Committee for Risk Assessment

RAC

Annex 1

Background document

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

spirodiclofen (ISO); 3-(2,4-dichlorophenyl)-2-oxo-1-xaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutyrate

EC Number: -
CAS Number: 148477-71-8

CLH-O-0000001412-86-135/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

9 December 2016

CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name: Spirodiclofen

EC Number: **Not applicable**

CAS Number: **148477-71-8**

Index Number: **Not applicable**

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Spirodiclofen*** ***3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutyrate*** *(IUPAC-name)*
EC number:	***Not applicable***
CAS number:	***148477-71-8***
Annex VI Index number:	***Not applicable***
Degree of purity:	***≥ 96.5%***
Impurities:	• ***3-(2,4-dichlorophenyl)-4- hydroxy-1-oxaspiro[4.5] dec-3-en-2-one (BAJ-2740 enol)*** • ***N,N-dimethylacetamide***

1.2 Harmonised classification and labelling proposal

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

	CLP Regulation
Current entry in Annex VI, CLP Regulation	None
Current proposal for consideration by RAC	Carc. 1B H350: May cause cancer Repr. 2 H361f: Suspected of damaging fertility Skin sens. 1B

	H317: May cause an allergic skin reaction STOT RE 2 H373: May cause damage to organs, through prolonged or repeated exposure Aquatic chronic 1 H410: very toxic to aquatic life with long-lasting effects M-factor (chronic): 10
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 1B H350: May cause cancer Repr. 2 H361f: Suspected of damaging fertility Skin sens. 1B H317: May cause an allergic skin reaction STOT RE 2 H373: May cause damage to organs, through prolonged or repeated exposure Aquatic chronic 1 H410: very toxic to aquatic life with long-lasting effects M-factor (chronic): 10

As spirodiclofen is a plant protection product that is proposed for harmonized classification for the first time, RAC is requested also to assess the correctness of this proposal that no classification is needed for all other hazard classes.

1.3 Proposed harmonised classification and labelling based on CLP Regulation

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	Not classified	conclusive but not sufficient for classification
2.2.	Flammable gases	None	None	Not classified	Not applicable
2.3.	Flammable aerosols	None	None	Not classified	Not applicable
2.4.	Oxidising gases	None	None	Not classified	Not applicable
2.5.	Gases under pressure	None	None	Not classified	Not applicable
2.6.	Flammable liquids	None	None	Not classified	Not applicable
2.7.	Flammable solids	None	None	Not classified	conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	None	None	Not classified	No data
2.9.	Pyrophoric liquids	None	None	Not classified	Not applicable
2.10.	Pyrophoric solids	None	None	Not classified	No data
2.11.	Self-heating substances and mixtures	None	None	Not classified	No data
2.12.	Substances and mixtures which in contact with water emit flammable gases	None	None	Not classified	No data
2.13.	Oxidising liquids	None	None	Not classified	Not applicable
2.14.	Oxidising solids	None	None	Not classified	conclusive but not sufficient for classification
2.15.	Organic peroxides	None	None	Not classified	No data
2.16.	Substance and mixtures corrosive to metals	None	None	Not classified	No data
3.1.	Acute toxicity - oral	None	None	Not classified	conclusive but not sufficient for classification
	Acute toxicity - dermal	None	None	Not classified	conclusive but not sufficient for classification
	Acute toxicity - inhalation	None	None	Not classified	conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	None	None	Not classified	conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	None	None	Not classified	conclusive but not sufficient for classification
3.4.	Respiratory sensitisation	None	None	Not classified	Data lacking

3.4.	Skin sensitisation	**Skin sens. 1B; H317**	**-**	Not classified	
3.5.	Germ cell mutagenicity	None	None	Not classified	conclusive but not sufficient for classification
3.6.	Carcinogenicity	**Carc. 1B; H350**	**-**	Not classified	
3.7.	Reproductive toxicity	**Repr. 2; H361f**	-	Not classified	
3.8.	Specific target organ toxicity –single exposure	None	None	Not classified	conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	**STOT RE 2; H373**	None	Not classified	
3.10.	Aspiration hazard	None	None	Not classified	Data lacking
4.1.	Hazardous to the aquatic environment	**Aquatic chronic 1; H410**	**Chronic M-factor: 10**	Not classified	
5.1.	Hazardous to the ozone layer	None	None	Not classified	Data lacking

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Danger

Hazard statements: H317: May cause an allergic skin reaction
H350: May cause cancer,
H361f: Suspected of damaging fertility,
H373:May cause damage to organs, through prolonged or repeated exposure
H410: very toxic to aquatic life with long-lasting effects

Precautionary statements: Not required as precautionary statements according to CLP are not included in Annex VI of Regulation EC no. 1272/2008.

Proposed notes assigned to an entry:

: none

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

According to the data presented in the DAR, the classification of spirodiclofen is: Xn; R40, R43, R52/53.

The conclusions on the peer review of pesticide risk assessment of spirodiclofen was published as an EFSA scientific report (2009; 339:1-86). The classification was unchanged. The DAR can be requested via: http://dar.efsa.europa.eu/dar-web/provision. The final addendum is available via the EFSA website (http://www.efsa.europa.eu/en/efsajournal/pub/339r).

Spirodiclofen was added to Annex I of Directive 91/414/EEC (Council Directive 2010/27/EU of 23 April 2010) from 1 August 2010.

Spirodiclofen has not previously been assessed for harmonised classification by RAC or TC C&L.

The current proposal for harmonised classification differs from the conclusions drawn by EFSA during their assessment. An additional classification for fertility (Repro. 2; H361f: Suspected of damaging fertility) and repeated dose toxicity as STOT RE 2 (H373: May cause damage to organs, through prolonged or repeated exposure) is proposed. In addition, classification for carcinogenicity is currently proposed using a more potent classification category (Carc. 1B; H350: May cause cancer). Further, the current proposal for classification as aquatic toxicity (chronic) includes a more potent classification category (Aquatic Chronic 1; H410: very toxic to aquatic life with long-lasting effects; including an M-factor of 10).

Spirodiclofen is not registered under REACH (10 August 2015).

2.2 Short summary of the scientific justification for the CLH proposal

According to the criteria in the CLP annex 1 chapters 3.4, 3.6, 3.7, 3.9 and 4.1, classification for Skin sens. 1B (H317: May cause an allergic skin reaction), Carc. 1B (H350: May cause cancer), Repro. 2 (H361F: Suspected of damaging fertility), STOT RE (H373: May cause damage to organs, through prolonged or repeated exposure), Aquatic chronic 1 (H410: very toxic to aquatic life with long-lasting effects; including a M-factor of 10) can be assigned to spirodiclofen.

- Given that the response in the guinea pig Maximization test is 40% at an intradermal induction dose of 5%, classification of spirodiclofen for skin sensitisation as Skin sens. 1B (H317: May cause an allergic skin reaction) is proposed. The data are considered sufficient for sub-classification seen the low response of 40% at an intradermal induction concentration of 5%.

- With respect to the potential carcinogenic effect of spirodiclofen in humans, the following justification is presented:

  Classification in category 1A is not considered for spirodiclofen as there are no human data regarding carcinogenicity.

  Based on the available data, it can be concluded that there is an appropriate combination of benign and malignant neoplasms in two or more species. For spirodiclofen there is an increase in malignant neoplasms in a single species (i.e. uterus adenocarcinoma in rat), in combination with an increase in benign neoplasms in a second species (i.e. hepatocellular adenomas in mouse). In addition, given that in mouse 1) the incidences of hepatocellular neoplasms were

statistically significantly increased for both the benign tumor types a well as the combined benign + malignant tumor types, and 2) also a dose-related increase (though not statistically significant) in malignant tumor types was observed, this strengthens the evidence for potential carcinogenicity of spirodiclofen.

It can therefore be concluded there is sufficient evidence for carcinogenic effects of spirodiclofen. Classification for carcinogenicity as Carc 1B (H350: May cause cancer) is therefore proposed.

Classification in category 2 is not considered for spirodiclofen. The available data cannot be considered as limited evidence, as the available data on spirodiclofen point towards an appropriate combination of benign and malignant neoplasms in two or more species.

- With respect to the potential reproductive toxicity (fertility) of spirodiclofen in humans, the following justification is presented:

  Classification in category 1A is not considered for spirodiclofen as there are no human data regarding reproductive toxicity (fertility).

  Effects on the reproductive organs were observed in the repeated dose toxicity studies and the carcinogenicity studies in all species tested (mouse, rat, dog), though the effects were most pronounced in dogs at lower dose levels as compared to rat and mouse. An overview of effects on reproductive organs in available repeated dose toxicity studies with spirodiclofen is presented in Table 57 of the CLH report. Observed effects included changes of weight of uterus/oviduct and ovaries in female animals, however without histopathalogical changes. Further, in male animals effects such as increased testis weight (absolute and/or relative), hyperplasia, hypertrophy and vacuolisation of testis cells, but also oligospermia and aspermia (in 4- and 14-week dog studies, 18-month mouse study) were observed. These latter effects are considered relevant for classification. The mechanistic studies showed that spirodiclofen has a direct effect on steroid hormone synthesis, which is probably mediated by effects on general pathways (interference with formation of NADPH). Further, it was shown that spirodiclofen might have a direct effect on the enzymes involved in the steroidogenesis in testis. These data indicate that the observed effects are not secondary to general toxic effects, but rather a direct effect of spirodiclofen. The dog was shown to be the most sensitive species for the effects on the reproductive organs which occurred at relevant dose levels. No oligo-/aspermia was observed in rat. Further, these adverse effects (aspermia) were observed in mice, although only at very high dose levels. These data indicate that species-differences in toxicodynamics occur, which might be related to potential species-differences in toxicokinetics. Based on the available data it is not clear whether or not the dog is the most relevant species for evaluating potential classification of spirodiclofen for effects on sexual function and fertility for humans. Given the observations that the effects were only observed at high dose levels in mice and the effects were not observed in rat, might indicate that the dog should not be regarded as the most relevant species for evaluating potential fertility effects of spirodiclofen in humans. However, clear effects on sexual function were observed, and as it cannot be excluded that the observed effects in dogs (including the underlying mechanism) are relevant for evaluating potential fertility effects of spirodiclofen in humans, the observed effects should be taken into account for potential classification for effects on sexual function and fertility.

  It can be concluded there is some evidence for reproductive effects of spirodiclofen, and spirodiclofen can be considered as a suspected human reproductive toxicant. Classification for effects on sexual function and fertility as Repro 2 (H361f: Suspected of damaging fertility) is proposed.

Classification in category 1B is not considered for spirodiclofen, as 1) fertility effects on reproductive organs were observed in dogs (a single species), 2) effects on reproductive organs and on fertility were not observed in rats, 3) in mice, these effects (aspermia) were observed at very high dose levels, 4) the observed accompanying testis enlargement and vacuolisation are considered adaptive.

- With respect to the potential specific target organ toxicity (repeated exposure) of spirodiclofen in humans, the following justification is presented:

  Effect levels in the available dog repeated dose toxicity studies were around or below the upper limit for STOT RE 2 classification (300 mg/kg bw/day for a 4-week study, 150 mg/kg bw/day for a 8-week study, 100 mg/kg bw/day for a 90-day study, 25 mg/kg bw/day for a 1-year study). In most studies the highest dose tested was below the upper limit for STOT RE 2 classification. At the highest dose level tested many parameters were effected. Most effects individually would not fufil the classification criteria. However, according to 3.9.1.4 of the CLP criteria also generalised changes of a less severe nature involving several organs should be taken into account. Further, the reduction in Hb and Ht in the 14 week dog study was around 20% at the highest dose level (83 mg/kg bw/day) which was below the upper limit for STOT RE 2. As it cannot be excluded that the observed effects in dogs are not relevant for evaluating potential effects of spirodiclofen in humans, the observed effects should be taken into account for potential classification for STOT RE. Together, the available information in dogs warrant classification for STOT RE 2 (H373: May cause damage to organs, through prolonged or repeated exposure). This classification applies to all routes as comparable effects after inhalation exposure cannot be excluded. As the classification is mainly based on the general effects on several organs and not to a primary target organ of toxicity (note to table 3.9.1 of CLP-regulation), no specific target organ is proposed.

  Given that no human data are available and the effective dose levels in the repeated dose animal studies were above the upper limit for STOT RE 1 (i.e. 30 mg/kg bw/day for a 4-week study, 15 mg/kg bw/day for a 8-week study, 10 mg/kg bw/day for a 90-day study, 2.5 mg/kg bw/day for a 1-year study), classification for category 1 is not applicable.

- Spirodiclofen is considered not rapidly degradable in the environment and does not fulfil the criterion BCF > 500. Chronic aquatic toxicity is available for all three trophic levels. The lowest NOEC of 0.00195 mg/L was obtained in fish (*Oncorhynchus mykiss*). This value is below the classification threshold value of 1 mg/L. Spirodiclofen does therefore fulfil the criteria for classification as chronic category 1 hazard to the aquatic environment. The available data indicates that the lowest NOEC value of 0.00195 mg/L falls within the range 0.001< NOEC ≤ 0.01 mg/L and is not rapidly degradable. Therefore spirodiclofen fulfils the criteria for classification as Aquatic Chronic category 1 (H410: very toxic to aquatic life with long-lasting effects) with an M-factor of 10.

2.3 Current harmonised classification and labelling

2.3.1 Current classification and labelling in Annex VI, Table 3.1 in the CLP Regulation

Spirodiclofen has currently no harmonised classification.

2.3.2 Current classification and labelling in Annex VI, Table 3.2 in the CLP Regulation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

2.4 Current self-classification and labelling

2.4.1 Current self-classification and labelling based on the CLP Regulation criteria

Table 4 below provides an overview of the self-classification for spirodiclofen according to the ECHA C&L inventory (accessed March 14th, 2014).

Table 4. Overview of the self-classification of spirodiclofen (CAS 148477-71-8) by the notifiers

Classification		Labelling			Specific Concentration limits, M-Factors	Notes	Number of notifiers
Hazard Class and Category Code(s)	Hazard Statement Code(s)	Hazard Statement Code(s)	Supplementary Hazard Statement Code(s)	Pictograms, Signal Word Code(s)			
Skin Sens. 1	H317	H317		GHS07 Wng			23
Skin Sens. 1	H317	H317		GHS07 GHS08 Wng			1
Carc. 2	H351	H351					

2.4.2 Current self-classification and labelling based on DSD criteria

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

RAC general comment

Spirodiclofen (ISO) is an active substance used in plant protection products approved under Regulation (EC) No 1107/2009. It is mainly used as an acaricide or insecticide on various crops and fruits. The draft assessment report (DAR) has been peer reviewed by EFSA (EFSA, 2009). The degree of purity is ≥ 96.5% with five major impurities among which N,N-dimethylacetamide (DMAC, EC 204-826-4) has an harmonised classification as Repr. 1B (H360D) in Annex VI of the CLP Regulation.

Spirodiclofen (ISO) has not previously been assessed for harmonised classification and has no entry in Annex VI of the CLP Regulation (CLP). The current opinion differs however, from the

conclusions drawn by EFSA during their assessment of the hazard classification of this substance. The EFSA peer review report was first issued in 2007 and re-issued in July 2009 at the request of Commission. Carc. 2 (R40) was proposed by EFSA (2009) who compared the data at that time against the Dangerous Substances Directive (67/548/EC) criteria which are qualitatively different to those of Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP). No classification for fertility effects was proposed since all reproductive and endocrine-mediated toxicity effects were concluded to be of low potency and not considered for risk assessment by EFSA.

Additional information (some generated since 2009), was made available to RAC for the development of the current opinion. This comprised the full reports of all key Guideline studies, additional mechanistic studies & reviews, revised historical control data and statistical analysis of the tumours.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Spirodiclofen is currently (d.d. 01/08/2010) approved as an active substance for authorisation as a plant protection product. Spirodiclofen has currently no harmonised classification.

The available data show that there is a need for classification for skin sensitisation, carcinogenicity, reproduction toxicity (fertility), specific target organ toxicity-repeated exposure, and aquatic toxicity (chronic).

Part B.

SCIENTIFIC EVALUATION OF THE DATA

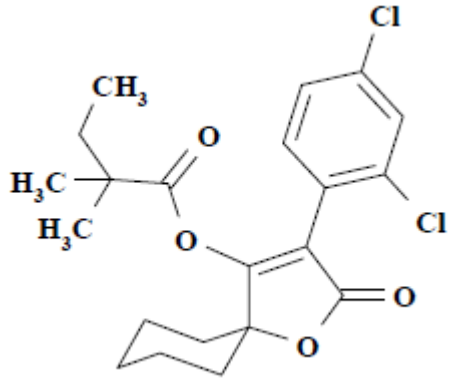
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	Not applicable
EC name:	Not applicable
CAS number (EC inventory):	148477-71-8
CAS number:	148477-71-8
CAS name:	butanoic acid, 2,2-dimethyl-, 3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl ester
IUPAC name:	3-(2,4-dichlorophenyl)-2-oxo-1-oxaspiro[4.5]dec-3-en-4-yl 2,2-dimethylbutyrate
ISO name	spirodiclofen
CLP Annex VI Index number:	Not applicable
Molecular formula:	$C_{21}H_{24}Cl_2O_4$
Molecular weight range:	411.3

Structural formula:



1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
Spirodiclofen	96.5%		

Current Annex VI entry: None

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
3-(2,4-dichlorophenyl)-4-hydroxy-1-oxaspiro[4.5] dec-3-en-2-one (BAJ- 2740 enol)	≤ 6 g/kg (0.6%)		
N,N-dimethylacetamide	≤ 4 g/kg (0.4%)		

Current Annex VI entry*:

N,N-dimethylacetamide: Repr. 1B (H360D; SCL≥5%)

Acute Tox. 4 (H332)

Acute Tox 4 (H312)

* A CLH proposal for N,N-dimethylacetamide (prepared by NL) to remove the current SCL of 5% was recently accepted by RAC (RAC opinion, d.d. 12-09-2014). The GCL of 0.3% for Repro 1B will be applicable.

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
confidential				not relevant for classification

Current Annex VI entry:

1.2.1 Composition of test material

The test material concerns spirodiclofen, unless otherwise specified.

The production process of spirodiclofen has changed during the registration procedure for inclusion of spirodiclofen in Annex I of Directive 91/414/EEC. The revised production process resulted in:

- an increase of the concentration of the active compound spirodiclofen (old: 955 mg/kg, new: 965 mg/kg)
- concentration shift of existing impurities (see Confidential Annex)
- the identification of three new impurities: N,N-dimethylacetamide (≤ 4 g/kg (0.4%)) and two confidential impurities (see Confidential Annex)

In the Confidential Annex 1, the modified specification as copied from the DAR is presented including a (eco)toxicological assessment of the new specification focussing on the new impurities.

Overall conclusion regarding the new impurities:

- ecotoxicity: Based on the submitted studies on toxicity of the active substance (together with the new impurities) and the new impurities itself, it can be concluded that the new impurities are not of ecotoxicological significance.
- human toxicity: The argumentation provided by the notifier on the toxicological equivalence of the new production process is considered acceptable. The applied maximal concentrations for the new impurities are toxicologically acceptable.

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	**Value**	**Reference [a]**	**Comment (e.g. measured or estimated)**
State of the substance at 20°C and 101,3 kPa	Solid	Krohn, 1997	White powder, odourless
Melting point	94.8°C	Krohn, 1997	
Boiling point	The boiling point could not be determined due to thermal decomposition	Treckmann, 1997a	
Relative density	1.2854 g/cm^3	Treckmann, 1997b	
Vapour pressure	3×10^{-7} Pa at 20°C 7×10^{-7} Pa at 25°C	Krohn, 1997	
Surface tension	Not required because water solubility is < 1 mg/L		
Water solubility	At pH 4 and 20°C : 50 µg/L At pH 7 and 20 °C: 0.19 mg/L = 190 µg/L	Krohn, 1997 Bogdoll and Strunk (2005)	
Partition coefficient n-octanol/water	Log Pow = 5.83 at 20°C and pH4. Log Pow = 5.1 at 23 ˚C and pH 7.	Krohn, 1997 Bogdoll and Wiche (2005)	
Flash point	-		
Flammability	Not highly flammable. Does not undergo spontaneous combustion.	Eberz, 1998	
Explosive properties	Not explosive	Eberz, 1998	Remark: Substance is dust explosible. The lower explosible limit of the substance is specified as 50 g/m^3
Self-ignition temperature	-		
Oxidising properties	The structural formula of the active substance does not contain any of the chemical groups characteristic of oxidizing agents. Therefore, it is regarded by the registrant as incapable of reacting exothermically with combustible material. This information justifies the not determining the oxidizing properties.		

Granulometry	-		
Stability in organic solvents and identity of relevant degradation products	-		
Dissociation constant	Not determinable, due to the instability of the active substance in aquous solutions with pH values > pH 4	Krohn, 1997	
Viscosity	-		

[a] As summarised in the DAR (Volume 3, annex B.2), April 2004.

2 MANUFACTURE AND USES

2.1 Manufacture

This information is confidential.

2.2 Identified uses

Spirodiclofen is an active substance of a plant protection product (containing 240 g/L spirodiclofen).

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 10: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference [a]
EC A.14	Not explosive	No classification required	Eberz, 1998
EC A.10 EC A.16	Not highly flammable Does not undergo spontaneous combustion	No classification required	Eberz, 1998

[a] As summarised in the DAR (Volume 3, annex B.2), April 2004.

3.1 Explosive properties

Spirodiclofen is not explosive. Classification for this endpoint is not required.

3.2 Flammability

Spirodiclofen is not highly flammable and does not undergo spontaneous combustion. Classification for this endpoint is not required.

3.3 Oxidising potential

The oxidising potential of spirodiclofen was not tested.

The structural formula of spirodiclofen does not contain any of the chemical groups characteristic of oxidizing agents. Therefore, it is regarded as incapable of reacting exothermically with combustible material.

Classification for this endpoint is not required.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

The Dossier Submitter (DS) did not propose classification for physical hazards. The data on physico-chemical properties did not indicate any concerns and as such, spirodiclofen does not meet the criteria for classification.

Comments received during public consultation

There were no comments regarding the classification for physico-chemical hazards.

Assessment and comparison with the classification criteria

Tests applied according to methods EC A. 14, EC A. 10 and EC A. 16 showed that spirodiclofen is not explosive, is not highly flammable and does not undergo spontaneous combustion. In addition, the structural formula of spirodiclofen does not contain any of the chemical groups characteristic of oxidizing agents. Therefore, it is regarded as incapable of reacting exothermically with combustible materials. Therefore RAC is in agreement with the DS that classification is not required for physico-chemical hazards.

4 HUMAN HEALTH HAZARD ASSESSMENT

The human health hazards of spirodiclofen were assessed in the Draft Assessment Report and the Addendum to the Draft Assessment Report prepared in the context of the possible inclusion of spirodiclofen in Annex I of Council Directive 98/8/EEC (Draft Assessment Report, April 2004 and subsequent final addendum June 2009, RMS The Netherlands) concerning the placing of plant protection products on the market. The summaries included in this proposal are copied from the DAR (and its addenda and assessment reports when these contain updated information). Additional information is added with regard to the reproductive toxicity studies.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

STUDY 1

Characteristics

Reference	:	I. Andersch, J. Köster, (2000a)	exposure	:	single low and high dose and repeated low dose
type of study	:	Absorption, distribution, excretion and metabolism	doses	:	1, 2, 100 mg/kg bw
year of execution	:	1997-2000	vehicle	:	0.5% carboxymethyl cellulose
test substance	:	[Dihydrofuranone-3-^{14}C] BAJ 2740 (spirodiclofen) (purity >89%)	GLP statement	:	yes
Route	:	Oral (by gavage)	guideline	:	In accordance with OECD 417
Species	:	rat (Wistar Hsd/Cpb: Wu)	acceptability	:	Acceptable
group size	:	4-6/male/dose/time point 1 experiment 4 female/dose/time point			

Study design

Wistar rats (4-6 male/dose/time point; in one experiment 4 female/dose/time point) received a single oral dose (1, 2 or 100 mg/kg bw) of ^{14}C-BAJ 2740 (spirodiclofen) or a repeated oral dose (14 daily doses of 2 mg/kg bw unlabelled spirodiclofen followed by a single oral dose of 2 mg/kg bw ^{14}C-spirodiclofen on day 15). Urine was collected at 4, 8, 24 h and daily after exposure to the labeled test compound. In the bile-duct cannulated rats urine and bile were collected at 1, 2, 3, 4, 6, 8 and 24 h after dosing. Feces was collected once per day. Following administration of ^{14}C-spirodiclofen, blood was sampled from the tail vein, at 0.08, 0.16, 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8 24, 32, 48 h and daily thereafter. Excretion of radiolabel in air was assessed at 4, 8, 24 and 48 h after administration in one experimental group. At sacrifice organs and tissues were dissected and, after combustion, radioactivity levels were measured by LSC (LOQ of 0.0023 μg/g). From urine-, feces- and bile samples metabolites were isolated by solid phase extraction and HPLC, followed by identification by LC-MS, LC-MS/MS and NMR. An overview of the experiments included in this study is presented in Table 11.

Table 11 Overview of experiments on kinetics and metabolism of spirodiclofen in Wistar rats (Andersch, 2000a).

Experiment	Dose (mg/kg bw)	Animals	No. of doses	sampling[1]	Time of Sacrifice (hr) after last dose
ADME	100	4 male	1	P, U, F	168
	2	4 male	1	U, F, A	48
	2	4 male	1	P, U, F	48 [2]
	2	4 female	1	P, U, F	48 [2]

2	4 male	15	P, U, F	48 [2]
1	6 male	1	U, F, B	24

1 radioactivity measured in P=plasma, U=urine, F=feces, A=expired air, B=bile

2 At sacrifice the following organs and tissues samples were collected and prepared for measurement of radioactivity levels: erythrocytes, plasma, spleen, gastrointestinal tract, liver, kidney, renal fat, adrenal gland, skeletal muscle, bone (femur), heart, lung, brain, thyroid gland, skin, carcass. Additionally in males radioactivity levels were measured in testes and in females in uterus and ovary.

In male and female rats plasma radioactivity levels rose quickly following single oral administration of a dose of 2 mg/kg bw, reaching maximum concentrations of 2.1-2.7 µg eq/g after 2-6 h in males, and after 1.5-4 h in females. In the 100 mg/kg bw group a peak plasma level of 51.3 µg eq/g was reached after 8 h, i.e. slightly later than after the low dose administration. In the low dose groups calculated half-lives for elimination of radioactivity from plasma were 4.2 h (males) and 3.4 h (females), in the males of the 100 mg/kg bw group calculated half-live was 3.1 h.

Recovery of radioactivity, as percentage of dose administered ranged from 89-107. Excretion of administered radioactivity was fast. Following administration of 2 mg ^{14}C-spirodiclofen/kg bw to male rats more than 99 % of recovered radioactivity was excreted within 48 h, i.e. 64-66 % in urine (59-61 % after 24h) and 33-35 % in feces (31-32% after 24 h). As compared to males, in females relatively more radioactivity was excreted in the urine (76 % in 48 h) and less in feces (24% in 48 h). After administration of 100 mg ^{14}C-spirodiclofen/kg bw to male rats 100 % of the recovered radioactivity was excreted within 168 h, i.e. 36.7% via urine and 63.3 % via feces. The relatively high amount of excretion in the feces indicates incomplete absorption at this high dose. Following administration of 1 mg ^{14}C-spirodiclofen/kg bw to bile-duct cannulated male rats 62.8 % of recovered radioactivity was excreted within 24 h, i.e. 22.8% in urine, 28.7 % in feces and 11.3 % in bile. The presence of a substantial amount of radioactivity in feces of bile-duct cannulated rats suggests that also at this low dose absorption of spirodiclofen is incomplete. In view of the radioactivity levels in urine and bile it can be assumed that in male rats more than 64 % of spirodiclofen, administered at a dose of 2 mg/kg bw, is absorbed, although it has to be noted that in the bile experiment the urinary excretion was low. In female rats the absorption may be even higher. After administration of 2 mg ^{14}C-spirodiclofen/kg bw less then 0.05 % of radioactivity was expired in air within 48 h.

Levels of radioactivity in tissues and organs were low. In male rats treated with a single dose of 2 mg ^{14}C-spirodiclofen/kg bw, at 48 h after administration the highest equivalent concentrations were observed in liver (0.049 µg/g), kidneys (0.024 µg/g), plasma (0.015 µg/g), gastro-intestinal tract (0.0135 µg/g) and skin (0.0107 µg/g). Concentrations in erytrocytes, spleen, skeletal muscle, heart, lung and carcass were below 0.01 µg eq/g. Testis concentrations were 0.0029 µg/g, and concentrations in renal fat, adrenal gland, bone femur, brain and thyroid gland were below LOD. In male rats treated for 15 days with spirodiclofen at a daily dose of 2 mg/kg bw, the relative distribution of radioactivity over the tissue was similar to that of male rats after single treatment. However, the concentrations in organs and tissue were approximately 4 times lower (liver 0.0105 µg/g, kidneys 0.0064 µg/g, plasma 0.0037 µg/g, gastro-intestinal tract 0.0061 µg/g and carcass 0.0020 µg/g). Concentrations in erytrocytes, heart and lung were below 0.0020 µg eq/g. Testis concentrations were 0.0008 µg/g, and no radioactivity (< LOD) was found in spleen, renal fat, adrenal gland, skeletal muscle, bone femur, brain, thyroid gland and skin. In females rats treated with a single dose of 2 mg ^{14}C-spirodiclofen/kg bw, at 48 h post administration concentrations were even 5 to 15 times lower than in males after a single dose, although the relative distribution of radiolabel was similar. In these females only in liver (0.0047 µg/g), kidney (0.0044 µg/g), gastrointestinal tract (0.0033 µg/g), plasma (0.0010 µg/g) and lung (0.0009 µg/g) radiolabel concentrations were above level of detection. The concentration in erytrocytes was below LOD. No

radioactivity (< LOD) was found in spleen, renal fat, adrenal gland, uterus, ovary, skeletal muscle, bone femur, brain, thyroid gland, skin, and carcass.

The study authors report that no measurable radioactivity was detected at 168h post administration of 100 mg ^{14}C-spirodiclofen/kg bw (data not shown).

Metabolites were isolated by solid phase extraction and HPLC, followed by identification by LC-MS, LC-MS/MS and NMR. BAJ 2740 (i.e. spirodiclofen) M01, M02, M03, M06 and M16 were included as reference compounds. In total 14 metabolites of spirodiclofen were identified (see Table 13) and 2 metabolites were partially identified. Between 78-90 % of the administered radioactivity could be identified. A proposed metabolic pathway is depicted in figure 1. The quantitative excretion of the main metabolites in feces, urine and bile, expressed as percentage of recovered radioactivity is presented in Table 12. In male rats treated with 100 mg ^{14}C-spirodiclofen/kg bw mainly the parent compound and BAJ-enol (M01) were found in the feces. In feces of rats treated with 2 mg ^{14}C-spirodiclofen/kg bw less than 5 % was unmetabolized spirodiclofen. In these rats the main metabolites in feces and urine were BAJ-enol (M01) and the equatorial (e) and axial (a) 3- and 4-hydroxy-BAJ-enol isomers (M02 and M03). There was a striking sex difference with respect to the excretion of BAJ-enol in urine: in males treated once with 2 mg^{14}C-spirodiclofen/kg bw 2.3-3.8 % of the recovered radioactivity corresponded to BAJ-enol while in females receiving the same treatment this was 54.8 %. Accordingly, in urine the levels of the 3- and 4-hydroxy-BAJ-enol isomers were high (55.1-57.4%) in males and lower (17.3%) in females. There were no obvious differences in quantitative distribution of metabolites between male rats receiving one dose of spirodiclofen or male rats repeatedly dosed with spirodiclofen. In bile of male rats the main metabolites were OH-enol-glucuronide and 3- and 4-hydroxy-BAJ-enols. No parent compound was detected in bile. In feces of bile-duct cannulated male rats 28.7 % of recovered radioactivity was found, of which only 0.6 % corresponded with the parent compound. The main metabolites were 3- and 4-hydroxy-BAJ-enols and BAJ-enol. Furthermore, the MA-cyclohexyl ester and dichlorobenzoic acid were almost exclusively found in feces. This suggests that substantial metabolisation of spirodiclofen (BAJ 2740) may take place inside the gastrointestinal tract.

Table 12 Quantitative excretion of BAJ 2740 (i.e. spirodiclofen) and metabolites, expressed as % recovered radioactivity.

Dose	100 mg/kg bw		2 mg/kg bw		2 mg/kg bw		2 mg/kg bw		1 mg/kg bw		
sex	male		male		female		male #		male		
duration of experiment	168h		48h		48 h		48 h		24 h		
	feces	urine	feces	urine	feces	urine	feces	urine	feces	urine	bile
BAJ2740 (spirodiclofen)	16.6	n.d.	2.0-4.8	n.d.	0.7	n.d.	2.5	n.d.	0.6	n.d.	n.d.
BAJ-enol	16.9	5.9	5.1-7.5	2.3-3.8	4.9	54.8	4.0	5.2	13.9	0.0	0.2
4-hydroxy-BAJ-enol (e)	1.6	8.1	3.1-5.3	14.2-16.6	7.6	8.9	6.2	16.2	6.6	12.8	0.9
4-hydroxy-BAJ-enol (a)	n.d.	3.5	0.6-1.0	6.5-7.5	0.6	2.7	0.6	6.1	0.2	3.2	0.5
3-hydroxy-BAJ-enol (e)	1.8	13.2	2.7-3.4	29.2-30.3	1.1	4.5	3.2	32.1	0.7	4.5	3.8
3-hydroxy-BAJ-enol (a)	0.4	1.8	0.5-0.7	4.1-4.2	0.7	1.3	0.6	5.2	0.3	0.9	0.4
OH-BAJ-enol-glucuronide	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.2
dichlorobenzoic acid	1.6	0.1	0.5-1.3	n.d.	0.5	n.d.	0.7	n.d.	0.1	n.d.	n.d.

MA-cyclohexyl ester	7.8	n.d.	2.6-3.3	n.d.	1.4	n.d.	1.7	n.d.	2.0	n.d.	n.d.

#14 daily doses of 2 mg/kg bw unlabelled spirodiclofen followed by a single oral dose of 2 mg/kg bw ^{14}C-Spirodiclofen on day 15.

Acceptability

The purity of the unlabeled compound is not reported. It is stated in appendix 17 to the study report that the data from rat no. 873 in the bile-duct experiment were excluded from calculation "due to lower activity in the bile". The appendix, however, shows that these data fall well within the range of the data from the other rats. This exclusion is expected to have no major consequences for the interpretation of the data.
The study is considered acceptable for the overall toxicological evaluation.

