proliferation at 3 days was 551.2% compared to 266.6% at 600 ppm and at 7 days it was 256.6% compared to 191.7% at 600 ppm. In addition, an increased number of mitoses was observed in 4/15 females dosed for 3 days treatment with fluopyram. Furthermore a slight, though statistically significant increase in the expression of Tacstd1 (a marker for cell proliferation; 3 day treatment only) and Gadd45b (a marker for apoptosis, following 3 and 7 days treatment) was recorded. Significant increases in mean absolute and relative liver weight were recorded at both time points, which could be associated with the centrilobular to panlobular hypertrophy observed in 6/15 females following 3 day treatment (minimal) and in 14/15 females following 7 days treatment (minimal to slight). The increases in enzyme activity observed at 600 ppm were more marked at 1500 ppm with statistically significant increases in EROD as well as total P450 content also being recorded at this dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced changes in the liver pertinent for a compound that activates the CAR/PXR nuclear receptors. Thus, increased cell proliferation was observed following both 3 and 7 days treatment, with the effects being more apparent in the centrilobular region (+217.7% and +603.4% increases compared to controls at 3 and 7 days, respectively) than in the perilobular region (+58.0% and +54.7% increases compared to the controls at 3 and 7 days, respectively). In addition an increased number of mitoses was observed in 3/15 females at both time points. Furthermore Gadd45b (a marker for apoptosis, following 3 and 7 days treatment) gene expression was statistically significantly increased at both time points. Liver weight (absolute and relative to brain and body weight following 3 days treatment; relative to body weight only following 7 day treatment) was statistically significantly increased following phenobarbital treatment, which could be associated with the centrilobular to panlobular hypertrophy observed in 3/15 females following 3 day treatment (minimal) and in 9/15 females following 7 days treatment (minimal to slight). BROD, PROD and UDPGT-nitrophenol activity were statistically significantly increased due to 7 days treatment with phenobarbital. Furthermore, statistically significant increases in the gene expression of Cyp2b1, Cyp3a3 and Udpgtr2 were recorded (at both time points).

Overall, clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed following fluopyram treatment. These changes were recorded as early as following 3 days of treatment and starting from 150 ppm. The dose of 75 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of Cyp3a3 at this dose level) and 30 ppm as a No Observed Effect Level (NOEL).

Materials and methods

Test Material:	Fluopyram
Description:	Light beige solid
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle:	None
Stability of test compound:	Stable for a period covering the study duration
Positive control	Phenobarbital, white crystalline powder, CAS 50-06-6
Lot	Lot No. 06100228,
Purity	99.6 %
	Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration
Species:	Rat
Strain:	Wistar Rj: WI (IOPS HAN) – females only
Age / weight:	10 weeks approx.; at study start: 218 to 263 g
Source/breeder:	R. Janvier, Le Genest St Isle, France
Acclimation period:	12 days
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.
Environmental conditions:	Temperature: 20 □□24°C
	Humidity: 40 □□70 %
	Air changes: Approximately 10-15 changes per hour
	Photoperiod: Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum,* except for overnight fasting prior to scheduled necropsy
Water:	Tap water, *ad libitum*

Seven groups of female rats were dosed for up to 7 days with the appropriate compound by the appropriate route of administration. Each group consisted of 30 female rats, 15 of which were sacrificed the morning after three days of treatment (Subgroup 1). The remaining 15 animals were sacrificed the morning after seven days of treatment (Subgroup 2).

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietary level (30, 75, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with fluopyram and were set after evaluation of the results from previous studies conducted with fluopyram and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the

top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrome P-450 activity and cell proliferation in the rat.

Table 197: Study design

Test group	Concentration in diet (ppm) of AE C656948	Dose per animal (study averages)		Animals assigned	
		Females Subgp 1 Days 1-3 (mg/kg bw/day)	Females Subgp 2 Days 1-7 (mg/kg bw/day)	Females Subgp 1	Females Subgp 2
1	0	0	0	15	15
2	30	2.4	2.3	15	15
3	75	6.2	5.6	15	15
4	150	12.0	11.6	15	15
5	600	46.1	44.1	15	15
6	750	117.6	118.5	15	15
7	Phenobarbital 80 mg/kg bw/day by gavage	-	-	15	15

Subgp = Subgroup

Fluopyram (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

A single formulation of phenobarbital was prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which covered the period of usage and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean value obtained in homogeneity check was used as measured concentration.

Appropriate statistics were used to analyse the data obtained (see study for details).

Observations: The animals were observed twice daily for moribundity and mortality (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals and detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of any clinical signs were recorded.

Body weight: Each animal was weighed during the acclimatization period. Body weights were also measured on study Day 1 and 3 for Subgroup 1 and on study Day 1 and 7 for Subgroup 2. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight).

Food consumption and compound intake: The weight of food supplied and of that remaining at the end of the food consumption period was recorded on study Day 3 for all animals in Subgroup 1 and on study Day 7 for all animals in Subgroup 2. Any food spillage was noted. From these records, the mean achieved dosage intake in mg/kg/day for each Subgroup was calculated.

Sacrifice and pathology

Necropsy procedure – Organ sampling: On study Day 4 (Subgroup 1) and study Day 8 (Subgroup 2), all animals were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane (Baxter, Maurepas, France). Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Brain and liver were weighed fresh at scheduled sacrifice only.

Duodenum and two central sections of the liver taken from the left and medial lobes from each animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74°C + 10°C until used for qPCR investigations. From the Subgroup 2 animals, the remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsomal preparations.

Histotechnology

Conventional histopathological examination: Duodenum and the two central sections of the liver were embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared for each animal in all groups. Histopathological examinations were performed on the liver samples from all animals in all groups.

Ki67 staining for cell proliferation assessment: For each animal in each group a section of a formalin-fixed paraffin-embedded block containing 2 liver samples and one sample of duodenum was prepared. The duodenum was included to serve as a positive control for staining as it has a high rate of cell proliferation. The immunohistochemical reaction included incubation with a monoclonal antibody raised against Ki67, amplification with a secondary biotinylated antibody and a streptavidin-horseradish complex, detection of the complex with diamino-benzidine (DAB) and nuclear counter staining with hematoxylin. The immunohistochemical staining for Ki67 and determination of the labeling index was performed on all surviving animals showing sufficient Ki67 staining (estimated by duodenal Ki67 labeling) to assess cell cycling in the liver. The zonal labeling index, expressed as the number of Ki67-positive hepatocytes per thousand cells, were measured separately on random fields comprising of at least 1000 centrilobular and perilobular cells using an automatic image analysis system. The mean and standard deviation were calculated for each group.

Hepatotoxicity testing

At final necropsy (ie Day 8), the remaining portions of the liver from were five randomly chosen females per treatment group (Subgroup 2) were weighed and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) and UDPGT specific isoenzyme profiles.

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample.

Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry (SAFAS SP2000 version 6.10.7.4) using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

Cytochrome P-450 dependent dealkylation of resorufin derivatives was followed over a period of 2, 5 or 7 minutes at 37°C.

Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate (method adapted from Zakim and Vessey. The enzymatic kinetic (disappearance of the colored 4-nitrophenol) was followed at 405 nm during 3 min. at 30°C. Three replicates from each sample were assayed. UDPGT with bilirubin as substrate was also determined using a spectrophotometry method (adapted from Heirwegh et al.) consisting in the determination of conjugated bile pigments after its conversion into azo-pigment derivatives. Absorbance was measured at 530 nm. Three replicates from each sample were assayed.

Quantitative PCR Analyses

Total cytoplasmic RNA was isolated from the liver of all surviving individual control and treated animals using RNeasy Midi kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies).

Ten µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, Fast Start Universal Probe Master mix (Roche) on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

Table 198: The list of Taqman assays used is as follows:

Gene (Major function)	**Abbreviation**	**Refset ID**	**Taqman assay ID (Applied Biosystems)**
METABOLISM: Phase I			
P450 (cytochrome)oxidoreductase	POR	NM_031576.1	Rn00580820_m1
Cytochrome P450 1a1	1a1	NM_012540.2	Rn00487218_m1
Cytochrome P450 2b1	Cyp2b2	275205*	Cyp2b1_tc5
Cytochrome P450 3a3	Cyp3a3 (Cyp3a23)	NM_013105.2	Rn01640761_g1
Cytochrome P450 4a1	Cyp4a1	NM_175837.1	Rn00598510_m1
METABOLISM: Phase II			
Glutathione S-transferase A2	Gsta2	NM_017013.4	Rn00566636_m1
Glutathione S-transferase mu3	Gstm4	NM_020540.1	Rn01789233_m1
UDP glucoronosyltransferase 2 family, polypeptide B1	Udpgtr2	NM_173295.1 NM_057105.3	Rn00756519_m1
Epoxide hydrolase 1, microsomal	Ephx1	NM_012844.2	Rn00563349_m1
Epoxide hydrolase 2, cytoplasmic	Ephx2	NM_022936.1	Rn00576023_m1
Sulfotranferase family 1E, member 1	Sult1e1	NM_012883.1	Rn00820646_g1
CELL PROLIFERATION/APOPTOSIS			
Epithelial cell adhesion molecule	Tacstd1	NM_138541.1	Rn01473202_m1
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	NM_001008321.1	Rn01452530_g1
Retinoblastoma 1	Rb1	XM_344434.3	Rn01753308_m1

*: Transcript made to order

Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman assay ID: Rn00560865_m1) was selected as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

Results and discussion

Mortality:

There was no mortality in any group during the course of the study.

Clinical signs:

Three day treatment (Subgroup 1):

Fluopyram: There were no treatment-related clinical signs during the three day treatment period.

Phenobarbital: All females displayed reduced motor activity starting from Day 2 of treatment. In addition, three females had ocular discharge in either one eye or both eyes on Day 3 of treatment.

Seven day treatment (Subgroup 2):

Fluopyram: There were no treatment-related clinical signs during the seven day treatment period.

Phenobarbital: All females displayed reduced motor activity starting from Day 1 of treatment. Two females had ocular discharge starting from Day 2 of treatment.

Body Weight and Body Weight Gain:

Three day treatment (Subgroup 1):

Fluopyram: There were no treatment-related changes to any of the body weight parameters between Day 1 and Day 3.

Phenobarbital: There were no relevant changes in any of the body weight parameters between Day 1 and Day 3 compared to the controls.

Seven day treatment (Subgroup 2):

Fluopyram: There were no relevant changes in any of the body weight parameters between Day 1 and Day 7 compared to the controls.

Phenobarbital: Between Days 1 and 7, there was a reduced mean absolute body weight gain compared to the control group (10g compared to 13g in the control group; -23%, not statistically significant).

Food consumption and compound intake:

There were no treatment-related effects on food consumption for either Fluopyram or Phenobarbital following treatment for either 3 or 7 days.

The mean achieved dietary intakes of Fluopyram expressed in mg/kg/day received by the females during the study were as follows:

Table 199: Mean achieved intake of fluopyram

	Mean achieved dietary intake of fluopyram	
Diet Concentration (ppm)	**SUBGROUP 1** **Days 1 – 3 (mg/kg/day)**	**SUBGROUP 2** **Days 1 – 7 (mg/kg/day)**
30	2.4	2.3
75	6.2	5.6
150	12.0	11.6
600	46.1	44.1
1500	117.6	118.5

Sacrifice and Pathology

Terminal body weight

Three day treatment (Subgroup 1):

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. The mean absolute and mean liver to body weight ratio were statistically significantly higher from 600 ppm when compared to controls. In addition, the mean liver to brain weight ratio was also statistically significantly higher at 1500 ppm when compared to controls. These liver weights changes were considered to be toxicologically relevant.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weights were statistically significantly higher when compared to controls. These liver weights changes were considered to be toxicologically relevant.

Table 200: Mean absolute and relative liver weight changes following 3 day treatment with fluopyram or phenobarbital

Mean liver weight ±SD at scheduled 3 Day sacrifice(% change when compared to controls)							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Mean absolute liver weight (g)	5.84 ±0.30	5.93 ±0.35 (+2%)	5.97 ±0.40 (+2%)	6.16 ±0.26 (+5%)	6.23* ±0.49 (+7%)	6.84** ±0.38 (+17%)	6.36** ±0.57 (+9%)
Mean liver to body weight ratio (%)	2.69 ±0.08	2.70 ±0.15 (0%)	2.75 ±0.13 (+2%)	2.79 ±0.10 (+4%)	2.83* ±0.17 (+5%)	3.15** ±0.17 (+17%)	2.93** ±0.22 (+9%)
Mean liver to brain weight ratio (%)	313.30 ±17.26	318.52 ±18.34 (+2%)	321.00 ±25.37 (+2%)	331.36 ±18.03 (+6%)	333.10 ±25.17 (+6%)	365.96** ±32.54 (+17%)	341.02** ±28.83 (+9%)

* $p \leq 0.05$; ** $p \leq 0.01$

Seven day treatment (Subgroup 2):

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean absolute and relative liver weights were statistically significantly higher at 1500 ppm when compared to controls. These liver weight changes were considered to be toxicologically relevant.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls. Mean liver to body weight ratio was statistically significantly higher when compared to controls. This change was considered to be toxicologically relevant.

Table 201: Mean absolute and relative liver weight changes following 7 day treatment with fluopyram or phenobarbital

Mean liver weight ±SD at scheduled 7 Day sacrifice(% change when compared to controls)							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Mean absolute liver weight (g)	6.18 ±0.91	5.88 ±0.53 (-5%)	5.96 ±0.43 (-4%)	5.96 ±0.35 (-4%)	6.29 ±0.49 (+2%)	7.17** ±0.51 (+16%)	6.66 ±0.37 (+8%)
Mean liver to body weight ratio (%)	2.67 ±0.34	2.52 ±0.13 (-5%)	2.59 ±0.14 (-3%)	2.61 ±0.20 (-2%)	2.76 ±0.16 (+3%)	3.16** ±0.16 (+18%)	2.95** ±0.17 (+11%)
Mean liver to brain weight ratio (%)	332.98 ±53.94	317.53 ±27.19 (-5%)	321.09 ±23.25 (-4%)	320.04 ±23.75 (-4%)	336.49 ±22.32 (+1%)	392.26** ±29.24 (+18%)	361.88 ±20.36 (+9%)

** $p \leq 0.01$

Gross pathology

Three day treatment (Subgroup 1):

Enlarged liver was found in 2/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

Table 202: Macroscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital

Incidence and severity of macroscopic changes in the liver- scheduled 3 day sacrifice							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Number examined	15	15	15	15	15	15	15
Enlarged	0	0	0	0	0	2	0

Seven day treatment (Subgroup 2):

Enlarged liver was found in 3/15 females at 1500 ppm fluopyram. There were no macroscopic changes recorded for the phenobarbital treated females.

Table 203: Macroscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital

Incidence and severity of macroscopic changes in the liver- scheduled 7 day sacrifice							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Number examined	15	15	15	15	15	15	15
Enlarged	0	0	0	0	0	3	0

Microscopic pathology

Three day treatment (Subgroup 1):

Fluopyram: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 6/15 females at 1500 ppm. In addition, an increased number of mitoses in hepatocytes was found at the same dose level for 4/15 females.

Phenobarbital: Minimal centrilobular to panlobular hepatocellular hypertrophy was found for 3/15 females. In addition, an increased number of mitoses in hepatocytes was found for 3/15 females.

Table 204: Microscopic changes in the liver following 3 days treatment with fluopyram or phenobarbital

Incidence and severity of microscopic changes in the liver- scheduled 3 day sacrifice							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Number examined	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	0	0	0	0	0	6	3
Total	0	0	0	0	0	6	3
Increased number of mitoses							
Present	0	0	0	0	0	4	3
Total	0	0	0	0	0	4	3

Seven day treatment (Subgroup 2):

Fluopyram: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy was found from 600 ppm (1/15 females at 600 ppm; 14/15 females at 1500 ppm).

Phenobarbital: Minimal to slight centrilobular to panlobular hepatocellular hypertrophy in 9/15 females was found as well as an increased number of mitoses in hepatocytes in 3/15 females.

Table 205: Microscopic changes in the liver following 7 days treatment with fluopyram or phenobarbital

Incidence and severity of microscopic changes in the liver- scheduled 7 day sacrifice							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Sex	Females						
Number examined	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	0	0	0	0	1	6	6
Slight	0	0	0	0	0	8	3
Total	0	0	0	0	1	14	9
Increased number of mitoses							
Present	0	0	0	0	0	0	3
Total	0	0	0	0	0	0	3

Cell proliferation

Three day treatment (Subgroup 1):

Fluopyram: Dose-related increases in the centrilobular, perilobular and, therefore, the global proliferation indexes were observed, which were statistically significantly higher from 150 ppm when compared to controls.

Table 206: Mean cell proliferation index following 3 days treatment with fluopyram

Mean cell proliferation index ±SD following three days treatment (% change when compared to controls)					
Test Substance	Dose		Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	14.6± 7.4	11.1 ± 5.1	12.8± 5.8
Fluopyram	30 ppm	N	14	14	14
		Mean ± SD	11.8 ± 8.0	10.7± 5.8	11.2 ± 6.4
		% Change	-19%	-3.5%	-12.5%
	75 ppm	N	15	15	15
		Mean ± SD	13.4 ± 5.9	15.4± 8.2	14.4 ± 5.8
		% Change	-8.2%	39.3%	12.3%
	150 ppm	N	15	15	15
		Mean ± SD	25.1 ± 11.1*	22.6 ± 13.4**	23.8± 10.6**
		% Change	71.9%	104.2%	86.0%
	600 ppm	N	15	15	15
		Mean ± SD	57.3± 20.1**	36.6 ± 15.7**	47.0 ± 15.8**
		% Change	293.8%	230.7%	266.6%
	1500 ppm	N	15	15	15
		Mean ± SD	99.5 ± 62.3**	67.4 ± 30.0**	83.4 ± 38.3**
		% Change	583.2%	509.0%	551.2%

* p≤0.05; ** p≤0.01

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: Centrilobular, perilobular and, therefore, the global proliferation indexes were statistically significantly higher, when compared to controls. The magnitude of the response was greater in the centrilobular region (217.7% increase compared to the corresponding controls) than that observed in the perilobular region (58% increase compared to the corresponding controls).