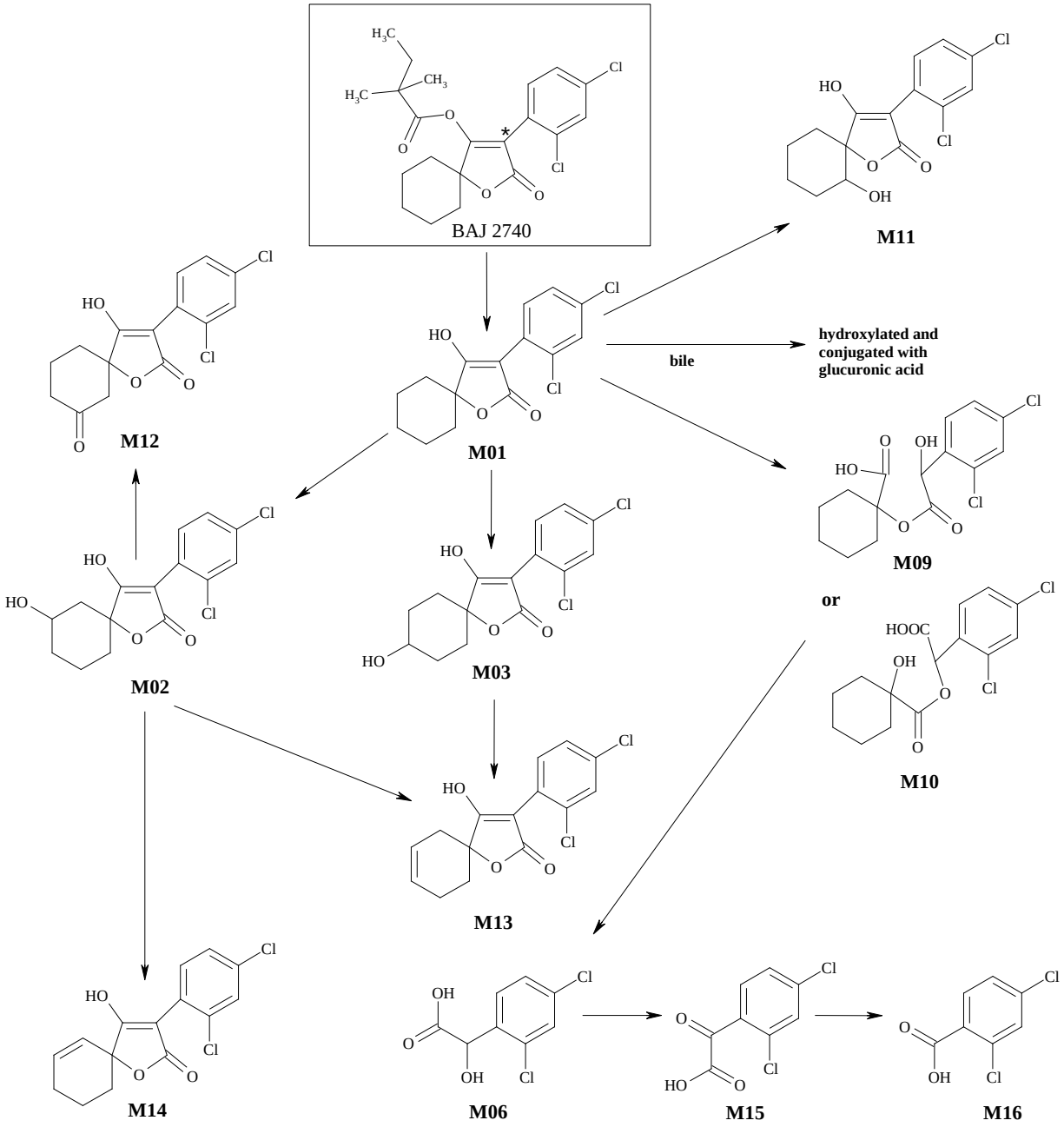
Table 13 List of identified compounds

Code	Compound	Occurrence*
BAJ 2740	spirodiclofen	F
M01	BAJ 2740-ENOL	F, U, B
M02	3-HYDROXY (EQ.)-BAJ 2740-ENOL	F, U, B
	3-HYDROXY (AX.)-BAJ 2740-enol	
M03	4-HYDROXY (EQ.)-BAJ 2740-ENOL	F, U, B
	4-HYDROXY (AX.)-BAJ 2740-ENOL	
M06	2,4-DICHLORO-MANDELIC ACID	F
M09	2,4-DICHLORO-MANDELIC ACID CYCLOHEXYL ESTER (ISOMER OF M10)	F
M10	2,4-DICHLORO-MANDELIC ACID CYCLOHEXYL ESTER (ISOMER OF M9)	F
M11	2-HYDROXY (EQ.)-BAJ 2740-ENOL	F, U, B
M12	3-KETO-BAJ 2740-ENOL	U
M13	3-ENE-BAJ 2740-ENOL	U
M14	2-ENE-BAJ 2740-ENOL	U
M15	2,4-DICHLORO-GLYOXYLIC ACID	F, U
M16	2,4-DICHLOROBENZOIC ACID	F, U

* F=feces, U=urine, B=bile

Molecular structures of spirodiclofen (BAJ 2740) and its metabolites are depicted in figure 1.

Figure 1 Proposed metabolic pathway of spirodiclofen (BAJ 2740) in the rat



STUDY 2

Characteristics

Reference	:	J. Köster (2000)	exposure	:	single dose
type of study	:	Absorption, Distribution	dose	:	3 mg/kg bw (actual 2.53 mg/kg bw)
year of execution	:	1997-2000	vehicle	:	0.5% carboxymethyl cellulose
test substance	:	[Dihydrofuranone-3-^{14}C]BAJ 2740 (spirodiclofen) (purity > 98%)	GLP statement	:	yes
Route	:	Oral (gavage)	guideline	:	in accordance with OECD 417
Species	:	male rat (Wistar Hsd/Cpb: Wu)	acceptability	:	acceptable
group size	:	1 per time point			

Study design

Five male rats received a single oral dose (3 mg/kg bw, actual dose 2.53 mg/kg bw) of ^{14}C-spirodiclofen. At 1, 4, 8, 24 and 48 h post administration 1 rat was sacrificed and prepared for whole body quantitative radioluminography. Urine was collected from periods 0-4, 4-8, 8-24 and 24-48 h, feces was collected from periods 0-24 or 0-48 h.

Results

The autoradiograms showed that already after 1 h there is uptake in the body, notably in the liver and kidney. The levels of radioactivity peaked at 4–8 h after administration in all tissues. At 24 h tissue levels already had markedly decreased and at 48 h all tissues were almost devoid of radiolabel except for liver, kidney and parts of the intestines. Quantitation of the radioluminograms revealed peak levels of 5.5 µg eq/g wet tissue in liver at 4 and 8 h post administration, 2.3 µg eq/g wet tissue in the renal cortex at 4 h post administration, and 2.7 µg eq/g wet tissue in brown fat. The study confirms the data from the biokinetics study (Study 1) demonstrating that spirodiclofen (BAJ 2740) is readily absorbed, distributed and excreted.

Acceptability

The study authors state that the relatively increased blackening of the radiograms at the site of the gastric mucosa and the skin, and uniform blackening at the site of the small intestine indicate extrabiliary secretion of radioactivity in these tissues. Alternative explanations for this blackening are not presented. To substantiate this hypothesis further research is necessary. It should be noted that only male rats were used in this study.

The study is considered acceptable for the overall toxicological evaluation.

STUDY 3

Characteristics

Reference	:	I. Andersch, J. Köster, (2000b)	exposure	:	15 weeks feeding followed by a single oral dose
type of study	:	Absorption, distribution, excretion and metabolism	doses	:	feed: 50 ppm, 2500 ppm (2.5, 125 mg/kg bw/day, unlabeled) oral dose: 2mg/kg bw (^{14}C-labeled)
year of execution	:	1998-2000	vehicle	:	0.5% carboxymethyl cellulose (for labeled compound)
test substance	:	[Dihydrofuranone-3-^{14}C]BAJ 2740 (spirodiclofen) (purity > 98%)	GLP statement	:	yes
Route	:	Oral (by gavage)	guideline	:	in accordance with OECD 417
Species	:	rat (Wistar Hsd/Cpb: Wu)	acceptability	:	acceptable
group size	:	4/sex/dose			

Study design

Rats (4/sex/dose) received unlabeled spirodiclofen (BAJ 2740) in the food at doses of 50 or 2500 ppm (equivalent to 2.5 and 125 mg/kg bw/day) for about 15 weeks prior to an oral dose of 2 mg/kg bw of [dihydrofuranone-3-^{14}C]spirodiclofen. Urine was collected at 4, 8, 24 and 48h. Feces was collected once per day. Following administration of ^{14}C-spirodiclofen blood was sampled from the tail vein, at 0.17, 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8 24, 32 and 48 h. The metabolite pattern in urine and feces were assessed by HPLC.

At sacrifice organs and tissues were dissected and, after combustion, radioactivity levels were measured by LSC. Metabolites in urine- and feces samples were analyzed by solid phase extraction and HPLC.

Results

In male and female rats pretreated with feed containing 50 or 2500 ppm spirodiclofen, plasma radioactivity levels rose quickly following administration of ^{14}C-spirodiclofen. In the 50 ppm group maximum plasma concentrations of about 2.1 µg eq/g and 3.2 µg eq/g were reached after 3-6 h in males and females respectively. In the 2500 ppm group in males a maximum plasma concentration of about 1.6 µg eq/g was reached after 2-4 h while in females a maximum plasma concentration of about 1.7 µg eq/g was reached after 6-8 h.

Recovery of radioactivity after 48 h, as percentage of dose administered ranged from 86-103. Excretion of administered radioactivity was fast. Following administration of 2 mg ^{14}C-spirodiclofen/kg bw more than 97.8 % of recovered radioactivity was excreted within 48 h in rats of all groups. In males of the 50 ppm group after 48 h about 70 % and 30 % of recovered radioactivity were excreted in urine and feces respectively. As compared to males, in females of this group relatively more radiolabel was excreted in urine (77 %) than in feces (22 %). In the 2500 ppm group excretion after 48 h was about 65 % (urine) and 32% (feces), both for males and females.

At sacrifice low levels (0.17-1.05% of recovered radioactivity) of radioactivity remained in the carcass without gastrointestinal tract. The radioactivity levels in the specific organs and tissues were not presented.

In total 14 metabolites of spirodiclofen were identified. In rats of the 50 ppm group 12 % (males) and 2% (females) of the radioactivity found in the feces represented parent compound. In rats of the 2500 ppm group 18 % (males) and 50 % (females) of the radioactivity found in the feces represented parent compound. This indicates that, in particular in females sub chronic pretreatment with a high dose of spirodiclofen reduces the absorption of this substance. The main metabolites in feces and urine were BAJ-enol and the equatorial (e) and axial (a) 3- and 4-hydroxy-BAJ-enol isomers, with a marked sex difference with respect to the excretion of BAJ-enol and the hydroxy-BAJ-enol isomers in urine. In males of the two treatment groups the major metabolites in urine were (e)- and, to a lesser extent, (a)-hydroxy-BAJ-enol (42-50% of recovered radioactivity), while only a small fraction (5-6%) represented BAJ-enol. In urine of females of both treatment groups BAJ-enol accounted for 46-54 % of recovered radioactivity, while 12-15 % corresponded to the (e)-isomers of hydroxy-BAJ-enol and 5 % to the (a)-isomers of hydroxy-BAJ-enol. In feces the major metabolites were 4-hydroxy-BAJ-enol (e) (4-9% of recovered radioactivity), BAJ-enol (1-5%) and 3-hydroxy-BAJ-enol (e) (1-4%). Qualitatively there was no difference in metabolism of spirodiclofen between the pretreated rats of this study and the untreated rats described in study 1. A proposed metabolic pathway is depicted in figure 1.

In rats pretreated with unlabeled spirodiclofen (this study) as well as in untreated rats (study 1), absorption and elimination of spirodiclofen is quick, with almost complete excretion of radiolabel within 48 h. Pretreatment with unlabeled spirodiclofen does not markedly change the metabolism of ^{14}C-spirodiclofen. There is a striking sex difference with respect to the metabolism; in females the major metabolite is BAJ-enol while in males the major metabolites are the (e) and (a) 3- and 4-hydroxy-BAJ-enols. Subchronic pretreatment with a high dose of spirodiclofen decreases its absorption from the gastrointestinal tract, in particular in females.

Acceptability

The study is considered acceptable for the overall toxicological evaluation.

STUDY 4

Characteristics

Reference	:	J. Köster & E. Weber (2002)	exposure	:	single dose (by gavage)
type of study	:	Absorption, distribution, excretion and metabolism	doses	:	2 mg/kg bw
year of execution	:	2002	vehicle	:	0.5% carboxymethyl cellulose
test substance	:	[Dihydrofuranone-3-^{14}C]BAJ2740 (spirodiclofen) (purity >98%)	GLP statement	:	yes
Route	:	oral	guideline	:	Japanese MAFF guideline 12 Nousan 8147
Species	:	rat (Wistar Hsd/Cpb: Wu)	acceptability	:	Acceptable
group size	:	4/sex/dose/time point			

Study design

Rats received, by gavage, a single oral dose (2 mg/kg bw) of ^{14}C-BAJ2740 (^{14}C-BAJ). Four animals/sex were killed at 3, 6 and 24 h. Urine and faeces were collected from time of administration to termination. Blood and tissue (liver, kidney, skin, GIT including faeces, carcass) samples were obtained at sacrifice and radioactivity levels were measured by LSC. Metabolites in urine and plasma samples were analyzed by HPLC, followed by LC-MS and LC-MS/MS. Metabolites in kidney and liver samples were analysed by HPLC.

Results

The distribution of radioactivity over urine and body compartments and the concentration, expressed as µg equivalents/g tissue, following administration of ^{14}C-BAJ to the rats is presented in Table 14.

Table 14 Radioactivity in % of dose administered and tissue levels[1] as µg equivalents/g.

Sex	Male			Female		
Sacrifice	3h	6h	24h	3h	6h	24h
Urine	1.83	20.85	57.66	5.11	34.91	74.78
Erythrocytes	0.51 *(0.58)*[1]	0.47 *(0.52)*	0.04 *(0.04)*	0.43 *(0.53)*	0.25 *(0.32)*	0.00 *(0.00)*
Plasma	3.99 *(4.5)*	2.61 *(3.5)*	0.20 *(0.24)*	2.83 *(3.8)*	2.1 *(2.8)*	0.02 *(0.02)*
Liver	26.00 *(13.4)*	17.94 *(8.4)*	1.45 *(0.70)*	13.52 *(9.4)*	12.44 *(7.3)*	0.09 *(0.4)*
Kidney	1.36 *(3.4)*	0.88 *(2.5)*	0.13 *(0.33)*	1.44 *(4.8)*	1.17 *(3.8)*	0.01 *(0.05)*
Carcass	12.81	10.22	0.93	9.27	7.34	0.13
Sum organs	*44.67*	*32.11*	*2.75*	*27.49*	*23.30*	*0.26*
Skin	7.22	5.71	0.54	4.34	3.67	0.05

GIT+ faeces	43.57	35.61	40.37	52.86	33.05	23.62
Total recovery	97.30	94.29	101.30	89.77	94.94	98.71

[1] Tissue levels are presented in brackets.

Total recovery of radioactivity ranged from 90-101%. The radioactivity levels in urine and organs demonstrate that at least 60 and 75 % of the administered ^{14}C-BAJ is absorbed within 24 h in male and female rats respectively. As a part of the absorbed radioactivity may be excreted in bile, total absorption may be higher. Excretion in urine was rapid, tending to be faster in females than in males. Highest tissue levels were found 3 h after administration. Tissue levels were low 24 h after administration.

The main metabolites in urine, plasma, liver and kidney in males and females are presented in Table 15.

Table 15 Radioactivity in urine, plasma, liver and kidney in percent of dose administered

	male				female			
	urine	plasma	liver	kidney	urine	plasma	liver	kidney
sacrifice	24h	3h	3h	3h	24h	3h	3h	3h
4-hydroxy-enol (e)	18.22	0.14	0.51	0.12	9.47	-	0.07	0.06
3-hydroxy-enol (a)	3.23	0.06	0.83	0.04	1.68	-	0.04	0.01
3-hydroxy-enol (e)	24.21	0.31	2.68	0.28	4.13	-	0.15	0.04
4-hydroxy-enol (a)	7.08	0.41	0.58	0.04	2.56	-	0.10	0.02
Enol	1.55	2.99	20.40	0.85	56.18	2.83	13.14	1.3
unidentified	3.38	0.08	1.00	0.02	0.78	-	0.02	-
% of radioactivity identified	94	98	96	98	99	100	100	100

The levels of the five major metabolites were assessed in samples of plasma, liver and kidney collected at 3 h after administration, i.e. at the time of peak radioactivity levels, and in urine collected over a 24 h period. It appeared that 94 – 100% of radioactivity found in these samples represented these 5 metabolites. There was a striking sex difference with respect to the levels of the 5 metabolites in urine. In female rats the main metabolite in urine was enol, accounting for 56% of the administered radioactivity. In male urine only 1.6% of administered radioactivity corresponded to enol. Here the bulk of the radioactivity (53%) represented the 3- and 4-hydroxy-enol isomers. In plasma, liver and kidney samples of males as well as females the radioactivity mainly represented enol. In these samples the levels of the 3- and 4-hydroxy-enol isomers were higher in males than in females, indicating a higher capacity in further metabolisation of the enol metabolite in male rats. No marked differences in the relative levels of the 5 metabolites between the 3h, 6h and 24h samples were observed.

Acceptability

The study is considered acceptable for the overall toxicological evaluation. The proposed metabolic pathway is presented in figure 1. For explanation of the metabolite codes see Table 13.

STUDY 5

Characteristics

Reference	:	Z. Wu (2002)	exposure	:	single dose (occlusion)
type of study	:	*In vivo* dermal absorption	doses	:	151 µg/animal; ca. 6.3 µg/cm^2
year of execution	:	2002	vehicle	:	BAJ2740 SC 240 Blank suspension in water
test substance	:	[Dihydrofuranone-3-^{14}C]BAJ2740 (spirodiclofen) (radiochemical purity 99%)	GLP statement	:	yes
Route	:	dermal	guideline	:	US-EPA 870.7600
Species	:	Rhesus Monkey	acceptability	:	Acceptable
group size	:	5 males			

Study design

The sponsor provided a ready-to-use formulation BAJ2740 SC 240 containing radiolabeled ^{14}C BAJ 2740 (1.51 µg/µL).

Five naïve male rhesus monkeys received a dermal application, under occlusion, of 100 µL of the test substance, containing in total 151 µg 14C-BAJ2740, to the shaven skin (4 cm x 6 cm). Subsequently, the animals were restrained in a primate chair for 8 h and then placed in metabolism cages. At 8 h after dosing the patch was removed, the application site was washed with cotton swabs dipped in soapy water. Next the application site was tape-stripped 16 times, and wiped with isopropyl alcohol swabs and soapy water swabs. Urine, faeces and samples of cage rinse and the final cage wash were collected up to 144 h post dosing. Following removal from the primate chair samples of chair wash were collected. All samples were analyzed for radioactive content.

Results

At 8 h after application 84.53 % of radioactivity was recovered in the dermal washes, with 58.75% being recovered in the first 4 cotton swabs. In tape strips and isopropylalcohol swabs 0.11 and 1.46 % were recovered respectively. From the securing materials and application site patch 2.19 and 1.93 % of radioactivity were recovered respectively. In urine, faeces, cage rinse/wash and chair wash a total of 2.12% (range 1.31 – 3.48%) of administered radioactivity was recovered over 144 h, with about 1.7% being excreted within the first 24 h. Total recovery of radioactivity was 92.34 %.

Table 16 Total recoveries of BAJ 2740-dihydrofuranone-3-[^{14}C]-derived radioactivity at 144 hours following dermal administration of BAJ 2740 SC 240 containing ^{14}C BAJ 2740 to male Rhesus monkeys at a target dose of 151 µg/animal

Recovery	Sample	Percentage of dose (%)

		Animal number					Mean	SD
		1001	1002	1003	1004	1005		
Elimination	Urine	1.56	1.98	1.21	1.92	0.50		
	Faeces	0.00	0.07	0.06	0.29	0.08		
	Cage debris/rinse	0.08	1.33	0.00	0.24	0.79		
	Chair/urine pan wash/wipe	0.12	0.1	0.04	0.24	0.00		
	Cage wash/wipe	0.00	0.00	0.00	0.00	0.00		
	Subtotal	**1.76**	**3.48**	**1.31**	**2.69**	**1.37**	**2.12**	**0.94**
Residual	Patch/securing material	4.89	5.96	4.14	4.52	1.08		
	Swabs	81.43	80.72	89.61	84.98	93.21		
	Tape strips	0.08	0.15	0.04	0.14	0.13		
	Subtotal	**86.40**	**86.83**	**93.79**	**89.64**	**94.42**	**90.22**	**3.77**
Total		**88.16**	**90.13**	**95.10**	**92.33**	**95.79**	**92.34**	**3.21**

In some animals elimination still occurred in the last study period (120-144 hours after application): 0.02% in urine and 0.02% in faeces in one animal, 0.14% in faeces in one animal, and 0.10% in cage debris/rinse in one animal.

Table 17 Elimination of BAJ 2740-dihydrofuranone-3-[^{14}C]-derived radioactivity by male Rhesus monkeys following dermal administration of BAJ 2740 SC 240 containing ^{14}C BAJ 2740 at a target dose of 151 µg/animal

Sample	Time (hours)	Percentage of dose (%)						
		Animal number					Mean	SD
		1001	1002	1003	1004	1005		
Urine	0-4	0.15	0.19	0.00	0.19	0.02		
	4-8	0.60	NS	0.29	0.81	NS		
	8-12	0.55	1.16	0.52	0.46	0.00		
	12-24	0.26	0.30	0.30	0.25	0.34		
	24-48	0.00	0.15	0.10	0.11	0.14		
	48-72	0.00	0.08	0.00	0.08	0.00		
	72-96	0.00	0.02	0.00	0.00	0.00		
	96-120	0.00	0.06	0.00	0.02	0.00		
	120-144	0.00	0.02	0.00	0.00	0.00		
	Subtotal	**1.56**	**1.98**	**1.21**	**1.92**	**0.50**	**1.43**	**0.61**
Faeces	0-4	NS	NS	NS	NS	NS		
	4-8	0.00	0.00	NS	0.00	NS		

	8-12	0.00	NS	NS	NS	NS		
	12-24	0.00	0.00	0.02	0.03	0.00		
	24-48	0.00	0.00	0.04	0.10	0.07		
	48-72	0.00	0.05	0.00	0.00	0.01		
	72-96	0.00	0.00	0.00	0.00	0.00		
	96-120	0.00	0.00	0.00	0.02	0.00		
	120-144	0.00	0.02	0.00	0.14	0.00		
	Subtotal	**0.00**	**0.07**	**0.06**	**0.29**	**0.08**	**0.10**	**0.11**
Cage debris/	8-12	0.00	0.22	0.00	0.24	0.69		
rinse	12-24	0.00	0.32	0.00	0.00	0.00		
	24-48	0.08	0.00	0.00	0.00	0.00		
	48-72	0.00	0.00	0.00	0.00	0.00		
	72-96	0.00	0.00	0.00	0.00	0.00		
	96-120	0.00	0.79	0.00	0.00	0.00		
	120-144	0.00	0.00	0.00	0.00	0.10		
	Subtotal	**0.08**	**1.33**	**0.00**	**0.24**	**0.79**	**0.49**	**0.56**

Acceptability

Areas of concern with the monkey study included the fact that levels of radioactivity in the skin and body were not determined, and the level of total radioactivity recovered (92%). The low level of variation in individual animals supported the theory that the 8% of radioactivity lost may have been absorbed, and that this should be incorporated into the dermal absorption to give a value of 2-10%.

Conclusion

The *in vivo* dermal absorption of BAJ2710 in rhesus monkeys is approximately 2 - 10%.

STUDY 6

Characteristics

Reference	:	C. Sebesta (2002)	exposure	:	single dose (occlusion)
type of study	:	*In vivo* dermal absorption	doses	:	ca. 20 µg/cm^2 for the dermal application
year of execution	:	2001	vehicle	:	BAJ2740 SC 240 Blank suspension in water
test substance	:	[Dihydrofuranone-3-^{14}C]BAJ2740 (spirodiclofen) (radiochemical purity 99%)	GLP statement	:	yes
Route	:	Dermal and intravenous	guideline	:	-
Species	:	Rhesus Monkey	acceptability	:	Acceptable as exploratory study

group size : 1 male

Study design

The purpose of this study was to determine the rate and route of elimination of BAJ2740-dihydrofuranone-3-^{14}C derived radioactivity following a single intravenous or dermal administration to male rhesus monkeys. This exploratory study was to aid in the design of a definitive dermal absorption and mass balance study.

The study consisted of two groups of one male rhesus monkey per group. BAJ 2740-dihydrofuranone-3- ^{14}C ([^{14}C]-spirodiclofen) was intravenously administered at a target dose of 50 µCi to the animal in Group 1. For the Group 2 animal, an equivalent dermal dose (50 µCi or ca. 20 µg/cm^2) of BAJ 2740 SC 240, containing [^{14}C]-spirodiclofen, was applied, under occlusion, to the shaven skin (4 cm x 6 cm). Subsequently, the animals were restrained in a primate chair for 8 h and then placed in metabolism cages. At 8 h after dermal administration the patch was removed, the application site was washed with 16 cotton swabs dipped in soapy water, 4 isopropyl alcohol swabs and 4 additional cotton swabs dipped in soapy water. At 24 hours and at 48 hours post-dose the application site for the Group 2 animal was tape-stripped 16 times, and and alcohol washes were collected at 48, 168 and 192 hours, and a dermal wipe at 192 hours.

Urine and feces were collected at specified time intervals up to 240 hours post-dose for Group 1 and up to 192 hours post-dose for Group 2. Following the removal of the animal from the primate chair, a chair/urine pan wash/wipe was conducted at specified intervals up to 8 hours post-dose. A cage debris/ cage rinse sample was conducted after each fecal collection up to 240 hours post-dose for Group 1 and up to 192 hours post-dose for Group 2. A cage wash/cage wipe was conducted following the final timepoint. All samples were analyzed for radioactive content by LSC.

Results

Intravenous administration of [^{14}C]-spirodiclofen to a male rhesus monkey resulted in excretion of radioactivity primarily in urine. Total recoveries through 240 hours post-dose were 87.12% in urine, 4.66% in feces, and 15.05% in cage debris/rinse samples. The cage debris/rinse radioactivity can be attributed primarily to urinary excretion based on the fact that the majority of the cage debris/rinse radioactivity was recovered during the first 12 hours after dosing, during which time the animal excreted no fecal matter. Excretion was rapid, with >70% of the dose recovered within 8 hours of dosing and approximately 95% by 24 hours post-dose.

Following dermal application of [^{14}C]-spirodiclofen formulated as 240 SC total recoveries of radioactivity were 1.11% in urine, 0.25% in feces (of which 0.22% was from the fecal-contaminated glove sample), and 0.34% in cage debris/rinse samples through 192 hours post-dose, suggesting minimal systemic exposure to [^{14}C]-spirodiclofen-derived radioactivity. The majority of the excreted radioactivity was recovered within 24 hours of dosing.

The overall recovery of radioactivity for the intravenously dosed animal was 107.27%. The overall recovery of radioactivity for the dermally dosed animal was 108.30%, with the large majority associated with the residual radioactivity recovered from the application site. The dermal absorption was estimated to be 1.77% of the administered dose.

Table 18 Total recoveries of [^{14}C]-spirodiclofen-derived radioactivity at 240 hours following intravenous administration and 192 hours following dermal administration to male Rhesus monkeys at a target dose of 50 µCi

Recovery	Sample	Percentage of dose (%)	
		Intravenous (Group 1)	Dermal (Group 2)
Elimination	Urine	87.12	1.11
	Faeces	4.66	0.25 [a]
	Cage debris/rinse	15.05	0.34
	Chair/urine pan wash/wipe	NA	0.07
	Cage wash	0.20	0.00
	Cage wipe	0.24	0.00
	Subtotal	**107.27**	**1.77**
Residual	Patch/securing material	NA	8.67
	Swabs	NA	97.78
	Tape strips	NA	0.08
	Subtotal	**NA**	**106.53**
Total		**107.27**	**108.30**

NA Not applicable

a residual radioactivity from the glove sample (0.22%) included in the data

After intravenous administration, elimination still occurred in the last study period (216-240 hours after dosing): 0.03% in urine. After dermal application there were serial nondetects starting 72 hours post-dose.

Table 19 Elimination of [^{14}C]-spirodiclofen-derived radioactivity by male Rhesus Monkeys following intravenous administration of [^{14}C]-spirodiclofen and dermal administration of BAJ 2740 SC 240 containing [^{14}C]-spirodiclofen at a target dose of 50 µCi

Recovery	Sample	Percentage of dose (%)	
		Intravenous (Group 1)	Dermal (Group 2)
Urine	0-4	39.85	0.03
	4-8	24.50	0.53
	8-12	10.13	0.00
	12-24	8.01	0.30
	24-48	3.49	0.18
	48-72	0.41	0.07

	72-96	0.14	0.00
	96-120	0.16	0.00
	120-144	0.10	0.00
	144-168	0.17	0.00
	168-192	0.10	0.00
	192-216	0.03	NA
	216-240	0.03	NA
	Subtotal	**87.12**	**1.11**
Faeces	0-4	NS	NS
	4-8	NS	0.22 [a]
	8-12	NS	NS
	12-24	0.10	0.00
	24-48	2.88	0.02
	48-72	1.48	0.00
	72-96	0.20	0.00
	96-120	0.00	0.00
	120-144	0.00	0.00
	144-168	0.00	0.00
	168-192	0.00	0.01
	192-216	0.00	NA
	216-240	0.00	NA
	Subtotal	**4.66**	**0.25**
Cage debris/ rinse	0-4	5.23	NS
	4-8	4.10	NS
	8-12	2.09	0.34
	12-24	1.12	0.00
	24-48	0.90	0.00
	48-72	0.41	0.00
	72-96	0.50	0.00
	96-120	0.39	0.00
	120-144	0.16	0.00
	144-168	0.00	0.00
	168-192	0.00	0.00
	192-216	0.15	NA
	216-240	0.00	NA
	Subtotal	**15.05**	**0.34**

NS No sample excreted

NA Not applicable

a residual radioactivity from the glove sample (0.22%) included in the data

Acceptability

The study is acceptable as exploratory study.

Conclusion

Based on the results obtained in one animal, the *in vivo* dermal absorption of spirodiclofen in rhesus monkeys is approximately 2% (rounded value).

STUDY 7

Characteristics

reference	:	Odin-Feurtet, M. (2008)	exposure	:	8 h, unoccluded
type of study	:	*in vitro* dermal absorption	doses	:	2.4 mg/cm^2 (concentrate), 16 µg/cm^2 (intermediate dose) and 0.5 µg/cm^2 (low dose)
year of execution	:	2008	vehicle	:	Blank formulation Envidor SC 240
test substance	:	Radiolabelled test substance: [Dihydrofuranone-3-^{14}C]-spirodiclofen, radiochemical purity 99%, specific activity 3.89 MBq/mg. Unlabelled test substance: spirodiclofen, purity 99.2%.	GLP statement	:	yes
route	:	Dermal	guideline	:	OECD guideline 428
species	:	Human and rat	acceptability	:	acceptable
group size	:	4, 5 or 6 replicates (see Table 20 for details per group)	**Result**	:	Human skin: 0.33% (concentrate; 2.4 mg/cm^2), 1.66% (at 16 µg/cm^2) and 3.1% (at 0.5 µg/cm^2) Rat skin: 8.2% (concentrate; 2.4 mg/cm^2), 24.4% (at 16 µg/cm^2) and 15.7% (at 0.5 µg/cm^2)

Study design

The percutaneous absorption of [^{14}C]-triflumizole was studied *in vitro*, using flow-through diffusion cells. Dermatomed skin from rats and humans was exposed to either the neat product (240 g spirodiclofen/L formulation resulting in an area dose of 2.4 mg/cm^2), or two spray dilutions (1.6 g spirodiclofen/L formulation resulting in an area dose of 16 µg/cm^2 and 0.05 g spirodiclofen/L formulation resulting in an area dose of 0.5 µg/cm^2). The integrity of the skin membranes was established prior to the application of the test substance, by measuring the trans-epidermal water loss (TEWL). Aliquots of 10 µl were applied to an area of 1 cm^2 of unoccluded skin samples. The test substance remained in contact with the skin for 8 hours. The receptor fluid was Eagle's medium supplemented with 5% bovine serum albumine and gentamycin. Samples of receptor fluid were collected hourly for 24 hours. At the end of the exposure period, the test compound was removed from the skin surface with 1% v/v Tween 80 in PBS using natural sponge swabs. At the end of the study (24 hours after application) the skin samples were swabbed again and each skin sample was

tape stripped to remove the *stratum corneum*. The tape strips were individually collected and analysed. The receptor fluid, skin swabs, tape strips, skin membranes (skin and surrounding skin) and diffusion cell components were analysed using LSC.

Results

The solubility of spirodiclofen in the receptor fluid was sufficient. The dose formulations were considered to be homogeneous and acceptable for use in the study.

Table 20 presents the distribution of radioactivity for the human and rat dermatomed skin following a single topical application of the high, intermediate and low dose concentration of [^{14}C]-spirodiclofen in Envidor SC 240 formulation.

Table 20 Recovery of radioactivity for rat and human skin membranes

Absorption is expressed as percentage of the administered radiolabel.

High dose (240 g/L)				
	Human (n = 5)		Rat (n = 5)	
Samples	Mean	SD	Mean	SD
Surface dose (tape strips 1+2)	0.27	0.13	3.54	2.94
Skin swabs [a]	104.81	2.14	93.01	4.66
Donor chamber	0.03	0.05	0.17	0.16
Total % non absorbed	105.44	2.14	96.72	4.05
Skin [b]	0.11	0.11	0.65	0.39
Stratum corneum [c]	0.23	0.20	7.48	3.05
Total % at dose site	0.33	0.23	8.13	3.23
Total % directly absorbed [d]	0.002	0.005	0.085	0.03
Total % potentially absorbable [e]	0.33	0.31	8.22	3.25
Total % Recovery	105.44	2.16	104.93	1.09
Intermediate Dose (1.6 g/L)				
	Human (n = 4)		Rat (n = 4)	
Samples	Mean	SD	Mean	SD
Surface dose (tape strips 1+2)	1.19	0.89	3.94	1.87
Skin swabs [a]	98.33	1.32	72.15	10.15
Donor chamber	0.08	0.07	0.10	0.11
Total % non absorbed	99.60	1.27	76.20	8.37
Skin [b]	0.70	0.63	7.02	5.67
Stratum corneum [c]	0.95	0.60	13.58	8.82
Total % at dose site	1.65	1.22	20.61	8.84
Total % directly absorbed [d]	0.012	0.01	3.82	1.27
Total % potentially absorbable [e]	1.66	1.22	24.42	8.43
Total % Recovery	101.27	2.03	100.62	1.19
Low Dose (0.05 g/L)				
	Human (n = 6)		Rat (n = 4)	
Samples	Mean	SD	Mean	SD
Surface dose (tape strips 1+2)	3.82	2.78	5.56	2.27
Skin swabs [a]	88.64	2.61	74.38	4.19
Donor chamber	0.00	0.00	1.45	1.18
Total % non absorbed	92.46	2.02	81.39	1.18
Skin [b]	1.01	0.53	3.08	2.61
Stratum corneum [c]	2.02	1.54	8.71	3.58
Total % at dose site	3.03	1.92	11.79	4.34
Total % directly absorbed [d]	0.11	0.25	3.91	2.53
Total % potentially absorbable [e]	3.14	1.96	15.70	1.89
Total % Recovery	95.60	3.14	97.09	1.13

[a]: including swabs at 8 and 24 hours + surrounding swabs

[b]: Sum of skin after tape-stripping procedure and surrounding skin

[c]: tape-strips excluding numbers 1 & 2 which are considered to be non-absorbed dose.

[d]: including receptor fluid (0 to 24h) receptor fluid at termination time and receptor chamber

[e]: total % directly absorbed + total % at dose site

SD: standard deviation

n: number of skin cells used for calculation

Total mean recovery of the high, intermediate and low dose was 105.4, 101.3 and 95.6% for human skin and 104.9, 100.6 and 97.1% for rat skin. Results are given in Table 20. At the high dose 0.002% AR (applied radioactivity) had penetrated through the human skin at the 24 hour time point. Rat skin exposed under the same conditions was more permeable, as 0.085% AR penetrated within 24 hours. At the intermediate dose 0.012% and 3.82% of the applied dose penetrated within 24 hours through human and rat skin, respectively. At the low dose 0.11% and 3.91% of the applied dose penetrated within 24 hours through human and rat skin, respectively.

Acceptability

The study was performed in accordance with draft OECD 428 and is considered acceptable.

Conclusion

Since the swabbing procedure was intended to reflect a simple washing regimen at the end of the working day, the amount of radioactivity retrieved in this compartment was considered to be nonabsorbed. Since the material recovered in the surface tape-strips (first two tape-strips) could be associated with surface residues following incomplete removal of the dose after an 8-hour exposure period and/or material from the superficial *stratum corneum*, the amount of radioactivity retrieved in this compartment was considered to be non-absorbed (this is in line with the EFSA list of decisions).

Good recovery data were obtained, with mean total recoveries of radioactivity in the range of 95.6% to 105.4% of the applied dose.

For both the neat and diluted formulations, the majority of the radioactivity was removed by swabbing and by removal of the surface dose (first two tape strips).

An overview of the different compartments is presented in Table 21.

The mean percentage of [^{14}C]-spirodiclofen formulated as Envidor 240 SC considered to be potentially absorbable (directly absorbed plus total remaining at dose site) over a period of 24 hours for the neat formulation was 0.334% and 8.22% for the human and rat skin, respectively, yielding a factor difference of 25 between the two species for the neat product.

The mean percentage of [^{14}C]-spirodiclofen formulated as Envidor 240 SC to be potentially absorbable for the intermediate dose formulation was 1.66% and 24.4% for the human and rat skin respectively, yielding a factor difference of 15 between the two species for the intermediate dose formulation.

The mean percentage of [^{14}C]-spirodiclofen formulated as Envidor 240 SC to be potentially absorbable for the low dose formulation was 3.14% and 15.7% for the human and rat skin respectively, yielding a factor difference of 5 between the two species for the low dose formulation.

Table 21 Recovery of radioactivity for rat and human skin membranes

Absorption is expressed as percentage of the administered radiolabel.

	Distribution of radioactivity					
Dose levels	Neat formulation (SYP13262, 240 g/L)		Spray dilution: Intermediate dose (SYP13264, 1.6 g/L)		Spray dilution: Low dose (SYP13265, 0.05 g/L)	
Species	Human (n = 5)	Rat (n = 5)	Human (n = 4)	Rat (n = 4)	Human (n = 6)	Rat (n = 4)
SURFACE COMPARTMENT						
Skin swabs [a]	104.808	93.008	98.335	72.154	88.643	74.379
Surface Dose (tape-strips1&2)	0.266	3.536	1.187	3.938	3.817	5.561
Donor chamber	0.032	0.173	0.081	0.105	ND	1.446
Total % non-absorbed	***105.106***	***96.717***	***99.603***	***76.197***	***92.459***	***81.385***
SKIN COMPARTMENT						
Stratum corneum [b]	0.226	7.478	0.947	13.581	2.024	8.709
Skin [c]	0.106	0.654	0.704	7.024	1.005	3.082
Total % at dose site	***0.332***	***8.132***	***1.651***	***20.605***	***3.029***	***11.791***
RECEPTOR COMPARTMENT						
Total % directly absorbed [d]	***0.002***	***0.085***	***0.012***	***3.816***	***0.112***	***3.910***
TOTAL ABSORBABLE						
Total % potentially absorbable [e]	***0.334***	***8.217***	***1.663***	***24.421***	***3.141***	***15.702***
Total % Recovery	**105.441**	**104.934**	**101.266**	**100.618**	**95.600**	**97.087**

[a]: including swabs at 8 and 24 hours + surrounding swabs

[b]: tape-strips excluding number 1 & 2 which are considered to be non-absorbed dose.

[c]: sum of skin after tape-stripping procedure and surrounding skin

[d]: including receptor fluid (0 to 24 h), receptor fluid at termination time and receptor chamber

[e]: total % directly absorbed + total % at dose site

n: number of skin cells used for calculation

ND: not detected (below the limit of detection)

4.1.2 Human information

No data available

4.1.3 Summary and discussion on toxicokinetics

Absorption

The excretion of radioactivity in urine indicates that after a single oral dose of 2 mg/kg bw at least 64 % (males) or 76% (females) of spirodiclofen is absorbed within 48 h. In another study in which a single oral dose of 2 mg/kg bw was adminstered to rats, the radioactivity levels in urine and organs demonstrated that at least 60 and 75 % of the administered spirodiclofen was absorbed within 24 h. The urinary excretion over a 168 h period following a single oral dose of 100 mg/kg bw showed that absorption in male rats was at least 37 % at this dose. The fecal excretion of radioactivity in bile-duct cannulated rats that were treated with a single oral dose of 1 mg/kg bw of spirodiclofen

suggests incomplete absorption at this dose. The higher level of fecal excretion after administration of 100 mg spirodiclofen /kg bw indicates that the level of absorption decreases with increasing oral doses. In a 15 week feeding study similar absorption values were observed.

Dermal absorption studies were also available for spirodiclofen. In the first *in vivo* study in monkey dermal absorption was demonstrated to be 2%, though 8% of the administered dose could not be recovered and might also be absorped. The second *in vivo* study showed a dermal absorption percentage of 2%. Further, an *in vitro* study (human and rat) with spirodiclofen tested as Envidor SC240 (i.e. the representative formulation evaluated in the EU) is available. Dose levels were representative for the intended uses. The results show that for human skin the amount absorped is very low (concentrate: 0.4%; spray dilution: 3%).

Distribution

Levels of radioactivity in organs and tissues were low. Depending on the time of sampling peak tissue levels were observed at 3 h after administration (as measured by LSC) or at 4-8 h after administration (as assessed by autoradiography). At 48 h after oral administration of 2 mg spirodiclofen/kg bw, highest levels of radioactivity were observed in liver, kidney, plasma, gastrointestinal tract and skin. In females, organ and tissue levels were 5-15 times lower than in males. In male rats treated with spirodiclofen for 15 days, tissue levels were about 4 times lower than in males treated with a single dose of spirodiclofen. Relative distribution of radioactivity was similar for all experimental groups.

Metabolism

There was a marked sex difference in the metabolite profile. Analysis of urine collected over a 48 h period revealed that in male rats the main urinary metabolites (55-57% of total recovered radioactivity) were the 3- and 4- hydroxy-BAJ-enol isomers. In females the main urinary metabolite (55% of total recovered radioactivity) was BAJ-enol. In urine collected over a 24 h period a similar sex difference in metabolites was found. In plasma, liver and kidney samples of both male and female rats the main metabolite was BAJ-enol. In these samples the levels of the 3- and 4-hydroxy-enol isomers were higher in males than in females, indicating a higher capacity in the metabolisation of BAJ-enol in male rats. The differences in the metabolite profile were quantitative rather than qualitative. Such a sexual dimorphism in metabolism of endogenous substances or xenobiotics is not uncommon. Pretreatment of rats for 15 days or 15 weeks with unlabeled spirodiclofen did not markedly change the metabolism of spirodiclofen. In feces of male and female rats treated with 2 mg spirodiclofen /kg bw low levels of the parent compound, BAJ-enol and hydroxy-BAJ-enol isomers were found. The observation that the MA-cyclohexyl ester and dichlorobenzoic acid are almost exclusively found in feces suggests that substantial metabolisation of spirodiclofen may take place inside the gastrointestinal tract.

Excretion

Excretion of radioactivity was fast. After a single administration of spirodiclofen at a dose of 2 mg/kg bw 58 % (males) and 75% (females) of the administered radioactivity was excreted in urine within 24 h. At least 88 % of the administered dose (99% of recovered radioactivity) was excreted within 48 h. At this dose, excretion in expired air was negligible. In male rats, 168h after

administration of 100 mg spirodiclofen /kg bw, 96 % of the administered dose (100 % of recovered radioactivity) was excreted. In a 15-week feeding study similar excretion rates were observed.

4.2 Acute toxicity

Table 22: Summary table of relevant acute toxicity studies

Method	Results	Remarks	Reference [a]
Oral, rat *OECD 423*	LD_{50} > 2000 mg/kg bw	Purity: 99.1% Vehicle: 0.5% carboxymethyl cellulose	Krötlinger, F., (1996a)
Inhalation, rat Nose-only exposure *OECD 403*	LC_{50} > 5030 mg/m^3	Purity: 99.8% Doses: 520 and 5030 (limit concentration) mg/m^3 air MMAD: 3.4. and 6.7 resp., GSD: 1.6 and 2.0 resp.	Pauluhn, J. (1997)
Dermal, rat *OECD 402*	LD_{50} > 2000 mg/kg bw	Purity: 99.1% Single dose tested (2000 mg/kg bw)	Krötlinger, F., (1996b)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

Characteristics

reference/notifier	:	Krötlinger, F. (1996a)	exposure	:	once (by gavage)
type of study	:	Acute oral toxicity study, limit test	dose	:	2000 mg/kg bw
year of execution	:	1996	vehicle	:	0.5% carboxymethyl cellulose (CMC)
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	oral	guideline	:	In accordance with OECD 423
species	:	rat, Wistar (HsdCpb:WU)	acceptability	:	Acceptable
group size	:	3/sex	**LD_{50} rats**	:	>2000 mg/kg bw

Study design

The study was in accordance with "OECD Draft New Guideline - Acute Oral Toxicity – Acute Toxic Class Method", drafted 28th April 1995 (as mentioned by the study author), presently known as OECD 423.

Results

Mortality: none

Symptoms of toxicity: none

Body weight: normal

Pathology: no toxicologically relevant findings.

Acceptability

The study was considered acceptable.

Conclusions

The acute oral LD_{50} of spirodiclofen in Wistar rats was > 2000 mg/kg bw.

4.2.1.2 Acute toxicity: inhalation

Characteristics

reference/notifier	:	Pauluhn, J. (1997)	exposure	:	4 h (nose only)
type of study	:	Acute inhalation toxicity study.	doses	:	520 and 5030 (limit concentration) mg/m^3 air, MMAD 3.4. and 6.7 resp., GSD 1.6 and 2.0 resp.
year of execution	:	1997	vehicle	:	none (aerosol generation)
test substance	:	BAJ 2740 (spirodiclofen, purity 99.8%)	GLP statement	:	yes
route	:	Inhalation	guideline	:	In accordance with OECD 403
species	:	rat, Wistar (HsdCpb:WU)	acceptability	:	acceptable
group size	:	5/sex/dose	**LC_{50}**	:	> 5030 mg/m^3 air

Study design

The study was in accordance with OECD 403.

Results

Mortality: none

Symptoms of toxicity: Statististically significantly decreased rectal temperatures were measured in females of both dose groups.

Body weight: normal

Pathology: no toxicologically relevant findings.

Acceptability

The study was considered acceptable. At the dose levels aerosol particles < 3 μm were 41% and 13% in the low and high dose group respectively (considered to be respirable).

Conclusions

The acute 4-hour LC_{50} of spirodiclofen in Wistar rats was > 5030 mg/m^3 air (limit concentration).

4.2.1.3 Acute toxicity: dermal

Characteristics

reference/notifier	:	Krötlinger, F. (1996b)	exposure	:	24 h
type of study	:	Acute dermal toxicity study, limit test	dose	:	2000 mg/kg bw
year of execution	:	1996	vehicle	:	Demineralized water
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	Yes
route	:	Dermal	guideline	:	In accordance with OECD 402
species	:	rat, Wistar (HsdCpb:WU)	acceptability	:	Acceptable
group size	:	5/sex	**LD_{50} rats**	:	> 2000 mg/kg bw

Study design

The study was in accordance with OECD 402.

Results

Mortality: none

Symptoms of toxicity: none

Body weight: normal

Pathology: no toxicologically relevant findings.

Acceptability

The study was considered acceptable.

Conclusions

The acute dermal LD_{50} of spirodiclofen in Wistar rats was > 2000 mg/kg bw.

4.2.1.4 Acute toxicity: other routes

No information available

4.2.2 Human information

No information available

4.2.3 Summary and discussion of acute toxicity

Spirodiclofen was shown to be not acutely toxic if swallowed, after inhalation or by skin contact.

Oral LD50 and dermal LD50 were >2000 mg/kg bw and inhalation LC50 was >5030 mg/m^3 air (limit concentration).

4.2.4 Comparison with criteria

The oral LD_{50} is above the classification cut-off of 2000 mg/kg bw, therefore no classification is proposed for the oral route.

No deaths were observed in rats after inhalation at the maximal attainable concentration, therefore no classification is proposed for the inhalation route

The dermal LD_{50} is above the classification cut-off of 2000 mg/kg bw, therefore no classification is proposed for the dermal route.

4.2.5 Conclusions on classification and labelling

No classification for acute toxicity is required for spirodiclofen under the CLP regulation.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

A total of three guideline studies, one for each acute toxicity endpoint, are discussed.

Oral

Spirodiclofen was tested for acute oral toxicity in Wistar rats, according to OECD test guideline (TG) 423, GLP-compliant study (Krottlinger, 1996a). No deaths were observed at the dose tested (2000 mg/kg bw). No treatment related clinical signs of toxicity or effects on body weight were observed. No pathological abnormalities were observed at necropsy.

No classification for acute oral was proposed by the DS, as the LD_{50} was >2000 mg/kg bw for rats.

Dermal

Spirodiclofen was tested for acute dermal toxicity in the Wistar rats, according to OECD test guideline (TG) 402, GLP-compliant study (Krottlinger, 1996b). No deaths were observed at the single dose tested, 2000 mg/kg bw. No treatment related clinical signs of toxicity or effects on body weight were observed. No pathological abnormalities were recorded at necropsy.

No classification for acute dermal was proposed by the DS, as the LD_{50} was >2000 mg/kg bw for both males and females.

Inhalation

In an OECD TG 403 (GLP-compliant study) acute inhalation study (Pauluhn, 1997), rats (5/sex/dose) were nose-only exposed to two doses(520 and 5030 mg/m^3) of spirodiclofen for 4 hours. No deaths were observed at the limit concentration dose of 5030 mg/m^3. Statistically significantly decreased rectal temperatures were measured in females of both dose groups. No other treatment related clinical signs of toxicity or effects on body weight were observed. No

pathological abnormalities were recorded at necropsy.

No classification for acute inhalation was proposed by the DS, as the LC_{50} was >5.03 mg/L for both male and female rats.

Comments received during public consultation

There was one comment from a MSCA received during the public consultation supporting the DS's proposal not to classify spirodiclofen for acute toxicity.

Assessment and comparison with the classification criteria

Comparison with CLP criteria

Oral

Taking into account that the oral LD_{50} value in male and female rats as reported in Krottlinger (1996a) exceeds the value for which classification for acute oral toxicity is justified (2000 mg/kg bw), RAC agrees with the DS, that spirodiclofen should **not be classified for acute oral toxicity** according to the CLP criteria.

Dermal

Taking into account that the dermal LD_{50} value in male and female rats as reported in Krottlinger (1996b) is above the threshold value for classification (2000 mg/kg bw), RAC agrees with the DS, that spirodiclofen should **not be classified for acute dermal toxicity** according to the CLP criteria.

Inhalation

Taking into account that the inhalation LC_{50} value in male and female rats as reported in Pauluhn(1997), is above the threshold value for classification (5 mg/L/4h), RAC agrees with the DS, that spirodiclofen should **not be classified for acute inhalation toxicity** according to the CLP criteria.

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

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

In the available acute toxicity studies (see section 4.2), no specific organ effects were observed after single acute exposure via the oral, inhalation or dermal route.

In addition, the available acute oral neurotoxicity study (see section 4.12) does not show any acute neurotoxic effects upto and including exposure to 2000 mg/kg bw.

4.3.2 Comparison with criteria

Substances should be classified for STOT-SE when specific target organ toxicity (Cat 1 or 2) or narcotic effects or respiratory tract irritation (Cat 3) are observed.

As no specific organ effects fulfilling the classification criteria for specific organ toxicity – single exposure (STOT SE) were observed after single acute exposure via the oral, inhalation or dermal route, classification of spirodiclofen for STOT-SE is not required.

4.3.3 Conclusions on classification and labelling

Classification for specific organ toxicity – single exposure (STOT-SE) is not required.

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

Summary of the Dossier Submitter's proposal

In the available acute toxicity studies (Krottlinger, 1996a; Krottlinger, 1996b and, Pauluhn, 1997), no specific organ effects were observed after single acute exposure via the oral, inhalation or dermal route. In addition, in an available GLP compliant acute oral neurotoxicity study in Wistar rats (Sheets *et al.*, 2000), no compound related effects were observed up to the highest tested dose of 2000 mg/kg bw.

Therefore, based on the acute toxicity studies no classification is proposed by the DS for specific target organ toxicity (single exposure).

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen for specific target organ toxicity (single exposure) - STOT SE.

Assessment and comparison with the classification criteria

According to CLP criteria, 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 are classified in STOT SE 1 or 2. Classification should be supported by evidence associating single exposure to the substance with a consistent and identifiable toxic effect. Classification as STOT SE 3 is reserved for transient target organ effects and is limited to substances that have narcotic effects or cause respiratory tract irritation.

In the acute toxicity studies there were no clinical signs of toxicity following oral (Krottlinger, 1996a) and dermal exposure (Krottlinger, 1996b) to spirodiclofen. The decreased core temperature of females following inhalation of spirodiclofen aerosol particles that was not accompanied by any other pathological findings (Pauluhn, 1997) is not regarded relevant for classification. In addition, no effects were observed in the neurotoxicity study (Sheets *et al.*, 2000).

Therefore, there was no clear evidence of specific toxic effects at any target organ or tissue and no signs of respiratory tract irritation or narcotic effects were observed. RAC concludes that **no classification for specific target organ toxicity (single exposure) is warranted**.

4.4 Irritation

4.4.1 Skin irritation

Table 23: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference[a]
Rabbit OECD 404	Not irritating to the skin	Purity: 99.1%	Leuschner (1997a)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.4.1.1 Non-human information

Characteristics

reference/notifier	:	Leuschner, F. (1997a)	exposure	:	4 h (semi-occlusive)
type of study	:	Skin irritation study	dose	:	500 mg
year of execution	:	1996	vehicle	:	Moistened with water
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	dermal	guideline	:	In accordance with OECD 404
species	:	Rabbit, Himalayan	acceptability	:	acceptable
group size	:	3 m	**Effect**	:	Not irritating to skin

Study design

The study was in accordance with OECD 404.

Results

No skin irritation was observed in any of the rabbits.

Acceptability

The study was considered acceptable.

Conclusions

Spirodiclofen does not need to be classified as irritating to skin.

4.4.1.2 Human information

No information available.

4.4.1.3 Summary and discussion of skin irritation

No local irritation was observed after topical cutaneaous application of spirodiclofen to the skin of rabbits for 4 h under semi-occlusive conditions. Spirodiclofen is not a skin irritant.

4.4.1.4 Comparison with criteria

The CLP criteria for classification of substances for skin irritation are as follows:

- At least 2 of 3 tested animals have a mean score of ≥ 2.3 - ≤ 4.0 for erythema/eschar or for oedema from gradings at 24, 48 and 72 hours after patch removal or, if reactions are delayed, from grades on 3 consecutive days after the onset of skin reactions; or
- 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
- In some cases where there is pronounced variability of response among animals, with very definite positive effects related to chemical exposure in a single animal but less than the criteria above.

Considering that no cutaneous irritation was observed during the available skin irritation study, no classification is required for spirodiclofen under the CLP Regulation

4.4.1.5 Conclusions on classification and labelling

No classification for skin irritation is required for spirodiclofen under the CLP regulation.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

In the available skin irritation study (Leuschner, 1997a) conducted in accordance to OECD TG 404 (GLP compliant), no local irritation effects were observed after topical cutaneous application of spirodiclofen to the skin of Himalayan rabbits for 4h under semi-occlusive conditions.

Therefore, no classification was proposed by the DS for skin irritation/corrosion.

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen as irritating or corrosive to the skin.

Assessment and comparison with the classification criteria

Spirodiclofen was tested in a guideline compliant rabbit skin irritation study (Leuschner,

1997a).

Considering that no cutaneous irritation was observed during the available skin irritation study, no classification is required for spirodiclofen under the CLP Regulation. Therefore, RAC agrees with the DS's proposal that spirodiclofen **should not be classified as a skin irritant**.

4.4.2 Eye irritation

Table 24: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference[a]
Rabbit OECD 405	Not irritating to the eyes	Purity: 99.1%	Leuschner (1997b)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.4.2.1 Non-human information

Characteristics

reference/notifier	:	Leuschner, F. (1997b)	exposure	:	single instillation in conjunctival sac
type of study	:	Eye irritation study	dose	:	100 mg
year of execution	:	1996	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	ocular	guideline	:	in accordance with OECD 405
species	:	Rabbit, Himalayan	acceptability	:	acceptable
group size	:	3m	**Effect**	:	not irritating to eyes

Study design

The study was in accordance with OECD 405.

Results

The cornea, iris and conjunctiva were not affected by instillation of the test compound (all scores were zero at all time points).

Acceptability

The study was considered acceptable.

Conclusions

Spirodiclofen does not need to be classified as irritating to eyes.

4.4.2.2 Human information

No information available.

4.4.2.3 Summary and discussion of eye irritation

No eye irritation was observed after ocular application of spirodiclofen to the eye of rabbits.

4.4.2.4 Comparison with criteria

A substance shall be classified as a substance which could induce reversible eye irritation, classified in Category 2 (irritating to eyes), if when applied to the eye of an animal, a substance produces:

at least in 2 of 3 tested animals, a positive response of:

- Corneal opacity ≥ 1 and/or
- Iritis ≥1 and/or
- Conjunctical redness ≥ 2 and/or
- Conjunctival oedema ≥ 2

Calculated as the mean scores following grading at 24, 48, 72 hours after instillation of the test material, and which fully reverse within an observation period of 21 days.

Considering that no eye irritation was observed during the available eye irritation study, no classification is required for spirodiclofen under the CLP Regulation.

4.4.2.5 Conclusions on classification and labelling

No classification for eye irritation is required for spirodiclofen under the CLP regulation.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

In the available eye irritation study in accordance to OECD TG 405 (GLP compliant) (Leuschner, 1997b), no eye irritation effects were observed after ocular application of spirodiclofen to the eyes of Himalayan male rabbits. The cornea, iris and conjunctiva were not affected by instillation of the test compound (all scores were zero at all time points).

Therefore, the DS proposed no classification for eye irritation for spirodiclofen under the CLP regulation.

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen as irritating to the eye.

Assessment and comparison with the classification criteria

A substance which has the potential to induce reversible eye irritation shall be classified in Category 2 (irritating to eyes) if when applied to the eye of an animal, it produces:

- at least in 2 of 3 tested animals, a positive response of:
  - Corneal opacity ≥ 1 and/or
  - Iritis ≥1 and/or
  - Conjunctival redness ≥ 2 and/or
  - Conjunctival oedema ≥ 2
- Calculated as the mean scores following grading at 24, 48, 72 hours after instillation of the test material, and which fully reverse within an observation period of 21 days.

Considering that no eye irritation was observed during the available eye irritation study, no classification is required for spirodiclofen under the CLP Regulation for serious eye damage/eye irritation. Therefore, RAC agrees with DS for **no classification for eye damage/irritation** since neither irreversible nor reversible effects were observed.

4.4.3 Respiratory tract irritation

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015. In the CLP regulation, respiratory tract irritation is included in STOT-SE (cat 3).

4.4.3.1 Non-human information

4.4.3.2 Human information

4.4.3.3 Summary and discussion of respiratory tract irritation

4.4.3.4 Comparison with criteria

4.4.3.5 Conclusions on classification and labelling

4.5 Corrosivity

See table 23 of paragraph 4.4.1 for information on the available study on skin irritation.

4.5.1 Non-human information

See paragraph 4.4.1.1 for the available non-human information (Leuschner, 1997a).

4.5.2 Human information

No data available

4.5.3 Summary and discussion of corrosivity

No local irritation or corrosion was observed after topical cutaneaous application of spirodiclofen to the skin of rabbits for 4 h under semi-occlusive conditions. Spirodiclofen is not a skin irrititant or corrosive.

4.5.4 Comparison with criteria

The CLP criteria for classification of substances for skin corrosion are as follows:

A corrosive substance is a substance that produces destruction of skin tissue, namely, visible necrosis through the epidermis and into the dermis, in at least 1 tested animal after exposure up to a 4 hour duration. Corrosive reactions are typified by ulcers, bleeding, bloody scabs and, by the end of observation at 14 days, by discoloration due to blanching of the skin, complete areas of alopecia and scars. Histopathology shall be considered to discern questionable lesions. Three subcategories are provided within the corrosive category: subcategory 1A – where responses are noted following up to 3 minutes exposure and up to 1 hour observation; subcategory 1B – where responses are described following exposure between 3 minutes and 1 hour and observations up to 14 days; and subcategory 1C – where responses occur after exposures between 1 hour and 4 hours and observations up to 14 days.

Considering that no cutaneous corrosion was observed during the available skin irritation study, no classification is required for spirodiclofen under the CLP Regulation.

4.5.5 Conclusions on classification and labelling

No classification for skin corrosion is required for spirodiclofen under the CLP regulation.

4.6 Sensitisation

4.6.1 Skin sensititsation

Table 25: Summary table of relevant skin sensitisation studies

Method	Results	Remarks	Reference [a]
Guinea pig Skin sensitisation (Maximization test) OECD 406	Skin reactions after 48 and 72 h in: First challenge: 4/10 and 1/10 animals Second challenge: 1/10 and 4/10 animals	Purity: 99.1%	Stropp (1996)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.6.1.1 Non-human information

Characteristics

reference/notifier	:	Stropp, G. (1996)	exposure	:	Intradermal and topical induction, topical challenge
type of study	:	Skin sensitization study (Maximization test)	doses	:	5% intradermal injection (physical saline containing 2% Cremophor EL®); 50% topical induction and challenges *
year of execution	:	1996	vehicle	:	physical saline containing 2% Cremophor EL®
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	dermal	guideline	:	In accordance with OECD 406
species	:	Guinea pig, Hsd Poc:DH	acceptability	:	acceptable
group size	:	10 test animals (f), 5 controls (f)	**Effect**	:	positive

* Selected dose levels were based on a dose range-finding study using dermal[1] applications of 0%, 1%, 2.5% and 5% spirodiclofen, topical induction concentrations of 0%, 12%, 25% and 50% and challenge concentrations of 0%, 12%, 25% and 50%.

Study design

The study was in accordance with OECD 406 (first and second challenge).

Results

Skin reactions were observed in 4/10 and 1/10 test group animals after 48h and 72h respectively. After the second challenge skin reactions were observed in 1/10 and 4/10 animals after 48h and 72h respectively. One animal reacted in both challenge procedures. No skin reactions were observed in the control group.

Acceptability

The study was considered acceptable. A skin reaction was observed in 40% of the animals in the test group after the first and second challenge (0% in control group), and therefore the effect of the test substance is considered positive. Remarkable is that only one animal showed a positive reaction in both challenge treatments.

Conclusions

Under the test conditions spirodiclofen exhibits a skin sensitization potential.

4.6.1.2 Human information

No data available.

[1] In the DAR it is stated that this concerns dermal application. However, it is assumed that this should be *intra*dermal application.

4.6.1.3 Summary and discussion of skin sensitisation

Under the conditions of the Maximization test, spirodiclofen induced a positive response in 40% of the animals, at an intradermal induction dose of 5%.

4.6.1.4 Comparison with criteria

Substances shall be classified as skin sensitizers in accordance with the following criteria:

- If there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons, or
- If there are positive results from an appropriate animal test (adjuvant type guinea pig test: ≥ 30% responding at ≤ 0.1% intradermal induction dose or ≥ 60% responding at > 0.1 to ≤ 1% intradermal induction dose (Cat 1A); or ≥ 30% to 60% responding at > 0.1 to ≤ 1% intradermal induction dose or ≥ 30% responding at > 1% intradermal induction dose (Cat 1B))

Given that the response in the guinea pig Maximization test is 40% at an intradermal induction dose of > 1%, classification of spirodiclofen for skin sensitisation as Skin Sens. 1B is required. The data are considered sufficient for sub-classification seen the low response of 40% at an intradermal induction concentration of 5%.

4.6.1.5 Conclusions on classification and labelling

Classification of spirodiclofen for skin sensitisation (Skin sens. 1B, H317: May cause an allergic skin reaction) is required.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

In a Guinea PigMaximisation Test (GPMT) according to OECD TG 406 (GLP compliant) (Stropp, 1996), spirodiclofen was found to be a skin sensitiser since a skin reaction was observed in 40% of the animals in thetest group after the first and second challenge (0% in control group) at an intradermal induction dose of 5%. Therefore the effects of the test substance were considered positive and the DS proposed to classify spirodiclofen as a Skin Sensitiser 1B; H317: May cause an allergic skin reaction.

Comments received during public consultation

Three comments were received from different MSCAs during public consultation supporting the DS's proposal to classify spirodiclofen as a Skin Sensitiser 1B; H317: May cause an allergic skin reaction.

Assessment and comparison with the classification criteria

There is one Guinea Pig Maximization Test (GPMT, OECD TG 406) available to assess the skin sensitisation properties of spirodiclofen. The study is a summarised in the Table below.

Table: Summary of the results of the GPMT study.

Dose/group	**First challenge***		**Second challenge***	
	48h	**72h**	**48h**	**72h**
5% intradermal injection (physical saline 2% Chemophor EL); challenge concentration 50% topical induction and challenges	4/10 animals	1/10 animals	1/10 animals	4/10 animals
Control group	0		0	

**One animal showed a positive reaction in both challenge treatments.*

When the data available is derived only from animal studies (GPMT), substances shall be classified as skin sensitizers in accordance with the following criteria:

- If there are positive results from an appropriate animal test (adjuvant type GPMT)
  - Cat. 1A: ≥ 30% responding at ≤ 0.1% intradermal induction dose or ≥ 60% responding at > 0. 1 to ≤ 1% intradermal induction dose; or
  - Cat. 1B: ≥ 30% to 60% responding at > 0.1 to ≤ 1% intradermal induction dose or ≥ 30% responding at > 1% intradermal induction dose.

The positive response in the Guinea Pig Maximization Test was 40% with an intradermal induction dose of > 1%. The data are considered sufficient for sub-classification: the response of 40% animals with positive skin reactions is comparably low at such a high intradermal induction dose of 5%. Therefore, RAC concludes that classification of spirodiclofen for skin sensitisation as **Skin Sens. 1B (H317: May cause an allergic skin reaction)** is appropriate.

4.6.2 Respiratory sensitisation

No data available.

4.6.2.1 Non-human information

4.6.2.2 Human information

4.6.2.3 Summary and discussion of respiratory sensitisation

4.6.2.4 Comparison with criteria

4.6.2.5 Conclusions on classification and labelling

No classification is proposed for respiratory sensitisation due to lack of data.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

No classification is proposed by the DS due to lack of data.

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen as a respiratory sensitiser.

Assessment and comparison with the classification criteria

No assessment and comparison with the classification criteria is possible due to lack of data.

4.7 Repeated dose toxicity

Table 26: Summary table of relevant repeated dose toxicity studies

Species	Route	Concentration, dose mg/m³, mg/kg bw/day	Exposure time and period	Observations and remarks	Reference [a]
ORAL ROUTE					
Mouse (CD-1) 10/sex/dose	Oral (feed)	0 - 100 – 1000 – 10000 ppm (corresponding to 0, 15.3, 163.8 and 1629.9 mg/kg bw/day in males and 0, 30.1, 233.6 and 2685.2 mg/kg bw/day in females) Purity: 99.1%	13-week	Centrilobular hepatocellular hypertrophy in males NOAEL < 15.3 mg/kg bw/day	Leser (1998)
Rat (Wistar) females 5/dose	Oral (feed)	0, 100, 500 and 5000 ppm (corresponding to 0, 10.0, 49.6 and 569.3 mg/kg bw Purity 98.2%	4-week	Effects on haematology (lymphocytes↓, segmented neutrophils↑), clinical chemistry (ASAT↑, ALAT↑, cholesterol↓, triglycerides↓, protein↓) , immune system (IgG antibodies↓, splenic and lymph node T-cells↓). Hepatic ECOD activity↑, periportal liver cell proliferation↑ NOAEL = 10 mg/kg bw/day	Krötlinger (2000)
Rat (Wistar) 10/s ex/dose	Oral (feed)	0, 100, 500, 2500 and 12500 ppm (corresponding to 0, 6.6, 32.1, 166.9, 851.4 mg/kg bw/day for males and 0, 8.1, 47.1, 215.3 , 995.8 mg/kg bw/day for females) Purity 99.1%	14-week	Adrenal cortical vacuolation accompanied by increased grading of vacuolation. NOAEL < 8.1 mg/kg bw/day	Wirnitzer (1998)
Dog (beagle) 2/sex/dose	Oral (feed)	0, 400, 2000 and 10000 ppm (corresponding to 11.3, 65.5 and 284.5 mg/kg bw/day)	4-week	Effects on haematology, increased ALAT, increased enzyme activities in liver tissue (N-DEM, O-DEM, P450, ECOD, ALD, EH, Glu-T), decreased kidney weight, increased weights of uterus, adrenals and	Wetzig (2000)

Species	Route	Concentration, dose mg/m^3, mg/kg bw/day	Exposure time and period	Observations and remarks	Reference [a]
		Purity 99.1%		brains, Leydig cell vacuolation, cytoplasmic vacuolation adrenal cortex. NOAEL = 11.3 mg/kg bw/day	
Dog (beagle) 5 males/dose	Oral (feed)	0, 100, 2000 ppm (corresponding to 0, 2.9, 55.9 mg/kg bw/day) Purity 97.9%	8-week	Increased AP, increased organ weights of liver, thyroid, adrenals, thymus and pancreas, decreased prostate weight, increased cytoplasmic vacuolation of the adrenal cortex NOAEL < 2.9 mg/kg bw/day	Wetzig (2001b)
Dog (beagle) 4/sex/dose	Oral (feed)	0, 200, 630, 2000 ppm (corresponding to 0, 8.0, 27.3, 82.8 mg/kg bw/day, for male and female together) Purity 98.6%	14-week	Effects on haematological parameters, clinical biochemistry, liver microsomal enzymes, changes relative prostate weight and histopathological changes in the adrenal gland NOAEL < 8.0 mg/kg bw/day	Wetzig (2001a)
Dog (beagle) 4/sex/dose	Oral (feed)	0, 20, 50, 150, 500/600[b] ppm (corresponding to 0, 0.57, 1.45, 4.54. 16.9 mg/kg bw/day) Purity 97.8%	52-week	Increased adrenal weight and adrenal vacuolation NOAEL = 1.45 mg/kg bw/day	Wetzig (2001c)
Mouse (CD-1)	Oral (feed)	25, 3500, 7000 ppm (4.1, 610, 1216 mg/kg bw/day in males and 5.1, 722, 1495 mg/kg bw/day in females) Purity: 97.6-98.6%	18 months *(combined chronic toxicity/carcinogenicity study)*	Non-carcinogenic effects: Effects on BW↓, haematological parameters (MCHC↓, WBC↓, segmented neutrophils↓, lymphocytes↑, atypical lymphocytes↓), organ weights of adrenals↑, liver↑, testes↑ and kidney↓. Increased incidence of adrenal pigmentation and vacuolation in females, increased incidence of amyloid in several tissues and increased incidence of hepatocytomegaly in males.	Wahle, 2000 (see section 4.10.1)

Species	Route	Concentration, dose mg/m^3, mg/kg bw/day	Exposure time and period	Observations and remarks	Reference [a]
				LOAEL (chronic toxicity) = 4.1 mg/kg bw/day	
Rat (Wistar)	Oral (feed)	0, 50, 100, 350, 2500 ppm (0, 2.04, 4.11, 14.72, 110.14 mg/kg bw/day for males, and 0, 2.87, 5.93, 19.88, 152.90 mg/kg bw/day for females) Purity: 97.6-98.6%	108 weeks *(combined chronic toxicity/carcinogenicity study)*	Non-carcinogenic effects: effects in high dose group on BW↓, food consumption↑, haematology (leukocytes↓, lymphocytes↓), and clinical chemistry (AP↑, T4↑, TSH↑). Increased adrenal weight (m) and decreased spleen weights (f) in all dose groups (however, not dose-realed and not accompanied by histopathological changes); increased (absolute/relative) thymus and ovaries weights in two highest dose groups. NOAEL (chronic toxicity) = 5.93 mg/kg bw/day	Wirnitzer, 2000 (see section 4.10.1)
Rat (Wistar)	Oral (feed)	0, 70, 350, 1750 ppm (F0: 5.2, 26.2, 134.8 mg/kg bw/day for males and 5.5, 27.6, 139.2 mg/kg bw/day for females; F1: 6.4, 30.2, 177.6 mg/kg bw/day for males and 7.0, 34.4, 192.7 mg/kg bw/day for females) Purity: 98.6%	F0: 12 weeks pretreatment F0; F1 offspring untill weaning of F2. *(2-generation study)*	Non-reprotoxic effects (F0-animals): BW↓ (m: all dose groups; f: mid nd high dose group), organ weights of brain (m,f), adrenals↑ (m/f), liver↓ (m), kidney↓ (f). Vacuolisation of adrenal glands (f, mid and hig dose groups) LOAEL (systemic effects) = 5.2 mg/kg bw/day	Eiben 2000 (see section 4.11.1.1)
Rat (Wistar)	Oral (feed)	Nominal: 0, 100, 1000, 12500 ppm (0, 7.2, 70.3, 1088.8 mg/kg bw/day in males and 0, 9.1, 87.3, 1306.6 mg/kg bw/day in females) Purity: 97.4-97.8%	13 weeks *(subchronic neurotoxicity screening study)*	BW↓ (m/f, high dose), food consumption ↓ (m/f, high dose), urine stain, red thinged paws, decreased foot splay (mf/, high dose), decreased forlimb/hindlimb grip strength (m/f, high dose) NOAEL (neurotoxicity) = 70 mg/kg bw/day	Sheets 2001 (see section 4.12.1.1)
Rat (Wistar)	Oral (feed)	0, 50, 100, 350, 2500 ppm (2.04, 4.11, 14.72, 110.14 mg/kg bw/day in males and 0, 2.87, 5.93, 19.88, 152.90 mg/kg bw/day in females) Purity: 97.6-98.6%)	77 weeks *(Functional observation battery)*	No neurotoxic effects observed. NOAEL (neurotoxicity) = 110.14 mg/kg bw/day	Wirnitzer 2000 (see section 4.12.1.1; this study is also part of the combined chronic toxicity /carcinogenicity study of Wirnitzer

Species	Route	Concentration, dose mg/m^3, mg/kg bw/day	Exposure time and period	Observations and remarks	Reference [a]
					2000 as described in section 4.10.1.1)
Rat (Wistar)	Oral (feed)	0, 100, 5000, 2500, 12500 ppm (0, 8.6, 44.7, 232.4, 1284.9 mg/kg bw/day for males and 0, 9.3, 45.0, 237.6, 1466.1 mg/kg bw/day for females)	4 weeks *(subacute immunotoxicity study)*	BW↓ (two highest dose groups), decreased immunological parameters (two highest dose groups) NOAEL = 45 mg/kg bw/day	Wirnitzer 1998 (see section 4.12.1.2; this study is also part of the 14-week oral toxicity study of Wirnitzer 1998)
DERMAL ROUTE					
Rat (Wistar) 5/sex/dose	dermal	0 and 1000 mg/kg bw/day Purity 97.9%	22 applications during a 28-day period	Decreased Hb, Ht, ALAT and triglycerides and decreased relative adrenal weight NOAEL < 1000 mg/kg bw/day	Kröttlinger (1999)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).
[b] The concentration in the 500 ppm group was increased to 600 ppm after about 3 weeks of exposure.