Table 207: Mean cell proliferation index following 3 days treatment with phenobarbital

Mean cell proliferation index ±SD following three days treatment (% change when compared to controls)					
Test substance	Dose		Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	14.6± 7.4	11.1 ± 5.1	12.8± 5.8
Phenobarbital	80 mg/kg/day	N	14	14	14
		Mean ± SD	46.25± 36.24**	17.48± 8.95*	31.87± 19.80**
		% Change	217.7%	58.0%	148.8%

p≤0.05; ** p≤0.0

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Seven day treatment (Subgroup 2):

Fluopyram: Centrilobular, perilobular and, therefore, the global proliferation indexes were statistically significantly higher from 150 ppm when compared to controls.

Table 208: Mean cell proliferation index following 7 days treatment with fluopyram

Mean cell proliferation index ±SD following seven days treatment (% change when compared to controls)					
Test Substance	Dose		Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	8.3± 5.3	10.5 ± 6.1	9.4 ± 5.1
Fluopyram	30 ppm	N	15	15	15
		Mean ± SD	11.2± 7.6	15.3 ± 11.2	13.2± 8.6
		% Change	34.5%	45.6%	40.7%
	75 ppm	N	15	15	15
		Mean ± SD	12.0 ± 6.1	10.3± 5.1	11.1± 5.1
		% Change	43.5%	NC	18.5%
	150 ppm	N	15	15	15
		Mean ± SD	20.4 ± 13.0**	18.4 ± 8.1**	19.4 ± 9.6**
		% Change	144.5%	75.2%	106.0%
	600 ppm	N	15	15	15
		Mean ± SD	27.8± 12.1**	27.0± 13.0**	27.4± 9.8**
		% Change	234.1%	157.6%	191.7%
	1500 ppm	N	15	15	15
		Mean ± SD	32.2 ± 19.6**	34.9 ± 17.2**	33.5± 15.0**
		% Change	286.3%	232.5%	256.6%

NC: no change; ** p≤0.01

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: Centrilobular and the global proliferation indexes were statistically significantly higher when compared to controls. The magnitude of response was greater in the centrilobular region (603.4% increase compared to controls) compared to that recorded for the perilobular region (54.7% increase compared to controls).

Table 209: Mean cell proliferation index following 7 days treatment with phenobarbital

Mean cell proliferation index ±SD following seven days treatment (% change when compared to controls)					
Test Substance	Dose		Centrilobular	Perilobular	Total
Control	0 ppm	N	15	15	15
		Mean ± SD	8.3± 5.3	10.5 ± 6.1	9.4 ± 5.1
Phenobarbital	80 mg/kg/day	N	15	15	15
		Mean ± SD	58.6 ± 33.9**	16.2± 10.2	37.4± 19.6**
		% Change	603.4%	54.7%	297.9%

p≤0.05; ** p≤0.01

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Hepatotoxicity testing

Investigation of hepatotoxicity was only conducted on 5 randomly chosen females/group from those animals dosed for 7 days (Subgroup 2).

Fluopyram: No statistically significant changes in total P450 content or enzyme activity was observed up to 150 ppm. Statistically significant increases in PROD (p≤0.05 at 600 ppm and p≤0.01 at 1500 ppm), BROD and both UDPGT isoforms (p≤0.01) were observed from 600 ppm. At 1500 ppm, total P450 content was also statistically significantly increased (p≤0.05). In addition, EROD activity was statistically significantly increased (p≤0.01) at 1500 ppm, however the increase (63%) was considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls (9).

Phenobarbital: A statistically significant increase (p≤0.05) in total P450 was recorded as well as statistically significant increases BROD (p≤0.05), PROD (p≤0.01) and UDPGT-nitrophenol (p≤0.01) activity.

Table 210: Mean cytochrome P-450 content and enzymatic activities in the liver following 7 days treatment with fluopyram or phenobarbital

Fluopyram dose	Mean Increase Compared to Controls (Statistical Significance)					
	Total P450 Content	EROD	BROD	PROD	UDPGT-4-nitrophenol	UDPGT-bilirubin
30 ppm	x 0.93	x 1.05	x 1.09	x 1.34	x 1.05	x 1.05
75 ppm	NC	x 0.94	x 1.17	x 1.46	x 1.10	x 1.28
150 ppm	x 1.11	x 1.02	x 1.44	x 1.41	x 1.27	x 1.44
600 ppm	x 1.02	x 1.12	x 2.42 (**)	x 2.16 (*)	x 1.52 (**)	x 1.91 (**)
1500 ppm	x 1.34 (*)	x 1.62 (**)	x 5.89 (**)	x 4.56 (**)	x 3.01 (**)	x 2.65 (**)
Phenobarbital 80 mg/kg/day	x 1.65 (*)	x 1.12	x 27.7 (*)	x 10.59 (**)	x 1.86 (**)	x 1.37

NC: no change; * p≤0.05; ** p≤0.01

Gene Transcript Analyses

Three day treatment(Subgroup 1):

Fluopyram: At 30 ppm, no statistically significant changes in the expression of any of the genes investigated were observed.At 75 ppm a marginal but statistically significant increase in the expression of Cyp3a3 (+48.4%; p≤0.05) was recorded. From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 (+230.7% at 150 ppm up to +24270% at 1500 ppm) and Cyp3a3 (+159.1% at 150 ppm up to +2051% at 1500 ppm). From 600 ppm, genes coding for Phase II enzymes (i.e. Gsta2, Gstm4, Udpgtr2 and Ephx1) were statistically significantly increased. In addition, Cyp1a1 was statistically significantly increased (+631.1%; p≤0.01) from this dose level. A marginal, though statistically significant increase (+45%; p≤0.05) in the marker for apoptosis (Gadd 45b) was also observed. Finally, at 1500 ppm, marginal, though statistically significant increases in those genes associated with cell proliferation/apoptosis (Tacstd 1, Gadd 45b) were recorded.

There were no clear effects on the expression of POR, Cyp4a1, Ephx2 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (61/90 control and fluopyram treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 211: Mean gene transcript analyses following 3 day treatment with fluopyram

	Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values)					
Gene transcripts	Control	30 ppm	75 ppm	150 ppm	600 ppm	1500 ppm
METABOLISM: Phase I						
POR	0.87 ± 0.24	0.82 ±0.29 NC	0.96 ±0.37 (+10.3)	0.71 ±0.21 (-18.4)	0.95 ±0.30 (+9.2)	0.98 ±0.39 (+12.6)
Cyp1a1	1.35 ± 1.14	1.12 ± 0.68 (-17)	1.51 ± 1.61 (+11.9)	2.3 ± 2.3 (+70.4)	9.87 ± 8.29** (+631.1)	84.6 ± 51.6** (+6167)
Cyp2b1	1.27 ± 1.87	0.81 ± 0.56 (-36.2)	1.44 ± 0.86 (+13.4)	4.2 ± 3.38** (+230.7)	63.0 ± 71.2** (+4861)	309.5 ± 176.7** (+24270)
Cyp3a3	0.93 ± 0.49	1.01 ± 0.50 (+8.6)	1.38 ± 0.68* (+48.4)	2.41 ± 0.89** (+159.1)	7.64 ± 2.23** (+721.5)	20.0 ± 6.45** (+2051)
Cyp4a1	1.46 ± 0.33	1.37 ± 0.38 (-6.2)	1.51 ± 0.4 (+3.4)	1.3 ± 0.4 (-11.0)	1.43 ± 0.46 (NC)	1.14 ± 0.27 (-21.9)
METABOLISM: Phase II						
Gsta2	0.51 ± 0.45	0.58 ± 0.60 (+13.7)	0.52 ± 0.34 (NC)	0.83 ± 0.54 (+62.7)	1.02 ± 0.67** (+100)	2.35 ± 1.31** (+360.9)
Gstm4	0.83 ± 0.35	1.05 ± 0.69 (+26.5)	1.24 ± 0.78 (+49.4)	1.01 ± 0.99 (+21.7)	2.15 ± 1.23** (+159.0)	3.86 ± 2.09** (+365)
Udpgtr2	1.77 ± 0.93	1.68 ± 0.79 (-5.1)	2.0 ± 0.89 (+13)	3.0 ± 2.13 (+69.5)	4.27 ± 2.03** (+141.2)	6.66 ± 3.68** (+276.3)
Ephx1	0.85 ± 0.24	0.91 ± 0.30 (+7.1)	0.84 ± 0.31 (NC)	1.0 ± 0.36 (+17.6)	1.73 ± 0.44** (+103.5)	3.12 ± 0.93** (+267.1)
Ephx2	1.14 ± 0.31	0.92 ± 0.29 (-19.3)	0.94 ± 0.21 (-17.5)	1.02 ± 0.26 (-10.5)	1.18 ± 0.33 (NC)	0.95 ± 0.32 (-16.7)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.19 ± 0.17	1.1 ± 0.16 (-7.6)	1.1 ± 0.21 (-7.6)	1.12 ± 0.23 (-5.9)	1.26 ± 0.22 (+5.9)	1.66 ± 0.34** (+39.5)
Gadd45b	0.8 ± 0.26	0.8 ± 0.29 (NC)	0.84 ± 0.36 (+5)	0.9 ± 0.23 (+12.5)	1.16 ± 0.45* (+45)	1.34 ± 0.57** (+67.5)
Rb1	1.01 ± 0.15	0.93 ± 0.15 (-7.9)	0.96 ± 0.16 (-5)	1.05 ± 0.3 (+4)	1.04 ± 0.26 (+3)	0.82 ± 0.14 * (-18.8)

NC: no change; * p≤0.05; ** p≤0.01; ND: not detected. In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The gene expression data indicated that three days treatment with phenobarbital leads to dose-related increased expression of Phase 1 and Phase 2 enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutathione (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant ($p \leq 0.01$) down-regulation of Cyp4a1 and Ephx2 expression.The marker for cell proliferation (Gadd45b) was also marginally but statistically significantly increased.There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (9/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 212: Mean gene transcript analyses following 3 day treatment with phenobarbital

	Mean Relative Quantity ± standard deviation of gene transcripts (3 day treatment) (% change compared to control mean values)	
Gene transcripts	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.87 ± 0.24	0.7 ± 0.24 (-19.5)
Cyp1a1	1.35 ± 1.14	1.65 ± 2.05 (+22.2)
Cyp2b1	1.27 ± 1.87	962.8 ± 892.5** (+75711)
Cyp3a3	0.93 ± 0.49	10.12 ± 7.18** (+988.2)
Cyp4a1	1.46 ± 0.33	0.52 ± 0.16** (-64.4)
METABOLISM: Phase II		
Gsta2	0.51 ± 0.45	2.22 ± 1.63** (+335.3)
Gstm4	0.83 ± 0.35	3.62 ± 3.82* (+336.1)
Udpgtr2	1.77 ± 0.93	7.83 ± 5.43** (+342. 4)
Ephx1	0.85 ± 0.24	3.3 ± 2.3** (+288.2)
Ephx2	1.14 ± 0.31	0.53 ± 0.23** (-53.5)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.19 ± 0.17	1.08 ± 0.18 (-9.2)
Gadd45b	0.8 ± 0.26	1.31 ± 0.67** (+63.75)
Rb1	1.01 ± 0.15	0.82 ± 0.15** (-18.8)

NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$; ND: not detected

In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Seven day treatment (Subgroup 2):

Fluopyram: A similar gene expression profile was observed following 7 days treatment with fluopyram as that observed following 3 days treatment.

At 30 ppm, no significant changes in the expression of any of the genes investigated were observed.

At 75 ppm, a marginal but statistically significant increase in the expression of Cyp3a3 (+95%; $p \leq 0.01$) was recorded.

From 150 ppm, clear dose-related increases were recorded for the expression of the Phase I genes Cyp2b1 (+1336% at 150 ppm up to +143300% at 1500 ppm), Cyp3a3 (+262.6% at 150 ppm up to +2756% at 1500 ppm) and Cyp1a1 (+356.2% at 150 ppm up to +22192% at 1500 ppm).

From 600 ppm, genes coding for Phase II enzymes (i.e. Gsta2, Gstm4, Udpgtr2 and Ephx1) were statistically significantly increased.

Finally, at 1500 ppm, a marginal, though statistically significant increase (+82.5%; $p \leq 0.05$) in Gadd45b was recorded. Cyp4a1 (-37%; $p \leq 0.01$) and Ephx2 (-39.5%; $p \leq 0.05$) were marginally though statistically significantly down-regulated at this dose level.

There were no clear effects on the expression of POR and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (59/90 control and fluopyram treated liver samples) was greater than the cut-off limit of 35.

Table 213: Mean gene transcript analyses following 7 day treatment with fluopyram

	Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values)					
Gene transcripts	Control	30 ppm	75 ppm	150 ppm	600 ppm	1500 ppm
METABOLISM: Phase I						
POR	0.85 ± 0.35	0.81 ± 0.29 (-4.7)	0.81 ± 0.26 (-4.7)	0.91 ± 0.3 (+7.1)	1.03 ± 0.42 (+21.2)	0.99 ± 0.36 (+16.5)
Cyp1a1	2.26 ± 1.3	3.07 ± 3.87 (+35.8)	4.0 ± 3.87 (+77)	10.31 ± 11.01** (+356.2)	143.7 ± 81.0** (+6258)	503.8 ± 170.5** (+22192)
Cyp2b1	0.95 ± 0.74	2.43 ± 1.85 (+155.8)	2.93 ± 3.87 (+208.4)	13.64 ± 12.06** (+1336)	310.2 ± 322.0** (+32553)	1362.3 ± 1422.2** (+143300)
Cyp3a3	0.99 ± 0.59	1.46 ± 0.84 (+47.5)	1.93 ± 0.79** (+95)	3.59 ± 1.65** (262.6)	12.32 ± 3.75** (+1144)	28.27 ± 10.15** (+2756)
Cyp4a1	0.73 ± 0.26	0.70 ± 0.22 (-4.1)	0.62 ± 0.14 (-15.1)	0.64 ± 0.24 (-12.3)	0.64 ± 0.19 (-12.3)	0.46 ± 0.11** (-37.0)
METABOLISM: Phase II						
Gsta2	2.08 ± 1.7	1.37 ± 0.56 (-34.1)	1.97 ± 1.45 (-5.3)	2.74 ± 1.78 (+31.7)	3.6 ± 1.83** (+73.1)	7.03 ± 4.57** (+238)
Gstm4	2.02 ± 1.02	2.42 ± 1.76 (+19.8)	1.81 ± 0.97 (-10.4)	2.78 ± 1.54 (+37.6)	4.2 ± 1.82* (+108)	11.3 ± 19.5** (+459.4)
Udpgtr2	2.53 ± 1.28	3.58 ± 1.32 (+41.5)	3.91 ± 1.16** (+54.5)	3.46 ± 1.5 (+36.8)	7.33 ± 3.22** (+189.7)	12.01 ± 4.25** (+374.7)
Ephx1	1.2 ± 0.41	1.46 ± 0.25 (+21.7)	1.34 ± 0.32 (+11.7)	1.2 ± 0.39 (NC)	2.12 ± 0.69** (+77)	4.21 ± 2.34** (+250.8)
Ephx2	1.19 ± 0.44	1.18 ± 0.38 (NC)	1.09 ± 0.48 (-8.4)	1.03 ± 0.43 (-13.4)	0.95 ± 0.38 (-20.2)	0.72 ± 0.35 * (-39.5)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.41 ± 0.32	1.55 ± 0.37 (+10)	1.36 ± 0.23 (-3.5)	1.1 ± 0.26 (-22.0)	1.38 ± 0.45 (-2.1)	1.51 ± 0.34 (+7.1)
Gadd45b	1.2 ± 0.45	1.41 ± 0.43 (+17.5)	1.13 ± 0.34 (-5.8)	1.25 ± 0.65 (+4.2)	1.55 ± 0.53 (+29.2)	2.19 ± 1.27* (+82.5)
Rb1	0.90 ± 0.18	0.98 ± 0.15 (+8.9)	0.89 ± 0.13 (NC)	0.83 ± 0.17 (-7.8)	0.87 ± 0.14 (-3.3)	0.79 ± 0.15 (-12.2)

NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$; ND: not detected In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: A similar gene expression profile was observed following 7 days treatment with Phenobarbital as that observed following 3 days treatment, although for some genes the expression was more marked after this prolonged treatment. Thus, increased expression of Phase 1 (Cyp2b1; Cyp3a3) and Phase 2 enzymes (Gstm4, Gsta2, Ephx1 and Udpgtr2) was recorded after 7 days with the magnitudes of induction for Cyp2b1 and Cyp3a3 being almost 3-fold and 1.6-fold respectively greater than those observed at the earlier time point. Similarly the induction of Gstm4 was 5.1-fold greater after 7 days compared to that recorded after 3 days.