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

STUDY 1

Characteristics

reference/notifier	:	Leser, K.H., A. Romeike (1998)	exposure	:	13 weeks
type of study	:	13 week oral toxicity study	doses[1]	:	0, 100, 1000 and 10000 ppm (equal to 15.3, 163.8 and 1629.9 mg/kg bw/day in males and 30.1, 233.6 and 2685.2 mg/kg bw/day in females)
year of execution	:	1996	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	Oral (diet, with 1% peanut oil)	guideline	:	In accordance with OECD 408
species	:	mouse, CD-1	acceptability	:	acceptable
group size	:	10/sex/dose	**NOAEL**	:	<15.3 mg/kg bw/day

[1] Dose levels were based on on the results of a pilot study with CD-1 mice in which 5/sex/dose animals were dosed with 0, 100, 1000 and 10000 ppm during 4 weeks (study no. T4060765, 1996).

Study design

The study was performed in accordance with OECD guideline 408 (deviation: no ophthalmoscopy performed).

Acceptability

The study was considered acceptable.

Results

The results are summarized in Table 27. No mortality or clinical signs were observed. No statistically significant effect on food- and water consumption and body weights were observed, though the body weights were slightly (not statistically significant) decreased in the mid dose male animals and high dose male and female animals. Haematological analysis revealed that Hb and Ht levels were decreased in female animals of the high dose group (Hb: statistically significant). Cholesterol levels were decreased in the high dose group (statistically significant for female animals only). Changes in organ weights were observed in the mid and high dose group animals. Adrenal weights (absolute and relative) were (not significantly, dose related) increased in females. Kidney weight (absolute and relative) was statistically significant decreased in mid- and high-dose male animals. Testes weights (absolute and relative) were (not significantly, dose related) increased. Histopathological analysis revealed some significant effects. Histopathological changes in liver included centrilobular hepatocellular hypertrophy (low-, mid- and high-dose males, and high-dose females) and periportal cytoplasmic vacuolisation (high-dose males and females). Changes to the adrenal glands included cytoplasmic vacuolisation (mid-dose females, high-dose males and females), degeneration of cortical cells (high dose female animals) and mononuclear infiltrate (high dose females). Testes effects were hypertrophy/activation and vacuolisation of Leydig cells.

Based on the results of this study, a NOAEL could not be derived (< 15.3 mg/kg bw/day). The LOAEL is considered 15.3 mg/kg bw/day.

Table 27. Overview of the results of a 13-week oral toxicity study in mice (Leser (1998))

Dose	**0 ppm (0 mg/kg bw/d)**		**100 ppm (m/f: 15.3/30.1 mg/kg bw/d)**		**1000 ppm (m/f: 163.8/ 233.6 mg/kg bw/d)**		**10000 ppm (m/f: 1629.9/2685.2 mg/kg bw/d)**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none								
Clinical signs	No toxicologically relevant effects								
Body weight					d		d	d	
Food and water consumption	No toxicologically relevant effects								
Ophthalmoscopy	Not performed								
Haematology									
-Hb								ds	
-Ht								d	
Urinalysis	Not performed								
Clinical chemistry[1]									
- cholesterol							d	ds	
Organ weights[2, 3]									
-adrenals						i[a/r]		i[a/r]	f
-spleen[4]								i[r]	
-kidneys					ds[a,r]		ds[a,r]		
-testes					i[a/r]		i[a/r]		m
-ovaries								i[r]	
Pathology									
macroscopy	No toxicologically relevant effects								
microscopy									
liver									
-centrilobular hepatocellular hypertrophy			3/10		3/10		6/10	2/10	
grade 2			3/3		3/3		5/6	2/2	
grade 3							1/6		
-periportal cytoplasmic vacuolisation							1/10	3/10	
Adrenal glands									
-cytoplasmic vacuolation						6/10	8/9	10/10	
-degeneration of cortical cells								9/10	
-mononuclear infiltrate								9/10	
Testes[5]									
-hypertrophy/activation Leydig cells	1/10		1/10		9/10		10/10		
-hypertrophy/activation Leydig cells, severity							i		
-vacuolation Leydig cells							7/10		

dr dose related
d/i decreased/ increased
ds/is decreased significantly/ increased significantly
a/r absolute/ relative organ weight

1 plasma glucose determination was performed in non-fasting animals.

2 notable is the observed higher relative weights of brain, adrenals and spleen in female animals compared to males.

3 the observed significantly decreased liver weight (a, r) of male mice dosed 1000 ppm is considered incidental, since no effect on liver weight was observed in the highest dose group.

4 mean spleen weight in male controls were high and showed a great SD, due to one animal with a high spleen weight.

5 male sex organs (testes, epididymides, prostates, seminal vesicles) were re-examined, based on the observed Leydig cell alterations in a subacute and a subchronic study in dogs. Results of the reexamination were presented in a first amendment in the study.

STUDY 2

Characteristics

reference/notifier	:	Krötlinger, F., V. Geiβ (2000)	exposure	:	28 days
type of study	:	4 weeks oral toxicity study	doses	:	0, 100, 500 and 5000 ppm (0, 10.0, 49.6 and 569.3 mg/kg bw)
year of execution	:	1994	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 98.2%)	GLP statement	:	yes
Route	:	Oral (diet, with 1% peanut oil)	guideline	:	not completely in accordance with OECD 407
Species	:	rat, Wistar	acceptability	:	Not acceptable
group size	:	5f/dose	**NOAEL**	:	10 mg/kg bw/day

Study design

A 4-week repeated dose oral toxicity study was performed in female Wistar rats. Animals (5/dose) were exposed to spirodiclofen via the diet in doses of 0, 100, 500 and 5000 ppm (0, 10.0, 49.6 and 569.3 mg/kg bw). The study was performed not completely following OECD 407. Only 5 animals/dose group and only females were tested, FACS-scan analyses were performed with pooled samples for each dose group and compared to "historical control values", which data were not included. Results with FACS-scan were presented without dimensions of the determined parameters. The following immunotoxicological investigations were performed: cell counts in spleen, lymph nodes and bone marrow, FACScan analyses for determination of subpopulations in spleen cells and lymph node cells (pooled samples for each dose group) with surface markers for B cells (PanB; RLN-9D3), T-helper cells (CD4; OX-35), lymphocytes (CD45; OX-22), interleukin-2 receptor expressing cells (CD25; OX-39) and T cell marker CD2 (OX-34). Double labeling was done with CD4/CD45R and CD2/CD25. In addition, cell proliferation in liver and kidney were determined.

Acceptability

Since the study was performed with only 5 female rats per dose group, and the FACSscan analyses were performed with pooled group samples which were compared with (not included) historical control values, the study was considered to be supportive.

Results

The results are summarized in Table 28. No mortality or other clinical symtoms were observed. Body weight, food and water consumption were not affected, though food and water consumption were slightly (not statistically significant) increased and decreased respectively in all treatment groups. Haematological analysis showed that the number of lymphocytes were (dose related, not statistically significant) decreased and the number of segmented neutrophils were (dose-related, not statistically significant) increased. Clinical chemistry analysis revealed that ASAT and ALAT were statistically significant increased in the high-dose group, whereas cholesterol, triglycerides and proteins were statistically significant decreased in the high-dose group. In hepatic tissue, a dose-related increase in 7-ethoxycoumarin deethylase (ECOD) activity was observed in the mid- and high-dose groups. Cell proliferation studies (performed with controls and highest dose group only) showed an increase in periportal liver cells (+32%), whereas in kidneys, proliferation of both

medulla (+76%) and cortex (+169%) cells was observed. Effects on the immune system included reduction of IgG antibodies and reduction of splenic and lymph node T-cells at 500 ppm and 5000 ppm. No histopathological changes were observed.

Based on the results of this study, a NOAEL of 100 ppm (10.0 mg/kg bw/day) was derived.

Table 28 Overview of the results of 28-day oral toxicity study in Wistar rats (Krötlinger (2000))

Dose	**0 ppm (0 mg/kg bw/d))**	**100 ppm (10.0 mg/kg bw/d)**	**500 ppm (49.6 mg/kg bw/d)**	**5000 ppm (569.3 mg/kg bw/d)**	**dr**
	f	**f**	**f**	**f**	
Mortality	none				
Clinical signs	No toxicologically relevant effects				
Body weight	No toxicologically relevant effects				
Food consumption		i	i	i	
Water consumption		d	d	d	
Haematology					
-reticulocytes		d	d	d	
-platelet count				d	
-hepato-quick (sec)				i	
-lymphocytes			d	d[1]	f
-segmented neutrophiles			i	i[1]	f
Clinical chemistry					
-ASAT				is	
-ALAT				is	
-AP				i[2]	
-cholesterol				ds	
-triglycerides				ds	
-protein				ds	
Liver tissue					
-ECOD			is	is	f
-EH				i	
Organ weights[3]					
-ovaries				d	
-thymus				d	
Immunotoxicity[4]					
-cell counts lymph nodes				d	
-cell counts spleen			d	d	
-cell counts after mitogen (LPS) stimulation lymph nodes				d	
-spleen surface marker, T helper/lymphocytes			d	ds[5]	
-lymph nodes surface marker, T helper/lymphocytes			d	ds[5]	
-spleen, surface marker CD2 T cell			d	ds[5]	
-lymph nodes, IL-2 expressing cells			d		
-IgG antibody titer[6]			ds	ds	

Dose	**0 ppm (0 mg/kg bw/d))**	**100 ppm (10.0 mg/kg bw/d)**	**500 ppm (49.6 mg/kg bw/d)**	**5000 ppm (569.3 mg/kg bw/d)**	**dr**
	f	**f**	**f**	**f**	
Cell proliferation liver[4]					
-periportal				i	
Cell proliferation kidney[4]					
-cortex				i	
-medulla				i	
Pathology					
macroscopy	No toxicologically relevant effects				
microscopy	No toxicologically relevant effects				

dr dose related
i increased
d decreased
is increased significantly
ds decreased significantly

[1] in the highest dose group, all individual values were below control values (lymphocytes) and higher than control values (segmented neutrophiles) with the exception of animal no 20. This animal also showed highest food intake and lowest water intake in that dose group.

[2] not significantly higher, but all values were higher than control values.

[3] decreased absolute and relative organ weights of ovaries and thymus were observed.

[4] no individual data were presented.

[5] since analyses in the FACScan were performed with pooled (group) samples, the statement 'significant' by the study author is made on the basis of the range of variance of their 'historical data' of control animals However, historical data were not included.

[6] According to the study author, an unusual high variation of the IgA titer was detected in the control animals, so dose level related effects were not discernible

STUDY 3

Characteristics

reference/notifier	:	Wirnitzer, U., A. Romeike (1998)	exposure	:	14 weeks (4 weeks for immunotox)
type of study	:	14 week oral toxicity study with 4 week recovery phase	Doses[1]	:	0, 100, 500, 2500 and 12500 ppm (0, 6.6, 32.1, 166.9, 851.4 mg/kg bw/day for males and 0, 8.1, 47.1, 215.3 , 995.8 mg/kg bw/day for females)
year of execution	:	1996	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	Oral (diet, with 1% peanut oil)	guideline	:	in accordance with OECD 408
species	:	Rat, Wistar (Hsd Cpb:WU)	acceptability	:	acceptable
group size	:	10/sex/dose (5/sex/dose for immunotox)	**NOAEL**	:	< 8.1 mg/kg bw/day

[1] doses are based on results of a subacute study with female rats (0 to 5000 ppm).

Study design

In a 14-week oral toxicity study, Wistar rats were exposed to spirodiclofen via the diet. Animals (10/sex/dose, except for immunotox) were exposed to 0, 100, 500, 2500 and 12500 ppm (0, 6.6, 32.1, 166.9 and 851.4 mg/kg bw/day for males, and 0, 8.1, 47.1, 215.3 and 995.8 mg/kg bw/day for females. A recovery group was included with a 4-week non-exposure period. The study was performed in accordance with OECD guideline 408, with the following deviations: sensory reactivity to stimuli of different types was not investigated; glucose was determined in blood of non-fasting animals. For immunotoxicological investigations, satellite groups of only 5/sex/dose were orally exposed to spirodiclofen for 4 weeks only. No immunotoxicological investigation was performed after 13 weeks of exposure.

Acceptability

The study was considered acceptable.

Results

The results of the study are summarized in Table 29. Details on incidence and grading of small cortical vacuolation in the adrenal gland are presented in Table 30.

Table 29. Overview of the results 14-week oral toxicity study in rats (Wirnitzer (1998))

Dose	**0 ppm (m/f: 0 mg/kg bw/d)**		**100 ppm (m/f: 6.6/8.1 mg/kg bw/d)**		**500 ppm (m/f: 32.1/47.1 mg/kg bw/d)**		**2500 ppm (m/f: 166.9/215.3 mg/kg bw/d)**		**12500 ppm (m/f: 851.4/995.8 mg/kg bw/d)**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none										
Clinical signs	No toxicologically relevant effects										
Body weight (gain)[1]							d	d	ds	ds	m, f
Food consumption[2]									ds	ds	
Water consumption									d	d	
Ophthalmoscopy	No toxicologically relevant effects										
Urinalysis											
-volume									is		
-density									ds		
Haematology											
-leukocytes[3]							d		d	ds	m
-ery's										is	
-Hb									d		
-MCV										ds	
-MCH										ds	
-thrombocytes									ds		
-Hepato Quick (anticoagulation)							is	is	is	is	m, f
-lymphocytes (%)									d	d	
-segm (%)									i	i	
-eos (%)									i	is	
Clinical chemistry											
-AP							is		is	is	m
-GLDH[4]									ds	d	
-Cholesterol							ds		ds	ds	m
-Triglycerides								d	ds	ds	f
-Bili-t							ds		ds	ds	m
-Protein									ds	d	
-Phosphate									ds		
-Chloride										ds	
-TSH								is		is	f
Organ weights											
-liver									ds^a	ds^a	
-spleen							d^a/ds^r		$d^{a/r}$	$ds^{a/r}$	
-adrenals									i^a/is^r	$is^a/^r$	
-testes									i^r		
-thymus									d^r		
Pathology											

Dose	0 ppm (m/f: 0 mg/kg bw/d)		100 ppm (m/f: 6.6/8.1 mg/kg bw/d)		500 ppm (m/f: 32.1/47.1 mg/kg bw/d)		2500 ppm (m/f: 166.9/215.3 mg/kg bw/d)		12500 ppm (m/f: 851.4/995.8 mg/kg bw/d)		dr
	m	f	m	f	m	f	m	f	m	f	
macroscopy	No toxicologically relevant effects										
microscopy											
Liver											
-reduced glycogen content	-	-	-	-	-	-	-	-	-	4/10	
Adrenals											
-small cortical vacuolation	5/10	4/10	9/10	3/10	8/10	8/10	10/10	8/10	10/10	10/10	
-grading	0.7	0.6	1.5	0.5	1.1	1.4	3.3	1.6	3.3	3.8	
-mixed cortical vacuolation	7/10	-	7/10	-	6/10	-	9/10	-	10/10	-	
-grading	1.6		1.2		0.9		1.2		2.9		
Duodenum											
-epithelial vacuolation	-	-	-	-	-	-	-	-	2/10	1/10	
Jejenum											
-epithelial vacuolation	-	-	-	-	-	-	8/10	7/10	8/10	7/10	
Ileum											
-epithelial vacuolation	-	-	-	-	-	-	1/10	-	-	2/10	

dr dose related
ds/is statistically significantly decreased/increased.
d/i decreased/increased.
a/r absolute/relative organ weight
- no abnormality detected
[1] body weights of satellite groups were more affected.
[2] food intake was significantly reduced during the first 4 days of treatment at 2500 and 12500 ppm in both sexes. During further treatment daily food intake was occasionally significantly reduced at 12500 ppm at both sexes. During the recovery period food intake in females was increased.
[3] Observed at weeks 13, effect also observed (not per se statistically different) at week 5.
[4] control values between male/females differed, without explanation of the study author. In addition, a great variance was observed in the female values. However, without explanation of the study author, the observed decrease in females in the highest dose group is considered relevant.

Table 30 Incidence and grading of small cortical vacuolation in the adrenal gland (Wirnitzer (1998))

Males

	0 ppm (0 mg/kg bw/d)	100 ppm (6.6 mg/kg bw/d)	500 ppm (32.1 mg/kg bw/d)	2500 ppm (166.9 mg/kg bw/d)	12500 ppm (851.4 mg/kg bw/d)
No. examined	10	10	10	10	10
Small cortical vacuolation					
Grade 1	3	4	5		1
Grade 2	2	4	3	1	1
Grade 3		1		5	3
Grade 4				4	4
Grade 5					1
Total no. of tissues affected	**5**	**9**	**8**	**10**	**10**
Average grade/no. of animals per group	**0.7**	**1.5**	**1.1**	**3.3**	**3.3**
Average grade/no. of tissues affected	**1.4**	**1.7**	**1.4**	**3.3**	**3.3**

Females

	0 ppm (0 mg/kg bw/d)	100 ppm (8.1 mg/kg bw/d)	500 ppm (47.1 mg/kg bw/d)	2500 ppm (215.3 mg/kg bw/d)	12500 ppm (995.8 mg/kg bw/d)
No. examined	10	10	10	10	10
Small cortical vacuolation					
Grade 1	2	1	3	2	
Grade 2	2	2	4	4	2
Grade 3			1	2	1
Grade 4					4
Grade 5					3
Total no. of tissues affected	**4**	**3**	**8**	**8**	**10**
Average grade/no. of animals per group	**0.6**	**0.5**	**1.4**	**1.6**	**3.8**
Average grade/no. of tissues affected	**1.5**	**1.7**	**1.8**	**2.0**	**3.8**

The submitted historical data for small cortical vacuolation in the male rat indicated that in 9 studies, including three with a recovery period, the average grade/no. of animals per group was between 0.1 and 1.1 (in 4 cases, the average grade was >0.7) and the average grade/no. of tissues affected was between 1.0 and 2.0 (in 6 cases, the average was >1.4) (Hartmann, 2005).

Conclusions

Since immunotoxicologal investigation was performed after 4 weeks of exposure instead of after 14 weeks, the results of those investigations are not considered in this 14 week oral toxicity study, but evaluated as a separate study (see section 4.12.1.2 of this CLH report).

Test substance induced changes were mainly observed at and above 2500 ppm, and included decreased bw and leukocyte number, increased coagulation time, AP and TSH and decreased cholesterol, triglycerides and bilirubin concentrations. Absolute and relative weight of spleen was decreased. Histopathological examination showed increased incidence of epithelial vacuolation in jejenum (m, f) and increased incidence and severity of adrenal cortical vacuolation in males dosed 100 ppm and above, whereas in females this effect was observed at and above 500 ppm. Additional submitted historical data for male rats indicated that the observed cortical vacuolation in males at 100 and 500 ppm was within the range of historical data, and was therefore not considered to be an adverse effect. No historical data for females were presented. During the recovery period, effects were observed on body weight, ASAT, AP, cholesterol, triglycerides, protein, albumin, Cl, T3, T4 and TSH.

Based on the observed increased incidence and severity of adrenal cortical vacuolation in females at 500 ppm and above, the NOAEL in this study is 100 ppm, equal to 8.1 mg/kg bw/day.

STUDY 4

Characteristics

reference/notifier	:	Wetzig, H., A. Romeike, E. Sander (2000) (revised report to report PH 29421 from 03-01-2000)	exposure	:	28 days
type of study	:	4 week oral toxicity study (range finding study)	doses	:	0, 400, 2000 and 10000 ppm (equal to 11.3, 65.5 and 284.5 mg/kg bw/day)
year of execution	:	1996	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	no
Route	:	Oral (diet)	guideline	:	Not in accordance with OECD 409
Species	:	Dog, Beagle	acceptability	:	Acceptable as range-finding study
group size	:	2/sex/dose	**NOAEL**	:	11.3 mg/kg bw/day (range-finding study)

Study design

A 4-week oral toxicity study was performed in beagle dogs. Animals (2/sex/dose) were exposed daily for 28 days to 0, 400, 2000 and 10000 ppm spirodiclofen via the diet (equal to 11.3, 65.5 and 284.5 mg/kg bw/day, for male and female animals together). The study was performed following OECD guideline 409, though with the following deviations: 2/sex/dose (instead of 4/sex/dose), age of the dogs 24-46 weeks (instead of maximally 9 months = 39 weeks), body weight range 6.9 to 15.4 kg.

Acceptability

The study was considered acceptable as range finding study.

Results

Results are summarized in Table 31. Treatment-related effects were observed in the mid- and high-dose groups. Since only 2 animals/sex were exposed in each dose group, results from males and females were considered together and results are only indicative for the effects observed. Treatment-related effects were observed on haematological parameters (erythrocytes↓, Hb↓, Ht↓, lymphocytes (%)↓, thromboplastin time↑). Changes in clinical biochemical parameters indicate treatment-related effects on liver and immune system. Effects on the liver were confirmed by induction of liver enzymes and increased liver weight in the mid- and high-dose groups, and periportal single cell necrosis in the high dose group, thus indicating liver as target organ. Other target organs were the sex organs (increased organ weight and histopathological changes), adrenals (increased organ weights, histopathological examination) and jejunum (histopathological examination). Details on organ weight are specified in Table 32.

Based on the results of this study, the NOAEL is 400 ppm (equal to 11.3 mg/kg bw/day).

Table 31. Overview of the results of a 4-week oral toxicity study in dogs (Wetzig (2000))

Dose	**0 ppm (0 mg/kg bw/d)**	**400 ppm (11.3 mg/kg bw/d)**	**2000 ppm (65.5 mg/kg bw/d)**	**10000 ppm (284.5 mg/kg bw/d)**	**dr**
	m/f	**m/f**	**m/f**	**m/f**	
Mortality	none				
Clinical signs[1]	No toxicologically relevant effects				
Body weight	No toxicologically relevant effects				
Food consumption[2]	No toxicologically relevant effects				

Dose	0 ppm (0 mg/kg bw/d)	400 ppm (11.3 mg/kg bw/d)	2000 ppm (65.5 mg/kg bw/d)	10000 ppm (284.5 mg/kg bw/d)	dr
	m/f	m/f	m/f	m/f	
Water consumption	No toxicologically relevant effects				
Haematology					
-ery			d	d	
-Hb			d	d	
-Ht			d	d	
-lymphocytes (%)				d	
-thromboplastin time				i	
Clinical chemistry					
-ASAT				i	
-ALAT			i	i	
-AP				i	
-GLDH				i	
-LDH				i	
-cholesterol				d	
-triglycerides				d	
-protein				d	
-albumin				d	
-Fe				d	
-T_4				d	
-albumin (electrophoresis)				d	
-α_1-globulin				i	
-β-globulin				i	
-γ-globulin				i	
Urinalysis					
-volume				d	
Liver tissue					
N-Dem			i	i	
O-Dem			i	i	
P_{450}			i	i	
-ECOD		i	i	i	dr
-ALD			i	i	dr
-EH			i	i	
-Glu-T^3			i	i	dr
Organ weights					
-liver			¡$^{a/r}$	¡$^{a/r}$	
-kidneys			¡$^{a/r}$	¡$^{a/r}$	
-ovaries				¡$^{a/r}$	
-uterus			¡$^{a/r}$	¡$^{a/r}$	
-adrenals			¡$^{a/r}$	¡$^{a/r}$	dr
-brain			i^{r}	i^{r}	dr
Pathology					
macroscopy	No toxicologically relevant effects				
microscopy					
liver					
-periportal single cell necrosis				4	
testes					
-Leydig cell vacuolation			2	2	
-Leydig cell hypertrophy/activation				1	
-immature testes/prostate				1	
Epididymides					
-massive oligo-/aspermia, slight spermic debris				1	
Adrenal glands					
-cytoplasmic	1	1	4	4	

Dose	0 ppm (0 mg/kg bw/d)	400 ppm (11.3 mg/kg bw/d)	2000 ppm (65.5 mg/kg bw/d)	10000 ppm (284.5 mg/kg bw/d)	dr
	m/f	m/f	m/f	m/f	
vacuolation cortex *jejenum* Vacuolation superficial mucosal epithelial cells			1	3	

dr dose related
i/d increased /decreased
a/r absolute /relative organ weight
[1] in the results section, the study authors mention one animal to be judged as slim in the high dose group. However, in the table of the revised report, all animals were judged to be normal, whereas in the original report this animal was judged to be meager.
[2] one animal of the highest dose group showed reduced feed intakes throughout the study period and week –1, according to the study authors probably due to the low bw (6.9 kg).
[3] Increase observed in males only.

Table 32 Organ weight of several organs (Wetzig (2000))

Dose (ppm)	0 ppm (0 mg/kg bw/d)	400 ppm (11.3 mg/kg bw/d)		2000 ppm (65.5 mg/kg bw/d)		10000 ppm (284.5 mg/kg bw/d)	
	m+f	m+f		m+f		m+f	
Terminal body weight (kg)	11.63	12.88		11.78		11.00	
Liver							
- absolute (g)	390.50	464.25	19%	467.50	20%	429.25	10%
- relative (g/kg BW)	33.5	36.12	8%	39.97	19%	39.79	19%
Kidneys							
- absolute (g)	55.75	68.75	23%	63.50	14%	65.50	17%
- relative (g/kg BW)	4.78	5.35	12%	5.50	15%	6.10	28%
Ovaries							
- absolute (g)	1.000	1.100	10%	0.900	-10%	1.300	30%
- relative (g/kg BW)	0.0700	0.0886	27%	0.0844	21%	0.1259	80%
Uterus							
- absolute (g)	4.5	5.0	11%	6.0	33%	5.0	11%
- relative (g/kg BW)	0.3	0.403	34%	0.525	75%	0.467	56%
Adrenals							
- absolute (g)	1.248	1.488	19%	1.615	29%	1.560	25%
- relative (g/kg BW)	0.108	0.116	7%	0.141	31%	0.158	46%
Brain							
- absolute (g)	75.25	78.75	5%	84.00	12%	78.50	4%
- relative (g/kg BW)	6.598	6.125	-7%	7.397	12%	7.679	16%

STUDY 5

Characteristics

reference/notifier	:	Wetzig, H., E. Hartmann (2001b)	exposure	:	8 weeks
type of study	:	8 week oral toxicity study	doses	:	0, 100, 2000 ppm (0, 2.9, 55.9 mg/kg bw/day)
year of execution	:	1998	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 97.9%, 98.6%)	GLP statement	:	yes[1]
route	:	Oral (diet)	guideline	:	Unknown
species	:	beagle dogs	acceptability	:	Acceptable
group size	:	5 males/dose	**NOAEL**	:	< 2.9 mg/kg bw/day

1 Deviations according to the study author: the testosterone hydroxylase assay was not performed under GLP conditions; the hormone determinations were not performed under GLP conditions, and the investigations were not mentioned in the study plan, and there is no documentation of the sample collection; the measurements of tocopherol, ubiquinone and dolichol concentrations were not conducted according to GLP principles, and they were reported separately, which means a deviation from the study plan.

Study design

An 8-week oral toxicity study was performed in beagle dogs. Animals (5 males/dose) were exposed via the diet to 0, 100, 2000 ppm (0, 2.9, 55.9 mg/kg bw/day) spirodiclofen.

The study was performed to study the testicular toxicity in male Beagle dogs. For this purpose, cholesterol-lowering and hormonal effects were examined (liver enzymes: cytochrome P-450 dependent monooxygenases (ECOD, EROD, ALD), epoxide hydroxylase (EH), conjugation enzymes (GLU-T, GS-T) and testosterone metabolism asssay (controls and high dose groups only). Besides standard parameters also testosterone and LH determinations in serum were included. It should be noted, that for the LH and testosterone assay no specifications on sensitivity and specificity of the assay were included, and no detailed description on the performance of the assays were given. Due to the small number of animals per group only mean values and standard deviations were calculated, although liver enzymes were statistically evaluated.

Acceptability

The study was considered acceptable.

Results

The results of the study are summarized in Table 33. At 2000 ppm, increased activities of ALAT, ASAT, AP and GLDH were measured together with decreased levels of cholesterol and triglycerides. At both dose-levels weights (absolute and relative) of liver, thyroid, adrenals, thymus and pancreas were increased (dose-related, except for thyroid weight), and prostate weight (absolute and relative) was decreased. At the high dose level also cytoplasmatic liver changes and Leydig cell changes were seen. Induction of 16α-hydroxylation was shown in the testosterone hydroxylation assay. LH concentrations were significantly increased in the 2000 ppm group.

Based on the results of this study, a NOAEL could not be derived (< 100 ppm). The LOAEL is 100 ppm, equal to 2.9 mg/kg bw/day.

Table 33. Overview of the results of a 8-week oral toxicity study in dogs (Wetzig, 2001b)

Dose	**0 ppm (0 mg/kg bw/d)**	**100 ppm (2.9 mg/kg bw/d)**	**2000 ppm (55.9 mg/kg bw/d)**	**dr**

	m	m	m	
Mortality		none		
Clinical signs		No toxicologically relevant effects		
Body weight		No toxicologically relevant effects		
Food consumption		No toxicologically relevant effects		
Ophthalmoscopy		Not performed		
Haematology		No toxicologically relevant effects		
Clinical chemistry				
- ALAT			i	
- ASAT			i	
- AP		i	i	
- GLDH			i	
- cholesterol			d	
- triglycerides			d	
Urinalysis		Not performed		
Organ weights				
- liver		i[a,r]	i[a,r]	m
- prostate		d[a,r]	d[a,r]	
- thyroid		i[a,r]	i[a,r]	
- adrenals		i[a,r]	i[a,r]	m
- thymus		i[a,r]	i[a,r]	m
- pancreas		i[a,r]	i[a,r]	m
Histopathology				
Liver				
- increased cytoplasmic granulation			3/5	
- hepatocellular single cell necrosis			3/5	
Adrenals				
- increased cytoplasmic vacuolation adrenal cortex		4/5	5/5	m
- mononuclear cell infiltration adrenal cortex		1/5	3/5	m
Testes				
- hypertrophy and vacuolation Leydig cells			5/5	
- degeneration germinal epithelium		1/5	4/5	
Liver enzymes				
- ECOD			is	
- ALD			is	
testosterone hydroxylation assay				
- 16α-hydroxylation activity			i	
Serum enzymes				
- LH			is	

dr dose related
i/d increased/decreased
a/r absolute/relative organ weight

STUDY 6

Characteristics

Reference/notifier	:	Wetzig, H., E. Hartmann (2001a)	exposure	:	14 weeks
Type of study	:	14 week oral toxicity study	Doses[1]	:	0, 200, 630, 2000 ppm (0, 8.0, 27.3, 82.8 mg/kg bw/day, for male and female together)

Year of execution	:	1997	vehicle	:	-
Test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%)	GLP statement	:	yes
Route	:	Oral (diet)	guideline	:	In accordance with OECD 409
Species	:	Dog, Beagle	acceptability	:	acceptable
Group size	:	4/sex/dose	**NOAEL**	:	< 8.0 mg/kg bw/day

[1] dose levels were based on the results of a subacute study with beagle dogs dosed 0, 400, 2000 and 10000 ppm .

Study design

A 14-week oral toxicity study was performed in beagle dogs. Animals (4/sex/dose) were exposed to spirodiclofen via the diet in doses of 0, 200, 630, 2000 ppm (equal to 0, 8.0, 27.3, 82.8 mg/kg bw/day, for male and female animals together). Due to the small number of animals per group, only mean values and standard deviation were calculated. In spite of the small number of animals per group, liver enzyme activities were statistically tested by using the Student's t-test.

Acceptability

The study was considered acceptable.

Results

A complete overview of the results is summarized in Table 34. Dose-related decreased in Hb, Ht, and erythrocytes were observed in all dose groups, which were about 20% lowered in the highest dose group. Clinical biochemical analysis showed several changes pointing towards the liver as target organ. Dose-related changes, starting at the lowest dose, were observed for AP (f), GLDH (m) and cholesterol (m). Determination of microsomal liver enzymes showed dose-related increases in all dose groups of N-DEM, O-DEM, ECOD, and ALD. Organ weight changes were also observed and included dose-related increases in relative liver (m/f), relative kidney (m) and relative adrenal weights (f) as observed in the mid- and high-dose groups. Furthermore, dose-related decreases in relative prostrate-weight (m) were observed in in all dose-groups. Histopathological analysis revealed changes in liver, kidney, testes, epididymis, prostate and thymus in animals of 630 ppm and 2000 ppm groups. Adrenal glands showed at and above 200 ppm vacuolization in the cortex (f), accompanied by mononuclear cell infiltration (m/f).

Based on the results of this study, a NOAEL could not be established (< 200 ppm). A LOAEL of 200 ppm (equal to 8.0 mg/kg bw/day) was derived.

Table 34. Overview of the results of a 14-week oral toxicity study in dogs (Wetzig, 2001a).

Dose	**0 ppm (0 mg/kg bw/d)**		**200 ppm (8.0 mg/kg bw/d)**		**630 ppm (27.3 mg/kg bw/d)**		**2000 ppm (82.8 mg/kg bw)**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none								
Clinical signs	No toxicologically relevant effects								
Body weight (gain)[1]					d	d	d	d	
Food consumption[2]	No toxicologically relevant effects								
Ophthalmoscopy	No toxicologically relevant effects								

Dose	0 ppm (0 mg/kg bw/d)		200 ppm (8.0 mg/kg bw/d)		630 ppm (27.3 mg/kg bw/d)		2000 ppm (82.8 mg/kg bw)		dr
	m	f	m	f	m	f	m	f	
Haematology									
-leukocytes					i		i		m
-neutrophils				i	i	i	i	i	m
-lymphocytes				d		d	d	d	
-monocytes							i		
-eosinophils						d		d	
-erythrocytes				d	d	d	d	d	m, f
-Hb				d	d	d	d	d	m, f
-Ht				d	d	d	d	d	m, f
-MCV				i		i	i	i	
-MCH								i	
-MCHC							d		
-reticulocytes							d		
-thrombocytes					i		i		
Clinical chemistry									
-ASAT					i	i	i	i	m, f
-ALAT						i	i	i	f
-AP				i		i	i	i	f
-GLDH			i		i		i	i	m
-CK							d		
-Albumin					d		d	d	
-calcium					d	d	d	d	
-Chloride					i	i	i	i	
-inorg. P							d		
-Magnesium								i	
-iron				d		d	d	d	
-T3							i	i	
-T4							d	d	
-Thyroxine Binding Capacity (TBC)							d	d	
-Glucose						i		i	
-Cholesterol			d		d		d	d	m
-Triglycerides					i		i	d	
-Creatinine							d		
-HST urea					i		i		
-Bilirubin-t					d		d	d	
-Protein					d	d	d	d	f
Liver examination									
-N-DEM			i	i	i	i	i	i	m, f
-O-DEM			i	i	i	i	i	i	m, f
-P450					i	i	i	i	m, f
-ECOD[3]			i	i	i	i	i	i	m, f
-ALD[3]			i	i	i	i	i	i	m
-EH[3]						i		i	
Urinalysis									
-volume				d	d	d	d	d	
-creatinine							d	d	
-Na							d	d	
Organ weights									
-liver					i^r	i^r	i^r	i^r	m, f
-kidneys					i^r		i^r	i^r	m
-spleen					d^r	d^r	d^r	d^r	
-prostate			d^r		d^r		d^r		m
-thyroid							i^r		
-adrenals					i^r	i^r	i^r	i^r	f
-thymus							d^r		
-brain					i^r	i^r	i^r	i^r	
-pituitary					i^r	i^r	i^r	i^r	
-uterus				d^r		d^r		d^r	
-lung								i^r	
Pathology									
macroscopy	No toxicologically relevant effects								
microscopy									
Liver									

Dose	0 ppm (0 mg/kg bw/d)		200 ppm (8.0 mg/kg bw/d)		630 ppm (27.3 mg/kg bw/d)		2000 ppm (82.8 mg/kg bw)		dr
	m	f	m	f	m	f	m	f	
-cytoplasmic changes								3/4	
-inflammatory infiltrates	1/4							4/4	
-periportal single cell necrosis								1/4	
Kidneys									
-dilation proxmal tubules, renal cortex	1/4			1/4	1/4		3/4	2/4	
Testes									
-degeneration germinal epithelium							2/4		
-vacuolisation Leydig cells					2/4		4/4		m
-hypertrophy Leydig cells					2/4		3/4		m
Epididymides									
-aspermia					1/4		2/4		m
-oligospermia					2/4				
Prostate									
-immature					1/4		4/4		m
Adrenal glands									
-vacuolisation zona fasciculata, cortex				2/4	3/4	3/4	4/4	4/4	f
-mononuclear cell infiltration			1/4	2/4	1/4		4/4	4/4	
Thymus									
-cortical atrophy					1/4		2/3	1/4	m

dr dose related
d/i decreased/increased compared to the controls
r relative organ weight
[1] decreased body weights were more pronounced in males.
[2] data on water consumption were not included
[3] observed changes were statistically significant by Student's t-test. Since the study was performed with a restricted number of animals per dose group it is not clear whether Student's t-test is an appropriate statistical test. Therefore, statistical significance is not indicated in the tabel.