As with the earlier time point, there was a statistically significant ($p \leq 0.01$) down-regulation of Cyp4a1 and Ephx2 expression and the marker for cell proliferation (Gadd45b) was also marginally but statistically significantly ($p \leq 0.05$) increased.

There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which for the majority of samples (8/15 phenobarbital treated liver samples) was greater than or equal to the cut-off limit of 35.

Table 214: Mean gene transcript analyses following 7 day treatment with phenobarbital

	Mean Relative Quantity ± standard deviation of gene transcripts (7 day treatment) (% change compared to control mean values)	
Gene transcripts	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.85 ± 0.35	0.66 ± 0.20 (-22.4)
Cyp1a1	2.26 ± 1.30	1.83 ± 1.24 (-19.0)
Cyp2b1	0.95 ± 0.74	2776.8 ± 1842.7** (+292195)
Cyp3a3	0.99 ± 0.59	16.26 ± 10.45** (+1542.4)
Cyp4a1	0.73 ± 0.26	0.40 ± 0.08** (-45.2)
METABOLISM: Phase II		
Gsta2	2.08 ± 1.70	4.8 ± 2.3** (+130.8)
Gstm4	2.02 ± 1.02	18.3 ± 11.8** (+805.9)
Udpgtr2	2.53 ± 1.28	13.1 ± 7.8** (+418)
Ephx1	1.2 ± 0.41	4.76 ± 2.43** (+297)
Ephx2	1.19 ± 0.44	0.81 ± 0.15** (-31.9)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.41 ± 0.32	1.13 ± 0.18** (-19.9)
Gadd45b	1.2 ± 0.45	2.07 ± 0.89** (+72.5)
Rb1	0.90 ± 0.18	0.71 ± 0.12** (-21.1)

NC: no change; * $p \leq 0.05$; ** $p \leq 0.01$; ND: not detected In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Conclusion

Overall, clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression) were observed following fluopyram treatment. These changes were recorded as early as following 3 days of treatment and starting from 150 ppm. The dose of 75 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of Cyp3a3 at this dose level) and 30 ppm as a No Observed Effect Level (NOEL).

Report:	KIIA 5.5.4 /24;Tinwell, H.;2012
Title:	Fluopyram (AE C 656948): Mechanistic investigations in the liver of female rats following dietary administration M-427431-01, ASB2012-7371
Guidelines:	No specific guideline
Deviations:	None
GLP	Yes

The objective of the present study was to investigate the mode of action by which fluopyram gives rise to liver tumors in the female rat following chronic exposure. The effects of different doses of fluopyram on the liver were determined following continuous dietary administration for at least 28 days. In addition, the reversibility of any effects observed at the highest dose level was assessed following a recovery period of one month on untreated control diet. Liver cell proliferation, hepatic total cytochrome P-450 as well as enzymatic activities and gene expression profiles for inducible P-450 and UDPGT enzymes were assessed. Liver histopathology was also evaluated. The CAR/PXR nuclear receptor activator, phenobarbital, was administered by oral gavage and was used as a positive control for the liver effects. The reversibility of any effects induced by phenobarbital was also assessed following a recovery period of one month on untreated control diet.

Consequently, groups of adult female Wistar rats were exposed to fluopyram (batch number: Mix-batch: 08528/0002: a light beige powder, 94.7% w/w) for at least 28 days in the diet at dose levels of 0, 30, 75, 150, 600 and 1500 ppm, which equated to 2.2, 5.6, 11.3, 44.5 and 111.4 mg/kg/day respectively. A group of animals dosed with 80 mg/kg/day phenobarbital by gavage acted as a positive control for the parameters investigated in this present study. Each group consisted of 15 females, apart from the negative control and 1500 ppm fluopyram group, together with phenobarbital group, where an additional 15 animals per group following the 28 days of treatment were maintained for a 1 month recovery period on untreated control diet.

Treatment Phase

There were no relevant changes in any of the body weight parameters during the treatment phase for either fluopyram or phenobarbital. Fluopyram treatment caused ocular discharge and red conjunctiva in some females of Groups 3 (75 ppm), 4 (150 ppm) and 5 (600 ppm). In addition, food consumption was statistically significantly reduced for females treated with 1500 ppm fluopyram during week 3 ($p \leq 0.05$) and week 4 ($p \leq 0.01$) of treatment.

All females dosed with phenobarbital displayed reduced motor activity and some females had ocular discharge and/or lacrimation in either one or both eyes. Other clinical signs included increased salivation and soiling around the mouth or the head. Food consumption was increased during treatment with phenobarbital ($p \leq 0.05$ for weeks 1 and 4; $p \leq 0.01$ for week 2).

Fluopyram had no effect on terminal body weight but induced treatment-related changes in all liver parameters investigated. The number of parameters affected and also the magnitude of the responses were dose-related as described below.

At 30 ppm, a statistically significant ($p \leq 0.01$) increase in the expression of Cyp3a3 was recorded (+81%), which increased with increasing dose up to +4943% at 1500 ppm. This increase in gene expression was, however, not associated with any statistically significant increase in corresponding enzyme (BROD) activity until 600 ppm.

At 75 ppm, enlarged liver was observed in 3/15 females and centrilobular cell proliferation was marginally, but statistically significantly ($p \leq 0.05$), increased (+47%) compared to the controls.

At 150 ppm, slight but statistically significant increases in mean liver weight relative to body weight (+7%; p≤0.01) and brain weight (+11%; p≤0.05) were recorded. This increased liver weight could be associated with enlarged liver in 5/15 females. Centrilobular and global hepatic cell proliferation (+106% and +70% respectively) were statistically significantly (p≤0.01) increased compared to the controls. In addition, statistically significant (p≤0.01) increases were recorded for the expression of the Phase I genes Cyp1a1 (+711%), Cyp2b1 (+990%) and for the Phase II genes Gstm4 (+136%) and Udpgtr2 (+64%). Statistically significantly (p≤0.01) increased hepatic enzyme activity was, however, only observed for UDPGT-bilirubin (corresponding to Udpgtr2) and EROD (corresponding to Cyp1a1) starting from this dose level.

At 600 ppm, marked changes to the liver were observed as evidenced by statistically significantly increased absolute and relative liver weights (+10 to +15%), coupled with enlarged liver in 4/15 animals and minimal hepatocellular hypertrophy in 6/15 females. However, despite these macroscopic and microscopic changes, hepatic cell proliferation was similar to that observed at 150 ppm with centrilobular and global proliferation being statistically significantly increased (+106% and +67% respectively; p≤0.01). Genes coding for additional Phase II enzymes were statistically significantly increased at this dose level (Gsta2: +53% and Ephx1: +116%). A marginal, though statistically significant (+75%; p≤0.01), increased expression of the marker for cell proliferation (Gadd 45b) was also observed. Increased activity of BROD (p≤0.01), PROD (p≤ 0.05) and UDPGT-4-nitrophenol (p≤0.01) were recorded from this dose level.

At 1500 ppm, the hepatic effects were more marked than those recorded at the lower doses. Specifically, for cell proliferation the % increase (compared to controls) in global cell proliferation was 188% compared to 67% at 600 ppm. Statistically significant increases in mean absolute and relative liver weight (+29 to +33%) were also recorded at this dose level as well as enlarged liver in 14/15 animals, which were associated with the centrilobular to panlobular hypertrophy observed in 14/15 females. Changes in gene expression and enzyme activity observed at the lower doses were also more marked at this top dose level.

Phenobarbital was used as a positive control for the various parameters measured in the present study and as such induced changes in the liver pertinent for a compound that activates CAR/PXR nuclear receptors. Liver weight (absolute and relative to brain and body weight) was statistically significantly increased following phenobarbital treatment and enlarged liver was noted in 10/15 animals, which could be associated with the centrilobular to panlobular hypertrophy observed in 12/15 females and an increased number of mitoses was observed in 1/15 females. Increased cell proliferation was observed with the effects being more apparent in the centrilobular region (+335% compared to controls) than in the perilobular region (+23% compared to controls). Furthermore, the gene expression of Gadd45b was statistically significantly increased (+68%; p≤0.01). Statistically significant (p≤0.01) increases in the gene expression of Cyp2b1, Cyp3a3 and Udpgtr2 were recorded, which could be associated with the statistically significantly increased hepatic enzyme activity of PROD (+8.44-fold; p≤0.01), BROD (+88-fold; p≤0.01) and UDPGT-bilirubin (+1.38-fold; p≤0.05) respectively. Enzyme activity of the second isoform of UDPGT (UDPGT-4-nitrophenol) was also statistically significantly increased (+1.81-fold; p≤0.01) and increased expression of genes coding for additional Phase II enzymes (Gstm4, Gsta2 and Ephx1) was also recorded following phenobarbital treatment.

Recovery Phase:

For the females previously treated with 1500 ppm fluopyram, there were no relevant changes in any of the body weight parameters during the recovery phase. A statistically significant reduction ($p \leq 0.05$) in food consumption was, however, recorded during the second and third week of the recovery phase. There was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Centrilobular and global hepatic cell proliferation were still statistically significantly increased compared to the controls (+81%, $p \leq 0.01$ and +51%, $p \leq 0.05$, respectively); however these observed increases were lower than those observed immediately following treatment with 1500 ppm fluopyram (215% and 188% for centrilobular and global cell proliferation, respectively). Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity/gene expression for BROD/Cyp3a3, PROD/Cyp2b1, EROD/Cyp1a1 and UDPGT-bilirubin. Marginal increases in the expression of Gstm4, Ephx2 and Gadd45b were also recorded. As with the cell proliferation parameters, the magnitude of these molecular and enzymatic changes was much lower than those recorded immediately following treatment.

A similar profile was observed for the females previously treated with 80 mg/kg/day phenobarbital. In these animals a loss or a reduction in body weight gain was recorded during the first two weeks of the recovery phase, but there was no effect on terminal body weight or any organ weights at the end of the recovery phase nor were there any macroscopic or microscopic changes recorded in the liver. Hepatic cell proliferation was still statistically significantly increased compared to the controls; however the magnitude of the increases were generally lower than those observed immediately following treatment with phenobarbital. Hepatic molecular and enzymatic changes were still apparent at the end of the recovery phase as evidenced by increased enzyme activity and/or gene expression for, PROD/Cyp2b1, EROD, Cyp3a3 and UDPGT-bilirubin. Marginal increases in the expression of Cyp4a1, Gstm4 and Ephx1 were also recorded. As with the cell proliferation parameters, the magnitude of these molecular and enzymatic changes was generally lower than those recorded immediately following treatment.

Overall, treatment with fluopyram for at least 28 days induced clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression). These changes were dose-related beginning from 75 ppm. The dose of 30 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of Cyp3a3, with no other correlated findings, at this dose level). The hepatic changes appeared to be reversible as evidenced by the reduced hepatic responses recorded in females previously treated with 1500 ppm fluopyram following the recovery period.

Materials and methods

Test Material:	Fluopyram, CAS 658066-35-4
Description:	Light beige solid
Lot/Batch:	Mix-Batch:08528/0002
Purity:	94.7 %
Vehicle:	None
Stability of test compound:	Stable for a period covering the study duration
Positive control	Phenobarbital, white crystalline powder, CAS 50-06-6
Lot	09050075
Purity	99 %
	Stable in 0.5% aqueous solution of methylcellulose for a period covering the study duration

Species:	Rat	
Strain:	Wistar Rj: WI (IOPS HAN) – females only	
Age / weight:	10 weeks approx.; at study start: 215 to 261 g	
Source/breeder:	R. Janvier, Le Genest St Isle, France	
Acclimation period:	6-8 days	
Housing:	Animals were caged individually in suspended stainless steel wire mesh cages.	
Environmental conditions:	Temperature:	20 □□24°C
	Humidity:	40 □□70 %
	Air changes:	Approximately 10-15 changes per hour
	Photoperiod:	Alternating 12-hour light and dark cycles (7 am-7 pm)
Diet:	Certified rodent powdered and irradiated diet A04C-10 P1 from S.A.F.E. (Scientific Animal Food and Engineering, Epinay-sur-Orge, France), *ad libitum*, except for overnight fasting prior to scheduled necropsy	
Water:	Tap water, *ad libitum*	

Seven groups of female rats were dosed for at least 28 days with the appropriate compound by the appropriate route of administration.

Six groups were dosed by dietary administration. One group consisted of control animals that received untreated diet and the remaining five groups received fluopyram at the appropriate dietary concentration (30, 75, 150, 600 and 1500 ppm) at a constant level. The dose levels for fluopyram were set after evaluation of the results from previous studies conducted with the compound and following discussions with the US (EPA) and Canadian (PMRA) authorities. The top dose level of 1500 ppm used in the present study represents the top dose level used in the rat cancer bioassay in which an increase in liver tumors was observed in the females.

The seventh group was dosed by oral gavage with 80 mg/kg/day phenobarbital suspended in 0.5% aqueous methylcellulose 400 using a dosing volume of 5ml/kg bodyweight. The volume administered to each rat was based on the most recent recorded body weight. A dose level of 80 mg/kg/day was chosen for phenobarbital as this dose is known to clearly induce liver cytochrome P-450 activity and cell proliferation in the rat.

Each group consisted of 15 female rats with the exception of the control group, the highest dose fluopyram group and the phenobarbital group, where 15 additional females were fed control or test diet (1500 ppm fluopyram) or were orally dosed (80 mg/kg/day phenobarbital) for at least 28 days and were then allowed one month (at least 28 days) of recovery during which they were maintained on untreated control diet.

All animals were sacrificed in the morning after the last day of treatment or after the last day of the recovery phase.

Animal housing and husbandry were in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH publication N°86-23, revised 1985) and “Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, N°86/609/CEE du 24 Novembre 1986”.

Table 215: Study design

Group	Test Substance	Dose level	Number of animals per group
FEMALES			
1	Control	0	15 + 15*
2	Fluopyram	30 ppm	15
3		75 ppm	15
4		150 ppm	15
5		600 ppm	15
6		1500 ppm	15 + 15*
7	Phenobarbital	80 g/kg/day	15 + 15*

*Animals allocated to recovery phase

Diet preparation and analysis

Fluopyram (AE C656948) was incorporated into the diet by dry mixing to provide the required concentrations. There was one preparation for each concentration used in the study. The stability was demonstrated in an earlier study at concentrations of 20 and 10000 ppm for a time which covered the period of usage and storage for the current study. Homogeneity of test substance in diet was verified on the lowest and highest concentrations to demonstrate adequate formulation procedures. The mean value obtained in homogeneity check was used as measured concentration. For the remaining dietary levels, concentration was checked.

Two formulations of phenobarbital were prepared by suspending the test substance (w/w) in a 0.5% aqueous solution of methylcellulose. The stability of phenobarbital at 8 g/l was demonstrated in an earlier study, which covered the period of usage and storage for the current study. Homogeneity of phenobarbital in aqueous methylcellulose was verified to demonstrate adequate formulation formulation procedures. The mean value obtained in homogeneity check was used as measured concentration.

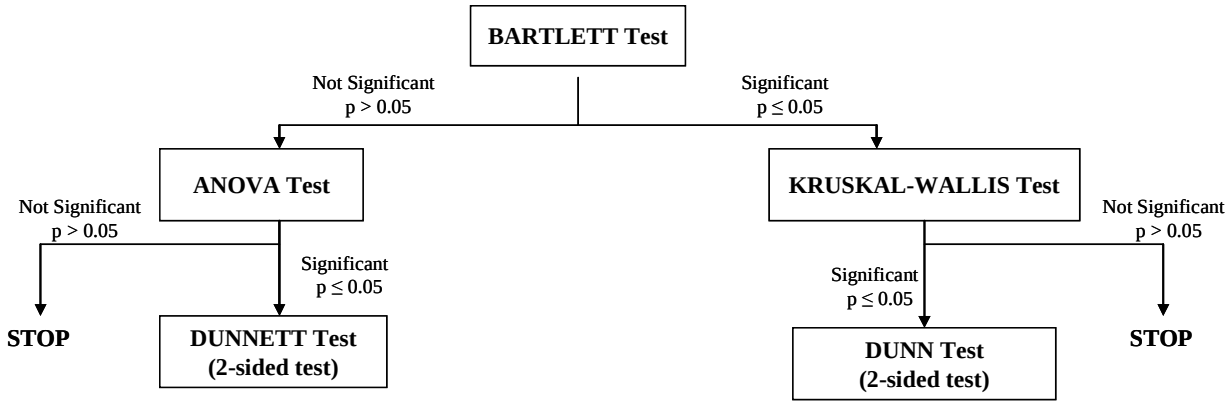
Statistics

Mean and standard deviation were calculated for each group. Group means were compared at the 5% and 1% levels of significance. The statistical analyses of the cell proliferation data were carried out by the statistician using the methods described in the appropriate paragraphs. All other statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics).

Dosing Phase: comparison between fluopyram groups and control group

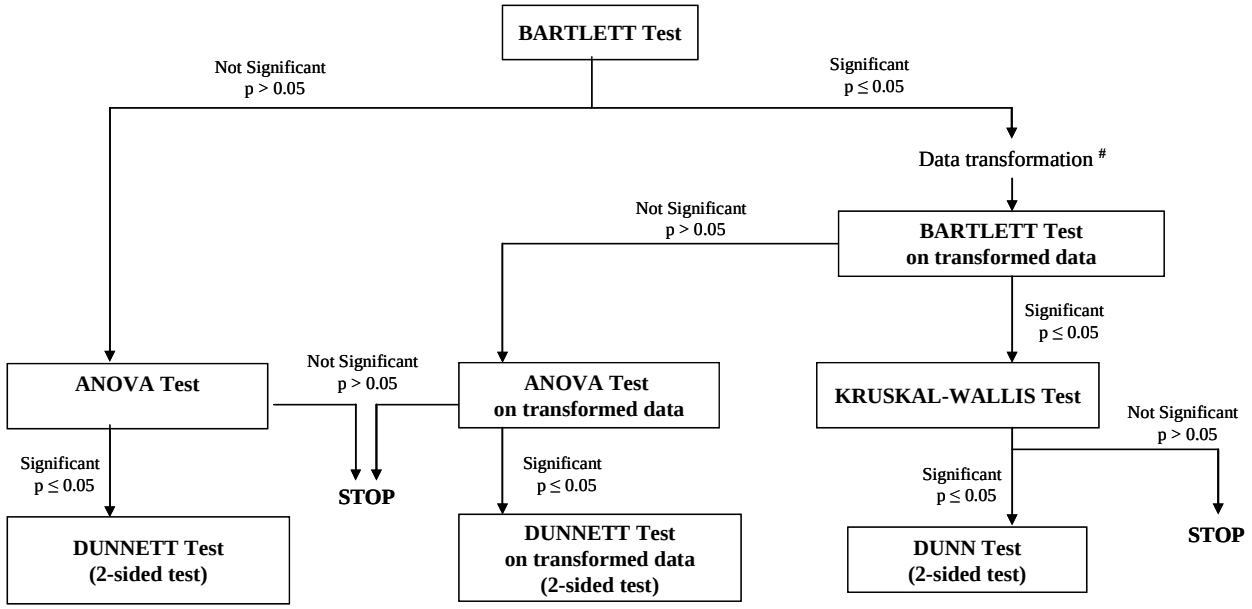
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

The mean and standard deviation will be calculated for each group and per time period for body weight change parameters.



- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



Data will be transformed using the log transformation for body weight parameters.

- Cell proliferation parameters

The Levene test was performed to compare the homogeneity of group variances. As the Bartlett test of homogeneity of variances is very sensitive to non-normality of data, the Levene test was preferred.

If the Levene test was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided).

If the Levene test was significant (p≤0.05), data were transformed using the log transformation.

If the Levene test on log transformed data was not significant (p>0.05), means of the exposed groups were compared to the mean of the control group using the Dunnett test (1-sided) on log transformed data.

If the Levene test was significant (p≤0.05) even after log transformation, means of the exposed groups were compared to the mean of the control group using the Dunn test (1-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

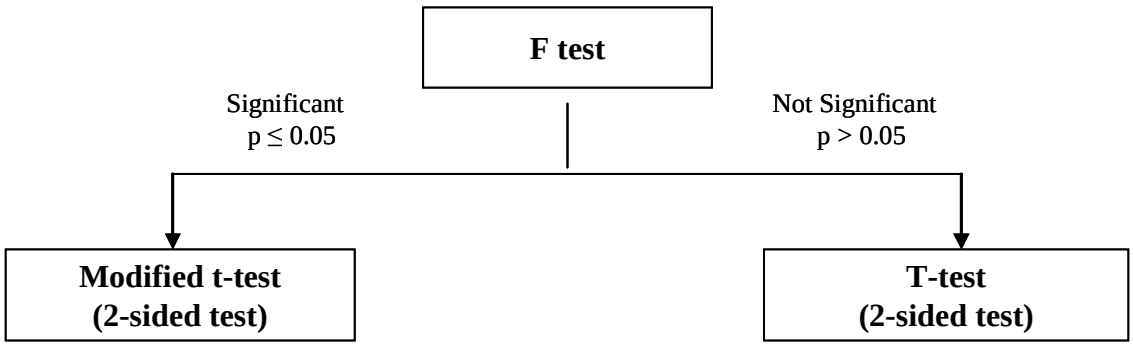
Dosing Phase: comparison between phenobarbital group and control group

Recovery Phase: comparison between high dose fluopyram group and control group

Recovery Phase: comparison between phenobarbital group and control group

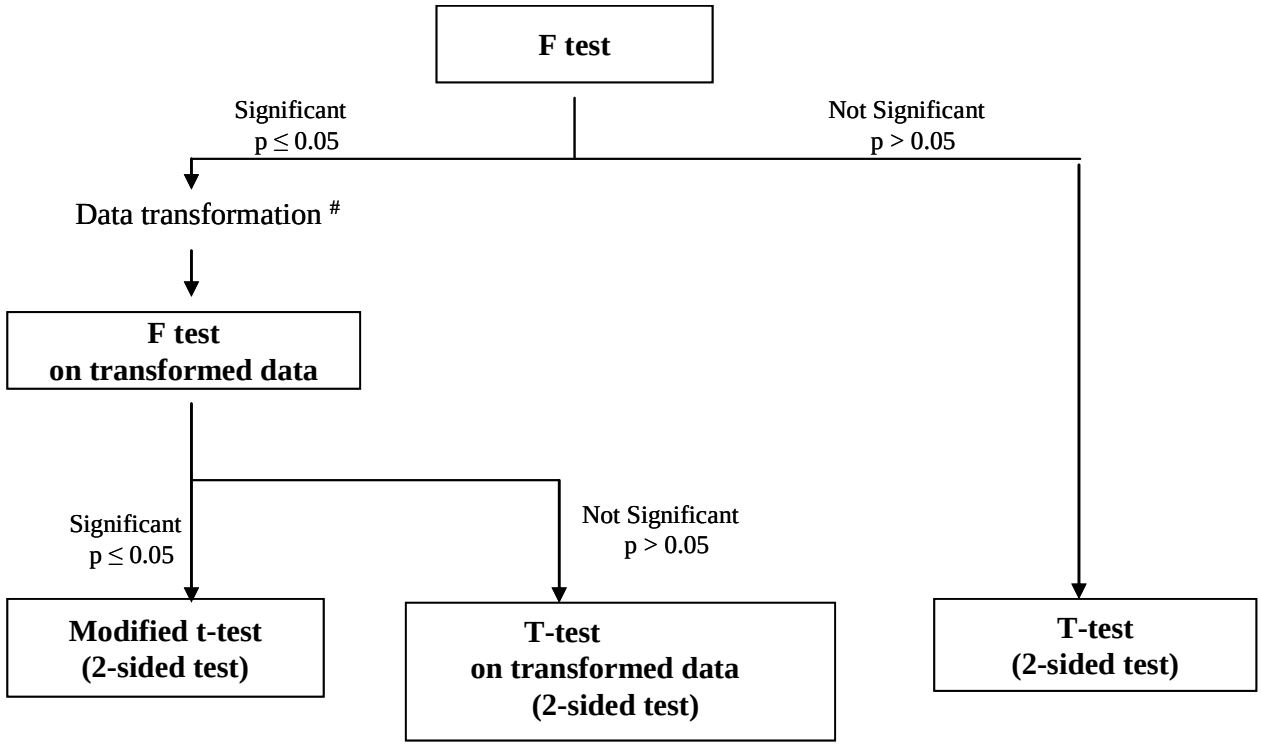
- Body weight change parameters,
- Terminal body weight, absolute and relative organ weight parameters,

The mean and standard deviation were calculated for each group and per time period for body weight change parameters.



- Body weight and average food consumption/day parameters
- Total cytochrome P450 content and liver enzyme activities
- Gene transcript analyses

The mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



Data will be transformed using the log transformation for body weight parameters.

- Cell proliferation 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 test group (high dose fluopyram or phenobarbital) was compared to the mean of the control group using the t-test (1-sided). If the F test was significant (p≤0.05), data were transformed using the log transformation. If the F test on log transformed data was not significant (p>0.05), mean of the test group (high dose fluopyram or phenobarbital) were compared to the mean of the control group using the t-test (1-sided) on log transformed data. If the F test was significant (p≤0.05), even after log transformation, mean of the test group were compared to the mean of the control group using the exact Mann-Whitney test (1-sided). If one or more group variance(s) equal 0, the means were compared using the non-parametric procedures. Group means were compared at the 5% and 1% levels of significance.

With the exception of those used for the cell proliferation data, all statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). SAS programs (version 9) were used for the cell proliferation data.

Observations

Observations were performed as described previously (Tinwell 2011).

Body weight

Each animal was weighed during the acclimatization period. Body weights were also measured on Day 1 and at least weekly during treatment and the recovery phase. Additionally, diet fasted animals were weighed before scheduled necropsy (terminal body weight).

Food consumption and compound intake

The weight of food supplied was measured on Day 1 and weekly thereafter. Empty feeder weights were measured weekly. The weight of food supplied to each animal and that remaining at the end of the food consumption period was recorded weekly. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for fluopyram for each week and for Weeks 1 to 4 was calculated.

Sacrifice and pathology

Necropsy procedure – Organ sampling

All animals were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane) in the morning of Day 30 of the treatment phase and Day 29 of the recovery phase. Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities.

Brain and liver were weighed fresh at scheduled sacrifice.

Duodenum and two central sections of the liver taken from the left and medial lobes from each animal were fixed by immersion in neutral buffered 10% formalin. In addition, a piece of the median and the left lobe of the liver from each animal were collected and flash frozen in liquid nitrogen, were stored at approximately -74°C + 10°C until used for qPCR investigations. At each scheduled sacrifice, the remaining portions of liver from each of 5 randomly selected females per group were weighed and homogenized for microsomal preparations.

Histotechnology

Conventional histopathological examination and Ki67 staining for cell proliferation assessment were performed as described above (Tinwell 2011).

Hepatotoxicity testing

At both scheduled sacrifice times, the remaining portions of the liver from five randomly selected females per group were weighed and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 isoenzyme profile (including EROD, BROD and PROD activities) to check the hepatotoxic potential of the test substance. Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with bilirubin as substrate. Results were compared to those generated with well known reference compounds.

Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry (Cary Win UV version 3.0 (182)) using a reduced CO differential spectrum. A single quantification was performed for each sample.

Specific cytochrome P-450 enzymatic activities were evaluated as described above (Tinwell 2011).

Phase II enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) with 4-nitrophenol as substrate as described above (Tinwell 2011).

Quantitative PCR Analyses

Total cytoplasmic RNA isolation, reverse transcription and quantitative realt time PCR was performed as described above (Tinwell 2011), except for that for Cyp2b1 a different Taqman assay (ID Cyp2b1_tc5, Refset ID 4331248) was used. Beta-microglobulin (B2m; Refset ID: NM_012512.1; Taqman assay ID: Rn00560865_m1) was selected as reference gene for the quantitative calculations of transcripts in the liver. The relative quantity (RQ) value of each test transcript was calculated as described above (Tinwel 2011).

Results and discussion

Mortality:

There was no mortality in any group during the course of the study.

Clinical signs:

Treatment phase:

Fluopyram: Four females in three different treatment groups had ocular discharge from Week 3 of treatment. One of the four females also had red conjunctiva in the same eye as the discharge.

Phenobarbital: All females displayed reduced motor activity starting from Week 2 of treatment. In addition, three females had ocular discharge and/or lacrimation in either one eye or both eyes starting from Week 2 of treatment. Other clinical signs included increased salivation for two females during the final week of treatment and soiling around the mouth or the head.

Recovery phase:

The few clinical signs (described below) recorded during the recovery phase were considered not to be treatment-related.

Fluopyram: Two females at 1500 ppm exhibited soiled fur around the head region during Week 2 of the recovery phase.

Phenobarbital: One female exhibited soiled fur around the nose during Week 4 of the recovery phase and one female had ocular discharge from one eye during Week 2 of the recovery phase.

Body weight and body weight gain:

Treatment phase:

Fluopyram: There were no relevant treatment-related changes in any of the body weight parameters compared to the controls during the treatment phase.

Phenobarbital: There were no relevant changes in any of the body weight parameters compared to the controls during the treatment phase.

Recovery phase:

Fluopyram: There were no relevant changes in any of the body weight parameters during the recovery phase compared to the controls.

Phenobarbital: Between Days 1 and 8 of the recovery phase, there was a mean absolute body weight loss compared to the control group (-2g compared to +8g in the control group; -125%, $p \leq 0.01$). Between Days 8 and 15 of the recovery phase there was a reduced mean absolute body weight gain compared to the control group (5g compared to 10g in the control group; -50%, not statistically significant). Thereafter, the mean absolute body weight gains were comparable to the controls.

Food consumption and compound intake:

Treatment phase:

Fluopyram: Food consumption was statistically significantly reduced at 1500 ppm during week 3 ($p \leq 0.05$) and week 4 ($p \leq 0.01$) of treatment. All other changes were considered non-relevant.

Phenobarbital: Food consumption was increased by up to 12% compared to the controls for all periods during the treatment phase. This increase was statistically significant for the first ($p \leq 0.05$), second ($p \leq 0.01$) and fourth ($p \leq 0.05$) week of treatment.

Recovery phase:

Fluopyram: A marginal, though statistically significant reduction ($p \leq 0.05$) in food consumption was recorded during the second and third week of the recovery phase.

Phenobarbital: A significant reduction ($p \leq 0.01$) in food consumption was recorded for the first week of the recovery phase, which could be attributed to two females with an apparent reduced food intake. Thereafter, food intake was comparable to the controls.

Table 216: Mean achieved intake of fluopyram

Diet Concentration (ppm) of fluopyram	Mean achieved dietary intake of fluopyram (mg/kg/day) for weeks 1-4
30	2.2
75	5.6
150	11.3
600	44.5
1500	111.4

Sacrifice and pathology

Terminal body weight and organ weight

Treatment phase:

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls.

At 600 and 1500 ppm, mean absolute and relative liver weight were statistically significantly higher when compared to controls.

At 150 ppm, mean liver to body weight ratio and mean liver to brain weight ratio were statistically significantly higher when compared to controls.

These changes were considered to be treatment-related.

Table 217: Mean absolute and relative liver weight changes following 28 days treatment with fluopyram

Mean liver weight ±SD at scheduled sacrifice (% change when compared to controls)						
Dose-level (ppm)	0	30	75	150	600	1500
Fluopyram						
Absolute liver weight (g)	6.14±0.53	6.10±0.26 (-1%)	6.48±0.46 (+6%)	6.64±0.77 (+8%)	6.78±0.49* (+10%)	7.90±0.53** (+29%)
Liver to body weight ratio (%)	2.38±0.16	2.42±0.11 (+2%)	2.49±0.13 (+5%)	2.55±0.11** (+7%)	2.69±0.15** (+13%)	3.16±0.14** (+33%)
Liver to brain weight ratio (%)	319.44 ±31.96	319.41 ±18.44 (0%)	342.46 ±24.12 (+7%)	353.35 ±39.95* (+11%)	368.06 ±28.60** (+15%)	424.55 ±30.85** (+33%)

*:$p \leq 0.05$, **:$p \leq 0.01$

The few other organ weight changes were considered to be incidental.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls.

Mean absolute and relative liver weight were statistically significantly higher when compared to controls following phenobarbital treatment.

Table 218: Mean absolute and relative liver weight changes following 28 days treatment with phenobarbital

Mean liver weight ±SD at scheduled sacrifice (% change when compared to controls)		
Dose-level	0 ppm	80 mg/kg
Phenobarbital		
Absolute liver weight (g)	6.14 ± 0.53	7.48 ± 1.01** (+22%)
Liver to body weight ratio (%)	2.382 ±0.160	2.936 ±0.352** (+23%)
Liver to brain weight ratio (%)	319.44 ± 31.96	412.05 ± 51.53** (+29%)

**:p≤0.01

The few other organ weight changes were considered to be incidental.

Recovery phase:

Fluopyram: There was no change in mean terminal body weight in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental.

Phenobarbital: There was no change in mean terminal body weight in treated animals when compared to the controls.

The few organ weight changes were considered to be incidental.

Gross pathology

Treatment phase:

Fluopyram: Enlarged liver was observed starting from 75 ppm and this observation could be correlated with microscopic findings starting from 600 ppm.

Other changes were considered as incidental and not treatment-related (no correlation with microscopic findings).

Table 219: Macroscopic changes in the liver following 28 days treatment with fluopyram

Incidence of macroscopic changes in the liver, scheduled sacrifice						
Dose-level (ppm)	0	30	75	150	600	1500
Fluopyram						
Enlarged	0/15	0/15	3/15	5/15	4/15	14/15

Phenobarbital: Enlarged livers were noted in 10/15 animals treated with phenobarbital.

Other changes were considered as incidental and not treatment-related.

Recovery phase:

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

Microscopic pathology

Treatment phase:

Fluopyram: Higher incidences of hepatocellular hypertrophy compared to the controls were noted at 600 and 1500 ppm and were considered to be treatment-related.

Phenobarbital: Higher incidences of hepatocellular hypertrophy and increased number of mitoses were noted and were considered to be treatment-related.

Table 220: Microscopic changes in the liver following 28 days treatment with fluopyram or phenobarbital

Incidence and severity of microscopic changes in the liver, all animals of the terminal sacrifice							
Dose level of Fluopyram (ppm)	0	30	75	150	600	1500	PB (80 mg/kg)
Number of examined animals	15	15	15	15	15	15	15
Hepatocellular hypertrophy: centrilobular to panlobular							
Minimal	0	0	0	0	6	7	6
Slight	0	0	0	0	0	6	5
Moderate	0	0	0	0	0	1	1
Total	0	0	0	0	6	14	12
Increased number of mitoses							
Present	0	0	0	0	0	0	1
Total	0	0	0	0	0	0	1

Recovery phase:

Fluopyram: All changes were considered as incidental and not treatment-related.

Phenobarbital: All changes were considered as incidental and not treatment-related.