STUDY 7

Characteristics

reference/notifier	:	Wetzig, H., Chr. Rühl-Fehlert (2001c)	exposure	:	52 weeks
type of study	:	1-year toxicity study.	doses	:	0, 20, 50, 150, 500/600[1] ppm (0, 0.57, 1.45, 4.54. 16.9 mg/kg bw/day)
year of execution	:	1998/1999	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 97.8%)	GLP statement	:	yes
route	:	Oral (diet)	guideline	:	According to OECD 452
species	:	Beagle dog	acceptability	:	acceptable
group size	:	4/sex/dose	**NOAEL**	:	1.45 mg/kg bw/day

1 The concentration in the 500 ppm group was increased to 600 ppm after about 3 weeks of exposure.

Study design

The study was in accordance with OECD guideline 453. In line with the small number of animals per group only mean values and standard deviations were calculated. For the parameters N-DEM, O-DEM, P450 and triglycerides a statistical evaluation was done for males and females/group together.

Acceptability

The study was considered acceptable.

Results

The results of the study are summarized in Table 35. Details of T3 concentrations in females and weights of heart, adrenals, liver and testes are specified in Table 36 and 37.

Table 35 Overview of the results of the 1-year chronic toxicity study in dogs (Wetzig, 2001c)

Dose	**0 ppm (0 mg/kg bw/d)**		**20 ppm (0.57 mg/kg bw/d)**		**50 ppm (1.45 mg/kg bw/d)**		**150 ppm (4.54 mg/kg bw/d)**		**600 ppm (16.9 mg/kg bw/d)**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none										
Clinical signs	No toxicologically relevant effects										
Body weight (gain)	No toxicologically relevant effects										
Food consumption	No toxicologically relevant effects										
Ophthalmoscopy	No toxicologically relevant effects										
Haematology[a]	No toxicologically relevant effects										
Urinalysis	No toxicologically relevant effects										
Clinical chemistry[b]											
- ALAT									d		
- AP					i		i		i	d	m
- GLDH							d		d	d	
- Glucose			i		i		i		i		
- Cholesterol[c]			i	d	i	d	i		i	d	
- Potasium									d		
- Ferrum									d		
- T3				i		i		i		i	
- T4								i		i	f
- TBC									i		
Organ weights[d]											
- heart				¡[a,r]		¡[a,r]	¡[a,r]	¡[a,r]	¡[a,r]	¡[a,r]	
- lung							¡[a,r]		¡[a,r]		m
- adrenals			¡[a,r]		¡[a,r]		¡[a,r]	¡[a,r]	¡[a,r]	¡[a,r]	f
- brain									d[r]	d[r]	
- kidneys						¡[a,r]		¡[a,r]	¡[a]	¡[a,r]	
- liver			¡[a,r]		¡[a,r]		¡[a,r]		¡[a,r]		
- thyroid							¡[a,r]		¡[a,r]		
- testes			¡[a,r]		¡[a,r]		¡[a,r]		¡[a,r]		
- prostate									¡[a,r]		
- epididymides					¡[a,r]		¡[a,r]		¡[a,r]		
- pancreas					¡[a]	¡[a,r]	¡[a]	¡[a,r]	¡[a]	¡[a,r]	
- thymus										¡[a,r]	
- uterus/oviduct										d[a,r]	
Liver examinations											
- N-DEM					i		i	i	i	i	m, f
- O-DEM					i		i		i	i	m
- P450									i	i	
Pathology											
Macroscopy	No toxicologically relevant effects										
Microscopy											
Duodenum											
- desquamation, epith.									2/4		
Liver											
- cytopl. inclusion					1/4		1/4		2/4		

Dose	0 ppm (0 mg/kg bw/d)		20 ppm (0.57 mg/kg bw/d)		50 ppm (1.45 mg/kg bw/d)		150 ppm (4.54 mg/kg bw/d)		600 ppm (16.9 mg/kg bw/d)		dr
	m	f	m	f	m	f	m	f	m	f	
- pigment								1/4		2/4	
Testes											
- vacuolat. Leydig cells									4/4		
- hypertrop. Leydig cells									1/4		
- focal tubular degener.							1/4		1/4		
Adrenals											
- vacuolation Z. fasc.	1/4	1/4	2/4	1/4			4/4	3/4	4/4	4/4	
- vacuolation Z. glom.								1/4		1/4	

dr dose related
a/r absolute/relative organ weight
[a] Hb and Ht in males of all dose groups lower; however: SD of the control group was large, and the observed decrease was not dose related. Fe in blood of males of the dosed groups were also slighly, not dose related, decreased.
[b] in females dosed 150 ppm, deviating values (compared to the other dose groups) were observed for GLDH, CK, glucose, cholesterol and triglycerides
[c] control cholesterol values differed for males and females (3 vs 6), whereas such difference could not be observed in historical values. The study author gives no explanation.
[d] ovaries and uterus/oviduct of females dosed 50 ppm were about half the weight of the ovaries of the other groups, with no explanation of the study author.

Table 36 T3 concentrations in females in nmol/l (Wetzig, 2001c)

Dose	0 ppm (0 mg/kg bw/d)	20 ppm (0.57 mg/kg bw/d)	50 ppm (1.45 mg/kg bw/d)	150 ppm (4.54 mg/kg bw/d)	600 ppm (16.9 mg/kg bw/d)
wk –3	2.07	1.76	1.81	1.97	1.79
wk 3	1.51	1.56	1.67	1.48	1.47
wk 6	1.61	1.77	1.66	1.75	1.78
wk 12	1.69	1.84	1.56	1.73	1.55
wk 26	1.42	1.68	1.58	1.75	1.52
wk 39	1.19	1.54	1.37	1.28	1.19
wk 52	1.49	2.27	1.85	1.88	1.82
Mean wk 3-52	1.49	1.78	1.62	1.65	1.56

Table 37 Weights of heart, adrenals, liver and testes in grams (Wetzig, 2001c)

Dose	0 ppm (0 mg/kg bw/d)	20 ppm (0.57 mg/kg bw/d)		50 ppm (1.45 mg/kg bw/d)		150 ppm (4.54 mg/kg bw/d)		600 ppm (16.9 mg/kg bw/d)	
Heart (females only)									
- absolute	106.0	114.0	8%	126.5	19%	118.3	12%	119.0	12%
- relative	7.48	8.45	13%	9.17	23%	8.24	10%	8.10	8%

Adrenals (males only)									
- absolute	1.638	1.763	8%	1.835	12%	1.738	6%	1.973	20%
- relative	0.1064	0.1208	14%	0.1191	12%	0.1154	8%	0.1232	16%
Liver (males only)									
- absolute	438.0	510.0	16%	548.8	25%	525.5	20%	552.5	26%
- relative	28.95	34.85	20%	35.83	24%	34.79	20%	34.24	18%
Testes (males only)									
- absolute	20.43	23.95	17%	22.30	9%	26.55	30%	29.13	43%
- relative	1.380	1.636	19%	1.438	4%	1.767	28%	1.790	30%

Conclusion

In the two lower dose groups, several clinical biochemical parameters and relative and absolute organ weights were affected, mainly without dose-relationship (i.e. effects are more or less constant or decreasing over the exposure groups). In the absence of associated histopathological changes in these dose groups and the fact that histopathological changes are only observed for a few organs at much higher doses, the observed effects at the lower doses are considered not clearly adverse. The adrenals are regarded to be the critical target organ. At and above 150 ppm, increased adrenal weights were observed accompanied by increased vacuolation of the adrenals, an effect which was also observed in several other toxicological studies with different species.

Based on the effects observed on the adrenals in the two highest dose groups, the NOAEL in this study is, in accordance with the opinion of the study author, 50 ppm, equal to 1.45 mg/kg bw/day.

In addition, an 18-month combined chronic toxicity/carcinogenicity study in mice and a 2 year combined chronic toxicity/carcinogenicity study in rat are available. Details can be found in paragraph 4.10.1.1. Information on the parental effects in the 2-generation study in rats is available in paragraph 4.11.1.1. Further, repeated dose studies for neurotoxicity and immunotoxicity are available in paragraphs 4.12.1.1 and 4.12.1.2 respectively.

4.7.1.2 Repeated dose toxicity: inhalation

No information available.

4.7.1.3 Repeated dose toxicity: dermal

STUDY 1

Characteristics

reference/notifier	:	Kröttlinger, F., E. Sander (1999)	exposure	:	22 applications/28 days, 6 h/day, semi-occlusive
type of study	:	4-week dermal toxicity study, limit test	doses	:	0 and 1000 mg/kg bw/day
year of execution	:	1998	vehicle	:	The substance was applied as solid onto wet gauze pad
test substance	:	BAJ 2740 (spirodiclofen, purity 97.9%; fine crystalline powder)	GLP statement	:	yes
Route	:	dermal	guideline	:	In accordance with OECD 410
Species	:	Rat, Wistar HsdCpb:WU	acceptability	:	Acceptable as screeening test
group size	:	5/sex/dose	**NOAEL**	:	<1000 mg/kg bw/day

Study design

A 4-week dermal toxicity study was performed in Wistar rats (5/sex/dose). Animals were exposed to control and 1000 mg/kg bw (limit dose) spirodiclofen in total on 22 days within a 28-day period for 6 hr/day under semi-occlusive conditions. The substance was applied as solid onto wet gauze pads. Since it is stated that 1000 mg/kg is applied to 30.25 cm^2, the applied dose is less than 10% of the body surface, based on a default body surface of 400 cm^2. Doses are based on a range-finding study with female rats (10 applications/14 days).

Acceptability

The study was considered acceptable as screening test, since effects were observed at the limit dose.

Results

The results of the study are summarized in Table 38. At the dose level tested, females showed a increased body weight compared to controls, whereas food consumption was lower in the dosed females. It should be noted that several females in the control group hardly gained weight during the study period. Other effects observed were statistically significant reduced Hb-, Ht, ALAT and triglyceride-levels in male animals. Adrenal weights of males in the 1000 mg/kg bw group were decreased (12%). No histopathological abnormalities were observed. There were no local effects in rats after 28 days dermal application of spirodiclofen at the dose level tested.

Based on the results of this study, a NOAEL could not be derived (< 1000 mg/kg bw/day). The LOAEL is considered 1000 mg/kg bw/day.

Table 38. Overview of result of a 4-week dermal toxicity study in Wistar rats (Kröttlinger, 1999)

Dose (mg/kg bw/day)	**0**		**1000**		**dr**
	m	**f**	**m**	**f**	
Mortality	None				
Clinical signs	No toxicologically relevant effects				
Body weight				i[1]	
Food consumption				d[2]	
Local skin findings	No toxicologically relevant effects				

Dose (mg/kg bw/day)	0		1000		dr
	m	f	m	f	
Haematology					
-Hb (g/l)	162	147	154 ds	146	
-Ht (l/l)	0.544	0.488	0.511 ds	0.478	
Clinical chemistry					
-ALAT			ds		
-Triglycerides (mmol/l)	1.36±0.29	0.61±0.20	0.85±0.34 ds	0.62±0.17	
Organ weights					
-adrenals[3]			d[ar]		
absolute (mg)	43	67	38 (-12%)	65	
relative (mg/100g bw)	15	32	14 (-7%)	30	
Pathology					
Macroscopy	No toxicologically relevant effects				
Microscopy	No toxicologically relevant effects				

dr dose related
i/ d increased/ decreased
ds statistically significantly decreased compared to the controls
r relative organ weight
[1] the body weights were significantly higher at day 7 and day 21
[2] food consumption was slightly decreased during weeks 1-3 in females, when expressed as mg/kg bw/d; there was no difference with control when expressed as mg/animal/d. The observed statistically significant decreased food consumption in males observed at day 7 is considered incidental.
[3] adrenal weights (absolute and relative) were higher (factor 2) in females compared to males

4.7.1.4 Repeated dose toxicity: other routes

No information available.

4.7.1.5 Human information

No information available.

4.7.1.6 Other relevant information

4.7.1.7 Summary and discussion of repeated dose toxicity

Oral

In a 28 day oral toxicity study with dogs, treatment-related effects were observed on haematological parameters. Also in dogs, changes in clinical biochemical parameters indicate treatment-related effects on liver and the immune system. Effect on the liver was confirmed by induction of liver enzymes in the mid-and high-dose groups and liver periportal cell necrosis in the high-dose group. In addition to the liver, effects were also observed on adrenals and jejenum (organ weight and cortical vacuolation) and sex organs (changed organ weights of uterus and ovaries, vacuolation of Leydig cells of the testes, oligo- and aspermia). The NOAEL in this range finding study is 11.3 mg/kg bw/day.

Further, two oral toxicity studies with dogs were performed, an 8 weeks study (males only) and a 14 weeks study. Comparable effects were observed in both studies and included clinical biochemical parameters as ALAT, ASAT, AP cholesterol and triglycerides, induction of liver enzymes (ECOD, ALD in both studies, N-DEM, O-DEM P450 in the 14 week study) and changed organ weights of liver, prostate, thyroid and adrenals, prostrate and uterus). Histopathological examination showed effects on liver, testes (hypertrophy and vacuolisation in testis, oligo- and aspermia) and adrenals. In both studies, a NOAEL could not be established, and the LOAELs are 2.9 mg/kg bw/day in the 8-week study and 8.0 mg/kg bw/day in the 14 week study.

In the 52 weeks chronic oral toxicity study with dogs, several clinical biochemical parameters and relative and absolute organ weights were affected in the two lower dose groups, mainly without dose-relationship (i.e. effects are more or less constant or decreasing over the exposure groups). In the absence of associated histopathological changes in these dose groups and the fact that histopathological changes are only observed for a few organs at much higher doses, the observed effects at the lower doses are considered not clearly adverse. Effects on reproductive organs were also observed and included increased testis, prostrate and epididymis weight and vacuolisation /hypertrophy of Leydig cells. At and above 150 ppm, increased adrenal weights were observed accompanied by increased vacuolation of the adrenals, an effect which was also observed in several other toxicological studies with different species. Based on the effects observed on the adrenals in the two highest dose groups, the NOAEL in this study is, in accordance with the opinion of the study author, 50 ppm, equal to 1.45 mg/kg bw/day.

In a 13 week oral toxicity study with mice, significant effects were observed on body weight (mid+high dose), haematology and cholesterol (high dose). Changed relative organ weights were observed for adrenals, spleen, kidneys, testes and ovaries in the mid and high dose groups (increased, except for kidneys weight which were significantly decreased). Histopathological examination showed hypertrophy and vacuolation of Leydig cells in the testes and in the adrenals cytoplasmic vacuolation, degeneration of cortical cells and mononuclear infiltrate in the (mid and) high dose group. The liver showed besides periportal cytoplasmic vacuolation in the high dose group also centrilobular hepatocellular hypertrophy in all dose groups. A NOAEL could not be established in this study, and the LOAEL is 15.3 mg/kg bw/day.

In a 14 week oral toxicity study with rats, substance-related effects were mainly observed in the two highest dose groups and included significant decreased bw and food consumption, significant changes in haematological parameters, significant effects on clinical chemistry including AP, GLDH, cholesterol, triglycerides and changed organ weights of liver, spleen, adrenals, testes and thymus. Histopathological examination showed epithelial vacuolation in jejenum in the highest two dose groups. Adrenal cortical vacuolisation accompanied by increased grading of this effect was observed in the highest three dose groups in females. Hence the NOAEL in this study is 100 ppm, equal to 8.1 mg/kg bw/day.

In an 18-month mouse combined chronic toxicity/carcinogenicity study, non-carcinogenic effects were observed. These effects included effects on BW (↓), haematological parameters (MCHC↓, WBC↓, segmented neutrophils↓, lymphocytes↑, atypical lymphocytes↓), organ weights of adrenals↑, liver↑, testes↑ and kidney↓. Further, increased incidence of adrenal pigmentation and vacuolation in females, increased incidence of amyloid in several tissues and increased incidence of hepatocytomegaly in males was observed. A NOAEL for chronic toxicity could not be derived. The LOAEL for chronic toxicity is 4.1 mg/kg bw/day. Full details of this study (including the carcinogenic effects) can be found in paragraph 4.10.1.1.

In a 2-year rat combined chronic toxicity/carcinogenicity, non-carcinogenic effects were observed. These effects included effects in high dose group on BW (↓), food consumption (↑), haematology

(leukocytes↓, lymphocytes↓), and clinical chemistry (AP↑, T4↑, TSH↑). Further, other non-carcinogenic effects which were observed included increased adrenal weight (m) and decreased spleen weights (f) in all dose groups (however, not dose-realed and not accompanied by histopathological changes) and increased (absolute/relative) thymus and ovaries weights in two highest dose groups. A NOAEL of 5.93 mg/kg bw/day for chronic toxicity was derived. Full details of this study (including the carcinogenic effects) can be found in paragraph 4.10.1.1.

In a 2-generation rat study in rats non-reprotoxic effects were observed in the F0-generation. These effects included BW↓ (m: all dose groups; f: mid and high dose group), changed organ weights of brain ↑ (m,f), adrenals↑ (m/f), liver↓ (m), kidney↓ (f). Further, vacuolisation of adrenal glands (f, mid and hig dose groups) was observed. A NOAEL for systemic effects could not be derived. A LOAEL for systemic effects of 5.2 mg/kg bw/day was derived. Full details of this study (including the reprotoxic effects) are available in paragraph 4.11.1.1.

In a 13-week rat neurotoxicity screening study, effects were observed. These effects included BW↓ (m/f, high dose), food consumption ↓ (m/f, high dose), urine stain and red thinged paws. Further, decreased foot splay (mf/, high dose) and decreased forlimb/hindlimb grip strength (m/f, high dose). For these latter effects, it could not be excluded that these were dose-related. A NOAEL for neurotoxicity of 70 mg/kg bw/day was derived. Full details of this study are available in paragraph 4.12.1.1.

In a 77-week chronic study which included a functional observation battery, no neurotoxic effects were observed. A NOAEL for neurotoxicity of 110.14 mg/kg bw/day was derived. Full details of this study are available in paragraph 4.12.1.1.

A 4-week immunotoxicity study revealed in addition to effects on BW also some immunotoxicological effects. These effects included decreased spleen and lymph node cell counts, decreased spleen T-helper cells and spleen lymphocytes. A NOAEL of 45 mg/kg bw/day was derived. Full details of this study are available in paragraph 4.12.1.2.

Dermal

A dermal exposure study during 28 days with rats showed statistically significant effects on haematological parameters, ALAT and triglycerides. Further, adrenal weight in the exposed group were slightly decreased. Similar effects were also observed in oral toxicity studies and considered substance-related. A NOAEL in this study could not be established, and the LOAEL systemic for dermal exposure is 1000 mg/kg bw/day. There were no local effects observed at the dose level tested.

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

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

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

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

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

This paragraph is considered irrelevant seen the repeal of Directive 67/548/EEC with effect from 1 June 2015.

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

Oral

In the available mouse 13-week repeated dose study, significant reductions in Hb-levels and cholesterol-levels (females) and kidney weight (males) were observed at 10000 ppm (M: 1629.9 mg/kg bw/day, F: 2685.2 mg/kg bw/day). In addition, some histological changes were observed which included centrilobular hepatocellular hypertrophy (males: 15.3, 163.8 and 1629.9 mg/kg bw/day), cytoplasmic vacuolisation of the adrenal glands (females: 233.6 and 2685.2 mg/kg bw/day, males: 1629.9 mg/kg bw/day), vacuolisation and hypertrophy/activation of Leydig cells in the testes (163.8 and 1629.9 mg/kg bw/day). In the 18-month chronic mouse study, non-neoplastic changes included increased adrenal pigmentation and vacuolisation (males: 610 and 1216 mg/kg bw/day, females: 5.1, 722 and 1495 mg/kg bw/day) combined with increased adrenal organ weight (males: 610 and 1216 mg/kg bw/day, females: 722 and 1495 mg/kg bw/day).

In the 4-week rat oral toxicity study, changes in clinical chemical parameters (a.o. ALAT and ASAT-levels and decreased cholesterol, triglycerides) at 569.3 mg/kg bw/day pointed towards potential liver damage. However, histopathological analysis did not reveal organ (liver) damage. The 14-week rat study revealed haematological changes, changes of clinical chemical parameters (mostly related to liver), mainly observed at the highest dose-level tested (males: 851.4 mg/kg bw/day, females: 995.8 mg/kg bw/day). Further, changes in organ weights were observed (reduced liver and spleen, increased adrenals) and histological changes including increased adrenal cortical vacuolisation. Non-neoplastic changes in the 2-year rat carcinogenicity study were mainly observed at the highest dose level and included changes in organ weights, increased adrenal hypertrophy/vacuolisation and focal Leydig cell hyperplasia.

The available repeated dose toxicity studies performed in dogs indicated that the adrenals are the main target organ. In a 4-week study in dogs, spirodiclofen induced changes of haematological parameters (65.5 and 284.5 mg/kg bw/day), various liver parameters (284.5 mg/kg bw/day), decreased kidney and increased uterus, adrenals and brain weight (65.5 and 284.5 mg/kg bw/day), and finally Leydig cell vacuolisation (65.5 and 284.5 mg/kg bw/day), and cytoplasmic vacuolisation of adrenal cortex (65.5 and 284.5 mg/kg bw/day). Also, the 8-week, 14-week and 1-year dog studies revealed effects on the adrenals (i.e. cytoplasmic vacuolisation of the adrenal cortex, increased adrenal weight). In addition, the 14-week study revealed a significant reduction of Hb and Ht-levels of 20% at the highest dose level (83 mg/kg bw/day).

Dermal

In the rat dermal repeated dose toxicity study, significant reductions in Hb and Ht-levels and decreased ALAT and triglycerides were observed at the dose-level of 1000 mg/kg bw/day. Overall, the severity of the effects and the effect-level do not fulfil the classification criteria for STOT RE.

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

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 (i.e. were observed in a 90-day repeated-dose study conducted in experimental animals below the guidance value range of 10 mg/kg bw/day or 20 mg/kg bw/day for oral and dermal exposure respectively).

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 observed in a 90-day repeated-dose study conducted in experimental animals within the guidance value ranges of 10-100 mg/kg/day for oral exposure or 20-200 mg/kg bw/day for dermal exposure.

Oral

Given that no human data are available and the effective dose levels in the repeated dose animal studies were above the upper limit for STOT RE 1 (i.e. 30 mg/kg bw/day for a 4-week study, 15 mg/kg bw/day for a 8-week study, 10 mg/kg bw/day for a 90-day study, 2.5 mg/kg bw/day for a 1-year study), classification for STOR RE 1 is not considered.

Effect-levels in the mouse repeated dose toxicity studies (13-week and 18 month) were mostly above the upper limit for STOT RE 2 classification (100 mg/kg bw/day for a 90-day study, 25 mg/kg bw/day for a 1-year study). However, in the 13-week study centrilobular hepatocellular hypertrophy was observed in 3 out of 10 male animals (grade 2: 3/3 animals) at a dose of 15.3 mg/kg bw/day. These effects do not fulfill the classification criteria based on the observed severity (as there is no evidence of marked organ damage as described in the CLP-guidance). Based on the mouse repeated dose toxicity studies, classification is not required.

Effect levels in the 4-week, 14-week and 2-year rat repeated dose toxicity studies were mostly above the upper limit for STOT RE 2 classification (300 mg/kg bw/day for a 4-week study, 100 mg/kg bw/day for a 90-day study, 12.5 mg/kg bw/day for a 2-year study). However, increased small cortical vacuolization of the adrenals (with increased grading) was observed in male animals at dose levels of 6.6 and 32.1 mg/kg bw/day and higher. However this was within the range of historical controls. Based on the rat repeated dose toxicity studies, classification is not required.

Effect levels in the available dog repeated dose toxicity studies were around or below the upper limit for STOT RE 2 classification (300 mg/kg bw/day for a 4-week study, 150 mg/kg bw/day for a 8-week study, 100 mg/kg bw/day for a 90-day study, 25 mg/kg bw/day for a 1-year study). In most studies the highest dose tested was below the upper limit for STOT RE 2 classification. At the highest dose level tested many parameters were effected. Most effects individually would not fufil the classification criteria. However, according to 3.9.1.4 of the CLP Guidance also generalised changes of a less severe nature involving several organs should be taken into account. Further, the reduction in Hb and Ht in the 14 week dog study was around 20% at the highest dose level (83 mg/kg bw/day) which was below the upperlimit for STOT RE 2. As it cannot be excluded that the observed effects in dogs are relevant for evaluating potential effects of spirodiclofen in humans, the observed effects should be taken into account for potential classification for STOT RE. Together, the available information in dogs warrant classification for STOT RE in category 2. The effects on the testes are taken into account in the assesment of effects on sexual function and fertility (paragraph 4.11).

The results of the combined chronic toxicity/carcinogenicity studies and 2-generation rat study do not warrant classification for STOT RE (see paragraph 4.10 and 4.11).

The results of the neurotoxicity and immunotoxicity studies do not warrant classification for STOT RE (see section 4.12).

Dermal

A single experimental dose was applied (i.e. 1000 mg/kg bw/day). This effect-level was above the upper limit for STOT RE 2 classification (600 mg/kg bw/day for a 28-day study) and the type and severity of the observed effects do not fulfill the criteria for classification for STOT RE. Classification for the dermal route is not required.

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

Based on the available studies, classification for specific organ toxicity – repeated exposure as STOT RE 2 (H373: May cause damage to organs, through prolonged or repeated exposure) is required. This classification applies to all routes as comparable effects after inhalation exposure cannot be excluded. As the classification is mainly based on the general effects on several organs, no specific target organ is proposed.

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

Summary of the Dossier Submitter's proposal

The evaluation of the specific target organ toxicity – repeated exposure (STOT RE) hazard point was based on two mouse (Leser & Romeike, 1998; Wahle, 2000), two rat (Krottlinger,

2000; Wirnitzer, 1998) and four dog (Wetzig, 2000;Wetzig, 2001a; Wetzig, 2001b; Wetzig, 2001c) studies by the oral route and one rat study by the dermal route. In addition, data from the combined chronic toxicity/carcinogenicity (Wirnitzer, 2000), 2-generation reproductive toxicity (Eiben, 2000), acute neurotoxicity (Wirnitzer, 1998) and subchronic immunotoxicity (Sheets, 2001) studies were also considered.

Oral

Mouse

In the mouse some histopathological changes were observed in the liver, the adrenal glands and Leydig cells in the testes along with increased organ weights (adrenals, liver, testes) and altered haematological parameters (MCHC, WBC, neutrophils, lymphocytes).

However, the effect levels were mostly above the guidance value with the exception of centrilobular hepatocellular hypertrophy at a dose of 15.3mg/kg bw/d in the 13 week study. However, these effects do not represent severe effects, as there is no evidence of marked organ damage or dysfunction, as specified in the Guidance on the Application of the CLP Criteria. The DS concluded that the mouse repeated dose toxicity studies were not sufficient to classify spirodiclofen for STOT RE.

Rat

In rats, haematological changes and changes of clinical chemical parameters (mostly related to liver) were mainly observed at the highest dose-level tested after 14 weeks of exposure (males: 851.4 mg/kg bw/d, females: 995.8 mg/kg bw/d). Further, changes in absolute organ weights were observed (reduced in liver and spleen, increased in adrenals) and histological changes including increased adrenal cortical vacuolation. Non-neoplastic effects in the 2-year rat carcinogenicity study were mainly observed at the highest dose level and included changes in organ weights, increased adrenal hypertrophy/vacuolation and focal Leydig cell hyperplasia.

The effect levels in the 4-week, 14-week and 2-year rat repeated dose toxicity studies were above the guidance values for STOT RE 2 classification. However, increased small cortical vacuolation of the adrenals (dose-dependent) was observed in male animals at lower dose levels but they were within the range of historical controls. Additionally, the results from the combined chronic toxicity/carcinogenicity study , the 2-generation reproduction toxicity study and the 13-week subchronic neurotoxicity screening study did not report severe effects below guidance values. Therefore the DS concluded that the rat studies are not sufficient to classify spirodiclofen for STOT RE.

Dog

In dogs, the available repeated dose toxicity studies performed in dogs indicated that the adrenal glands, the liver and haematological parameters are the main target organs. The 14-week study revealed a significant reduction of Hb and Ht levels of 20% at the highest dose level (83 mg/kg bw/d). In all dog studies except the 1 year study, liver necrosis was observed at doses relevant for STOT RE 2 classification.

Additional relevant toxicological effects were observed in some animals below the guidance value for STOT RE 2. However, according to 3.9.1.4 of the CLP Guidance, also generalised changes of a less severe nature involving several organs should be taken into account. For the effects affecting the adrenal glands in dogs, the DS concluded that the effects were not severe enough. By contrast, the reduction in Hb and Ht in the 14 week dog study was around 20% at the highest dose level (83mg/kg bw/d) which is below the guidance value for STOT RE 2 were considered relevant. In addition, liver necrosis was also observed in all dog studies at doses

relevant for STOT RE 2 classification. The latter two effects are considered by the DS as generalised changes of a less severe nature involving several organs and are taken into account. As it cannot be excluded that the observed effects in dogs are not relevant to humans, the DS proposed classification for STOT RE in category 2.

Dermal

A single experimental dose was applied (i.e. 1000 mg/kg bw/d) to Wistar rats for 22 times in a 28 days study according to OECD TG 410 and GLP (Kröttlinger, 1999). This effect-level was above the upper limit for STOT RE 2 classification (600 mg/kg bw/d for a 28-day study) and the type and severity of the observed effects do not fulfil the criteria for classification for STOT RE. The DS proposed no classification for the dermal route.

Comments received during public consultation

Two MSCAs commented on the STOT RE 2 proposal by the DS.

The first MSCA stated that the proposed classification for spirodiclofen as STOT RE 2 is mainly based on effects observed in dogs which were inconsistent. Haematological effects were observed in the 14-week dog study and consisted of a dose-related reduction of haemoglobin and haematocrit of about 20%. Such effects though were not reproduced in the other dog studies. In the 4-week dog study a non-dose related and not quantified decrease of Hb and Ht was observed. No such effects were found in the 8-week and 1-year studies. Given the inconsistencies of these haematological effects among the dog studies and the absence of haematological effects in other tested species (mouse and rat), the relevance of the classification as STOT RE for these effects was questioned.

In response to this statement the DS noted that although the effects on the haematological system were not observed in the 8-week and 1-year dog study, effects were observed in the 4-week and 14-week studies. Dose-related effects on Hb and Ht levels and % erythrocytes were observed in the 14-week oral dog study, in which a 20% decline of these parameters was observed at the highest dose level of 82.8 mg/kg bw/d (i.e. below the upper limit of 100 mg/kg bw/d for STOT RE 2). Additionally, in the 4-week oral dog study, haematological parameters were affected and reduced erythrocytes, Hb and Ht were observed at ≥ 65.5 mg/kg bw/d. However, no quantitative information is available in this 4-week dog study.

Moreover, the liver was found to be a target organ in the 4-week, 8-week, 14-week and 1-year dog studies. Effects included increased organ weight and increased biochemical parameters. Furthermore, hepatocellular necrosis was observed at 284.5 mg/kg bw/d in the 4-week study (i.e. below the upper limit of 300 mg/kg bw/d for STOT RE 2), at 55.9 mg/kg bw/d in the 8-week study (i.e. below the upper limit of 150 mg/kg bw/d for STOT RE 2) and 82.8 mg/kg bw/d in the 14-week study (i.e. below the upper limit of 100 mg/kg bw/d for STOT RE 2).

The DS acknowledged that some effects would not fulfil the classification criteria for STOT RE. However, according to section 3.9.1.4 of the CLP Guidance "Assessment shall take into consideration not only significant changes in a single organ or biological system but also generalised changes of a less severe nature involving several organs". Furthermore, according to section 3.9.2.5.2 of the CLP Guidance, a reduction in Hb of ≥20% would fulfil the classification criteria. In addition, necrosis is also one of the effects which fulfil the classification criteria as mentioned in and illustrated in an example of the Guidance on the application of the CLP criteria.

In summary, the DS proposed classification as STOT RE 2 since it cannot be excluded that the observed effects in dogs are relevant for humans. Given that the effective dose levels are below the upper limit of STOT RE 2, classification as STOT RE 2 was proposed.

The second MSCA noted that although effects seen on several target organs (liver, prostate) fulfil the criteria for being classified as STOT RE 2, there is one effect in particular (adrenal) that warrants classification in category 1 due to the rather low dose levels at which the effect occurs.

The DS responded with a review of the adrenal effects in all the species tested.

Most of the adrenal effects were observed at effective dose levels above the upper limit for STOT RE 2 and therefore do not warrant classification. However at individual level, some of the adrenal effects were observed below the upper limit for STOT RE 2 classification and even below the upper limit for STOT RE 1 classification. Effects included increased adrenal weight, cytoplasmic vacuolation and mononuclear cell infiltration in the adrenal cortex. Although these effects clearly point towards the adrenal glands as a target organ, they were considered by the MSCA not severe enough to fulfil the classification criteria (i.e. no evidence of marked organ damage or dysfunction).

Assessment and comparison with the classification criteria

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 (i.e. were observed in a 90-day repeated-dose study conducted in experimental animals below the guidance value range of 10 mg/kg bw/d or 20 mg/kg bw/d for oral and dermal exposure respectively).

Substances are classified in category 2 for target organ toxicity (repeated exposure) on the basis of observations from appropriate studies in experimental animals in which significant toxic effects, of relevance to human health, were observed in a 90-day repeated-dose study conducted in experimental animals within the guidance value ranges of 10-100 mg/kg bw/d for oral exposure or 20-200 mg/kg bw/d for dermal exposure.

Oral

Given that no human data are available and the effective dose levels in the repeated dose animal studies were above the upper limit for STOT RE 1 (i.e. 30 mg/kg bw/d for a 4-week study, 15mg/kg bw/d for a 8-week study, 10 mg/kg bw/d for a 90-day study, 2.5 mg/kg bw/d for a 1-year study), classification for STOR RE 1 is not considered.

Effect-levels in the mouse repeated dose toxicity studies (13-week and 18 month) were mostly above the upper limit for STOT RE 2 classification (100 mg/kg bw/d for a 90-day study, 25mg/kg bw/d for a 1-year study). However, in the 13-week study centrilobular hepatocellular hypertrophy was observed in 3 out of 10 male animals (grade 2: 3/3 animals) at a dose of 15.3mg/kg bw/d. These effects do not fulfil the classification criteria based on the observed severity (as there is no evidence of marked organ damage as described in the CLP guidance). Based on the mouse repeated dose toxicity studies, classification is not required.

Effect levels in the 4-week, 14-week and 2-year rat repeated dose toxicity studies were mostly

above the upper limit for STOT RE 2 classification (300 mg/kg bw/d for a 4-week study, 100 mg/kg bw/d for a 90-day study, 12.5 mg/kg bw/d for a 2-year study). However, increased small cortical vacuolation of the adrenal glands (with increased grading) was observed in male animals at dose levels of 6.6 and 32.1 mg/kg bw/d and higher. However, according to the DS, this was within the range of historical controls (Hartmann, 2005). Based on the rat repeated dose toxicity studies, classification is not required.

Effect levels in the available dog repeated dose toxicity studies were around or below the upper limit for STOT RE 2 classification (300 mg/kg bw/d for a 4-week study, 150 mg/kg bw/d for an 8-week study, 100 mg/kg bw/d for a 90-day study, 25 mg/kg bw/d for a 1-year study). In most studies, the highest dose tested was below the upper limit for STOT RE 2 classification. At the highest dose level tested many parameters were effected. Most effects individually would not fulfil the classification criteria. However, according to 3.9.1.4 of the CLP Guidance also generalised changes of a less severe nature involving several organs should be taken into account. Further, the reduction in Hb and Ht in the 14 week dog study was around 20% at the highest dose level (83 mg/kg bw/d) which was below the upper limit for STOT RE 2. In the same study there was a reduction in the reticulocytes exceeding 50% observed in males only. In the 4-week study in dogs at 2000 ppm a reduction of Hb up to 9% in 50% of the animals was observed while at 1000 ppm the decrease reaches 12 % in all animals. No statistical effects on reticulocytes was observed. In addition, in the 4-, 8- and 14 –week dog studies liver necrosis was observed in doses relevant for STOT RE 2 classification. The adrenal effects were not considered since histopathological analysis did not reveal severe organ damage and/or dysfunction. As it cannot be excluded that the observed haematological and liver effects in dogs are relevant for evaluating potential effects of spirodiclofen in humans, the observed effects should be taken into account for potential classification for STOT RE. Together,the available information in dogs warrant classification for STOT RE in category 2.

The results of the combined chronic toxicity/carcinogenicity studies and 2-generation rat study do not warrant classification for STOT RE.

The results of the neurotoxicity and immunotoxicity studies do not warrant classification for STOT RE.

Dermal

A single experimental dose was applied (i.e. 1000 mg/kg bw/d). This effect-level was above the upper limit for STOT RE 2 classification (600 mg/kg bw/d for a 28-day study) and the type and severity of the observed effects do not fulfil the criteria for classification for STOT RE.

Therefore, classification for the dermal route is not required.

In summary, based on the available studies, classification for specific organ toxicity – repeated exposure as STOT RE 2 (H373: May cause damage to organs, through prolonged or repeated exposure) is required. This classification applies to all routes as comparable effects after inhalation exposure cannot be excluded. As the classification is mainly based on the general effects on several organs, no specific target organ is proposed.