Cell proliferation

Treatment phase:

Fluopyram: Centrilobular and global (referred to as "Total" in tables below) proliferation indexes were statistically significantly higher from 150 ppm when compared to controls. Periportal proliferation index was also significantly higher at 1500 ppm when compared to controls. Centrilobular proliferation was statistically significantly increased at 75 ppm.

Phenobarbital: Centrilobular and global proliferation indexes were statistically significantly higher when compared to controls.

Table 221: Mean cell proliferation index following 28 days treatment with fluopyram or phenobarbital

		Mean ± SD[A] (% change compared to control mean values)		
Compound	Dose	Centrilobular	Periportal	Total
Control	0	4.93 ± 3.11	8.37 ± 4.75	6.65 ± 3.19
Fluopyram	30 ppm	4.23 ± 2.42 (-14%)	7.62 ± 3.66 (-9%)	5.93 ± 2.82 (-11%)
	75 ppm	7.23 ± 3.55* (+47%)	8.51 ± 3.86 (+2%)	7.87 ± 2.65 (+18%)
	150 ppm	10.16 ± 3.86** (+106%)	12.51 ± 3.97* (+49%)	11.33 ± 3.30** (+70%)
	600 ppm	10.14 ± 5.27** (+106%)	12.10 ± 8.36 (+45%)	11.12 ± 6.50** (+67%)
	1500 ppm	15.54 ± 7.33** (+215%)	22.80 ± 10.49** (+172%)	19.17 ± 7.20** (+188%)
Phenobarbital	80 mg/kg	21.46 ± 17.90** (+335%)	10.28 ± 7.24 (+23%)	15.87 ± 11.74** (+139%)

A: n = 15 females/group for cell proliferation determinations; *: p≤0.05; **: p≤0.01. Differences in data between table above and in Appendix K are due to rounding-up.

Recovery phase:

Fluopyram: Centrilobular and global proliferation indexes were statistically significantly higher when compared to the control group.

Phenobarbital: Centrilobular, periportal and global proliferation indexes were significantly higher when compared to the control group.

Table 222: Mean cell proliferation index following Recovery Phase

	Mean ± SD[A]		
	Centrilobular	**Periportal**	**Total**
Control	4.59 ± 2.44	8.25 ± 4.60	6.42 ± 3.29
1500 ppm	8.30 ± 3.75** (+81%)	11.12 ± 6.87 (+35%)	9.71 ± 4.77* (+51%)
PB (80 mg/kg)	6.91 ± 3.44* (+51%)	16.92 ± 9.83** (+105%)	11.92 ± 6.47** (+86%)

A: n = 15 females/group for cell proliferation determinations with the exception of the control group where n=14 instead of 15; *: p≤0.05; **: p≤0.01. Differences in data between table above and in Appendix K are due to rounding-up.

Hepatotoxicity testing

Treatment phase:

Fluopyram: A marginal, though not statistically significant increase in total P450 content was recorded at 1500 ppm. No statistically significant changes in enzyme activity were observed at 30 ppm and 75 ppm. Statistically significant increases in UDPGT-bilirubin (p≤0.01) were observed from 150 ppm. EROD activity was also statistically significantly increased from this dose level, however the increases (up to 96% at 1500 ppm) were considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls. BROD (p≤0.01), PROD (p≤0.05) and UDPGT-4-nitrophenol (p≤0.01) were statistically significantly increased from 600 ppm.

Table 223: Mean cytochrome P-450 content and enzymatic activities in the liver following

Fluopyram dose	**Mean Fold ChangeRelative to Controls**					
	Total P450 Content	**EROD**	**BROD**	**PROD**	**UDPGT-4-nitrophenol**	**UDPGT-bilirubin[A]**
30 ppm	0.94	1.13	1.24	0.92	0.82	1.09
75 ppm	0.97	1.08	1.44	1.27	0.95	1.21
150 ppm	1.24	1.34**	2.89	1.53	1.08	1.57**
600 ppm	1.05	1.31*	9.12**	1.87*	1.83**	2.14**
1500 ppm	1.42	1.96**	39.09**	4.76**	3.21**	2.76**
Phenobarbital 80 mg/kg	1.51**	1.07	87.4**	8.44*	1.81**	1.38*

A: n=4 instead of 5 for the control group due to one outlier animal. *: p≤0.05; **: p≤0.01

Recovery phase:

Fluopyram: EROD (p≤0.05), BROD (p≤0.05), PROD (p≤0.01) and UDPGT-bilirubin (p≤0.05) activity were statistically significantly increased following 1 month on control diet; however the increases were much lower than those recorded immediately following treatment.

Phenobarbital: PROD and UDPGT-bilirubin activity were statistically significantly increased (p≤0.01) following 1 month recovery. The increase in PROD activity was much lower than that recorded immediately after treatment; however UDPGT-bilirubin activity was marginally increased compared to immediately following treatment (1.56-fold following recovery compared to 1.38-fold following treatment). EROD was also significantly increased (p≤0.01) following the recovery phase despite there being no activity immediately following treatment. The increase (27%) was considered marginal in comparison with the positive control, beta-naphthoflavone, which induced EROD by 800% compared to controls.

Table 224: Mean cytochrome P-450 content and enzymatic activities in the liver following Recovery Phase

Dose	Mean Fold changeRelative to Controls					
	Total P450 Content	EROD	BROD	PROD	UDPGT-4-nitrophenol	UDPGT-bilirubin
Fluopram 1500 ppm	1.03	1.21*	1.47*	1.54**	1.01	1.39*
Phenobarbital 80 mg/kg/day	1.01	1.28**	0.85	1.93**	0.98	1.56**

*: p≤0.05; **: p≤0.01

Gene Transcript Analyses

Treatment phase:

Fluopyram: At 30 ppm, a clear and statistically significant (p≤0.01) dose-related increase in the expression of Cyp3a3 was recorded (+81% at 30 ppm up to +4943% at 1500 ppm).

From 150 ppm, clear dose-related and statistically significant (p≤0.01) increases were recorded for the expression of the Phase I genes Cyp2b1 (+990% at 150 ppm up to +154275% at 1500 ppm) and Cyp1a1 (+711% at 150 ppm up to +35372% at 1500 ppm). In addition, a statistically significant (p≤0.01) increase was recorded for the expression of genes coding for the Phase II enzymes Gstm4 (+136% at 150 ppm up to 1289% at 1500 ppm) and Udpgtr2 (+64% at 150 ppm up to +304% at 1500 ppm).

From 600 ppm, genes coding for additional Phase II enzymes were statistically significantly increased. Specifically, Gsta2 expression was increased by 53% (p≤0.05) at this dose and Ephx1 expression was increased by +116% (p≤0.01). A marginal, though statistically significant (+75%; p≤0.01), increased expression of the marker for cell proliferation (Gadd 45b) was also observed.

Finally, the expression of Ephx2 demonstrated a weak but dose-related decrease (-3%, -10%, -13%, -27% at 30, 75, 150 and 600 ppm respectively), which was statistically significant at 1500 ppm (-43%; p≤0.01).

There were no clear effects on the expression of POR, Cyp4a1, Tacstd1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which was greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples).

Table 225: Mean gene transcript analyses following 28 days treatment with fluopyram

	Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values)					
Gene transcripts	Control	30 ppm	75 ppm	150 ppm	600 ppm	1500 ppm
METABOLISM: Phase I						
POR	0.78 ± 0.34	0.70 ± 0.29 (-10%)	0.67 ± 0.29 (-14%)	0.77 ± 0.27 (NC)	0.81 ± 0.24 (+4%)	0.89 ± 0.30 (+14%)
Cyp1a1	1.06 ± 0.70	1.87 ± 1.25 (+76%)	2.43 ± 1.48 (+129%)	8.60 ± 7.02** (+711%)	107.0 ± 28.03** (+999%)	376.0 ± 130.43** (+35372%)
Cyp2b1	1.26 ± 1.15	3.38 ± 7.17 (+168%)	2.09 ± 1.91 (+66%)	13.74 ± 10.57** (+990%)	267.69 ± 193.39** (+21145%)	1945.13 ± 1518.28** (+154275%)
Cyp3a3	1.66 ± 0.65	3.01 ± 1.34** (+81%)	6.19 ± 2.66** (+273%)	8.72 ± 2.69** (+425%)	28.37 ± 7.22** (1609%)	83.72 ± 27.02** (+4943%)
Cyp4a1	0.78 ± 0.30	0.65 ± 0.25 (-17%)	0.69 ± 0.20 (-12%)	0.77 ± 0.16 (NC)	0.63 ± 0.18 (-19%)	0.55 ± 0.08 (-30%)
METABOLISM: Phase II						
Gsta2	3.62 ± 2.63	3.76 ± 2.19 (+4%)	3.43 ± 2.34 (-5%)	3.28 ± 2.37 (-9%)	5.54 ± 2.54* (+53%)	10.85 ± 6.21** (+200%)
Gstm4	0.56 ± 0.39	0.60 ± 0.36 (+7%)	0.92 ± 0.62 (+64%)	1.32 ± 0.48** (+136%)	3.42 ± 2.82** (+511%)	7.78 ± 6.64** (+1289%)

Udpgtr2	0.90 ± 0.40	1.08 ± 0.40 (+20%)	1.04 ± 0.58 (+16%)	1.48 ± 0.51** (+64%)	2.29 ± 0.92** (+154%)	3.64 ± 1.51** (+304%)
Ephx1	1.06 ± 0.22	1.34 ± 0.60 (+26%)	1.18 ± 0.31 (+11%)	1.49 ± 0.35 (+41%)	2.29 ± 0.58** (+116%)	4.49 ± 2.04** (+324%)
Ephx2	1.73 ± 0.64	1.67 ± 0.64 (-3%)	1.55 ± 0.74 (-10%)	1.51 ± 0.66 (-13%)	1.27 ± 0.54 (-27%)	0.98 ± 0.29** (-43%)
Sult1e1	ND	ND	ND	ND	ND	ND
CELL PROLIFERATION/APOPTOSIS						
Tacstd1	1.09 ± 0.31	1.30 ± 0.38 (+19%)	1.16 ± 0.23 (+6%)	1.35 ± 0.25 (+24%)	1.23 ± 0.28 (+13%)	1.35 ± 0.32 (+24%)
Gadd45b	0.79 ± 0.23	0.85 ± 0.44 (+8%)	0.76 ± 0.20 (-4%)	0.75 ± 0.20 (-5%)	1.38 ± 0.49** (+75%)	1.75 ± 0.89** (+122%)
Rb1	1.15 ± 0.26	1.14 ± 0.19 (NC)	1.10 ± 0.19 (-4%)	1.21 ± 0.18 (+5%)	1.16 ± 0.19 (NC)	1.16 ± 0.23 (NC)

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to thevariable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (59/90 control and fluopyram treated liver samples). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The gene expression data indicated that treatment with phenobarbital leads to dose-related increased expression of Phase I and Phase II enzymes. In particular, Cyp2b1, Cyp3a3, both isoforms of glutathione (Gstm4 and Gsta2), Ephx1 and Udpgt were all statistically significantly increased. In contrast there was a statistically significant (p≤0.01) down-regulation of Cyp4a1 and Ephx2 expression.

The marker for cell proliferation (Gadd45b) was also statistically significantly increased.

There were no clear effects on the expression of POR, Cyp1a1 and Rb1. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (8/15 phenobarbital treated liver samples).

Table 226: Mean gene transcript analyses following 28 days treatment with phenobarbital

	Mean Relative Quantity ± standard deviation of gene transcripts (28 day treatment) (% change compared to control mean values)	
Gene transcripts	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.78 ± 0.34	0.60 ± 0.25 (-23%)
Cyp1a1	1.06 ± 0.70	0.80 ± 0.41 (-25%)
Cyp2b1	1.26 ± 1.15	2930.45 ± 2578.47** (+232475%)
Cyp3a3	1.66 ± 0.65	54.21 ± 43.44** (+32%)
Cyp4a1	0.78 ± 0.30	0.41 ± 0.08** (-47%)
METABOLISM: Phase II		
Gsta2	3.62 ± 2.63	6.33 ± 4.30* (+75%)
Gstm4	0.56 ± 0.39	13.85 ± 11.39** (+2373%)
Udpgtr2	0.90 ± 0.40	3.92 ± 2.66** (+336%)
Ephx1	1.06 ± 0.22	3.67 ± 1.77** (+246%)
Ephx2	1.73 ± 0.64	0.95 ± 0.27** (-45%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.09 ± 0.31	1.16 ± 0.42 (+6%)
Gadd45b	0.79 ± 0.23	1.33 ± 0.59** (+68%)
Rb1	1.15 ± 0.26	1.28 ± 0.23 (+11%)

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to thevariable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (19/30 for controls and phenobarbital). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Recovery phase:

Fluopyram: The expression of genes coding for the Phase I enzymes Cyp1a1 (+83%), Cyp2b1 (+70%) and Cyp3a3 (+157%; p≤0.01) and the Phase II enzyme Gstm4 (+73%; p≤0.05) were still increased compared to the controls. Gadd45b (+42%; p≤0.01) and Rb1 (+13%; p≤0.05) were also statistically significantly increased compared to the controls. In all cases however; the % changes were much lower than those recorded immediately following treatment.

Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than the cut-off limit of 35 for the majority of samples (25/30 control and fluopyram treated liver samples).

Table 227: Mean gene transcript analyses following Recovery Phase in fluopyram treated group

	Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values)	
Gene transcripts	Control	1500 ppm
METABOLISM: Phase I		
POR	0.54 ± 0.24	0.62 ± 0.18 (+15%)
Cyp1a1	0.72 ± 0.23	1.32 ± 1.11 (+83%)
Cyp2b1	0.30 ± 0.24	0.51 ± 0.36 (+70%)
Cyp3a3	2.67 ± 1.61	6.86 ± 3.75** (+157%)
Cyp4a1	0.68 ± 0.29	0.76 ± 0.14 (+12%)
METABOLISM: Phase II		
Gsta2	1.55 ± 1.37	1.67 ± 1.12 (+8%)
Gstm4	0.74 ± 0.48	1.28 ± 0.95* (+73%)
Udpgtr2	0.83 ± 0.33	0.65 ± 0.25 (-22%)
Ephx1	0.80 ± 0.19	0.86 ± 0.31 (+8%)
Ephx2	0.85 ± 0.26	1.11 ± 0.29* (+31%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.21 ± 0.29	1.31 ± 0.27 (+8%)
Gadd45b	1.38 ± 0.42	1.96 ± 0.57** (+42%)
Rb1	1.02 ± 0.14	1.15 ± 0.17* (+13%)

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to thevariable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (25/30 for controls and fluopyram). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Phenobarbital: The expression of genes coding for the Phase I enzymes Cyp2b1 (+107%; p≤0.01), Cyp3a3 (+179%; p≤0.01) and Cyp4a1 (+46%; p≤0.01) and the Phase II enzyme Gstm4 (+181%; p≤0.01) were still increased compared to the controls. Ephx1 (+21%; p≤0.05) was also increased following the recovery phase. However, with the exception of Cyp4a1 and Ephx1, which were not increased immediately following treatment, the % changes were much lower than those recorded immediately following treatment. Sult1e1 was only weakly expressed in the liver as evidenced by the variable and high Ct (cycle threshold) values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 control and phenobarbital treated liver samples).

Table 228: Mean gene transcript analyses following Recovery Phase in phenobarbital treated group

	Mean Relative Quantity ± standard deviation of gene transcripts (Recovery Phase) (% change compared to control mean values)	
Gene transcripts	Control	Phenobarbital (80 mg/kg/day)
METABOLISM: Phase I		
POR	0.54 ± 0.24	0.76 ± 0.23* (+41%)
Cyp1a1	0.72 ± 0.23	0.87 ± 0.45 (21%)
Cyp2b1	0.30 ± 0.24	0.62 ± 0.46** (+107%)
Cyp3a3	2.67 ± 1.61	7.46 ± 5.02** (+179%)
Cyp4a1	0.68 ± 0.29	0.99 ± 0.29** (+46%)
METABOLISM: Phase II		
Gsta2	1.55 ± 1.37	1.98 ± 2.11 (+28%)
Gstm4	0.74 ± 0.48	2.08 ± 1.22** (+181%)
Udpgtr2	0.83 ± 0.33	0.69 ± 0.38 (-17%)
Ephx1	0.80 ± 0.19	0.97 ± 0.22* (+21%)
Ephx2	0.85 ± 0.26	1.03 ± 0.40 (+21%)
Sult1e1	ND	ND
CELL PROLIFERATION/APOPTOSIS		
Tacstd1	1.21 ± 0.29	1.20 ± 0.26 (NC)
Gadd45b	1.38 ± 0.42	1.57 ± 0.41 (+14%)
Rb1	1.02 ± 0.14	1.16 ± 0.22 (+14%)

NC: no change; *: p≤0.05; **: p≤0.01; ND: considered as not detected due to thevariable and high Ct values, which were greater than or equal to the cut-off limit of 35 for the majority of samples (24/30 for controls and phenobarbital). In the above table the presented % changes do not always calculate exactly from the presented mean data. This is due to rounding-up differences.

Conclusion

Overall, treatment with fluopyram for at least 28 days induced clear and statistically significant changes in the liver (cell proliferation, hypertrophy and enzyme activity as well as associated changes in gene expression). These changes were dose-related beginning from 75 ppm. The dose of 30 ppm was considered as a No Observed Adverse Effect Level (NOAEL; based on the increased gene expression of Cyp3a3, with no other correlated findings, at this dose level). The hepatic changes appeared to be reversible as evidenced by the reduced hepatic responses recorded in females previously treated with 1500 ppm fluopyram following the recovery period.

4.12.1.4 Human information

Medical surveillance on manufacturing plant personnel

Medical surveillance, not directly related to exposures, did not reveal any unwanted effects in the workers, nor have there been any medical complaints regarding the product.

Report on clinical cases and poisoning incidents

No poisoning incidents or clinical cases have been seen.

Observations on general population exposure & epidemiological studies

There has been no exposure of the general public so far.

Clinical signs and symptoms of poisoning and details of clinical tests

There are no reported cases in humans

4.12.2 Summary and discussion

In an acute neurotoxicity study, a single dose of fluopyram was administered by gavage to young adult Wistar rats at nominal doses of 0 (vehicle control), 125, 500 or 2000 mg/kg bw. Effects in males and/or females consisted of decreased motor and locomotor activity on the day of treatment, urine stain, and decreased body temperature. A clear NOAEL of 125 mg/kg bw could be established for male rats whereas in females motor and locomotor activity was still impaired at this lowest dose level. Therefore, a follow-up study was conducted under the same conditions but only in females at nominal doses of 0 (vehicle), 25, 50 or 100 mg/kg bw to establish an overall No-Observed-Adverse-Effect Level (NOAEL). Because slight decreases in (loco)motor activity became apparent at 100 mg/kg bw, the next lower dose of 50 mg/kg bw was considered the NOAEL for females. There were no gross or microscopic lesions in the nervous system up to the highest dose of 2000 mg/kg bw.

In a 90-day neurotoxicity study, fluopyram was administered in the diet for 13 weeks to Wistar rats at nominal concentrations of 0, 100, 500 and 2500 ppm. No evidence of neurotoxicity was observed at any treatment level and, accordingly, the highest dose (equivalent to a mean daily intake of 164 mg/kg bw by male rats and of 197 mg/kg bw by females) was considered the NOAEL for this endpoint. Treatment-related findings of general toxicty at the high dose consisted of decreases in body weight, total body weight gain and food consumption in males and females, some alterations in clinical chemistry and haematological parameters and an increase in the organ weights of liver, thyroid and kidneys. A few of these findings such as lower food consumption, haematological changes and an increase in relative liver weight were also observed at the next lower dose level but were rather minor in nature. Thus, this mid dose of 500 ppm (corresponding to a mean daily intake of about 33 mg/kg bw in males and 41 mg/kg bw in females) was considered the overall NOAEL in this study.

Based on the whole toxicological profile of fluopyram and because there was no evidence of a specific neurotoxic potential, further investigations such a test for delayed neurotoxicity or a developmental neurotoxicity study are not warranted.

A data package including an acute oral toxicity study, *in vitro* genotoxicity tests and a 28-day short term toxicity study was provided for each of two plant metabolites of fluopyram (fluopyram-pyridyl-carboxylic acid, AE C657188, and fluopyram-methyl-sulfoxide, AE 1344122) that had not been found in the rat. Accordingly, toxic properties of these biotransformation products to which consumers might become exposed via residues cannot be considered as covered by toxicity testing of the parent compound. The first one occurred in grapes, potatoes, beans, red bell peppers, and in rotational crops whereas the second was only detected in low amounts in a rotational crop study so far. Fluopyram-pyridyl-carboxylic acid and -methyl-sulfoxide are also known to be metabolites of the fungicide fluopicolide. These metabolites were of no toxicological concern and that further investigations were not warranted. Both substances exhibited a very low acute oral toxicity and proved negative in the genotoxicity test battery. The subacute feeding studies revealed a lower toxicity of these metabolites when compared to fluopyram active ingredient.

High doses of fluopyram proved carcinogenic in long-term studies in both rats and mice (see 0). A number of mechanistic studies were performed in rats and mice but also *in vitro* to elucidate potential similarities between fluopyram and the well known tumour promoter phenobarbital (PB). Overall the mechanistic data presented by the notifier are neither sufficient nor convincing that fluopyram follows a mode of action that is not relevant for humans, when liver tumours are concerned. For the thyroid tumours in male mice, there is more convincing evidence that the mode of action might be of less relevance to humans.

Medical surveillance, not directly related to exposures, did not reveal any unwanted effects in the workers, nor have there been any medical complaints regarding the product.

4.12.3 Comparison with criteria

Criteria for acute toxicity, STOT and mutagenicity are presented in Table 17, Table 18 and Table 77 and in 4.2, 4.3, 0, 4.8 and 0 above. The metabolites investigated did not meet any of the criteria set under DSD or CLP for classification and labelling for these endpoints.

The mechanistic studies were used to further elucidate the carcinogenic potential of fluopyram. Hence they were compared with criteria at a different paragraph of this report (see 0).

4.12.4 Conclusions on classification and labelling

No further classification and labelling of fluopyram is regarded necessary due to the findings summarised in the section on other effects above.

5 ENVIRONMENTAL HAZARD ASSESSMENT

5.1 Degradation

Table 229: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Aqueous hydrolysis at pH 4, 7 and 9 (OECD 111)	stable at pH 4, 7 and 9	--	Doble; Oddy 2006 CX/06/015
Photolysis in water in buffer at pH 7	DT_{50} = 23 d corresponding to DT_{50} = 89 d (June, Athens, Greece)	--	Oddy; Brett 2008 CX/06/016
Phototransformation in natural water	DT_{50} = 21 d corresponding to DT_{50} =135 d (June, Athens, Greece)	--	Koehn; Stupp 2007 MEF-07/227
Biodegradation in water/sediment systems (OECD 308)	$DissT_{50}$ = 648 – 1190 d (whole system) DissT50 = 14-26 d (water)	--	Allan; Shepherd 2007 1599531

5.1.1 Stability

Hydrolytic degradation:

- Doble, M. L.; Oddy, A. M.; 2007; CX/06/015, M-282473-01

The hydrolysis of [phenyl-UL-^{14}C] Fluopyram was studied at 1.0 mg/L in the dark at 50 °C in sterile buffer solutions at pH 4, pH 7 and pH 9 for five days. Fluopyram is hydrolytically stable under acidic, neutral and alkaline conditions. No major degradation products were detected at pH 4, pH 7 and pH 9. One minor unidentified degradation product was detected with a maximum of 1.6 and 1.2 % of applied amount at pH 7 and pH 9, respectively. Another degradation product was detected at pH 9 with a maximum of 0.5 % of applied amount. No half-life could be calculated as the test compound was stable to hydrolysis at all pH conditions.

Photolysis in water:

- Oddy, A. M. and Brett, R.; 2008; CX/06/016, M-297180-02

The photolysis of phenyl and pyridyl labeled [^{14}C]Fluopyram in aqueous buffer solution (0.02 M phosphate, pH 7) at an initial nominal concentration of 1.0 mg a.i./L has been investigated under sterile conditions, at 25 ± 2 °C, with continuous artificial sunlight (< 290 nm cut-off filter) for a period of 13 days (equivalent to natural summer sunlight of 32 days at Phoenix, Arizona or 50 days at Athens, Greece). The recovered radioactivity for both labels was above 90 % in all cases.

Fluopyram undergoes limited transformation by indirect photolytic processes at 25 °C in aqueous buffer solution of pH 7 to Fluopyram-lactam (14 % AR) and 14 unidentified transformation products. None of the unidentified transformation products reached amounts greater than 4.2 % AR by the end of the 312 hour study period. The DT_{50} for Fluopyram in aqueous buffer solution of pH 7 was found to be 21.0 days (phenyl ^{14}C]-labelled and 25.0 days (pyridyl ^{14}C]-labelled) of continuous irradiation. The mean DT_{50} values equivalent to June summer days of natural sunlight at Phoenix, Arizona and Athens, Greece were 57 and 89, respectively. Aquatic photolysis is not considered to be an important transformation route for Fluopyram in the environment.

- Koehn, D. and Stupp, H. P.; 2007; MEF-07/227, M-296408-01

The aqueous phototransformation of Fluopyram was studied in natural water from the river Rhine at 25 °C by continuous exposure to a xenon lamp with < 290 nm cut-off filter for eight days. Two labels, [phenyl-UL-^{14}C]Fluopyram and [pyridyl-2,6-^{14}C]Fluopyram were used as test items at an initial concentration of 0.7 mg/L.

Photolytic degradation of Fluopyram in natural water is relatively slow under environmental conditions. Fluopyram phototransformed slowly with a DT_{50} of 21days (equivalent to 42.0 days, assuming 12 hour days of continuous sunlight) in natural water under continuous irradiation. The predicted environmental DT_{50} was 87 solar summer days at Phoenix, AZ, USA and 135 summer days at Athens, Greece. No major transformation products were detected. Minor transformation product, Fluopyram-lactam was observed at a maximum of 1.2 % of applied radioactivity. Phototransformation would not be a principle route of transformation in natural waters.

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

5.1.2.2 Screening tests

Readily biodegradability:

No studies on ready biodegradability according to OECD 301 and on inherent biodegradability were submitted. However, these studies are not deemed to be necessary, since higher tiered studies, namely simulation tests for the relevant environmental compartments 'water/sediment', are available.

5.1.2.3 Simulation tests

Biodegradation in water/sediment systems

- Allan, J. G., Shepherd, J. J.; 2007; MEGMP064, M-290531-01

The aerobic degradation of [phenyl-UL-^{14}C]AE C656948 and [pyridyl-2,6-^{14}C]AE C656948 (fluopyram) was studied in two pond water-sediment systems – Leverkusen, Germany and Lawrence, Kansas, U.S.A., for 120 days according to OECD Guideline for Testing of Chemicals Number 308.

No major transformation products were detected in the water or sediment phases. Five minor transformation products were detected over both systems. No single metabolite exceeded 2.5 % of the applied radioactivity.

The DFOP DT_{50} dissipation times for [^{14}C]Fluopyram in aerobic water were depending on the ^{14}C-labelling 25 and 26 days for German system and 14 and 17 days for U.S systems, respectively. The DT_{50} values of [^{14}C]Fluopyram in the entire system were estimated to be greater than 648 days in both sediment/water systems, which indicate that Fluopyram is persistent in the aquatic environment . Aerobic biotransformation would not be an important transformation route of Fluopyram in the aquatic environment.

The results are summarised in Table 230, Table 231 and Table 232.

Table 230: Degradation of fluopyram in water/sediment-systems

Water / sediment system	pH water phase	pH sed.	t. °C	$DissT_{50}$ - $DissT_{90}$ whole sys. [d]	St. (r^2)	$DissT_{50}$ - $DissT_{90}$ Water [d]	St. (r^2)	DT_{50} - DT_{90} sed. [d]	St. (r^2)	Method of calculation
Anglerweiher, Leverkusen, Germany	6.8	5.6	24	[phenyl ^{14}C]-labelled:						SFO (whole system); DFOP (water phase)
				1190 - 3960	0.8	25 - 284	2.1	--		
				[pyridyl ^{14}C]-labelled						
				1470 - 4900	1.3	26 - 293	1.8	--		
Lawrence, Jefferson County, Kansas, USA	7.3	5.3	n.m.	[phenyl ^{14}C]-labelled:						SFO (whole system); DFOP (water phase)
				1000 - 3330	0.9	14- 215	1.5	--		
				[pyridyl ^{14}C]-labelled						
				648- 2150	1.7	17 - 221	4.8	--		

Diss … dissipation, n.m. … not measured

St.(r^2)..coefficient of.mean variation

Table 231: Distribution of Fluopyram in water/sediment-systems (expressed as percentage of applied radioactivity (AR)

Water / sediment system	pH water phase	pH sed.	% AR in Water	% AR in Sediment
Anglerweiher, Leverkusen, Germany	6.8	5.6	[phenyl ^{14}C]-labelled : 91.3 % day 0 26.3 % at day 120 [pyridyl ^{14}C]-labelled: 89.6 % at day 0 25.5 at day 120	[phenyl ^{14}C]-labelled: 4.4 at day 0 62.8 % at day 120 [pyridyl ^{14}C]-labelled: 6.7 at day 0 63.3 % at day 120
Lawrence, Jefferson County, Kansas, USA	7.3	5.3	[phenyl ^{14}C]-labelled : 94 % day 0 18.8 % at day 120 [pyridyl ^{14}C]-labelled: 94 % at day 0 19.8 at day 120	[phenyl ^{14}C]-labelled: 3.8 at day 0 69.9 % at day 120 [pyridyl ^{14}C]-labelled: 3.9 at day 0 67.2 % at day 120

Table 232: Mineralisation and non extractable residues

Water / sediment system	pH water phase	pH sed.	Mineralisation x % after n d (end of the study)	Non-extractable residues in sed. max x % after n d ([phenyl/pyridyl ^{14}C]-labelled)	Non-extractable residues in sed. max x % after n d (end of the study) ([phenyl/pyridyl ^{14}C]-labelled)
Anglerweiher, Leverkusen, Germany	6.8	5.6	< 0.4% (120 d)	2.9/3.7% (120 d)	2.9/3.7% (120 d)
Lawrence, Jefferson County, Kansas, USA	7.3	5.3	< 1.8% (90 d)	4.4/8.4% (120 d)	4.4/8.4% (120 d)

5.1.3 Summary and discussion of degradation

No studies on ready biodegradability according to OECD 301 and on inherent biodegradability were submitted. However, these studies are not deemed to be necessary, since higher tiered studies, namely simulation tests for the relevant environmental compartments 'water/sediment', are available.

In water/sediment systems it was shown that fluopyram was persistent with $DissT_{50}$ values greater than 648 days and mean of $DissT_{50}$ = 20.25 days (water).

Fluopyram is hydrolytically stable under acidic, neutral and alkaline conditions. Aquatic photolysis is not considered to be an important transformation route for Fluopyram in the environment with DT_{50} of 23 days corresponding to DT_{50} = 89 d (June, Athens, Greece). The result of phototransformation study is DT_{50} of 21 days corresponding to DT_{50} = 135 d (June, Athens, Greece).

The results of the test on the biodegradation of Fluopyram in the water/sediment system and abiotic degradation show that Fluopyram is considered not rapidly degradable (a degradation > 70 % within 28 days) for purposes of classification and labelling.

5.2 Environmental distribution

Not relevant for this dossier.

5.3 Aquatic Bioaccumulation

Table 233:Summary of relevant information on aquatic bioaccumulation for fluopyram

Method	Results	Remarks	Reference
OECD 305	$BCF_{steady\ statc}$ = 13 (whole fish, parent)	Lipid normalized to 5 %	Bruns and Weber, 13.03.2008 EBGMP116 M-298506-01

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

The log $P_{o/w}$ of fluopyram is 3.3 at 20 °C. So there is an indication for bioaccumulation potential of fluopyram. A bioconcentration study on fish is triggered.

5.3.1.2 Measured bioaccumulation data

Table 234: Fish bioaccumulation study (Bruns & Weber, 2008)

Guideline / Test method	Species	Exposure		Results	Remarks	Reference
		design	duration			
OECD 305	Lepomis macrochirus	Flow-through 6 µg as/L and 60 µg as/L Lipd content 7.03 %	42 d	6 µg/L: $BCF_{steady\ state}$ = 97.2 (whole fish, TRR) $BCF_{kinetic}$ = 87.9 (whole fish, TRR) normalized to 5 % lipid content: $BCF_{kinetic}$ = 62.5 (whole fish, TRR) 60 µg/L: $BCF_{steady\ state}$ = 79.2 (whole fish, TRR) $BCF_{kinetic}$ = 65.7 (whole fish, TRR) normalized to 5 % lipid content: $BCF_{kinetic}$ = 46.7 (whole fish, TRR) $BCF_{steady\ state}$ = 18 (whole fish, parent) normalized to 5 % lipid content: $BCF_{steady\ state}$ = 13 (whole fish, parent)	relevant for CLP: $BCF_{steady\ state}$ = 13 (whole fish, parent)	Bruns and Weber, 13.03.2008 EBGMP116 M-298506-01

TRR...total radioactive residue

The fish bioaccumulation study (Bruns & Weber, 2008) with bluegill sunfish indicated a rapid absorption by fish (time to reach 95% steady state was 7.7 to 14.8 days). Fluopyram was tested over 28 days uptake phase and 14 days depuration phase at two concentrations (6 and 60 µg/L) with radioactive 14C marking of fluopyram.The kinetic bioconcentration factor of 87.9 (whole fish, TRR) and 65.7 (whole fish, TRR) were determined from uptake and depuration rate constants. The observed steady state bioconcentration factors for both concentrations were 97.2 (whole fish, TRR) and 79.2 (whole fish, TRR). A mean lipid content (day 0-28) of 7.03 % for the used fish batch was found. The lipid normalized BCF values related to whole fish and TRR (total radioactive residue) of 62.5 and 46.7 were obtained after normalization to 5 % lipid content of fish. Related to unchanged parent a low bioconcentration factor (whole fish = 18; whole fish, normalized to 5% lipid content = 13) and a very rapid clearance half life (1.8 to 3.4 days) were determined. However, after 14 days in uncontaminated water, a maximum of 25% of the absorbed quantity of fluopyram remained in fish. This remaining quantity is still to be considered as very low and environmentally not relevant when compared to the plateau concentration of absorption that corresponds to a very low BCF value of 18.

5.3.2 Summary and discussion of aquatic bioaccumulation

Fluopyram has a log $P_{o/w}$ of 3.3 (20°C). The experimentally derived steady state BCF of 13 L/Kg ww for fluopyram were obtained after lipid normalization to 5 % lipid content related to parent is below the trigger of 100 (criterion for bioaccumulating potential conform Directive 67/548/EEC) for not rapidly

degradable substances and is also below the trigger of 500 (criterion for bioaccumulating potential conform Regulation EC 1272/2008) for not rapidly degradable substances.