Comparison with CLP criteria

In the table below, all the effects relevant for STOT RE observed in the three dog studies are summarised. Effects reported in mouse and rats studies are not reported since they were all above guidance values and not considered severe. However, the liver and the blood are also target organs in those species.

Table: Summary of effects on STOT RE parameters in available repeated dose toxicity in dogs

Dose/target organs	**≤ Guidance Value for STOT RE 2**
Dog 4-week, oral, 2m+2f, (0, 11.3, 65.5, 284.5 mg/kg bw/day) (STOT RE 2 ≤ 300 mg/kg bw/day)*	
general	Effects on haematology, ↓ kidney weight, ↑ weights of uterus, adrenals and brains, Leydig cell vacuolation, cytoplasmic vacuolation adrenal cortex.
hematology	↓ Hb, Ht, Lymphocytes (%)
adrenals	↑ar weight, cytoplasmic cortex vacuolation 1/4, 1/4, 4/4, 4/4 (1,2,1,3)
liver	increased ALAT, increased enzyme activities in liver tissue (N-DEM, O-DEM, P450, ECOD, ALD, EH, Glu-T), periportal single cell necrosis: 0/4, 0/4, 0/4, 4/4 (2,1,2,1)
clinical chemistry	↓cholesterol, triglycerides
Dog 8-week, oral, 5m, (0, 2.9, 55.9 mg/kg bw/day) (STOT RE 2 ≤ 150 mg/kg bw/day)*	
general	↑ AP, ↑ organ weights of thyroid, adrenals, thymus and pancreas, ↓ prostate weight
hematology	no toxicologically relevant effects
adrenals	cytoplasmic cortex vacuolation 0/5, 4/5, 5/5 mononuclear cell infiltration adrenal cortex 0/5, 1/5, 3/5
liver	Increased AP, increased organ weights of liver hepatocellular single cell necrosis 0/5, 0/5, 3/5
clinical chemistry	↓cholesterol, triglycerides, ↑LH
Dog 14-week, oral, 4/sex/dose, (0, 8.0, 27.3, 82.8 mg/kg bw/day) (STOT RE 2 ≤ 100 mg/kg bw/day)*	
general	Effects on haematological parameters, clinical biochemistry, changes relative prostate weight and histopathological changes in the adrenal gland
hematology	~ 20% ↓ Hb, Ht, and ↓lymphocytes (%),
adrenals	↑r adrenal weight (m, f) vacuolisation zona fasciculata, cortex 0/4, 2/4, 3/4, 4/4 (f), 0/4, 0/4, 3/4, 4/4 (m) mononuclear cell infiltration 0/4, 2/4, 0/4, 4/4 (f), 0/4, 1/4, 1/4, 4/4 (m)
liver	↑liver microsomal enzymes periportal single cell necrosis 0/4, 0/4, 1/4 (f)
clinical chemistry	various parameters affected at 27.3 mg/kg bw/day
organ weights	various organs affected at 27.3 mg/kg bw/day, thymus ↓r
Dog 1-year, oral, 4 sex/dose, (0, 0.57, 1.45, 4.54, 16.9 mg/kg bw/day) (STOT RE 2 ≤ 25 mg/kg bw/day)*	
general	↑ adrenal weight and adrenal vacuolation
hematology	no toxicologically relevant effects
adrenals	≥ 0.57 mg/kg bw/day ↑ar adrenal weight (m) ≥ 4.54 ↑ar adrenal weight (f) ↑ vacuolation 1/4, 2/4, 0/4, 4/4, 4/4 (m) ↑ vacuolation 1/4, 1/4, 0/4, 3/4, 4/4 (f)
liver	cytoplasmic inclusion 0/4, 0/4, 1/4, 1/4, 2/4 (m), pigment 0/4, 0/4, /4, 0/4, 1/4, 2,4 (f)
clinical chemistry organ weights	↑ cholesterol (m), ↓cholesterol (f) various organs affected at 0.57 mg/kg bw/day

* no general toxicity observed; a: absolute; r, relative; m, male; f, female.

In the oral repeated dose toxicity studies in the **mouse** (13-week & 18-month) there was no evidence for severe liver effects such as organ lesions or dysfunction. Therefore, RAC agrees with the DS that classification based on the mouse studies is not justified.

The effect levels in the 4-week, 14-week and 2-year **rat** repeated dose toxicity studies were mostly above the upper limit for STOT RE 2 classificationIncreased small cortical vacuolisation of the adrenals (with increased grading in function of the dose) was observed in male animals at dose levels of 6.6 and 32.1 mg/kg bw/d and higher in the 14-week study (below the guidance valuefor STOT RE 2 classification). However this was within the range of historical controls. Additionally, the results from the combined chronic toxicity/carcinogenicity, 2-generation reproductive toxicity, neurotoxicity and immunotoxicity studies did not warrant classification. therefore, based on the aforementioned rat studies, RAC agrees with the DS for classification for STOT RE is not justified.

In the available repeated dose toxicity studies in **dogs** (4-week, 8-week, 14-weekand 1-year) many parameters of various organ systems were affected including the haematological system, the liver and the adrenals. RAC is of the opinion and agrees with the DS that the observed adrenal effects in dogs (cytoplasmic vacuolisation and mononuclear cell infiltration adrenal cortex effects) are not severe and do not fulfil the CLP criteria for STOT RE classification.

Effects on the haematological system were not observed in the 8-week and 1-year dog studies. However, they were seen in the 4-week and 14-week dog studies. In both studies the effect-levels were below the upper limit for STOT RE 2. In the 4-week study reduced erythrocytes, Hb and Ht were observed. In the 14-week study though, a dose related effect on Hb and Ht levels and % erythrocytes was seen and at the highest dose level a 20 % decline of these parameters was observed which is considered a consistent and adverse effect in haematology (Guidance on the application of CLP criteria, Annex 3.9.2.7.3.(c)).

The liver was also identified as a target organ in dogs. Effects included increased organ weight and increased biochemical parameters. Also hepatocellular necrosis was observed although at effect-levels below the upper limit for STOT RE 2 classification.

In conclusion, RAC agrees with the DS's proposal to classify spirodiclofen as **STOT RE 2 (H373: May cause damage to organs through prolonged or repeated exposure)** based on the dog data. The classification is based on haematology and liver effects and apply to all routes of exposure with no specific organ specified.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 39: Summary table of relevant in vitro and in vivo mutagenicity studies

Method	Results		Remarks	Reference[a]
	+ activation	-activation		
In vitro				
Gene mutation Ames-test *S. typhimurium* (TA98, TA100, TA1535, TA1537, TA102) OECD 471(with deviations)	negative	negative	No cytotoxicity observed up to highest dose tested Purity: 99.1%	Herbold, 1996a
Gene mutation hprt – assay Chinese hamster cells (V79) OECD 476	negative	negative	Purity: 99.1%	Brendler-Swaab (1997)
In vitro chromosome aberration assay Chinese hamster cells (V79) OECD 473	equivocal	equivocal	Purity: 99.1%	Herbold (1996b)
In vivo				
In vivo micronucleus-test Mice OECD 474	negative		Only one dose tested Purity: 99.1%	Herbold, 1996c

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.9.1 Non-human information

4.9.1.1 In vitro data

STUDY 1

Study design and results

Type of study: Ames test, plate incorporation, preincubation.

Indicator cells	Endpoint	Res. - act.	Res. +act.	Activation		Dose range	Reference/ notifier
				Tissue	Inducer		
B: *S. typh.* TA 98 TA 100 TA 1535 TA 1537 TA 102	 point mut. point mut. point mut. point mut. point mut.	 - - - - -	 - - - - -	 Rat liver Rat liver Rat liver Rat liver Rat liver	 Aroclor 1254 Aroclor 1254 Aroclor 1254 Aroclor 1254 Aroclor 1254	0, 16, 50, 158, 500, 1581 and 5000 µg/plate[1, 2] solvent: DMSO	Herbold, B. (1996a)

[1] Up to and including 5000 µg/plate no cytotoxicity was observed.
[2] due to the range of stability of solutions with spirodiclofen in DMSO, 0.01 ml compound was added and 0.09 ml solvent additionally. (Stability of the test substance in vehicle: 0.4 mg/ml solution after 24h : –7%, 150 mg/ml solution after 24h: -15%).

Test substance: BAJ 2740 (spirodiclofen, purity 99.1%)
GLP: yes
According to OECD 471: yes, deviations: no information is given on the amount of viable cells in the bacterial cultures used (has to be approx. 10^8 viable cells/ 0.1 ml bacterial culture)

Acceptability

The study was considered acceptable.

Conclusions

Under the test conditions, spirodiclofen did not induce mutations in S.typhimurium.

STUDY 2

Study design and results

Type of study: mammalian cells *in vitro*, gene mutations, HPRT-assay

Indicator cells	**Endpoint**	**Res. - act.**	**Res. +act.**	**Activation**		**Dose range**	**Reference/ notifier**
				Tissue	**Inducer**		
Chinese hamster lung cells (V79)	gene mutation (HPRT)	-[3]	-[4]	Rat liver	Aroclor 1254	-S9: 4, 6, 8, 10, 15 or 20 μg/ml[1,2] S9: 10, 20, 40, 50, 60 or 80 μg/ml[1,2] Solvent : DMSO	Brendler-Swaab, S. (1997)

[1] Dose levels were based on a dose-range finding study in which concentrations ranging from 1.17 to 300 μg/ml (+/- S9) were tested. A dose-dependent decrease of cell viability was observed under both (+/- S9) conditions. Virtually all cells were killed at concentrations above 18.8 μg/ml (-S9) or above 75 μg/ml (+S9). Precipitation of the test substance was observed at a final concentration of 156 μg/ml.
(Stability of the test substance in vehicle: 0.4 mg/ml solution after 24h : –7%, 150 mg/ml solution after 24h: -15%).
[2] cytotoxicity was observed at concentrations at and above 15 μg/ml (-S9) or at and above 60 μg/ml (+S9).
[3] increased mutation frequency was observed in one culture at concentrations of 6, 8 and 10 μg/ml (mutant frequencies 5.5%, 5.8% and 9.7% resp., with vehicle control of 2.3%). These increases were neither reproduced in the parallel treated culture nor in the second trial, and therefore considered not toxicologically relevant.
[4] increased mutation frequency was observed in one culture at concentrations of 10 and 20 μg/ml (mutant frequencies 10.7% and 11.4% resp., with vehicle control of 2.4%). These increases were neither reproduced in the parallel treated culture nor in the second trial. The joint statistical assessment of the two trials resulted in a statisticallly significant increase of the mutation frequency over the vehicle controls at concentrations of 10, 20 and 50 μg/ml, however without dose-relationship. These observed increases in mutant frequencies are considered not toxicologically relevant.

Test substance: BAJ 2740 (spirodiclofen, purity 99.1%)
GLP: yes
According to OECD 476: yes (3 x 10^5 cells instead of 10^6 cells)

Acceptability

The study was considered acceptable.

Conclusions

In this test system, spirodiclofen did not induce gene mutations in mammalian cells *in vitro*.

STUDY 3

Study design and results

Type of study: mammalian cells *in vitro*, cytogenetic assay

Indicator cells	Endpoint	Res. - act.	Res. +act.	Activation		Dose range	Reference/ notifier
				Tissue	Inducer		
Chinese hamster V79 cells	chromosome aberrations	[2]+/-	[2]+/-	Rat liver	Aroclor 1254	[1]-S9 (18 h): 0.75, 1.5, 3 μg/ml -S9 (30 h): 3 μg/ml [1]+S9 (18 h): 20, 40, 80 μg/ml (18 h) +S9 (30 h): 80 μg/ml Solvent: DMSO	Herbold, B. (1996b)

[1] In a dose-range finding study, cytotoxicity was observed at and above 10 μg/ml (-S9) and 50 μg/ml (+S9). Based on these results in the first test concentrations in the range of 5 to 80 μg/ml (-S9) and 10 to 160 μg/ml (+S9) were used. However, no metaphase reading was possible at and above 20 μg/ml (-S9) and 160 μg/ml (+S9). Therefore, a second trial (-S9) was performed with concentrations of 0.75, 1.5, 3, 6 and 12 μg/ml (no metaphase reading possible at 12 μg/ml).
-S9: MI reduced at and above 0.75 μg/ml; cytotoxicity at and above 0.75 μg/m
+S9: MI reduced at and above 80 μg/ml (the observed reduced MI at 10 μg/ml is considered not relevant); cytoxicity at and above 20 μg/m.
[2] (-S9): statistically significant increase of aberrations was observed at 3 μg/ml (18 h).
(+S9): statistically significant increase of aberrations was observed at 80 μg/ml (30 h).

spirodiclofen was soluble in DMSO up to 167 mg/ml.
Precipitation of spirodiclofen was observed in the pre-test at and above 100 μg/ml.
(Stability of the test substance in vehicle: 0.4 mg/ml solution after 24h : –7%, 150 mg/ml solution after 24h: -15%).

Test substance: BAJ 2740 (spirodiclofen, purity 99.1%)
GLP: yes
According to OECD 473: yes

Acceptability

The study was considered acceptable.

Conclusions

The results of the *in vitro* chromosome aberration study are equivocal. Statistically significant increased values of aberrations were observed in the absence and in the presence of S9, but these increased values are within the range of historical control values. Therefore, the biological relevance of this observation is considered low and BAJ 2740 is considered as not-clastogenic in mammalian cells *in vitro*

4.9.1.2 In vivo data

STUDY 1

Study design and results

Type of study: micronucleus test

Species	Endpoint	Result	Dose range	Reference/notifier

mouse	micronuclei (bone marrow)	-	[1] 800 mg/kg bw, i.p.; sacrifice after 16, 24 and 48 hours Solvent: 0.5% aqueous Cremophor emulsion	Herbold, B. (1996c)
[1] Selection of the dose was based on a pilot test (250-1250 mg/kg bw i.p.), were 4/5 animals died in the 1250 mg/kg bw group, and in all dose groups the following symptoms were observed: apathy, roughened fur, spasm, difficulty in breathing and eyelids stuck together. In the main study, treated animals (m,f) showed the following symptoms until sacrifice: apathy, roughened fur, spasm and eyelids stuck together. 1/40 treated animals died during the test period. (spirodiclofen was stable in the vehicle at concentrations ranging from 10 to 100 mg/ml for at least 24 h). Test substance: BAJ 2740 (purity 99.1%) GLP: yes According to OECD 474: yes (only one dose tested)				

Acceptability

The study was considered acceptable. The test substance was administered i.p., systemic toxic effects were observed and P/N ratio was altered in the treated animals indicating that the test substance did reach the bone marrow.

Conclusion

In this test system, spirodiclofen did not induce micronuclei in mouse bone marrow cells.

4.9.2 Human information

No data available.

4.9.3 Other relevant information

No data available.

4.9.4 Summary and discussion of mutagenicity

Spirodiclofen did not induce mutations in bacteria nor gene mutations (hprt) *in vitro* in mammalian cells. The results of the chromosomal aberration study in mammalian cells *in vitro* were equivocal, since statistical increased values of aberrations were observed (+/-S9), but these increased values are within the range of historical values. Therefore the biological relevance of this observation is considered low and spirodiclofen is considered as not-clastogenic in mammalian cells *in vitro*. Moreover, spirodiclofen was found to be negative in an *in vivo* micronucleus test. Based on these results, spirodiclofen is considered to be non genotoxic.

4.9.5 Comparison with criteria

Considering that no positive response was observed in the available *in vitro* and *in vivo* mutagenicity tests, no classification is required for spirodiclofen under the CLP Regulation.

4.9.6 Conclusions on classification and labelling

Classification of spirodiclofen for mutagenicity is not required under the CLP regulation.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The evaluation of the germ cell mutagenicity hazard endpoint was based on three *in vitro* and one *in vivo* study.

- In the *in vitro* bacterial reverse mutation test according to OECD TG 471 (GLP compliant) spirodiclofen did not induce mutations in *S.typhimurium* (Herbold, 1996a).
- In the *in vitro* mammalian cell gene mutation test using the hprt assay according to OECD TG 476 (GLP compliant), spirodiclofen did not induce gene mutations (Brendler-Swaab, 1997).
- In the *in vitro* chromosome aberration study (OECD TG 473, GLP compliant) statistically significant increased values of aberrations were observed both in the absence and in the presence of S9 but these increased values were within the range of historical control values. Therefore, the biological relevance of this observation is considered low and spirodiclofen considered as not-clastogenic in mammalian cells *in vitro* (Herbold, 1996b).
- In the *in vivo* micronucleus test (bone marrow) (OECD TG 474, GLP compliant) spirodiclofen did not induce micronuclei in mouse bone marrow cells (Herbold, 1996c).

Based on the negative results of the available *in vitro* and *in vivo* mutagenicity tests the DS proposed no classification for mutagenicity for spirodiclofen.

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen for mutagenicity.

Assessment and comparison with the classification criteria

Considering that no positive responses were observed in the available *in vitro* and *in vivo* mutagenicity tests, RAC agrees that **no classification is warrented for spirodiclofen for mutagenicity** under the CLP Regulation.

4.10 Carcinogenicity

Table 40: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference[a]
Mouse (CD-1) 18 months Oral *OECD 451* 25, 3500, 7000 ppm (4.1, 610, 1216 mg/kg bw/day in males and 5.1, 722, 1495 mg/kg bw/day in females)	Increased incidence of adrenal pigmentation and vacuolation in females, increased incidence of amyloid in several tissues and increased incidence of hepatocytomegaly in males; hepatocellular neoplasia (significant for benign types) in males at and above 610 mg/kg bw/day	-	Wahle, 2000
Rat (Wistar) 108 weeks Oral *OECD 453* 0, 50, 100, 350, 2500 ppm (0, 2.04, 4.11, 14.72, 110.14 mg/kg bw/day for males, and 0, 2.87, 5.93, 19.88, 152.90 mg/kg bw/day for females)	Increased incidence of Leydig cell tumors at 110.14 mg/kg bw/day and adenocarcinomas in the uterus at 152.90 mg/kg bw/day	-	Wirnitzer, 2000

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

STUDY 1

Characteristics

Reference/notifier	:	Wahle, B.S. (2000)	Exposure	:	18 months
Type of study	:	Oncogenicity testing study	Doses[1]	:	25, 3500, 7000 ppm (4.1, 610, 1216 mg/kg bw/day in males and 5.1, 722,1495 mg/kg bw/day in females)
Year of execution	:	1997-1998	Vehicle	:	-
Test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%, 97.6%, 97.7%, 97.9%, 97.6%)	GLP statement	:	Yes
Route	:	Oral (diet, with acetone/corn oil)	Guideline	:	In accordance with OECD 451
Species	:	Mouse, CD-1	Acceptability	:	acceptable
Group size	:	50/sex/dose	**NOAEL**	:	< 4.1 mg/kg bw/day

1 Doses are based on a subchronic (6-weeks) range-finding toxicity study.

Study design

The study was performed in accordance with OECD guideline 451. Haematological examination included HDW and LUC, which were not mentioned in the list of abbreviations and determinations and are not clear to the dossier submitters.

Results

The results of the study are presented in Table 41.

Table 41 Overview of the results of the 18-month oral toxicity study in mice (Wahle, 2000)

Dose	**0 ppm (m/f: 0 mg/kg bw/d)**		**25 ppm (m/f: 4.1/5.1 mg/kg bw/d)**		**3500 ppm (m/f: 610/722 mg/kg bw/d)**		**7000 ppm (m/f: 1216/1495 mg/kg bw/d)**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	No toxicologically relevant effects								
Clinical signs									
Eyes									
-focal opacity	1/50		0/50		0/50		6/50		
Lesion									
-redness		7/50		5/50		13/50		14/50	
Behavior									
Spiller[1]		27/50		23/50		36/50		42/50	
Body weight (gain)[2]					d	d	d	d	
Food consumption[3]					i		i		
Ophthalmoscopy	Not performed								
Haematology									
- MCHC					ds		ds		
- platelets							d	d	
- WBC							ds		
- HDW[8]								d[4]	
- % monocytes							d[4]		
- % reticulocytes					i		i		
- % segmented neutrophils						ds		d	
- % lymphocytes						is		i	
- % atypical lymphocytes				ds		ds		ds	
- % Luc[8]								d	
Urinalysis	Not performed								
Clinical chemistry	Not performed								
Organ weights[5]									
- adrenals					Is^{a,r}	is^{a,r}	is^{a,r}	is^{a,r}	m, f
- kidneys					ds^{a,r}	ds^{a,r}	ds^{a,r}	ds^{a,r}	m, f
- liver					is^{a,r}	i^{a,r}	is^{a,r}	is^{a,r}	m, f
- testes					i^{a}/is^{r}		is^{a,r}		m
Pathology									
macroscopy									
- *heart*, enlarged							5/50		
- *adrenals*, enlarged					2/50	3/50	6/50	10/50	m, f
- *eyes*, opacity	3/50		1/50		1/50		8/50		
- *testicles*, discoloration					3/50		7/50		m

Dose	0 ppm (m/f: 0 mg/kg bw/d)		25 ppm (m/f: 4.1/5.1 mg/kg bw/d)		3500 ppm (m/f: 610/722 mg/kg bw/d)		7000 ppm (m/f: 1216/1495 mg/kg bw/d)		dr
	m	f	m	f	m	f	m	f	
microscopy									
nonneoplastic lesions									
Adrenals									
- amyloid	18/50	17/50	19/49	10/49	18/50	12/50	30/50	10/50	
- average severity	3.2	2.1	3.4	2.2	4.7	3.8	4.5	4.8	f
- lymphocytic infiltrate						2/50	2/50	5/50 is	f
- pigmentation[6]	7/50	11/50	5/49	20/49 is	11/50	45/50 is	37/50 is	42/50 is	
- average severity	1.3	2.2	1.6	2.4	1.8	2.7	2.4	2.8	m, f
- vacuolation		1/50		6/49	31/50 is	49/50 is	37/50 is	48/50 is	m
- average severity		2.0		1.0	2.1	2.4	2.5	2.9	m
Brain									
- mineralization		5/50		7/50		4/49		14/50 is	
Epididymides									
- aspermia, rel. or abs.	15/50		15/50		15/50		26/50 is		
- average severity	4.3		4.2		4.8		4.8		
Heart									
- amyloid	8/50		12/50		16/50		19/49		m
- average severity	2.4		2.0		2.5		3.3		
- thrombus, arterial			1/50		1/50		9/50 is		
Liver									
- amyloid	10/50	4/50	16/50	3/50	17/50	9/50	18/50	10/50	
- average severity	1.9	1.5	2.0	2.0	2.3	2.2	2.6	2.6	m, f
- hepatocytomegaly	2/50		6/50		17/50 is		21/50 is		m
- average severity	1.5		1.7		1.5		1.9		
Lymph node, mesenteric									
- congestion	1/49		6/48		7/49		8/49		
Pancreas									
- amyloid					2/50		5/49 is		m
- average severity					1.0		2.0		m
Parathyroids									
- amyloid	8/47		18/49		17/48		19/50		
- average severity	2.8		1.9		3.5		4.7		
Spleen									
- amyloid	5/50		5/50		13/50 is		14/48 is		
Stomach									
- amyloid	11/50		13/50		16/50		20/49		m
- average severity	2.5		1.5		2.6		3.2		
Testes									
- amyloid	10/50		10/50		13/50		17/50		
- average severity	2.8		3.0		3.5		3.7		m
- degeneration	18/50		18/50		22/50		28/50		m
- hypertrophy/hyperplasia interstitial cells[7]	6/50		6/50		26/50 is		31/50 is		m
- average severity	1.2		1.3		1.8		2.5		m
Thyroids									
- amyloid	11/50		19/50		18/49		19/49		
- average severity	3.2		3.0		3.8		4.7		m
microscopy									
neoplastic lesions									
Liver									
- adenoma, hepatocellular					5/50 is	3/50	6/50 is	1/50	
- carcinoma, hepatocellular	1/50		1/50		3/50	2/50	5/50	2/50	m
- Combined adenoma/carcinoma, hepatocellular	1/50	0/50	1/50	0/50	8/50	5/50	11/50	3/50	

dr	dose related
[a,r]	absolute organ weight, relative organ weight
1	the study author considers the increased incidence of feed spillage in females of the highest dose groups not relevant, since this observation did not include the number of times an animal had spilled. However, it cannot be excluded that it is a substance related observation, and is therefore considered relevant.
2	bw in males was decreased, and occasionally significantly decreased. In females bw was significantly decreased in weeks 1 to 4, and was decreased in the weeks thereafter, with occasionally significantly decreased bw. Terminal BW of female dosed 3500 or 7000 ppm was significantly decreased in tables of mean organ weights shown in the study.
[3]	Occasionally increased significantly. Increased food consumption was observed until day 63.
[4]	significantly decreased at 12 months.
[5]	Variation of ovary weights was very high in the contol females, which prevented evaluation of substance-related effects on ovary weight in this study.
[6]	The study author concludes, that the observed significant increased pigmentation in the adrenals of females dosed 25 ppm is not compound related, since historically, two studies showed comparable pigmentation in control tissue. . However, since pigmentation increases with age, the provided historical control values of a 92 weeks exposure study are not comparable with the values in the present 78 weeks exposure study. The provided historical control values of a 81 weeks exposure study showed pigmentation in 12/50 females, which is comparable with the control values in females of the present study. The observed increase in the 25 ppm female group (20/49) is therefore considered a substance related effect. The difference in frequency between the lowest dose and the two higher doses is considered to be related to the great jump in doses (25 ppm vs 3500 and 7000 ppm).
[7]	Testes of the control and 25 ppm animals showed hyperplasia only, whereas testes of males of the 3500 ppm and 7000 ppm showed both increased cell size and increased numbers of cells.
[8]	Not defined in the list of abbreviations

Acceptability

The study was considered acceptable.

Conclusions

In the two highest dose groups, body weight was decreased, effects were observed on haematological parameters and organ weights of adrenals, liver and testes were dose-relatedly increased and kidney weights were dose-relatedly decreased. Histopathological examination showed dose-related increased adrenal pigmentation in females of all dose groups (see remark 6 under Table 35) and in males of the two highest dose groups, accompanied by increased average severity. Adrenals also showed increased vacuolation in females of all dose groups (the difference in frequency between the lowest dose and the two higher doses is considered to be related to the great jump in doses (25 ppm vs 3500 and 7000 ppm)) and in males of the two highest dose groups. In males of all dose groups, a dose-related increase in hepatocytomegaly was observed.

A significantly increased frequency of hepatocellular adenomas over controls was observed in males of dose groups 3500 and 7000 ppm. Further, a dose-related increase (not statistically significant) of malignant hepatic tumor types (carcinomas) was observed in male animals as well. The combined frequency of hepatocellular neoplasms (adenomas and carcinomas) was also significantly increased with combined tumor frequencies of 1/50, 1/50, 8/50 and 11/50.

Remarkable was the increased incidence and/or increased average severity of amyloid in several tissues in the exposed animals. Increased incidence of amyloid was already observed in the lowest dose group in the heart, liver, thyroids and parathyroids of males. The historical control values of a 79-81 weeks exposure study are substantially lower than the observed increase in the 25 ppm group.

Based on the observed increased incidence of adrenal pigmentation and vacuolation in females, increased incidence of amyloid in several tissues of males and increased incidence of hepatocytomegaly in males of the lowest dose group, a NOAEL for chronic toxicity in this study could not be established. The LOAEL for chronic toxicity is 25 ppm, equal to 4.1 mg/kg bw/day.

Spirodiclofen is considered carcinogenic for inducing liver tumors in the mouse. Hepatocellular tumors were observed at and above 610 mg/kg bw/day, the NOAEL for neoplastic lesions is therefore 4.1 mg/kg bw/day.

STUDY 2

Characteristics

reference/notifier	:	Wirnitzer, U., U. Bach, E. Hartmann (2000)	Exposure	:	108 weeks (interim necropsy after 52 weeks), neurotoxicity tested at week 77
type of study	:	Combined study on chronic toxicity and carcinogenicity.	Doses[1]	:	0, 50, 100, 350, 2500 ppm (0, 2.04, 4.11, 14.72, 110.14 mg/kg bw/day for males and 0, 2.87, 5.93, 19.88, 152.90 mg/kg bw/day for females)
year of execution	:	1997/1998	Vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%, 98.5%, 97.9%, 97.8%, 97.6%, 97.8%)	GLP statement	:	Yes[2]
route	:	Oral (diet, with 1% peanut oil)	Guideline	:	In accordance with OECD 453, with some deviations (see study design)
species	:	Rat, Wistar (HSd Cpb:WU)	Acceptability	:	Acceptable, with the exception of neurotoxicity.
group size	:	50/sex/dose (additional animals for interim necropsy after 1 year 10/sex/dose)	**NOAEL**	:	5.93 mg/kg bw/day

1 Dose levels are based on the results of a 14 weeks study with rats. Check of the test compound content in the diet showed lower than the defined concentrations at 50 ppm (3 times observed) and 100 ppm (2 times observed).

2 two deviations were reported: one out of ten analytical contents check which was re-evaluated after the end of the in-life phase was found to be invalid due to insufficient documentation; determination of test substance concentration in plasma samples as well as experiments for method development (telomere measurements in different tissues) were not conducted according to GLP. According to the study author, results are reported separately, but are not included for the present evaluation.

Study design

The study was in accordance with OECD guideline 453, with the following deviations: no high dose satellite group (20/sex) and satellite control group (10/sex) for evaluation of pathology other than neoplasia were present; blood samples for hematological examination should be collected from 20 rats/sex of all groups; neurotoxicity was tested on week 77 (Functional Observational Battery) with the observer aware of the animal's treatment assignment and the test runs were done in in the same order (control-50 ppm-100 ppm- 350 ppm- 2500 ppm). Results of this 77 weeks neurotoxicity study are presented as a chronic neurotoxicity study in section 4.12.1.1.

Results

The results of the study are summarized in Table 42. Details on several organ weights at terminal sacrifice are presented in Tables 43 and 44.

Table 42 Overview of the results of the 2-year chronic toxicity/carcinogenicity study in rats (Wirnitzer, 2000)

Dose	0 ppm (m/f: 0 mg/kg bw/d)		50 ppm (m/f: 2.04/2.87 mg/kg bw/d)		100 ppm (m/f: 4.11/5.93 mg/kg bw/d)		350 ppm (m/f: 14.72/19.88 mg/kg bw/d)		2500 ppm (m/f: 110.14/152.90 mg/kg bw/d)		dr
	m	f	m	f	m	f	m	f	m	f	
Mortality	No toxicologically relevant effects										
Clinical signs	No toxicologically relevant effects										
Body weight (gain)[1]									ds	ds	
Food consumption (rel)									i	i	
Ophthalmoscopy											
-% eyes with post capsular lens opacity	21.9		24.2		24.7		39.4		31.7		
Haematology[2]											
-leukocytes									d	ds	
-lymphocytes									d	ds	
Urinalysis	No toxicologically relevant effects										
Clinical chemistry											
-AP									is	is	
-cholesterol									d	d	
-triglycerides									d	d	
-urea[3]										d	
-T4[4]									is		
-TSH[5]									i	is	
-P[6]									d	d	
Organ weights (12 months sacrifice)											
- brain										is^{r}	
- adrenals[7]									i^{r}		
- heart									is^{r}	ds^{a}	
- spleen									$d^{a,r}$	ds^{a}	
- thymus									i^{r}	is^{r}	
- testes									i^{r}		
Organ weights (24 months sacrifice)											
- adrenals			$i^{a,r}$		$i^{a,r}$		$i^{a,r}$		$i^{a,r}$		
- liver									ds^{a}		
- spleen				ds^{r}		d^{r}		ds^{r}	d^{a}	d^{a}/ds^{r}	
- thymus								$i^{a,r}$	$i^{a,r}$	$i^{a,r}$	f
- testes									i^{r}		
-ovaries								$i^{a,r}$		$i^{a,r}$	f
Pathology (12 months sacrifice)											
macroscopy	No toxicologically relevant effects										
Microscopy											
nonneoplastic lesions											
Liver											
-fat content single cells, cell clusters and periportal hepatocytes									d		
Eyes											
Degeneration/ retinal	2/10		0/10		0/10		0/10		5/10		
Adrenals											
- cytopl. vacuolation (Zona fasciculata), small											
Incidence	6/10		7/10		5/10		3/10		9/10		
average grading	1.3		1.0		1.0		1.0		2.2		
- cytopl. vacuolation (Zona fasciculata), large											
Incidence	6/10		5/10		4/10		5/10		10/10		
average grading	1.5		1.4		1.3		1.2		1.5		
- adrenocorticocellular hypertrophy											

Dose	0 ppm (m/f: 0 mg/kg bw/d)		50 ppm (m/f: 2.04/2.87 mg/kg bw/d)		100 ppm (m/f: 4.11/5.93 mg/kg bw/d)		350 ppm (m/f: 14.72/19.88 mg/kg bw/d)		2500 ppm (m/f: 110.14/152.90 mg/kg bw/d)		dr
	m	f	m	f	m	f	m	f	m	f	
Incidence	0/10		0/10		0/10		0/10		3/10		
average grading	0		0		0		0		1.7		
Spleen											
- hemosiderin decomposition											
mean severity	3.1	3.9	3.3	3.8	3.5	3.5	2.9	3.9	1.9	2.9	
Thyroid											
- C-cell hyperplasia	0/10		0/10		0/10		0/10		4/10		
Pathology (24 months sacrifice)											
macroscopy											
Liver											
- discoloration (intercurrent deaths)		3/21		4/18		0/19		1/15		8/24	
Microscopy											
nonneoplastic lesions											
Eyes											
- lenticular degeneration	5/31		11/30		is 14/36		is 13/31		11/41		
- retinal atrophy (deaths only)		7/21		7/18		7/19		5/15		13/24	
Jejenum											
- vacuolated enterocytes	3/31	2/29	0/30	0/32	4/36	0/31	3/31	0/35	is 18/41	is 14/26	
Adrenals											
-diffuse hypertrophy/vaculation cortex cells (Z. fasc.)	3/31		1/30		5/36		4/31		is 25/41		
- focal hypertrophy (Z. fasc.) (deaths only)	1/19		0/20		0/14		0/19		2/9		
Nasal cavity											
- atrophy.degeneration olfactory epithelium	8/31		14/30		12/36		11/31		is 25/41		
Thyroid											
-colloidal alteration	23/50		23/50		28/50		28/50		35/50		
- follicular cell hyperplasia		1/29		0/32		1/31		0/35		5/26	
Liver											
- tigroid basophilic focus	10/31		8/30		12/36		9/31		19/41		
- cholangiofibrosis	9/31		5/30		11/36		5/31		18/41		
- focal necrosis (deaths only)		0/21		2/18		1/19		1/15		is 5/24	
Testes											
- focal Leydig cell hyperplasia	4/31		4/30		4/36		6/31		is 19/41		
Microscopy											
neoplastic lesions[8]											
Testes											
- benign Leydig cell tumor (except deaths)	2/31		1/30		0/36		4/31		9/41		
- benign Leydig cell tumor (deaths only)	0/19		0/20		0/14		0/19		1/9		
Uterus											
- adenocarcinoma (except deaths)		2/29		3/32		2/31		0/35		3/26	
- adenocarcinoma (deaths only)		2/21		2/18		1/19		2/15		is 11/24	
Thyroid											
- C-cell adenoma (except deaths)		1/29		2/32		3/31		4/35		4/26	
- C-cell adenoma (deaths only)		1/21		0/18		0/19		1/15		2/24	

dr dose related
ds/is statistically significantly decreased/increased
d/i decreased/increased
a/r absolute/relative organ weight
1 The difference was significant nearly throughout the whole treatment period in males (up to 11%) and from week up to and including

week 53 in females (up to 8%). (Mean individual water intake was slightly lower at 2500 ppm in both sexes).

2 The dossier submitter does not subscribe the opinion of the study author, that the observed significantly decreased leukocyte and lymphocyte counts in females are mainly due to relative high control values, and thus not considered to be of toxicological relevance, since decreased lymphocyte and leukocyte numbers were observed at almost all time points in males and females, though not statistically significant.

3 Observed at weeks 79 and 105.

4 Significantly increased at weeks 53 and 105, increased at weeks 27 and 79.

5 Males: observed at weeks 53, 79 and 105; females: increased at week 53, significantly increased at weeks 79 and 105.

6 Males: significantly decreased at weeks 27 and 53, decreased at weeks 79 and 105; females: significantly decreased at weeks 27 and 79, decreased at weeks 53 and 105.

7 Mean absolute adrenal weights in males were 50 and 54 mg (interim sacrifice) in controls and highest dose group respectively. Taken into account that bw was significantly decreased in the highest dose group, the observed increase in adrenal weight is expected to be more pronounced considered relative to body weight. However, due to the presented relative organ weights in round numbers by the study author, the expected increased rel adrenal weights were not observed. In view of the present evaluator, organs with low weights should not be presented in round numbers. Rough calculations with the mean values resulted in rel. adrenal weights of 9.54 and 11.49 in males of control and highest dose group respectively, which is considered an increase in rel adrenal weight.

8 In females of the highest dose group that had died intercurrently, metastasis of carcinoma were observed in several organs, including spinal cord, forestomach, glandular stomach, liver, pancreas, kidneys, ovaries, lymph nodes, spleen femur, body cavities.

Table 43 Thymus and ovary weights at terminal sacrifice in grams ± SD (and % increase compared to control)

Dose	**0 ppm (m/f: 0 mg/kg bw/d)**	**50 ppm (m/f: 2.04/2.87 mg/kg bw/d)**	**100 ppm (m/f: 4.11/5.93 mg/kg bw/d)**	**350 ppm (m/f: 14.72/19.88 mg/kg bw/d)**	**2500 ppm (m/f: 110.14/152.90 mg/kg bw/d)**
Thymus (males)					
- absolute (mg)	200 ± 49.9	227 ± 72.4 (14%)	220 ± 70.5 (10%)	207 ± 67.4 (4%)	229 ± 66.1 (15%)
- relative (mg/100 g bw)	38 ± 8.1	44 ± 13.7 (16%)	40 ± 12.0 (5%)	40 ± 12.7 (5%)	46 ± 11.9 (21%)
Thymus (females)					
- absolute (mg)	172 ± 45.5	188 ± 59.3 (9%)	192 ± 83.2 (12%)	205 ± 129.4 (19%)	215 ± 70.4 (25%)
- relative (mg/100 g bw)	56 ± 15.3	58 ± 17.0 (4%)	59 ± 23.2 (5%)	62 ± 36.6 (12%)	67 ± 19.1 (20%)
Ovaries					
- absolute (mg)	153 ± 70.3	164 ± 76.9 (7%)	147 ± 32.2 (-4%)	172 ± 103.9 (12%)	206 ± 135.3 (34%)
- relative (mg/100 g bw)	49 ± 22.6	51 ± 22.2 (4%)	45 ± 11.3 (-8%)	55 ± 42.9 (12%)	64 ± 40.7 (31%)

Table 44 Adrenal and spleen weights at terminal sacrifice in grams and % increase compared to control

Dose	**0 ppm (m/f: 0 mg/kg bw/d)**	**50 ppm (m/f: 2.04/2.87 mg/kg bw/d)**		**100 ppm (m/f: 4.11/5.93 mg/kg bw/d)**		**350 ppm (m/f: 14.72/19.88 mg/kg bw/d)**		**2500 ppm (m/f: 110.14/152.90 mg/kg bw/d)**	
Adrenals (males)									
- absolute (mg)	65	77	18%	82	26%	73	12%	82	26%
- relative (mg/100 g bw)	13	16	23%	16	23%	14	8%	16	23%
Adrenals (females)									
- absolute (mg)	79	84	6%	80	1%	86	9%	75	-5%

- relative (mg/100 g bw)	26	26	0%	25	-4%	27	4%	24	-8%
Spleen (males)									
- absolute (mg)	1131	1130	0%	1191	5%	1129	0%	1016	-10%
- relative (mg/100 g bw)	217	222	2%	225	4%	220	1%	207	-5%
Spleen (females)									
- absolute (mg)	702	642	-9%	696	-1%	649	-8%	625	-11%
- relative (mg/100 g bw)	226	199	-12%	213	-6%	199	-12%	196	-13%

Acceptability

The study is considered acceptable for the evaluation of long term toxicity and carcinogenic potential of the substance. The Functional Observational Battery which was conducted in week 77, is not considered in this 2 year study, but separately as a neurotoxicity study (see section 4.12.1.1).

Conclusions

Effects on body weight, food consumption, haematology and clinical chemistry were observed in the highest dose group only. After 12 months of exposure, changed organ weights were only observed in the highest dose group. After 24 months of exposure, increased adrenal weights (m) and decreased spleen weights (f) were observed in all dose groups. These changed organ weights were not dose-related and not accompanied by histopathological observations in the lowest dose groups. Other observations in the lowest dose groups were lenticular degeneration of the eyes and degeneration of olfactory epithelium of the nasal cavity, which were both not dose-related. An increased incidence of benign Leydig cell tumors were observed (2/50, 2/50, 0/50, 4/50 and 10/50 for 0, 50, 100, 350 and 2500 ppm respectively). Further, the incidences of uterus adenocarcinoma were also increased (total of 4/50, 5/50, 3/50, 2/50, 14/50 for 0, 50, 100, 350, 2500 ppm respectively). Total observed thyroid C-cell adenomas were increased in the 350 and 2500 ppm dose groups, but based on historical control data not relevant. In these two highest dose groups, increased weights of thymus and ovaries were observed. Since in females the absolute and relative weights of thymus and ovaries were increased by > 10%, this effects is considered to be test substance related. Hence the NOAEL for chronic toxicity is set at 100 ppm, equivalent to 5.93 mg/kg bw/d.

Spirodiclofen is considered carcinogenic for inducing Leydig cell tumors and uterus adenocarcinomas in the rat. These tumors were observed at and above 110.14 mg/kg bw/day, the NOAEL for neoplastic lesions is therefore 14.72 mg/kg bw/day.

4.10.1.2 Carcinogenicity: inhalation

No data available.

4.10.1.3 Carcinogenicity: dermal

No data available.

4.10.2 Human information

No data available.

4.10.3 Other relevant information

No data available.

4.10.4 Summary and discussion of carcinogenicity

Long-term oral carcinogenicity studies were perfomed with mice and rats. In the study with mice, observed effects included decreased bw, changes in haematological parameters and organ weights of adrenals, kidneys, liver and testes in the mid and high dosed groups. Histopathological examination showed effects on testes (increased incidence and severity of interstitial cell hypertrophy/hyperplasia), epididymides (aspermia, accompanied by increased severity), adrenals (vacuolation and pigmentation, increased incidence and severity) and liver (hepatocytomegaly). Remarkable was the increased incidence/increased average severity of amyloid in several tissues in all dose groups (heart, liver, thyroids and parathyroids of males). Based on the observed adrenal pigmentation and vacuolation, hepatocytomegaly and amyloid in all dose groups, a NOAEL could not be established in this study, and the LOAEL for chronic toxicity is 4.1 mg/kg bw/day. Spirodiclofen is considered carcinogenic for inducing liver tumors in the mouse. Hepatocellular tumors were observed at and above 610 mg/kg bw/day. A significant increased combined (benign+malignant) frequency of hepatocellular neoplasms over controls was observed. The incidence of hepatocellular carcinoma was however not significantly increased upon exposure to spirodiclofen. The NOAEL for neoplastic lesions is therefore 4.1 mg/kg bw/day.

In the rat study, substance-related effects were mainly observed in the two highest dose groups and included decreased bw, changes in haematology and clinical biochemistry (AP, cholesterol, triglycerides) and organ weights of adrenals, liver, thymus, testes and ovaries. Histopathologically, effects were observed in testes (leydig cell hyperplasia), adrenals (hypertrophy/vacuolation cortex cells) and jejenum (vacuolation enterocytes), whereas neoplastic lesions were observed in the highest dose group in testes (leydig cell tumors) and uterus (adenocarcinoma). The NOAEL for chronic toxicity is set at 100 ppm, equivalent to 5.93 mg/kg bw/d. Spirodiclofen is considered carcinogenic for inducing (benign) Leydig cell tumors and uterus adenocarcinomas in the rat. These tumors were observed at and above 110.14 mg/kg bw/day, the NOAEL for neoplastic lesions is therefore 14.72 mg/kg bw/day.

<u>Potential mechanism and human relevance</u>

Based on the mutagenicity tests, the mechanism for the carcinogenic effect is probably non-genotoxic.

The spirodiclofen-induced testicular and uterine carcinogenicity, as observed in rat, could not be demonstrated in mice and dogs, which might point towards a species-specificity for the rat. The observed uterus tumors in rat were a malignant type. No information is available which might point towards a potential irrelevance of this tumour type for humans. The observed testes tumors (Leydig cells tumors) were a benign type. Spirodiclofen was shown to induce enlargement of the testes, hypertrophy and hyperplasia, which might be further related to the observed testes tumor formation (Leydig cells tumor) in rats. It is known that some tumor types occur with a high spontaneous tumor incidence or are not relevant for humans. Leydig cell adenomas are observed with a high

spontaneous tumour incidence in male F344 rats (according to section 3.6.2.2.6-a of the CLP Guidance), but this is not observed for male Wistar rats (i.e. the rat strain used in the combined chronic/carcinogenicity rat study). Further, Leydig cell adenomas induced by dopamine antagonists or gonadotropin-releasing hormones (GnRH) are considered not relevant for humans (according to section 3.6.2.2.6-k of the CLP Guidance). Mechanistic studies were performed to identify the underlying mechanism for the observed effects on for example testes and adrenals. The mechanistic studies (paragraph 4.12.1.3) showed that spirodiclofen clearly interferes with steroid hormone synthesis in the adrenals and gonads. Further, it was noted that the effect on steroidogenesis is probably mediated by effects on general biochemical pathways (interference with the formation of mitochondrial and cytoplasmatic NADPH, which is an important co-substrate in several steps of the biosynthesis of steroid hormones), and that no androgenic, antiandrogenic, estrogenic or anti-estrogenic effects were noted in mechanistic studies. Further, a direct effect of spirodiclofen on enzymes involved in the synthesis of steroidhormones in the testes (microsomal hydrogenases) could not be excluded. Taking all the data together, it cannot be excluded that the Leydig cell tumor formation (including the underlying mechanism) is relevant for humans.

In mice, a significantly increased frequency of hepatocellular adenomas over controls was observed in males. Further, a dose-related increase (not statistically significant) of malignant hepatic tumor types (carcinomas) was observed in male animals as well. The combined frequency of hepatocellular neoplasms (adenomas and carcinomas) was also significantly increased with combined tumor frequencies of 1/50, 1/50, 8/50 and 11/50. Liver hypertrophy as observed in the 13-week and chronic study (liver hepatocytomegaly) might be related to the observed hepatic neoplasms. It is known that some tumor types occur with a high spontaneous tumor incidence or are not relevant for humans. Liver tumors are observed with a high spontaneous incidence in B6C3F1 mice (according to section 3.6.2.2.6-a of the CLP Guidance). However, this is not clearly demonstrated for CD-1 mice (i.e. the mouse strain used in the combined chronic/carcinogenicity mouse study). Further, liver tumors in rodents conclusively linked to peroxisome proliferation are considered not relevant for humans (according to section 3.6.2.2.6-k of the CLP Guidance). This specific mechanism (peroxisome proliferation) is not demonstrated for spirodiclofen-induced liver tumors in mice. A potential mechanism for the hepatocellular adenomas and carcinomas is discussed by the notifer as follows (see section 4.12.1.3, study 13, Sittert et al., 2002): *"a potential mechanism for the hepatocellular adenomas and carcinomas found in mice might be an induction of CYP450-dependent liver enzymes, which might subsequently result in liver hypertrophy, hyperplasia and hepatic tumor formation as is also shown for organochlorine pesticides according the notifier. Tumor induction by organochlorine pesticides was not observed in other species, including humans. Spirodiclofen-induced liver tumors in mice are, therefore, deemed as mouse-specific and not of relevance to humans."* P450 induction was observed in the 4-week dog, 8-week dog, 14-week dog, 1-y dog study and the 4-week rat studies, however not in any of the available mouse studies. Further, no information is available which demonstrates that spirodiclofen-mediated CYP-induction and subsequent liver tumor formation might not be relevant for humans. Therefore, a potential irrelevance for humans is not clearly demonstrated for the spirodiclofen-induced liver tumours.

4.10.5 Comparison with criteria

The CLP criteria for classification as a category 1 Carcinogen are as follows:

"A substance is classified in Category 1 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 CLP criteria for classification as a category 2 Carcinogen are as follows:

"Substances are classified as a category 2 Carcinogen when evidence is obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies."

Classification in category 1A is not considered for spirodiclofen as there are no human data regarding carcinogenicity.

Classification in category 1B is required when there is sufficient evidence which is defined as "an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in two or more species" (CLP Regulation, section 3.6.2.2.3.b).

Based on the available mutagenicity tests, the mechanisms for the carcinogenic effect of spirodiclofen is probably non-genotoxic.

In rat (Wistar), spirodiclofen induced neoplastic effects in testes and uterus. The tumors in the testes (Leydig cell tumors) are benign. The tumors in the uterus (uterus adenocarcinoma) are malignant. For both tumor types it cannot be excluded that these are relevant for humans, and these should be taken into account for classification of spirodiclofen for carcinogenicity in humans.

In mice (CD-1), spirodiclofen induced a significantly increased frequency of hepatocellular adenomas over controls in males. Further, a dose-related increase (not statistically significant) of malignant hepatocellular tumor types (carcinomas) was observed in male animals as well. The combined frequency of hepatocellular neoplasms (adenomas and carcinomas) was also significantly increased with combined tumor frequencies of 1/50, 1/50, 8/50 and 11/50. As it cannot be excluded that the observed liver tumors are relevant for humans, these should be taken into account for classification of spirodiclofen for carcinogenicity in humans.

Based on these data, it can be concluded that there is an appropriate combination of benign and malignant neoplasms in two or more species. For spirodiclofen there is an increase in malignant neoplasms in a single species (i.e. uterus adenocarcinoma in rat), in combination with an increase in benign neoplasms in a second species (i.e. hepatocellular adenomas in mouse). In addition, given that in the mouse 1) the incidences of hepatocellular neoplasms were statistically significantly increased for both the benign tumor types a well as the combined benign + malignant tumor types,

and 2) also a dose-related increase (though not statistically significant) in malignant tumor types was observed, this strengthens the evidence for potential carcinogenicity of spirodiclofen.

It can therefore be concluded there is sufficient evidence for carcinogenic effects of spirodiclofen. Classification for carcinogenicity as Carc 1B (H350: May cause cancer) is therefore proposed.

Classification in category 2 is not considered. The available data cannot be considered as limited evidence, as the available data on spirodiclofen point towards an appropriate combination of benign and malignant neoplasms in two or more species.

4.10.6 Conclusions on classification and labelling

Classification of spirodiclofen for carcinogenicity as Carc. 1B (H350: May cause cancer) is required.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The evaluation of the carcinogenicity hazard endpoint by the DS was based on two long-term oral studies on rodents.

In the mouse study (Wahle, 2000, OECD TG 451, GLP compliant), observed effects included decreased body weight, changes in haematological parameters and organ weights of adrenal glands, kidneys, liver and testes in the mid and high dosed groups. Histopathological examination showed effects on testes (increased incidence and severity of interstitial cell hypertrophy/hyperplasia), epididymides (increased incidence of aspermia), adrenals (vacuolation and pigmentation, increased incidence and severity) and liver (hepatocytomegaly). Remarkable was the increased incidence/increased average severity of amyloid in several tissues in all dose groups (heart, liver, thyroids and parathyroids of males). Based on the observed adrenal pigmentation and vacuolation, hepatocytomegaly and amyloid in all dose groups, a NOAEL could not be established in this study and the LOAEL for chronic toxicity is 4.1 mg/kg bw/d. Spirodiclofen is considered carcinogenic for inducing liver tumours in the mouse. Hepatocellular tumours were observed at and above 610 mg/kg bw/d. A significant increased combined (benign and malignant) frequency of hepatocellular neoplasms over controls was observed. The incidence of hepatocellular carcinoma was however not significantly increased upon exposure to spirodiclofen. The NOAEL for neoplastic lesions is therefore 4.1 mg/kg bw/d.

In the rat study (Wirnitzer, 2000), substance-related effects were mainly observed in the two highest dose groups and included decreased body weight, changes in haematology and clinical biochemistry (AP, cholesterol, triglycerides) and organ weights of adrenal glands, liver, thymus, testes and ovaries. Histopathologically, effects were observed in testes (Leydig cell hyperplasia), adrenals (hypertrophy/vacuolation of cortex cells) and jejunum (vacuolation of enterocytes), whereas neoplastic lesions were observed in the highest dose group in testes (Leydig cell tumours) and uterus (adenocarcinoma). The NOAEL for chronic toxicity is set at 100 ppm, equivalent to 5.93 mg/kg bw/d. Spirodiclofen is considered carcinogenic for inducing (benign) Leydig cell tumours and uterus adenocarcinomas in the rat. These tumours were observed at and above 110.14 mg/kg bw/d; the NOAEL for neoplastic lesions is therefore 14.72 mg/kg bw/d.

Potential mechanism and human relevance

Based on the mutagenicity tests, the mechanism for the potential carcinogenic effect is probably nongenotoxic.

Spirodiclofen induced testicular and uterine carcinogenicity in rats. The same effect did not occur in mice and dogs, which might point towards a species-specificity for the rat. The observed uterus tumours in rats were of a malignant type. No information is available which might point towards a potential irrelevance of this tumour type for humans. The observed testes tumours (Leydig cell tumours) were of a benign type. Spirodiclofen was shown to induce enlargement of the testes, hypertrophy and hyperplasia, which might be further related to the observed testes tumour formation (Leydig cells tumour) in rats. It is known that some tumour types occur with a high spontaneous incidence or are not relevant for humans. Leydig cell adenomas are observed with a high spontaneous incidence in male F344 rats (according to section 3.6.2.2.6-of the CLP Guidance), but not in male Wistar rats which is the rat strain used in the combined chronic/carcinogenicity rat study. Leydig cell adenomas induced by dopamine antagonists or gonadotropin-releasing hormones (GnRH) are considered not relevant for humans (according to section 3.6.2.2.6-k of the CLP Guidance). Therefore, mechanistic studies were performed to identify the underlying mechanism of the observed effects on testes and adrenals. The mechanistic studies showed that spirodiclofen clearly interferes with steroid hormone synthesis in the adrenals and gonads. Additionally, it was noted that the effect on steroidogenesis is probably mediated by effects on general biochemical pathways (interference with the formation of mitochondrial and cytoplasmatic NADPH, which is an important co-substrate in several steps of the biosynthesis of steroid hormones), and that no androgenic, anti-androgenic, estrogenic or anti-estrogenic effects were noted in the mechanistic studies. Further, a direct effect of spirodiclofen on enzymes involved in the synthesis of steroid hormones in the testes (microsomal hydrogenases) could not be excluded. Considering all of the above data, it cannot be excluded that the Leydig cell tumour formation (including the underlying mechanism) is relevant for humans.

In mice, a significantly increased frequency of hepatocellular adenomas over controls was observed in males. Further, a dose-related increase (not statistically significant) of malignant hepatic tumour types (carcinomas) was observed in male animals. The combined frequency of hepatocellular neoplasms (adenomas and carcinomas) was also significantly increased. Furthermore, liver hypertrophy was observed in the 13-week and chronic study (liver hepatocytomegaly) which might be related to the observed hepatic neoplasms. It is known that some tumour types occur with a high spontaneous tumour incidence or are not relevant for humans. Liver tumours are observed with a high spontaneous incidence in B6C3F1mice (according to section 3.6.2.2.6-of the CLP Guidance). However, this is not the case for CD-1 mice which is the mouse strain used in the combined chronic/carcinogenicitymouse study. Further, liver tumours in rodents conclusively linked to peroxisome proliferation are considered not relevant for humans (according to section 3.6.2.2.6-k of the CLP Guidance). This specific mechanism (peroxisome proliferation) is not operating for spirodiclofen-induced liver tumours in mice. A potential mechanism for the hepatocellular adenomas and carcinomas is discussed by the notifier as follows: "a potential mechanism for the hepatocellular adenomas and carcinomas found in mice might be an induction of CYP450-dependent liver enzymes, which might subsequently result in liver hypertrophy, hyperplasia and hepatic tumour formation as it is also shown for organochlorine pesticides according to the notifier. Tumour induction by organochlorine pesticides was not observed in other species, including humans. Spirodiclofen induced liver tumours in mice are, therefore, deemed as mouse specific and not of relevance to humans. " P450 induction was observed in the 4-, 8-, 14-week, 1-year dog studies and the 4-week rat studies, however not in any of the available mouse studies. Further, no information is available which demonstrates that spirodiclofen-mediated CYP450-induction and subsequent liver tumour formation might not be relevant for humans. Therefore, a potential irrelevance for humans is not

clearly demonstrated for the spirodiclofen induced liver tumours.

Comments received during public consultation

Three MSCAs commented and supported the DS's proposal to classify spirodiclofen as Carc.1B (H350: May cause cancer).

The first MSCA noted that in the mouse study (CD-1), spirodiclofen induced a significantly increase of hepatocellular adenomas in males. Furthermore, a dose-related increase (not statistically significant) of malignant hepatocellular tumour (carcinomas) was observed in male animals. The combined frequency of adenomas and carcinomas was also significantly increased. As a potential irrelevance for humans is not clearly demonstrated, these effects should be taken into account for classification of spirodiclofen for carcinogenicity in humans. In rat (Wistar), spirodiclofen induced neoplastic effects in testes and uterus. The tumours in the testes (Leydig cell tumours) are benign, while the tumours in the uterus (uterus adenocarcinomas) are malignant. The mechanistic studies showed that spirodiclofen clearly interferes with steroid hormone synthesis in the adrenals and gonads. For both tumour types, it cannot be excluded that they could be relevant for humans, and should be taken into account for the classification of spirodiclofen.

In conclusion, there was a combination of benign and malignant neoplasms of potential relevance to humans in two species and therefore, there is sufficient evidence for classification of spirodiclofen as a Carc.1B.

The second MSCA noted that since the available long-term oral carcinogenicity studies showed that spirodiclofen induced adenocarcinomas in the uterus and benign Leydig cell tumours in rats, and hepatocellular carcinomas and adenomas in mice, there is evidence matching the criteria for classification of spirodiclofen as a Category 1B carcinogen. In addition, 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 two or more species of animals.

The third MSCA agreed with the proposed classification but identified two important points for further evaluation. Firstly, there is a new publication (Yoshida *et al.,* 2015) providing information on the mode of action of spirodiclofen which was not included in the CLH report. The study focused on predictive modes of action of pesticides in uterine adenocarcinoma development in rats. The DS evaluated the publication, a short summary is included below.

Yoshida *et al.*(2015) evaluated pesticides for potential uterine carcinogenicity and attempted to predict their mechanism using parameters from mechanistic and toxicity studies. Five pathways for uterine carcinogenesis in rodents were presented (of which the first three appear to be accepted as the major pathways): 1) estrogenic activity, 2) increased serum 17beta-estradiol (E2) to progesterone (P4) ratio, 3) modulation of oestrogen metabolism to produce 4-hydroxyestradiol via P450 induction, 4) inhibition of oestrogen excretion and 5) increased aromatase *in situ* in the tumour.

Their evaluation of a total of 300 pesticides revealed that seven chemicals increased uterine tumour formation in rats, and the pathways of 4 chemicals (including spirodiclofen) could be predicted based on various mechanistic studies. The mode of action of spirodiclofen was predicted to be increased serum 17beta-estradiol (E2) to progesterone (P4) ratio given that mechanistic studies showed that E2-levels did not change while P4-levels decreased.

The second point the MSCA focused on the historical control data (HCD). In the oncogenicity study in CD-1 mice (Wahle, 2000) significantly increased incidences of hepatocellular adenoma and a significantly increased combined frequency of hepatocellular adenomas and carcinomas were found in males of the mid- and high-dose group. A dose-related but not significant increase of hepatocellular

carcinomas was found in male mice.

In the combined chronic toxicity and carcinogenicity study in Wistar rats (Wimitzer*et al.,* 2000) increased incidences in benign Leydig cell tumours and malignant uterus adenocarcinomas (both not statistically significant) were found in the high-dose group. An occurrence of thyroid C-cell adenoma and carcinoma in female Wistar rats was considered to be irrelevant based on HCD. It is also well known though that Leydig cell tumours and hepatocellular adenomas occur spontaneously and with a high variability in certain rat and mice strains (Section 3.6.2.3.2 insee Guidance on the application of the CLP criteria), although not in the strains used in the specific studies. An inquiry by the commenting MSCA on publicly available HCD revealed (although certain limitations regarding differences in laboratories, animal specification, time window broadness and time window distance existed) that the combined multiplicity of data indicates a relatively high spontaneous occurrence and variability in incidences of hepatocellular tumours in CD-1 mice and of Leydig cell tumours in Wistar rats, the strains used in the carcinogenicity studies of the CLH report. Based on this HCD according to the MSCA all tumour types except the malignant uterus adenocarcinoma could be considered to lie within the HCD.

The DS addressed the HCD using the Guidance on the Application of the CLP criteria (November 2015): "*The historical data must be from the same animal strain/species, and ideally, be from the same laboratory to minimise any potential confounding due to variations in laboratory conditions, study conditions, animal suppliers, husbandry etc. It is also known that tumour incidences in control animals can change over time, due to factors such as genetic drift, changes in diagnostic criteria for pathological changes/tumour types, and husbandry factors (including the standard diet used), so the historical data should be contemporary to the study being evaluated (e.g. within a period of up to around 5 years of the study). Historical data older than this should be used with caution and acknowledgement of its lower relevance and reliability (RIVM, 2005; Fung et al., 1996; Greim et al., 2003)*". The DS concluded that the HCD claimed by the MSCA and not numerically presented in the PC, are not relevant and the afore mentioned tumours should be part of the carcinogenicity evaluation.

Additional key elements

The mode of action of spirodiclofen is not fully understood but it is considered relevant to humans given the consistent pattern of physiological and hormonal changes seen in mechanistic studies conducted in vitro and in toxicology studies in rats or dogs. Spirodiclofen has been shown to interfere with hormone production at the pituitary level, with increased concentration of LH in plasma of male dogs and increased concentration of TSH in male and female rats. It also possibly affects several reproductive organs by decreasing the concentrations of estradiol and progesterone in plasma of rats in subchronic studies. In particular, spirodiclofen :

a. Depletes cholesterol levels probably via the inhibition of its biosynthesis (Schmidt, 2001a; Schmidt, 2000)
b. Inhibits enzymes involved in the steroid hormone biosynthesis (Freyberger, 2001a; Freyberger, 2001c; Yoshida 2015)
c. Depletes NADPH levels in the mitochondria (Freyberger, 2002; Freyberger, 2003)

A summary of the available mechanistic studies conducted with spirodiclofen is presented in the table below.

Table: summary of the available mechanistic studies conducted with spirodiclofen

Overview of Mechanistic Studies						
Test substance	**Duration**	**Species**	**Doses**	**Goal**	**Result**	**Reference**
	Route					
Spirodiclofen (batch BAJ 2740)	8 weeks, oral	dog	0, 100, 2000 ppm	The study was performed to investigate the involvement of HMG-CoA reductase in spirodiclofen-induced decrease in cholesterol levels.	In this study, plasma cholesterol and triglycerides were reduced in the high dose group to 70% and 88% respectively. Plasma ubiquinone (Q10) was significantly reduced (to 64%) and also the dolichols and dolicholphosphates were (not-significantly) decreased in the testes (up to about 80%, dose-related). According to the study author, an effect on the HMG-CoA reductase as an inhibiting step in the cholesterol biosynthesis cannot be excluded, as indicated by the observed decrease in ubiquinone and dolichol levels.	U. Schmidt (2001a)
Spirodiclofen (batches BAJ 2740, BAJ 2510, 3-OH, BAJ 2510, 4-OH,BAJ 2510)	in vitro	rat (testes slices)		The study was performed in order to investigate a mechanism interfering with testosterone synthesis. Potential interaction of spirodiclofen and its main metabolites was tested in a steroidogenesis assay using rat testicular fragments.	The in vitro mechanistic study with testes-slices showed decreased (stimulated) testosterone secretion into the medium in the presence of all test substances, with BAJ 2510 as the most potent inhibitor.	A. Freyberger (2001a)
Spirodiclofen (batches BAJ 2510, OH-BAJ 2510)	in vitro	human cell lines	10^{-11}-10^{-5} M	This receptor binding study was performed to investigate receptor-mediated hormonal activities of the test compounds.	Under physiological pH values, spirodiclofen, BAJ 2510 and hydroxy-BAJ 2510 had no receptor mediated hormonal activity (neither estrogenic or antiestrogenic nor androgenic or antiandrogenic activity up to a concentration of 10 µM).	G. Schmuck (1999)
Spirodiclofen (batch BAJ 2740)	19 weeks, oral	rat	0,2500, 10000 ppm	Special subchronic toxicity study on hormone levels in female rats	Decreased bw, increased adrenal weight, decreased estradiol concentration and increased estradiol/progesterone ratio in all dose groups. In the highest dose decreased levels of LH were observed.	P. Andrews 2001
Spirodiclofen (Batches BAJ 2740 BAJ 2510)	in vitro	rat testicular microsomes		In order to study potential effects on cytochrome-P450-dependent microsomal monooxygenases involved in steroid hormone synthesis, the effects of spirodiclofen and its metabolite BAJ 2510 on steroid 17α-monooxygenase and C-17,	In this in vitro study with rat testicular microsomes, steroid 17α-monooxygenase and C-17, 20-lyase were neither affected by 50 µM spirodiclofen nor 300 µM BAJ 2510.	A. Freyberger (2001b)

			20-lyase were studied in vitro.		
Spirodiclofen (Batches BAJ 2740, BAJ 2510, 3-OH, BAJ 2510, 4-OH,BAJ 2510)	in vitro	rat testicular microsomes	The study was performed to evaluate potential effects on microsomal dehydrogenases involved in steroid hormone synthesis. The effects of spirodiclofen and its metabolites BAJ 2510, 3-OH-BAJ 2510 and 4-OH BAJ 2510 on 3β-hydroxysteroid dehydrogenase-Δ4,5-isomerase and 17β-hydroxysteroid dehydrogenase were studied in vitro.	Only spirodiclofen showed an inhibitory effect on 3β-hydroxysteroid dehydrogenase-Δ4,5-isomerase in vitro. It cannot be excluded that this effect may contribute to reduction of testosterone synthesis in spirodiclofen treated testicular tissue. 17β-hydroxysteroid dehydrogenase was not inhibited in the presence of the test substances.	A. Freyberger (2001c)
Spirodiclofen (batches BAJ 2740 BAJ 2510)	in vitro	Pancreatic cholesterol esterase (commercial available)	The study was performed to investigate the effects of the test sustances on cholesterol esterase.	In a study which investigated whether spirodiclofen or BAJ 2510 could act as competing substrate for cholesterol esterase it was shown that spirodiclofen inhibited pancreatic cholesterol esterase but did not act as a competing substrate for cholesterol esterase BAJ 510 showed a mild inhibitory potential on cholesterol esterase.	A. Freyberger (2000)
Batch BAJ 2510	in vitro	rat testicular microsomes	The study was performed to investigate the effects of BAJ 2510 on steroidogenesis and identificate malate dehydrogenase isoenzymes as molecular target.	BAJ 2510 interfered with cholesterol side chain cleavage in malate-supplemented mitochondria, but not in the presence of citrate. Compared to testosterone synthesis, progesterone synthesis in cultured rat testicular tissue was only moderately reduced by BAJ 2510. In vitro incubation of BAJ 2510 with commercially available purified malic enzyme and mitochondrial malate dehydrogenase resulted in inhibition of mitochondrial malate dehydrogenase, but not malic enzyme.	A. Freyberger (2001d)

Spirodiclofen (batch BAJ 2740)	82 weeks oral	rat		The study was performed to determine spirodiclofen and BAJ 2510 in plasma samples.	In a chronic oral toxicity study with rats, blood samples after 82 weeks of exposure were taken for determination of spirodiclofen and BAJ 2510 in plasma. In all samples, spirodiclofen concentrations were below the LOQ. BAJ 2510 was dose-dependently increased in plasma, with higher concentrations in females compared to males.	U. Schmidt (2000)
Spirodiclofen (batch BAJ 2740)	20/28 weeks oral	dog		The study was performed to determine spirodiclofen and BAJ 2510 in plasma and urine samples.	In a chronic oral toxicity study with dogs, samples were taken after 20 weeks and 28 weeks of blood and urine respectively for determination of spirodiclofen and BAJ 2510. In all samples, spirodiclofen concentrations were below the LOQ. Plasma concentrations of BAJ 2510 were lower 2h and 4h after feeding, whereas 7h and 24h after feeding, plasma concentrations were at the same level as before feeding. Urine samples were only determined from 3f and 1m, and the measured concentration BAJ 2510 is only indicative for the presence of the metabolite of spirodiclofen.	U. Schmidt (2001b)
BAJ 2510	in vitro	Rat testicular mitochondria		The study was performed to measure intramitochondrial NADH and NADPH and to study the effects of BAJ 2510 on NADH and NADPH concentrations in rat testicular mitochondria.	BAJ 2510 (300 µM) significantly decreased intramitochondrial NADH levels in the presence of 0. 05mM, 0. 5 mM and 5 mM malate. NADPH levels were significantly reduced in the presence of 0. 05 mM and 0. 5 mM malate, whereas in the presence of 5 mM malate NADPH levels were not significantly reduced. These results support the hypothesis of inhibition of malate dehydrogenase by BAJ 2510.	A. Freyberger (2002)
BAJ 2511	in vitro	Rat testicular mitochondria		The study was performed to measure intramitochondrial NADH and NADPH and to study the effects of BAJ 2510 on NADH and NADPH concentrations in rat testicular mitochondria.	Under conditions known to be associated with effective side chain cleavage, BAJ 2510 concentration-dependently decreased the overall amount of reducing equivalents and of levels of NADH and NADPH in mitochondria.	A. Freyberger (2003)
BAJ 2740		rat		300 pesticides were evaluated for uterine carcinogenicity and their mode of action was studies	spirodiclofen was predicted to act via the pathway of increased serum 17beta-estradiiol (E2) to progesterone (P4), thus showing a decrease in the levels of progesterone.	M. Yoshida (2015)

The mechanistic studies show that spirodiclofen could be responsible for the adrenal, testicular and uterine effects observed. Chronic stimulation of the pituitary hormone levels could lead to chronic stimulation of testicular Leydig cells and endometrial uterine cells resulting in hypertrophy, hyperplasia and eventual tumor formation. This is also supported by the fact spirodiclofen does not exhibit mutagenic properties. Regarding the adrenal effects observed mainly in the dog studies, the histopathological findings (dose dependent vacuolation and increased body weight) are however not considered critical effects for the organ function and/or organ damage.

Regarding the liver effects, biosynthesis of cholesterol in the liver accounts for approximately 20% of endogenous production. Study of the correlation between total serum cholesterol and liver tumor in humans, showed that the cholesterol level in patients who died of cancer was significantly lower than that in those who survived (Cambien *et al.,* 1980; Saito *et al.,* 2013). Tumor cells intake of cholesterol, synthesis and changes of membrane tumor cells content in cholesterol suggested that endogenous cholesterol may play an important role in tumor pathobiology (Dessì *et al.,* 1994). Cholesterol metabolic products participate in duplication of DNA and the regulation of oncogene proteins (Casey *et al.,* 1989). Cholesterol is not only considered as a structural component but also as a relevant lipid involved in the control of the intermediate metabolism of different liver cell types, such as hepatocytes, hepatic stellate cells and Kupffer cells. Cholesterol plays a significant role at the level of specific membrane domains, as well as modulating the expression of sterol-dependent proteins (Delgado *et al.,* 2011). Therefore, it could be argued that the depleted cholesterol synthesis due to spirodiclofen might influence tumorigenesis in the mice liver.

Assessment and comparison with the classification criteria

Based on the available mutagenicity tests, RAC agrees that the mechanism(s) for the carcinogenic effect of spirodiclofen is probably non-genotoxic.

Dose Selection

In the 18 months mouse study, doses were selected based principally upon the toxicological profile which emerged in the mouse over the course of a subchronic study conducted with the test chemical (Leser 1998). Based on a relatively weak toxicological response of the mouse through 7000 ppm (limit dose), it was estimated that the low and high doses chosen of 25 and 7000 ppm would constitute a no-observed effect level and a maximum tolerated dose (limit dose), respectively, with the intermediate dosage of 3500 ppm serving to establish possible dose response relationships.

In the 2 years rat study, the dose levels were selected based on results of a 14 weeks study followed by a 4 week recovery period with substance administration in food (0, 100, 500, 2500 and 12500 ppm). Lower body weights (2500 ppm males, and above both sexes) and reduced food intake (2500 ppm first 4 days, 12500 ppm both sexes) were determined. During recovery the body weight gain and food intake (females) increased. Thrombocyte counts were reversibly decreased (males 12500 ppm). Functional liver effects (500 ppm and above) were derived from increased plasma enzyme activities of aspartate and alanine aminotransferase (12500 ppm, both sexes), reduced plasma concentrations of cholesterol (2500 ppm males, 12500 ppm both sexes) and triglycerides (500 ppm females, 2500 and 12500 ppm, both sexes) as well as decreased plasma protein concentration (both sexes 12500 ppm). Histologically, hepatocellular glycogen content was reduced in four females at highest dose. After the recovery period activities of aspartate aminotransferase were still slightly increased and cholesterol, triglyceride and protein concentrations were reduced in plasma of males. Secondary effects on organs and tissues of the immune system were seen at 2500 and 12500 ppm:

decreased peripheral blood leucocytes (12500 ppm), cell counts (12500 ppm) and immunoglobulins as well as a shift (12500 ppm) and decreases in subclass composition. Absolute and relative spleen (females) and thymus weights (males) were reduced at 12500 ppm. Adrenal weights were increased (12500 ppm, both sexes); histologically, the incidence of cytoplasmic cortical vacuolation (500 ppm females, 2500 ppm and above, both sexes) was increased. This was reversible within the recovery period. Mucosal epithelial cells of the small intestine (mainly the jejunum) were vacuolated (2500 ppm and above, both sexes) and plasma alkaline phosphatase activity was increased during treatment (2500 and 12500 ppm, both sexes) and at the end of the recovery.