5.4 Aquatic toxicity

The most sensitive aquatic toxicity for fluopyram are summarised in Table 235.

Table 235: Summary of relevant information on aquatic toxicity

Group, specie	Time-scale (Test type)	Endpoint	Toxicity (mg a.s./L)	Reference
Fish				
C. variegatus	96 h (static)	Mortality, LC_{50}	> 0.98 mm*	Banman and Lam, 2006, EBGMP053 M-279167-01
L macrochirus	96 h (static)	Mortality, LC_{50}	>5.17 mm*	Nieden, 2006 EBGMP052 M-278441-02
P. promelas	33 d (flow through) ELS	Length and morphological / behavioral effects, NOEC	0.135 mm	Nieden, 2006 EBGMP054, M-279440-01
Aquatic invertebrates				
D. magna	48 h (static)	Immobility, EC_{50}	> 17 mm*	Bruns, 2006 EBGMP046 M-278709-01
A. bahia	96 h (flow trough)	Mortality, EC_{50}	> 0.51 mm*	Palmer, Kendall and Krueger, 2007 EBGMP043 M-282839-01
D. magna	21 d (semi-static)	Offspring production, offspring behavior and parental body length, NOEC	1.25 nom	Bruns, 2007 EBGMP047 M-282102-02
Algae and aquatic plants				
P. subcapitata	72 h (static)	Biomass, E_bC_{50} Growth rate E_rC_{50} NOEC	3.97 mm 8.9 mm 1.46 mm	Banman and Lam, 2007 EBGMP048 M-286541-01
Skeletonema costatum	96 h (static)	Biomass, Growth rate EC_{50} NOEC	> 1.13 mm* 1.13 mm	Banman and Lam, 2007 EBGMP050, M-287289-01
Lemna gibba	7 d (static)	Fronds E_yC_{50} Growth rate E_rC_{50} NOEC	2.32 nom 2.51 nom. 0.256 nom.	Dorgerloh, 2007 EBGMP051 M-283647-01
Other aquatic organism				
C. riparius	28 d (static, spiked water)	Emergence, NOEC	0.525 mm	Dogerloh, 2008 EBGMP121 M-298266-01

mm…mean measured

* effect concentration above practical limit of water solubility under test conditions

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

Table 236: Sort term toxicity of fluopyram to Cyprinodon variegatus

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
OECD 203 (rev. 1992), US EPA OPPTS 850.1075 (1996), FIFRA 72-3 (1982)	*C. variegatus*	mortality	static	96 h	LC_{50}> 0.98 mm	effect concentration above practical limit of water solubility under test conditions	Banman and Lam, 2006, EBGMP053 M-279167-01

mm… mean measured

In a 96-h acute toxicity test, sheepshead minnow (*Cyprinodon variegatus*) were exposed to Fluopyram with 94.7% purity at nominal concentrations of 0 (negative and solvent controls), 0.063, 0.125, 0.25, 0.5 and 1.0 mg ai/L; mean measured concentrations were <6 µg ai/L (<LOQ; controls), 0.072, 0.138, 0.25, 0.51 and 0.98 mg ai/L under static conditions. The solvent carrier was Dimethylformamide with 0.1 mL/L. Test levels were set based upon the functional limit of solubility in synthetic sea water (17‰ salinity). The limit of solubility was determined by conducting numerous solubility trials in the synthetic sea water identical to which was used during testing using stock solutions and solvent loads. Precipitates were detected in all test solutions prepared at higher concentrations (1.5 to 10 mg ai/L). The 96-h LC_{50} was >0.98 mg ai/L. The EC_{50} and NOAEC values, based on a lack of mortality or sub-lethal effects, were >0.98 and 0.98 mg ai/L, respectively.

Table 237: Short term toxicity of fluopyram to Lepomis macrochirus

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
OECD 203 (rev. 1992), US EPA OPPTS 850.1075 (1996), FIFRA 72-1 (1982)	*Lepomis macrochirus*	mortality	static	96 h	LC_{50}> 5.17 mm	effect concentration above practical limit of water solubility under test conditions	Nieden, 2006, EBGMP052 M-278441-02

mm… mean measured

In a 96-h acute toxicity limit test, bluegill sunfish (*Lepomis macrochirus*) were exposed to Fluopyram with 94.7% purity at nominal concentrations of 0 (negative and solvent controls) and 6.0 mg ai/L; mean measured concentration during test was 5.17 mg ai/L under static conditions. The solvent carrier was Dimethylformamide with 0.1 mL/L. Pre-experiments were performed to determine the solubility of fluopyram in the testwater under test conditions. It was found that a limit test at 6.0 mg ai/L (practical limit of water solubility under test conditions) seems to be adequate, because pretests showed precipitations at test concentrations higher than 6.0 mg ai/L. The 96-h LC_{50} was >5.17 mg a.i/L. The EC_{50} and NOAEC values, based on lack of mortality or sub-lethal effects, were >5.17 and 5.17 mg a.i/L, respectively. Test material residues were observed in the test vessels during the study, but there was no mention of sample centrifugation prior to analytical determination. However, the study indicates the material had little or toxicity up to its limit of solubility.

5.4.1.2 Long-term toxicity to fish

Table 238: Long term toxicity of fluopyram to Pimephales promelas

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
see footnote[1)]	P. promelas	length and morphological / behavioral effects/ ELS	Flow-through	33 d	NOEC = 0.135 mm	---	Nieden, 2006 EBGMP054, M-279440-01

[1)] EPA OPPTS 850.1400, OECD 210, SEP-EPA-560/6-82-002, ASTM E 1241-92

The 33-day chronic toxicity of fluopyram to the early life stage of fathead minnow (*Pimephales promelas*) was studied under flow-through conditions. The parameters measured in this study were egg hatchability (time to hatch, hatching success), larval survival, fry survival, fry growth (length and weight) and morphological and behavioural effects. Fertilized eggs/embryos (100/level, <24 hours old) of fathead minnow were exposed to AE C656948 at nominal concentrations of 0 (negative and 0.1 mL/L DMF solvent controls), 0.0185, 0.0370, 0.0740, 0.148, 0.296, 0.592, and 1.18 mg ai/L (adjusted for purity; i.e., 94.7% of nominal values based on technical material tested). Reviewer-calculated TWA concentrations were <0.00514 (<LOQ, controls), 0.0175, 0.0380, 0.0652, 0.135, 0.269, 0.560, and 1.05 mg ai/L, respectively. The test system was maintained at 24.7 to 26.8°C and a pH of 6.5 to 7.4. The 33-day NOEC and LOEC values were 0.135 and 0.269 mg ai/L, respectively, based on length of fry and morphological/behavioural effects to early life stages of fathead minnow, the most sensitive endpoints.

The time to hatch and hatching success were unaffected by exposure. For all levels, hatching occurred from days 3 to 5, and hatching success ranged from 89 to 95%. Prior to thinning (on day 5), larval mortality (5 %) was observed at the 1.05 mg ai/L treatment level and was considered a result of exposure; no other larval mortality occurred at any level. Treatment-related signs of toxicity were observed at the ≥0.269 mg ai/L levels from days 20 to 33; effects included deformed mouth, ventral hematoma, labored respiration, remaining at the water surface, dark coloration, swollen belly, loss of equilibrium (with lateral deviation from normal orientation), and/or lordosis.

A statistically-significant reduction in post-hatch survival was indicated at the two highest treatment levels compared to the negative control; mean fry survival (day 33) ranged from 87 to 97% for the control through 0.269 mg ai/L levels, 70% at the 0.560 mg ai/L level, and 15% at the 1.05 mg ai/L level. The growth of surviving fry was adversely affected by exposure. Mean lengths were statistically-reduced compared to the negative control (21.0 mm) at the three highest treatment levels 0.269, 0.560 and 1.05 mg ai/L levels (19.9, 16.7 and 10.8 mm, respectively). Mean dry weights were also statistically-reduced compared to the negative control (35.1 mg) at the 0.560 and 1.05 mg ai/L levels (22.1 and 4.1 mg, respectively).

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Table 239: Short term toxicity of fluopyram to Daphnia magna

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
see footnote[1)]	D. magna	Immobility	static	48 h	EC_{50} > 17 mm	effect concentration above practical limit of water solubility under test conditions	Bruns, 2006 EBGMP046 M-278709-01

[1)] OECD 202 (2004), EEC Directive 92/69/EEC (1992), JMAFF 12 Nousan No. 8147 (2000), FIFRA 72-2 (1982), EPA OPPTS 850.1010 (1996)

The 48-hour acute toxicity of fluopyram with 94.7% purity to *Daphnia magna* was studied under static conditions. Daphnids were exposed to nominal concentrations of 0 (negative and 0.1 mL/L DMF solvent controls), 3.05, 4.88, 7.81, 12.5 and 20 mg a.i./L; mean-measured concentrations were <0.1029 (negative and solvent controls), 2.9, 4.6, 7.0, 11.0 and 17.0 mg a.i./L. Mortality and sublethal effects were assessed daily. The 48-hour EC_{50} and NOAEC values were >17 mg a.i/L and 17 mg a.i/L, respectively, based on a lack of immobilization. No sublethal effects were observed among any daphnids in the negative or solvent control groups, or among any animals in any treatment groups exposed to AE C656948.

Table 240: Short term toxicity of fluopyram to Americamysis bahia

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
EPA OPPTS 850.1035	A. bahia	Mortality	Flow through	96 h	EC_{50} > 0.51 mm	surrogate EC_{50} based on 10% mortality in treatment, effect concentration above practical limit of water solubility under test conditions	Palmer, Kendall and Krueger, 2007 EBGMP043 M-282839-01

mm…mean measured

A 96-hour flow-through acute toxicity test with fluopyram with 94.7% purity was conducted with the saltwater mysid (*Americamysis bahia*). Nominal concentrations were 0.038, 0.075, 0.15, 0.30, and 0.60 mg ai/L. Treatment groups were compared to a negative and solvent control. The reviewer-calculated time-weighted average (TWA) concentrations were <LOQ (<0.025, negative and solvent control), 0.043, 0.080, 0.15, 0.27, and 0.51 mg ai/L. The solvent carrier was Dimethylformamide with 0.1 mL/L. Test levels were set based upon the functional limit of solubility in natural sea water (20‰ salinity) which was approximately 0.6 mg ai/L. No visible precipitates were observed in test chambers.After 96 hours, there was 10% mortality in the highest treatment group; no mortality was detected in the lower treatment levels or controls. The EC_{50} was >0.51 mg ai/L and the NOAEC was 0.27 mg ai/L.

5.4.2.2 Long-term toxicity to aquatic invertebrates

Table 241: Long term toxicity of fluopyram to Daphnia magna

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration	NOEC		
OECD 211, EEC Directive C.20, US EPA 72-4, OPPTS 850.1300	D. magna	Offspring production, offspring behavior and parental body length	semi-static	21 d	1.25 nom 1.22 mm	--	Bruns, 2007 EBGMP047 M-282102-02

mm…mean measured

The 21-day-chronic toxicity of fluopyram with 94.7% purity to *Daphnia magna* was studied under static renewal conditions. Daphnids were exposed to AE C656948 at nominal concentrations of 0 (dilution water and 0.1 mL/L DMF controls), 80, 200, 500, 1250, and 3125 µg ai/L. Time-weighted average (TWA) concentrations were <5.14 (<LOD, controls), 79, 196, 497, 1214, and 2996 µg ai/L, respectively. The 21-day EC_{50} for adult immobility (mortality) was >2996 µg ai/L. The 21-day NOAEC was 1214 µg ai/L, based upon treatment-related effects on offspring production and terminal body lengths of surviving females.

No mortality was observed at any level. However, five animals were identified as males (one each from the negative control, 79 µg ai/L, and 497 µg ai/L levels, and two from the 2996 µg ai/L level) and were excluded from any statistical evaluation. Offspring production was adversely affected at the highest concentration level. Specifically, the cumulative number of living offspring per surviving parent female averaged 76.4 at the 2996 µg ai/L level, compared to 185.2 for the negative control and 170.1 for the solvent control. Similarly, the mean daily offspring per surviving parent female was 5.6 at the 2996 µg ai/L level, compared to 13.8 and 12.9 for the negative and solvent controls, respectively. Of the 836 total offspring produced at the 2996 µg ai/L level, 225 neonates were dead, and 49 of the 611 living offspring (8.0%) showed unspecified sub-lethal signs of toxicity. No treatment-related effect was indicated for the age of first brood release, which ranged from 9.3 to 10.2 days for all levels.

Mean body lengths of surviving first-generation daphnia averaged 4.8 mm for the control through 1214 µg ai/L levels, and 4.5 mm for the 2996 µg ai/L level, with a statistically-significant difference observed at the 2996 µg ai/L level compared to the negative control. No statistically-significant differences were observed on final body mass (dry weight), which ranged from 0.80 to 0.96 mg for all test levels.

5.4.3 Algae and aquatic plants

Table 242: Toxicity of fluopyram to Pseudokirchneriella subcapitata

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
FIFRA Guideline 123-2 (1982), OPPTS Guideline 850.4500 (2006 draft), OECD Guideline 201 (2006)	*P. subcapitata*	Biomass, growth rate	static	96 h	72 h: E_bC_{50} = 3.97 mm E_rC_{50} = 8.9 mm NOEC = 1.46 mm 96 h: E_bC_{50} = 4.3 mm E_rC_{50} = 6.0 mm	--	Banman and Lam, 2007 EBGMP048 M-286541-01

mm … mean measured

In a 96-hour acute toxicity study, cultures of the freshwater green algae *Pseudokirchneriella subcapitata* were exposed to Fluopyram technical with 94.7% purity at nominal concentrations of 0 (negative and solvent controls), 0.102, 0.256, 0.64, 1.6, 4.0, and 10.0 mg a.i./L under static conditions. The solvent

carrier was Dimethylformamide with 0.1 mL/L. Mean-measured concentrations were <0.005 (<LOQ; controls), 0.093, 0.241, 0.584, 1.46, 3.78, and 9.53 mg a.i./L. The percent inhibition in cell density, in the treated algal culture as compared to the negative control, ranged from 10 to 99%. The % inhibition in biomass, in the treated algal culture as compared to the negative control, ranged from 9 to 99%. The % inhibition in growth rate, in the treated algal culture as compared to the negative control, ranged from 2 to 95%. The most sensitive endpoint was biomass, with NOEC and EC50 values of 1.46 and 4.3 mg a.i./L, respectively. Cell abnormalities were not reported.

Germany indicates that 1) 72 hours is the standard period evaluated by the EU and 2) based on pooled control data, after 72 hours the EC_{50} would be 3.97 mg ai/L based on effects on biomass and 8.9 mg ai/L based on growth rate.

Table 243: Toxicity of fluopyram to Skeletonema costatum

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
OECD Guideline 201 (2006), OPPTS 850.4500 (2006 draft), FIFRA 123-2 (1982)	Skeleto-nema costatum	cell density, biomass and growth rate	static	96 h	EC_{50} > 1.13 mm (biomass, growth rate) NOEC = 1.13 mm	effect concentration above practical limit of water solubility under test conditions	Banman and Lam, 2007 EBGMP050, M-287289-01

mm … mean measured

In a 96-hour acute toxicity study, cultures of the saltwater diatom *Skeletonema costatum* were exposed to Fluopyram technical with 94.7% purity at nominal concentrations of 0 (negative and solvent controls), 0.063, 0.125, 0.250, 0.50, and 1.0 mg a.i./L under static conditions. The highest testing concentration was set based upon functional limit of solubility of the test item in the testing system (enriched saltwater media with 26 ‰ salinity), because precipitates were formed in all solutions prepared above 1.0 mg ai/L. The solvent carrier was Dimethylformamide with 0.1 mL/L. Mean-measured concentrations were <0.006 (<LOQ; controls), 0.080, 0.152, 0.302, 0.621, and 1.13 mg a.i./L. The percent inhibition in cell density, in the treated algal culture as compared to the negative control, ranged from -26 to 29%. The % inhibition in biomass, in the treated algal culture as compared to the negative control, ranged from -32 to 27%. The % inhibition in growth rate, in the treated algal culture as compared to the negative control, ranged from -5 to 9%.

The most sensitive endpoint could not be determined, as no endpoint was affected by treatment. The NOEC and EC_{50} values for all three endpoints were 1.13 and >1.13 mg a.i./L, respectively. Cell abnormalities were not reported.

Table 244: Toxicity of fluopyram to Lemna gibba

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
OECD 221, EPA OPPTS 850.4400	Lemna gibba	frond area based on yield growth rate for frond number	static	7 d	E_YC_{50} = 2.32 nom (Fronds) NOEC = 0.256 nom. E_rC_{50} =2.51 nom. NOE_rC = 1.6 nom.	--	Dorgerloh, 2007 EBGMP051 M-283647-01

In a 7-day acute toxicity study, the freshwater floating aquatic vascular plant duckweed, *Lemna gibba*, was exposed to fluopyram with 94.7% purity at nominal concentrations of 0 (negative control and solvent controls), 0.256, 0.640, 1.60, 4.00, and 10.0 mg/L under static conditions. The solvent carrier was Dimethylformamide with 0.1 mL/L. Mean-measured concentrations were <1.7 µg/L, <5 µg/L (<LOD, <LOQ; controls), 0.278, 0.694, 1.65, 4.04, and 8.88 mg a.i./L. Based on frond number, the percent inhibition in the treated samples as compared to the negative control ranged from 1.2 to 92.9%. Based on frond area, the percent inhibition in the treated samples as compared to the negative control ranged from 3.8 to 90%. The study author conducted statistics for frond number based on growth rate and yield, and on frond area based on growth rate and yield. Based on the calculated EC_{50} values, the most sensitive endpoint was frond number based on yield, with NOAEC and EC_{50} values of 0.278 and 2.6 mg a.i./L (mean measured), respectively. There were no phytotoxic effects in the first three treatment levels. Small fronds and slight chlorosis were observed in the 4.04 mg a.i./L test concentration, while single fronds and slight to medium chlorosis were observed in the highest test concentration (8.88 mg a.i./L). There were certain deviations from OPPTS guidelines, but these are not considered sufficient to downgrade the study classification.

Measured concentrations were within 80 – 120% of nominal concentrations. Therefore, nominal concentrations could be used by the EU for risk assessment. In that case the most sensitive endpoint was frond area based on yield, with EC_{50} value of 2.32 mg a.i./L (nominal).

5.4.4 Other aquatic organisms (including sediment)

Table 245: Toxicity of fluopyram to Chironomus riparius

Guideline / Test method	Species	Endpoint / Type of test	Exposure		Results [mg a.s./L]	Remarks	Reference
			design	duration			
OECD 219	C. riparius	Emergence/ water sediment using spiked water	Static, spiked water	28 d	NOEC = 1.39 nom NOEC = 0.525 mm	--	Dogerloh, 2008 EBGMP121 M-298266-01

The 28-day sub-chronic toxicity of technical-grade fluopyram with 94.7% purity to the freshwater dipteran *Chironomus riparius* was studied under static (with aeration) conditions. First instar larvae were exposed to fluopyram at nominal overlying water concentrations of 0 (negative and solvent controls), 0.0139, 0.139, 0.32, 1.39, 3.20, and 32.0 mg ai/L. The solvent carrier was Dimethylformamide with 0.1 mL/L. TWA overlying water concentrations were <0.00110 (controls), 0.00628, 0.0516, 0.125, 0.525, 1.63, and 6.76 mg ai/L, respectively. Initial measured overlying water concentrations were <0.00110 (controls), and 0.0128, 3.11, and 5.52 mg ai/L for the nominal 0.0139, 3.20, and 32.0 mg ai/L levels, respectively (representing the low, middle, and high levels; other levels not measured on day 0).

The 28-day EC_{50} exceeded the highest concentration level for emergence ratio and development rate. The more sensitive endpoint was emergence ratio, which the study author reported was statistically-reduced ($p<0.05$) compared to the pooled control at the two highest treatment levels. Thus, the 28-day NOAEC was between 0.0128 and 3.11 mg ai/L based on initial measured concentrations (observed; actual level not measured on day 0) and 0.525 mg ai/L based on TWA concentrations. No treatment-related difference between sexes was indicated. The study author also reported that development rate was statistically-reduced ($p<0.05$) compared to the pooled control at the highest treatment level.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

The results of the test on the biodegradation of Fluopyram in the water/sediment system and abiotic degradation show that Fluopyram is considered not rapidly degradable (a degradation > 70 % within 28 days) for purposes of classification and labelling.

Fluopyram has a log $P_{o/w}$ of 3.3 (20°C). The experimentally derived steady state BCF value of 13 L/kg ww (with lipid normalization) related to unchanged parent is below the trigger of 100 (criterion for bioaccumulation potential conform Directive 67/548/EEC) for not rapidly degradable substances and also below the trigger of 500 (criterion for bioaccumulation potential conform Regulation EC 1272/2008) for not rapidly degradable substances.

In aquatic toxicity studies acute LC_{50} values for fish and EC_{50} values for invertebrates were obtained at fluopyram concentrations greater than solubility limit under test conditions. In aquatic toxicity studies with algae and aquatic plants ErC50 values were determined > 1 mg/L but < 10 mg/L. In long- term toxicity studies NOEC < 1 mg/L for fish, aquatic plants and sediment dwelling organisms were determined.

Criteria for classification for aquatic environmental hazard together with the most sensitive endpoints for fluopyram are summarised in Table 246.

Table 246: Critria for classification regarding environmental hazards for fluopyram

Criteria	Most sensitive endpoint for Fluopyram	Reference
Acute aquatic toxicity	E_rC_{50} = 2.51 mg a.s./L (*Lemna gibba*, 7 d, stat.) E_rC_{50} = 8.9 mg a.s./L (*P. subcapitata*, 3 d, static)	see Table 235
Chronic aquatic toxicity	NOEC = 0.135 mg a.s./L (*P. promelas*, 33 d, flow trhough) NOEC = 0.256 mg a.s./L (*Lemna gibba*, 7 d, stat.)	see Table 235
Potential for or actual bioaccumulation	BCF = 62.5	see Table 231
Degradation (biotic or abiotic) for organic chemicals	Not rapidly degradable	see Table 227

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Conclusion of environmental classification according to Regulation EC 1272/2008

The lowest acute aquatic toxicity of fluopyram with E_rC_{50} = 2.51 mg a.s./L(*Lemna gibba*, 7 d, stat.) is above the trigger for acute aquatic classification (1 mg/L).

Therefore no acute aquatic classification is necessary.

In the ELS study with *Pimephales promelas* the chronic aquatic toxicity of fluopyram was determined with a NOEC of 0.135 mg a.s./L. The study with aquatic plant *Lemna gibba* delivered a NOEC of 0.256 mg a.s./L. These values are higher than the trigger for chronic category 1 (NOEC ≤ 0.1 mg/L) but below the trigger for chronic category 2 (NOEC ≤ 1.0 mg/L)

The results of the test on the biodegradation of Fluopyram in the water/sediment system and abiotic degradation show that Fluopyram is considered not rapidly degradable (a degradation > 70 % within 28 days) for purposes of classification and labelling.

Fluopyram has a log $P_{o/w}$ of 3.3 (20°C). The experimentally derived steady state BCF of 13 L/Kg ww for fluopyram were obtained after lipid normalization to 5 % lipid content related to parent is below the trigger of 500 (criterion for bioaccumulating potential conform Regulation EC 1272/2008) for not rapidly degradable substances.

These results trigger the environmental classification as Aquatic Chronic Category 2 (H411). An M-factor is not requested.

Conclusion of environmental classification according to Directive 67/548/EEC

Fluopyram fulfils the criteria for classification with N; R51-53

Based on the toxicity data for *Lemna gibba* (ErC50 = 2.51 mg/L) in a 7-d static study the following specific concentration limits should be applied:

Concentration	Classification
C ≥ 25%	N; R51/53
2.5% ≤ C < 25%	R52/53

where C is the concentration of fluopyram in the preparation.

RAC evaluation of environmental hazards

Summary of the Dossier submitter's proposal

Fluopyram currently has no a harmonised classification according to CLP Regulation. The dossier submitter (DS) proposes to classify the substance as Aquatic Chronic Category 2 (H411).

Degradation

A hydrolysis study according to OECD guideline 111 and in compliance with GLP was run at pH 4, 7 and 9 at 50 °C for 5 days. Fluopyram was hydrolytically stable under acidic, neutral and alkaline conditions. No major degradation products were detected at pH 4, 7 and 9. No half-lives could be calculated as the substance was stable to hydrolysis at all pH conditions.

The photodegradation of radio-labelled fluopyram in water at pH 7 was studied according to the EPA-FIFRA 161-2 guideline. The study, in compliance with GLP, was carried out at 25 °C with continuous artificial light for 13 days. Fluopyram undergoes limited transformation by photolytic processes to fluopyram-lactam (14% of applied radioactivity) and 14 unidentified

transformation products (maximum 4.2% of applied radioactivity (AR). The DT_{50} value for fluopyram was found to be 23 days (mean) of continuous irradiation. Aquatic photolysis is not considered to be an important transformation route for fluopyram in the environment.

Photolytic degradation of fluopyram was also studied in natural water at 25 °C. The study was carried out according to the EPA-FIFRA 161-2 guideline and in compliance with GLP. Fluopyram phototransformed slowly with a DT_{50} of 21 days in natural water under continuous irradiation. No major transformation products were detected. A minor transformation product, fluopyram-lactam was observed at a maximum of 1.2% AR. Phototransformation would not be a principle route of transformation in natural waters.

No data on ready biodegradation are available.

A water/sediment simulation study, carried out according to OECD TG 308 and in compliance with GLP, was run for 120 days using two pond systems (Leverkusen, Germany and Lawrence, Kansas, U.S.A.). No major degradants were formed in either water/sediment systems. The maximum CO_2 evolved was 1,8% AR. The DT_{50} values of fluopyram in the entire system were estimated to be greater than 648 days in both sediment/water systems, which indicate that fluopyram is persistent in the aquatic environment. Aerobic biotransformation would not be an important transformation route of fluopyram in the aquatic environment.

Bioaccumulation

Fluopyram has a measured logK_{ow} of 3.3 (Method OECD 107, 20 °C).
The DS provided a bioaccumulation study on fluopyram. In this study (OECD TG 305) bluegill sunfish (*Lepomis Macrochirus*) were exposed to radio-labelled fluopyram over 28 days uptake phase and 14 days depuration phase. Two test concentrations of 6 and 60 µg/L were used.
Kinetic bioconcentration factor of 87.9 (whole fish, total radioactive residue-TRR) and 65.7 (whole fish, TRR) were determined from uptake and depuration rate constants. BCF values related to whole fish and TRR of 62.5 and 46.7 were obtained after normalization to 5 % lipid content of fish. Related to unchanged parent a low steady-state bioconcentration factor (BCF whole fish = 18; BCF whole fish normalized to 5% lipid content = 13) and a very rapid clearance half-life (1.8 to 3.4 days) were determined.

Based on these information the DS concludes that the fluopyram has a very low bioconcentration factor.

Aquatic toxicity

Several results on aquatic toxicity were provided for the three trophic levels (fish, aquatic invertebrates and algae/aquatic plants) other than for a sediment-dwelling organism.

Regarding toxicity to fish, two saltwater acute tests were available (*Cyprinodon variegatus* and *Lepomis macrochirus*). In both cases the LC_{50} values were above the practical limit of water solubility under test conditions and no effect were observed up to the highest measured concentration.

The DS provided a chronic test on *Pimephales promelas.* It is the key study for the chronic toxicity classification, with a **33-d NOEC=0.135 mg/L** (mean measured concentration).

Regarding toxicity to aquatic invertebrates, acute tests on *Daphnia magna* and *Americamysis bahia* showed EC_{50} values above the practical limit of water solubility under test conditions. No effect were observed up to the highest measured concentration for *D. magna*, while a 10% mortality was reported in the highest treatment group for *A. bahia.*

A 21-d semi-static chronic toxicity test on *D. magna* was provided, with a NOEC = 1.25 mg/L nominal (1.22 mg/L mean measured).

Regarding toxicity to algae and aquatic plants, the DS provided a 7-day acute toxicity study on

Lemna gibba and two 96-h acute toxicity studies (the freshwater green alga *Pseudokirchneriella subcapitata* and the saltwater diatom *Skeletonema costatum*). While for the *Skeletonema costatum* the DS stated that no effect was observed up to the functional limit of solubility in the test system, for the green alga, a 72-h EC_{50} = 3.97 mg/L was reported based on effects on biomass and 8.9 mg/L based on growth rate.

The results obtained with the test on *Lemna gibba* are related to two endpoints: frond area based on yield and growth rate for frond number. The most sensitive endpoint was frond area based on yield (E_YC_{50} = 2.32 mg/L nominal; NOEC=0.256 mg/L nominal).The NOE_rC values, based on the growth rate, is 1.6 mg/L and the **E_rC_{50} = 2.51 mg/L** (nominal) based on growth rate is proposed as the key value for the acute toxicity classification.

Moreover the DS provided a 28-day static toxicity study on the sediment-dwelling organism *Chironomus riparius* according to OECD TG 219. The most sensitive endpoint was the emergence ratio. The reported NOEC was based on the nominal concentration of 1.39 mg/L and on the corresponding Time Weight Average (TWA) concentration of 0.525 mg/L. All the measurements were based on overlying water concentrations.

A summary of the most reliable ecotoxicity results were as follows (the key studies for classification are highlighted in bold):

Method	**Test organism**	**Test type**		**Results**		**remarks**
			Endpoint	**LC_{50}/EC_{50} [mg/L]**	**NOEC [mg/L]**	
Fish						
OECD 203 (rev. 1992), US EPA OPPTS 850.1075 (1996), FIFRA 72-3 (1982)	*C. variegatus*	96-h (static)	Mortality, LC_{50}	>0.98 mg/L * mm		
OECD 203 (rev. 1992), US EPA OPPTS 850.1075 (1996), FIFRA 72-1 (1982)	*L .macrochirus*	96-h (static)	Mortality, LC_{50}	>5.17 mg/L * mm		
EPA OPPTS 850.1400, OECD 210, SEP-EPA-560/6-82-002, ASTM E 1241-92	*P. promelas*	**33-d (flow through) ELS**	**Length and morphological/ behavioral effects**		**0.135mg/L mm**	
Aquatic invertebrates						
OECD 202 (2004), EEC Directive 92/69/EEC (1992), JMAFF 12 Nousan No. 8147 (2000),	*D. magna*	48-h (static) 21-d (semi-static)	Immobility EC_{50} Offspring production, offspring behavior and parental body length,	>17 mg/L * mm	1.25 mg/L nom 1.22 mg/L mm	OECD TG 211, EEC Directiv C.20, US EPA 72-4,

FIFRA 72-2 (1982), EPA OPPTS 850.1010 (1996)						OPPTS 850.1300
EPA OPPTS 850.1035	*A. bahia*	96-h (flow through)	Mortality, EC_{50}	>0.51 mg/L * mm		
Aquatic algae and plants						
FIFRA Guideline 123-2 (1982), OPPTS Guideline 850.4500 (2006 draft), OECD Guideline 201 (2006)	*P. subcapitata*	72-h (static)	Biomass, E_bC_{50} Growth rate E_rC_{50} NOEC	3.97 mg/L mm 8.9 mg/L mm	 1.46mg/L mm	
OECD Guideline 201 (2006), OPPTS 850.4500 (2006 draft), FIFRA 123-2 (1982)	*S. costatum*	96-h (static)	EC_{50}	>1.13 mg/L * mm	1.13mg/L mm	
OECD 221, EPA OPPTS 850.4400	*L. gibba*	7-d (static)	Fronds, E_yC_{50} **Growth rate E_rC_{50}** NOEC (frond area based on yield) NOErC (growth rate for frond number)	2.32 mg/L nom **2.51 mg/L nom**	 0.256 mg/L Nom 1.6 mg/L nom	
Other aquatic organism						
OECD 219	*C. riparius*	28-d (static,spiked water)	Emergence		0.525 mg/L mm (TWA)	

mm: mean measured concentrations
nom: nominal concentrations

* effect concentration above practical limit of water solubility under test conditions

Comments received during public consultation

Three MSs and one Industry representative contributed during public consultation. The MSCA stated general agreement with the proposed environmental classification and Industry made only editorial remarks.

One of MS suggested that the NOEC value for *Chironomus riparius* of 1.39 mg/L, based on nominal concentrations and validated in the peer review of the pesticide risk assessment of the active

substance fluopyram (EFSA 2013), should be considered more relevant than the NOEC value of

0.525 mg/L, which seemed to be based on TWA concentrations and not on mean measured concentrations. In addition, they asked to explain why the NOEC of *Lemna gibba* used in the CLH report was based on the $NOEC_{yield}$ instead of the $NOEC_{rate}$. They concluded, however, that these comments will not change the conclusion of the classification proposal.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS proposal to consider fluopyram as not rapidly degradable. The substance is hydrolytically stable under acidic, neutral and alkaline conditions. In addition, aquatic photolysis is not considered to be an important transformation route for fluopyram in the environment. Although no studies on ready biodegradability according to OECD 301 are available, fluopyram is demonstrated to be not ultimately degraded to a level greater than 70% in a water/sediment simulation test.

Bioaccumulation

Based on experimental data, fluopyram has a logK_{ow} value of 3.3 (Method OECD 107, 20 °C).
The measured BCF of 13 (normalized to 5 % lipid content) based on the parent compound showed that the bioaccumulation potential of fluopyram is low. Therefore, the BCF value is below the decisive CLP criterion (BCF≥500).

Aquatic toxicity

Acute aquatic hazard

Acute toxicity data were available for all three trophic levels. The most sensitive aquatic species was *Lemna gibba*. The lowest and relevant reliable short-term aquatic toxicity result was **7d E_rC_{50} = 2.51 mg/L** (nominal concentration). This value is above the trigger for acute aquatic classification (1 mg/L), therefore no acute aquatic classification is necessary.

Chronic aquatic toxicity

Reliable and relevant long-term aquatic toxicity data are available for all three trophic levels. The lowest value is for *P. promelas*, with a **33 d NOEC=0.135 mg/L** (mean measured concentration). This value lies in the toxicity range of 0.1 < NOEC ≤ 1.0 mg/L.

Conclusion on classification

Fluopyram is considered not rapidly degradable and does not fulfil the criteria for bioaccumulation.
The lowest acute toxicity value falls above the trigger value of 1 mg/L and the lowest chronic toxicity value lies in the toxicity range of 0.1 < NOEC ≤ 1.0 mg/L.
RAC concludes that fluopyram fulfils the CLP criteria for classification as Aquatic Chronic category 2 (H411).

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8 ANNEXES