On the basis of these results the following dose levels were selected for the present combined chronic toxicity/carcinogenicity study: 0, 50,100, 350 and 2500 ppm

Historical Control Data (HCD)

Historical control data in mice from the literature suggest a rate of 0%-9.6% in male controls (n=499) and 0%-2.7% in females (n=497) in nominal 18-month studies. Data from five in-house studies conducted 1989-1998 show a rate for the combined hepatocellular neoplasms in controls of 4%-14% in male controls (n=250) and 0%-2% in female controls (n=250). While the male control numbers in the mouse carcinogenicity study are historically low, at 2% hepatocellular neoplasms occurrance, the female values are consistent with historical data. Male frequencies at 3,500 and 7,000 ppm (16% and 20% respectively), and corresponding female values of 10% and 6% are above the range seen in either in-house or literature historical data.

HCD references were provided during PC on hepatocellular adenomas in mice (Maita *et al.,* 1988; Chandra and Frith, 1992; Giknis and Clifford, 2000; Giknis and Clifford, 2001; Giknis and Clifford, 2005; Forster *et al.,* 2014) and on Leydig cell tumours in Wistar rats (Bomhard and Rinke, 1994; Eiben and Bomhard, 1999; Walsh and Poteracki, 1994; Poteracki and Walsh, 1998; Giknis and Clifford, 2003). They are shown in the two following tables for CD1 mice and Wistar rats, respectively.

Table: HCD for tumours in CD-1 mice (1988-2000)

Study HCD	**% liver adenomas**	**% liver carcinomas**	**% combined liver tumours**	**ODD study**	**Dose (ppm)**	**% liver adenomas**	**% liver carcinomas**	**% combined liver tumours**
Males								
Maita *et al.,* 1988	26	9.1	35.4	Wahle, 2000	3500	10	6	16
Chandra and Frith, 1992	11	5.7	16.7		7000	**12***	**10**	**22**
Giknis and Clifford, 2000	10.46	5.29	15.8					
Females								
Maita *et al.,* 1988	5.17	0.9	6.07	Wahle, 2000	3500	**6**	**4**	**10**
Chandra and Frith, 1992	1.8	0.7	2.48		7000	2	**4**	**6**
Giknis and Clifford, 2000	0.99	0.66	1.64					

**Bold shows values outside the HCD range.*

The table below presents HCD for tumours in Wistar rats.

Table: HCD for tumours in Wistar rats

Study HCD	**% Benign Leydig cell tumours**	**Uterus adenocarcinomas**	**ODD study**	**Dose (ppm)**	**% Benign Leydig cell tumours**	**Uterus adenocarcinomas**
Bomhard and Rinke, 1994	2.1-16.3 (7.0)	0.0-16.3 (7.8)	Wirnitzer et al.2000	350	8	4
Eiben and Bomhard, 1999 (Bayer AG rats)	7.0	6.5				
Walsh and Poteracki, 1994	3.9	1.6		2500	**20**	**28**
Giknis and Clifford, 2003	No reference	2.3				

The analysis of Historical Control Data shows:

1. High variation and inconsistencies;
2. Incidences of liver adenomas, carcinomas and combined tumours in the mouse carcinogenicity study are higher than the HCD in some cases (especially in females). A combination of benign and malignant hepatocellular tumours were observed in the CD-1 mice in both sexes.

In the available mouse carcinogenicity study, non-neoplastic findings included effects in the liver (hepatocytomegaly). The size of hepatocytes was significantly increased over control at dose levels of 3500 and 7000 ppm males. The change was essentially not seen in females (M: 2, 6, 17*, 21*; F: 0, 0, 0, 1). This finding correlates well with liver relative and absolute weights in males.

In addition, hypertrophy and/or hyperplasia of testicular interstitial cell was noted with significantly increased frequency in both the 3500 and 7000 ppm (male) groups (M: 6, 6, 26*, 31*). Some of the control and 25 ppm testes showed hyperplasia only. The lesion in the 3500 and 7000 ppm groups consisted of both greatly increased cell size and increased numbers of cells; thus, the dual diagnosis of hypertrophy and hyperplasia. Average severity increased with dose. The enlarged foamy cells in the two higher groups were sometimes accompanied by small numbers of inflammatory cells or occasional giant cells, but there was no significant increase in the frequency of inflammatory changes. Tubular degeneration of the testis was slightly, but not significantly increased in the high dose. The frequency of the background lesion of abnormal spermatozoa in the epididymis was directly related to the testicular changes (M: 15, 15, 15, 26*).

As far as neoplastic lesions are concerned, the combined frequency of hepatocellular neoplasm (hepatocellular adenoma and carcinoma) was significantly increased over controls in 3500 and 7000 ppm males and in 3500 ppm females. Data were corrected for one animal with both types of tumor. (M: 1, 1, 8*,10*; F: 0, 0, 5*, 3).

In the 2 years rat study in male rats a positive trend for Leydig cell adenomas was observed (Trend Test p=0.0010). The frequency of benign Leydig cell tumors was markedly increased at 2500 ppm. Concurrently, also the incidence of focal Leydig cell hyperplasia was significantly increased in males of the high dose group (Trend Test p=<0.0005+). The average severity per group was slightly increased in affected high dose males. The pooled incidence, i.e. the number of males with Leydig

cell adenomas and/or focal Leydig cell hyperplasias showed a highly significant increased incidence exclusively in the 2500 ppm-group. The majority of Leydig cell adenomas and focal hyperplasias were found in males at the termination of the study suggesting a late onset of these alterations. Diffuse Leydig cell hyperplasia was only seen in two males (-/-/-/1/1). According to the registrant, the Leydig cell is a target structure of spirodiclofen known from previous toxicity studies with mice and beagle dogs where Leydig cell hypertrophy and/or vacuolation was diagnosed at high dose levels. Spirodiclofen is assumed to exert an influence on steroid biosynthesis or metabolism. No indications for lesions influenced by the test compound were found in the epididymides, prostate, and seminal vesicles/coagulations glands.

In the ovaries of female rats, the frequency of females with metastases of carcinomas increased (1/-/-/1/5). These tumors originated from uterine adenocarcinomas. The incidence of primary ovarian neoplasms themselves was not influenced by the treatment.

In addition, in the uterus adenocarcinomas 8/14 tumors graded as malignant neoplasms probably fatal were metastatic to multiple organs an sites such as: lungs, forestomach, glandular stomach, liver pancreas, ovaries, kidneys, colon, lymphnotes etc. One adenocarcinoma was metastatic only to liver. Compared to controls and rats of the other groups, the number of females with metastases of carcinomas in the spleen (primary tumor uterus) was increased after 2500 ppm (incidence: -/1/1/-/3). In the 350 ppm dose, all uterus adenocarinomas were metastatic.

A positive trend was observed in the presence of corpora lutea. The incidences were 28/28/33/39*/32 (Trend Test, p=0.038). The average grading was similar in all groups including controls. The Exact Fisher Test was only significant in females at 350 ppm and not in the high dose females. However, since dose correlation and similar average severity were absent, an influence by the treatment can be ruled out. A significant negative trend was calculated for the incidence of ovarian atrophy (19/24/17/13/13, p=0.0154). The Exact Fisher Test revealed no significant decrease. Because the missing of a dose correlation and the great variance of this finding in aged rats, the slight decrease is regarded as incidental,

In the uterus, adenocarcinomas were increased at 2500 ppm (Trend Test: p=0.0088) when compared to controls and females dosed up to and including 350 ppm. This correlates with the increased gross incidence of uterine nodules in this group.

The majority of the adenocarcinomas (11 out of 14) was found in females which died or had to be sacrificed before the termination of the study. As already mentioned, many of the adenocarcinomas in these high dose females had metastasized in various organs namely of the abdominal cavity by invasion and also into the lungs (5 cases) and the bone marrow (two cases). The high prevalence of metastatic uterine adenocarcinomas at 2500 ppm might have contributed to the slightly increased overall mortality in females of this group (mortality: 21/18/19/15/24). The incidence of other epithelial or mesenchymal neoplasms of the uterus was not affected as was the incidence of possibly pre-neoplastic focal glandular hyperplasia.

In females, the incidence of c-cell adenomas of the thyroid gland was 2/2/3/5/6, being statistically significant in the Trend Test (p=0.0319+). The incidences of focal and diffuse c-cell hyperplasia were not influenced by the dosing with the test compound.

In males, c-cell adenomas (incidence: 4/5/5/4/7) were distributed evenly among the groups as were focal and diffuse c-cell hyperplasia. A significantly increased incidence of colloidal alteration was observed in high dose males (Trend Test p=0.0047). The incidence was 23/23/28/28/35*. In the Exact Fisher Test significance was achieved only for high dose males. There was no evidence of an

influence on hyperplastic or neoplastic lesions of the follicular epithelium in both sexes.

In conclusion, spirodiclofen was found to lead to an increase in the incidences of tumors in the testes and the uterus in rats after the administration of a very high dose. The no-effect-level for neoplastic lesions was considered at 350 ppm for both sexes.

Stastitical Analysis

A statistical analysis of the tumor incidences in mice and rats, respectively is shown in the tables below.

Table: statistical analysis of the tumor incidences in mice

CD-1 mouse						
Dose	Male			Female		
	Liver hepatocellular adenomas	Liver hepatocellular carcinomas	Combined tumours	Liver hepatocellular adenomas	Liver hepatocellular carcinomas	Combined tumours
Control (0 ppm)	0/50	1/50	1/50	0/50	0/50	0/50
25 ppm	0/50	1/50	1/50	0/50	0/50	0/50
3500 ppm	**5/50 (p=0.02)**	3/50 (p=0.31)	**8/50 (p=0.01)**	3/50 (p=0.08)	2/50 (p=0.16)	**5/50 (p=0.02)**
7000 ppm	**6/50 (p=0.01)**	5/50 (p=0.09)	**11/50 (p=0.002)**	1/50 (p=0.31)	2/50 (p=0.16)	3/50 (p=0.08)

Table: statistical analysis of the tumor incidences in rats

Wistar rat				
Dose		Male	Female	
		Benign Leydig cell tumours	Uterus adenocarcinomas	Thyroid C-Cell adenoma
Control (0 ppm)	Except deaths	2/31	2/29	1/29
	Only deaths	0/19	2/21	1/21
	Total	*2/50*	*4/50*	*2/50*
50 ppm	Except deaths	1/30 (p=0.57)	3/32 (p=0.72)	2/32 (p=0.61)
	Only deaths	0/20	2/18 (p=0.87)	0/18 (p=0.35)
	Total	*1/50 (p=0.56)*	*5/50 (p=0.73)*	*2/50 (p=1)*
100 ppm	Except deaths	0/36	2/31 (p=0.94)	3/31 (p=0.33)
	Only deaths	0/14	1/19 (p=0.61)	0/19 (p=0.34)
	Total	*0/50*	*3/50 (p=0.70)*	*3/50 (p=0.65)*
350 ppm	Except deaths	4/31 (p=0.39)	0/35 (p=0.11)	4/35 (p=0.24)
	Only deaths	0/18	2/15 (p=0.72)	1/15 (p=0.80)
	Total	*4/50 (p=0.40)*	*3/50 (p=0.40)*	*5/50 (p=0.24)*
2500 ppm	Except deaths	9/41(p=0.07)	3/26 (p=0.55)	4/26 (p=0.12)
	Only deaths	1/9 (p=0.16)	**11/24 (p=0.007)**	2/24 (p=0.63)

	Total	***10/50 (p=0.014)***	***14/50 (p=0.009)***	*6/50 (p=0.14)*

Bold *denotes statistical differences with the controls; P values have been calculated by RAC; Data in italics have been calculated by RAC.*

A combination of benign and malignant hepatocellular tumours were observed in the CD-1 mice in both sexes at 2 doses in a statistically significant manner and a dose-dependent way in the males. In Wistar rat, statistical significant benign Leydig cell tumours in the male at the highest dose and statistical significant metastatic uterus adenocarcinomas in the female at the highest dose, too, were observed.

In the following table a summary of carcinogenicity related neoplastic and non-neoplastic findings are presented for all three species tested for spirodiclofen.

Table: summary of carcinogenicity related neoplatic and non-neoplastic findings is mice, rats and dogs

	Mouse 18 month carcinogenicity study, 7000 ppm		**Rat 108 week carcinogenicity study, 2500 ppm**		**Dog, 52 week, repeated dose toxicity study, 600 ppm**	
	female	**male**	**female**	**male**	**female**	**male**
Epididymides		Aspermia				28 % ↑ wt
Leydig Cells		–		benign tumors		vacuolation 4/4 (grade 1,2,1,1) hypertrophy 1/4 (2) focal tubular degeneration 1/4 (2)
Liver	Hepatocellular adenomas and carcinomas, 14 % ↑ wt, hepatocytomegaly 1/50 grade 2.0	Hepatocellular adenomas and carcinomas, 18 % ↑ wt, hepatocytomegaly 21/50 grade 1.9	4 % ↓ wt, no neoplastic lesions	9 % ↓ wt, no neoplastic lesions	4 % ↓ wt, no neoplastic lesions	26 % ↓ wt, no neoplastic lesions
Ovaries	48 % ↓ wt, no neoplastic lesions		31 % ↑ wt, 5/50 carcinomas metastatic from uterus adenocarcinomas		vacuolation 1/4 (1), no neoplastic lesions	
Testes		↑20 % Degeneration - grade 3.8, 19 % ↑ wt, Hypertrophy/hyperplasia/Interstitial cell 31/50 - grade 2.5		6 % ↑ wt, no neoplastic lesions		30 % ↑ wt, no neoplastic lesions
Uterus	–		adenocarcinomas		150 ppm, mononuclear infiltration 3/4 (2), no neoplastic lesions	

General Toxicity

Additionally, RAC analysed the tumor incidences in mice and rats in relation to general toxicity issues. The results are shown in the table below.

Table: Overview of effects on carcinogenicity and general toxicity parameters in available repeated dose toxicity and carcinogenicity studies

Study		males	females
Leser, Romeike (1998)	**13-wk oral mouse repeated dose toxicity study** 0, 100, 1000, 10000 ppm	**≥ 1000 ppm** non significant ↓ in body weight **≥ 10000 ppm** non significant ↓ in body weight, no mortality, no clinical signs, no food & water consumtion effects	**≥ 1000ppm** no effects **≥ 10000 ppm** non significant decrease in body weight, no mortality, no clinical signs, no food & water consumtion effects
Wahle (2000)	**18-month mouse carcinogenicity study** 0, 25, 3500, 7000 ppm	**≥ 3500 ppm** no mortality, ↓ bw (statistically not consistent) ↑ food consumption **hepatocellular adenoma** 5/50 **hepatocellular carcinoma 3**/50 **hepatocellular combined adenoma/carcinoma** 8/50 **≥ 7000 ppm** no mortality, ↓ bw (statistically not consistent) increased food consumption **hepatocellular adenoma** 6/50 **hepatocellular carcinoma** 5/50 **hepatocellular combined adenoma/carcinoma** 11/50	**≥ 3500 ppm** no mortality, ↓ bw (statistically not consistent) terminal body weight was significantly ↓ **hepatocellular adenoma** 3/50 **hepatocellular carcinoma** 2/50 **hepatocellular combined adenoma/carcinoma** 5/50 **≥ 7000 ppm** no mortality, ↓ (statistically not consistent) body weight, terminal body weight was significantly ↓ **hepatocellular adenoma** 1/50 **hepatocellular carcinoma** 2/50 **hepatocellular combined adenoma/carcinoma** 3/50
Wirnitzer, Romeike (1998)	**14-week oral rat repeated dose toxicity study** 0, 100, 500, 2500, 12500 ppm	**≥ 2500 ppm** no mortality, no clinical signs, ↓ bw statistically not significant, no food & water consumtion effects **≥ 12500 ppm** no mortality, no clinical signs, ↓ bw statistically significant, ↓ food consumption(significant), ↓ water consumtion (non significant)	**≥ 2500 ppm** no mortality, no clinical signs, ↓ bw statistically not significant, no food & water consumtion effects **≥ 12500 ppm** no mortality, no clinical signs, ↓ bw statistically significant, ↓ food consumption significantly , ↓ water consumtion (non significant)
Wirnitzer (2000)	**108-week rat carcinogenicity study** 0, 50, 100, 350, 2500 ppm	**≥ 2500 ppm** no mortality, no clinical signs, ↓ bw (up to 11%) ↑food consumption **Liver Leydig cell tumors** 10/50	**≥ 2500 ppm** no mortality, no clinical signs, ↓ bw (up to 8%) ↑food consumption **Uterus adenocarcinomas** 14/50

* HCD and statistical analysis can be found in tables above.

In the 18 month mouse study there is no mortality in the two highest dose groups (3500 and 7000 ppm). There are some clinical signs, increased food consumption and decreased body weight in both male and female mice. However, there is no quantifiable data in the CLH report and the studies are not available to the rapporteurs since there are all industry studies. In the rat study, in the highest dose group (2500 ppm) there was a decrease of up to 11% in male and up to 8% in female body weight. No mortality and no clinical signs were observed but there was a slight increase in food consumption.

In addition, in the available repeated dose toxicity studies RAC noted the following:

1. In the 13-week mouse study at the 10000 ppm dose no mortality, no clinical signs and no food & water consumption effects were observed. There was a decrease in the body weight of both male and female mice but not in a significant way.
2. In the 14-week rat study at the 2500 ppm dose there was no mortality, no clinical signs, no food & water consumption effects and a non significant decrease in the body weight of both male and female rats.

Therefore, the rapporteurs do not believe that the relevant doses are at a MTD level in the carcinogenicity studies.

In conclusion, based on the HCD and statistical analysis RAC believes that the observed tumors should be considered for classification.

The hormonal and cholesterol disrupting properties of spirodiclofen could be responsible for the adrenal, testicular and uterine effects observed. Chronic stimulation of the pituitary hormone production could lead to chronic stimulation of testicular Leydig cells and endometrial uterine cells resulting in hypertrophy, hyperplasia and eventual tumor formation. This is further supported by the fact that spirodiclofen does not exhibit mutagenic properties. Therefore, the mode of action appears to be relevant to humans and should be considered in the weight of evidence for the classification of spirodiclofen. The available data cannot be considered as 'limited evidence', as they include an expected combination of benign and malignant metastatic neoplasms in two or more species. Classification in category 2 is thus not supported.

Overall, RAC concludes there is sufficient evidence for carcinogenic effects of spirodiclofen, and agrees with the DS to classify it as **Carc. 1B (H350: May cause cancer)**.

4.11 Toxicity for reproduction

Table 45: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference[a]
Rat (Wistar) Oral 2-generation study *OECD 416* 0, 70, 350, 1750 ppm (F0: 5.2, 26.2, 134.8 mg/kg bw/day for males and 5.5, 27.6, 139.2 mg/kg bw/day for females; F1: 6.4, 30.2, 177.6 mg/kg bw/day for males and 7.0, 34.4, 192.7 mg/kg bw/day for females)	F0: Decreased BW (m), decreased relative organ weights of liver, epididymides and seminal vesicles, increased relative weights of brain and prostate; F1: decreased blood cholesterol and triglyceride concentrations, decreased spermatogenesis NOAEL: <5.2 mg/kg bw/day (systemic toxicity) 26.2 (reproductive toxicity)	-	Eiben (2000)
Rabbit Oral Developmental study *OECD 414* 0, 100, 300, 1000 mg/kg bw/day during GD 6 to 28	Decreased bw (maternal) Increased incidence of liver lobulation in fetuses. NOAEL: Maternal: 100 mg/kg bw/day Fetal: 300 mg/kg bw/day	-	Holzum (1998)
Rat Oral Developmental study *OECD 414* 0, 100, 300, 1000 mg/kg bw/day during GD 6 to 19	No effects NOAEL: Maternal: 1000 mg/kg bw/day Fetal: 1000 mg/kg bw/day	-	Klaus (2000)

[a] As summarised in the DAR (Volume 3, annex B.6 April 2004 + addendum B.6 2005 +addendum B6 2009).

In addition, effects on the reproductive organs were observed in the repeated dose toxicity and carcinogenicity studies (as described in paragraph 4.7 and 4.10). Table 57 below (section 4.11.4) presents an overview of these (non-neoplastic) effects.

4.11.1 Effects on fertility

4.11.1.1 Non-human information

STUDY 1

Characteristics

reference/notifier	:	R. Eiben (2000)	Exposure	:	F0: 12 weeks pretreatment F0; F1 offspring untill weaning of F2.
type of study	:	Two-generation study.	Doses[1]	:	0, 70, 350, 1750 ppm (F0: 5.2, 26.2, 134.8 mg/kg bw/day for males and 5.5, 27.6, 139.2 mg/kg bw/day for females; F1: 6.4, 30.2, 177.6 mg/kg bw/day for males and 7.0, 34.4, 192.7 mg/kg bw/day for females)
year of execution	:	1997-1999	Vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%)	GLP statement	:	Yes
route	:	Oral (diet)	Guideline	:	OECD 416
species	:	Wistar rat (Crl: WI (WU) BR)	Acceptability	:	Acceptable
group size	:	25/sex/dose	**$NOAEL_{syst}$**	:	< 5.2 mg/kg bw/day
			$NOAEL_{repr}$		26.2 mg/kg bw/day

1 dose selection was based on the results of a one-generation pilot study with dietary administration of 0, 250, 2500 and 10000 ppm spirodiclofen, with an overall NOAEL of 250 ppm.

Study design

The study was performed in accordance with OECD guideline 416. The test substance was administered to parental (P) animals prior to and during their mating, during the resultant pregnancy and through the weaning of their F1 offspring. The substance was then administered to selected F1 offspring during their growth into adulthood, mating, and production of a F2 generation, until weaning of the F2 generation.

Dose-selection was based on the results of a one-generation range finding study in wistar rats with dietary administration of 0, 250 2500 and 10000 ppm spirodiclofen. In this pilot study an overall NOAEL was established at 250 ppm. Spirodiclofen administration at higher concentrations resulted in retarded body weights in 10000 ppm males and in females from 250 ppm onwards as well as in pups at 250 ppm and above.

Food intake was not determined during lactation period.

Parameters of reproduction in F0 parents included length of estrus cycle (determined about 3 weeks before mating of F0), insemination index, fertility index, gestation index and duration of pregnancy, and in males of dose groups 0 and 1750 ppm: sperm morphology, sperm motility, sperm count epididymides and spermatid head count in testis.

Results

The results of the study are presented in tables 46-53.

Table 46 Overview of the results of the rat 2-generation study (Eiben, 2000)

Dose (ppm)	**0**		**70**		**350**		**1750**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
F0 animals									
Mortality	None								

Dose (ppm)	**0**		**70**		**350**		**1750**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Clinical signs	No toxicologically relevant effects								
Body weight[1]			d		ds	d	ds	ds	m,f
Food consumption	No toxicologically relevant effects								
Mating/fertility/gestation	No toxicologically relevant effects								
Pathology									
Organ weight									
- brain			is[r]		is[r]		is[r]	is[r, a]	
- adrenals							is[r]	is[r]	
- liver			ds[a,r]		ds[a,r]		ds[a,r]		
- kidneys								ds[a, r]	
- testes							is[r]		
- prostate			is[r]		is[r]				
- epididymides (left)			ds[r]		ds[r]				
- seminar vesicles			ds[r]		d				
Pathology									
macroscopy	No toxicologically relevant effects								
microscopy									
Small intestine									
- epithelial vacuolation	4/25	6/25	0/25	7/25	5/25	5/25	17/25	10/25	
Adrenal glands									
- mean severity vacuolation	2.0	1.2	1.9	1.2	1.8	1.6	2.7	2.1	
Testes									
- diminished in size			1/25		1/25		4/25		
Epididymides									
- diminished in size			1/25		1/25		4/25		
F1 pups									
Litter size	No toxicologically relevant effects								
Survival index	No toxicologically relevant effects								
Sex ratio	No toxicologically relevant effects								
Body weight[2]			i		ds	ds	ds	ds	m, f
Organ weights F1 weanlings									
- brain					i[r]	i[r]	i[r]	i[r]	m,f
- spleen					d[r]		d[r]		m
Pathology									
macroscopy	No toxicologically relevant effects								
F1 animals									
Mortality	No toxicologically relevant effects								
Clinical signs	No toxicologically relevant effects								
Body weight					d		ds	ds[3]	m
Food consumption							is	is	
Mating/fertility/gestation									
- spermatids per mg testis			nd		-1%		-23%		

Dose (ppm)	**0**		**70**		**350**		**1750**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
- sperms per mg epididymides			nd		+10%		-18%		
Clinical chemistry									
- AP							is	is	
- Cholesterol			d		ds		ds	ds	m
- Triglycerides			ds	d	ds	d	ds	ds	m, f
- UFA (unesterified fatty acids)					d	d	ds	ds	m, f
- Creatinine						i		is	f
Organ weight									
- brain							is[a, r]		
- adrenals							is[a, r]	is[a, r]	
- liver							ds[a, r]		
- kidneys								ds[r]	
- ovaries								i[a, r]	
- uterus								i[a, r]	
Pathology									
macroscopy	No toxicologically relevant effects								
microscopy									
Testes									
- atrophy, diffuse	0/25		1/25		1/25		4/25		
Epididymides									
- oligospermia	0/25		1/25		1/25		4/25		
- atrophy	0/25		1/25		1/25		4/25		
Adrenal glands									
- vacuolation, mean severity	1.9	1.2	2.0	1.2	2.1	1.5	2.8	2.1	m,f
Prostate									
- atrophy	0/25		0/25		0/25		3/25		
Small intestine									
- epithelial vacuolation		10/25		12/25		13/25		17/25	f
Ovaries									
- vacuolation, mean severity		1.4		1.3		1.3		1.8	
F2 pups									
Litter size	No toxicologically relevant effects								
Survival index	No toxicologically relevant effects								
Sex ratio	No toxicologically relevant effects								
Body weight									
- at birth					ds	ds	ds	ds	m, f
- during lactation							ds	ds	
Organ weight[4]									
- spleen								d[r]	
- thymus			d[r]		d[r]	d[r]	d[r]	d[r]	f
Pathology									
macroscopy	No toxicologically relevant effects								
microscopy	Not performed								

dr dose related
i/ d increased/ decreased
ds/is statistically significantly decreased/increased compared to the controls
1 mean bw of females of dose group 70 ppm in week 0 was significantly higher compared to controls (about 6%), and mean bw of females of dose group 350 ppm was about 2% higher compared to mean control bw weight.
2 Mean pup weight at birth and during lactation (day 0 – day 28)
3 Significantly decreased bw from week 17 to 21 (lactation period)
4 Determined in 5/sex/dose group; no statistics performed.

Table 47 Details on organ weights of F0-generation, and clinical chemistry of F1-generation at terminal sacrifice (Eiben, 2000)

Dose (ppm)	**0**		**70**		**350**		**1750**	
	m	f	m	f	m	f	m	f
Organ weights (F-0 generation)								
Terminal body weight (g)	498	260	481	266	476	254	463	235
Liver								
- absolute (g)	18,826	11,842	16,605**	12,461	16,535**	11,944	15,991**	10,214**
			-12%	5%	-12%	1%	-15%	-14%
- relative (mg/100g BW)	3,78	4,55	3,45**	4,68	3,47**	4,70	3,45**	4,35
			-9%	3%	-8%%	3%	-9%	-4%
Clinical chemistry (F-1 generation)								
Cholesterol (mmol/l)	2.55	2.24	2.30	2.33	2.07**	2.20	1.58**	1.69**
	±0.229	±0.373	±0.366	±0.362	±0.236	±0.268	±0.261	±0.307
Triglyceriden (mmol/l)	2.62	1.36	1.84**	1.22	1.68**	1.02	0.96**	0.58**
	±0.667	±0.461	±0.469	±0.551	±0.712	±0.354	±0.193	±0.166

According to the study report, the values of cholesterol and triglycerides were within the ranges of historical control data during 1996-1997. However, only minimal details were provided (ranges were indicated, no mean values).

Table 48 Mean body weight of the F1 pups at birth and during lactation (Eiben, 2000)

Mean pup weight (g) at birth and during lactation							
Dose		**Day 0**	**Day 4**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
ppm	sex		After culling				
0	M	5.73	9.61	14.52	28.77	44.98	75.34
70	M	6.08**	10.00	14.71	30.40**	46.94*	77.45
350	M	5.66	9.01**	13.88*	28.12**	43.28*	71.27**
1750	M	5.62	8.73**	12.80**	23.64**	35.32**	58.20**
0	F	5.59	9.40	14.32	28.51	44.26	71.09
70	F	5.66	9.41	14.05	29.49*	45.25	71.40
350	F	5.41**	8.55**	13.17**	27.00**	41.89	67.13**
1750	F	5.30**	8.40**	12.42**	23.19**	34.85**	56.66**

* significant difference with control animals, P<0.05
** significant difference with control animals, P<0.01

Table 49 Mean body weight of the F2 pups at birth and during lactation (Eiben, 2000)

Mean pup weight (g) at birth and during lactation							
Dose		**Day 0**	**Day 4**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
ppm	sex		After culling				
0	M	6.39	10.68	15.27	30.09	46.00	74.17
70	M	6.44	10.48	16.23*	30.14	45.70	72.50
350	M	6.10*	10.11	15.59	29.50	45.58	72.19

1750	M	5.99**	9.90**	14.75	25.73**	39.31**	62.28**
0	F	6.17	10.44	15.39	30.03	45.51	70.31
70	F	6.06	10.03	16.06	29.19	44.44	68.72
350	F	5.72**	9.87	15.34	29.31	44.74	69.07
1750	F	5.37**	8.97**	13.52**	24.22**	35.92**	57.57**

* significant difference with control animals, P<0.05
** significant difference with control animals, P<0.01

Body weights of male F0 animals receiving 70 ppm were statistically significantly decreased during weeks 1 to 6, however, the deviations were below 5%. At 350 ppm statistically significant lower body weights were observed at several times with a maximum of –5.1% in weeks 12-15. In 1750 ppm males there was a statistically significant (-7%) retardation in body weights. In F0 females body weights were not affected up to 350 ppm. At 1750 ppm statistically significant lower body weights occurred mainly during the lactation period (-15.2% in week 19). In F1 animals, body weights of males and females receiving 70 or 350 ppm were not affected. At 1750 ppm decreased body weights were noted in males (up to –23% in week 1) and females during the lactation period (-10.3% on day 14).

At 1750 ppm there were more F1 males exhibiting a testicular and epididymal atrophy (0/1/1/4) than in the other groups. The testicular atrophy was reported with a mean severity score of -/4/4/3.5, suggesting a treatment-related effect in the high dose group. These findings were associated with epididymal oligospermia (0/1/1/4) and (partly) atrophic prostate and seminal vesicles. No such findings were found in the F0 generation. The F1-data are summarized in Table 50.

Table 50 Testicular and epididymal effects in F1 males.

Organ/finding	**0 ppm**	**70 ppm**	**350 ppm**	**1750 ppm**
Testes				
- Difuse atrophy	0	1	1	4
- Dif. Leydig hyperplasia	0	1	0	2
Epididymis				
- Atrophy	0	1	1	4
- Oligospermia	0	1	1	4
Male access. sex gland				
- Atrophy*	0	0	0	2

* incidence related to males with testicular lesions

The absolute mean weights of the male sexual organs were reduced in the high dose group as a result of the very low individual values of those four male animals exhibiting testicular and epididymal atrophy as well as extremely low body weights (i.e animal # 353, 366, 367, 372). These data are presented in Table 51.

Table 51 Body weight and testis weight in F1 males.

Dose (ppm)	**Body weight- week 19 (g)**		**Absolute testis wight (mg)**		**Relative testis weight (mg/100g)**	
	Mean with animal 353, 366, 367, 372	Mean without animal 353, 366, 367, 372	Mean with animal 353, 366, 367, 372	Mean without animal 353, 366, 367, 372	Mean with animal 353, 366, 367, 372	Mean without animal 353, 366, 367, 372
0	470		3587		751	
70	471		3395		700	
350	447		3458		755	
1750	391	410	3161	3619	768	863

The individual weights of the four high dose males which are given in Table 52.

Table 52 Individual body and testis weight in four high dose males with testicular atrophy.

Animal no	**Individual body weight – week 19 (g)**	**Individual absolute testis weight (g)**	**Individual relative testis weight (mg/100g)**
353[$]	227	838	357
366[*#]	357	744	203
367[*#]	354	753	224
372[#]	248	679	281

* litter mates

$ rat exhibited distinctly increased food intake values compared to the mean in the same week and group most probably due to spillage with the food

rat exhibited distinctly decreased food intake values compared to the mean in the same week and group

Two of the affected males (nos 366 and 367) were litter mates with nearly identical low body weights. Microscopically, these litter mates showed exactly the same type of testicular and epididymidal lesions. Lowest body weights at all were registered in animal no. 353 and 372 with tubular atrophy grade 3. The body weight in these two animals in week 19 was only 48.3 or 52.8% of the control mean.

Sperm motility and morphology as well as spermatid and sperm counts were not affected at 1750 ppm besides the four affected males which had no sperms. The testicular and epididymidal atrophy and oligospermia found in a single male each of the low and the mid dose group were considered incidental in the study report.

Historical control data for testicular degeneration (i.e. used as a synonym of testicul atrophy) of two-generation studies performed at the same laboratory with the same rat substrain are presented in Table 53.

Table 53 Historical control data testicular atrophy in Wistar rats

Study no	**year**	**generation**	**Finding/incidence**	**control**	**Low dose**	**Mid dose**	**High dose**
T1068962	2000	F0	**Tubular deg./atrophy** Individual severity scores Diffuse Leydig cell hypertrophy	1 3	5 3,1,1,5,2 1	2 1,1	1 5
T9063190	2004	F0	**Tubular deg./atrophy** Individual severity scores Diffuse Leydig cell hypertrophy	4 4,4,1,2	1* 5	1* 3	6 4,1,5,2,2,4
		F1	**Tubular deg./atrophy** Individual severity scores	3 1),1),4	1* 1	-	3 1),2),1)
T4071303	2003	F0	**Tubular deg./atrophy** Individual severity scores	6 1,1,1,2,3,4	6 1,1,2,2,2,2	7 1,1,2,2,3,4,5	10 1,1,1,1,1,2,2, 2,3,5
		F1	**Tubular deg./atrophy** Individual severity scores	4 1,1,2,31#	9 1,2,2,2,2,3,3, 4,4	9 1,1,1,1,2,3,3, 4,4	7 1,1,1,1,2,3,5
T4069306	2001	F0	**Tubular deg./atrophy** Individual severity scores	5 1,1,1,1,4	-	1* 4	1 1
		F1	**Tubular deg./atrophy** Individual severity scores	1 1	-	-	1 3
T5070099	2002	F0	**Tubular deg./atrophy** Individual severity scores	1 2	-	-	1 1
		F1	**Tubular deg./atrophy** Individual severity scores	8 1,1,1,1,1,2,3, 4	-	2 4,4	4 1,1,1,1

25 males per group
* only macroscopic lesions examined
) unilateral finding
this is considered a typographical error in the report of industry, and is assumed to be a score of 3

The historical control data show a large variation in incidences of testicular atrophy and severity scores. Incidences ranging from 1 to 8 were observed in untreated historical controls (25 animals/group) were observed. The observed effects in current 2-generation study are within the ranges of historical control data.

Acceptability

The study was considered acceptable.

Conclusions

Body weight in F0 animals was dose-relatedly decreased in males of all dose groups and in females at and above 350 ppm. In males of all dose groups a significantly decreased liver weight (not dr) and significantly increased brain weight was observed. In females dosed at and above 350 ppm adrenal gland vacuolation severity was increased.

In F1 pups, a significantly decreased body weight at birth (females) and during lactation (males and females) was observed at and above 350 ppm (dr). In F1 weanlings decreased relative spleen weights in males (dr) and increased brain weights (m/f, dose-related) at and above 350 ppm were observed.

F1 animals showed decreased blood cholesterol (10-40%, m) and triglyceride (30-70%) concentrations (dr) at all dose levels and decreased unesterified fatty acid levels in high dose groups, pointing towards a changed liver function. Further, a decreased body weight was observed (females: high dose group, males: mid and high dose group, dose related). In the high dose group, weights of brain, adrenals, liver, kidneys, ovaries and uterus had changed. Further, decreases were observed in the number of spermatids in the testes and the number of sperms in the epididymides in the high dose groups (4 animals). The observed effects on testicular atrophy are within the ranges of historical controls. Further, the testes effects were observed in presence of significant general toxic effects (reduced body weight).

The F2 pups showed significantly decreased body weight at birth at and above 350 ppm (dr) and also during lactation decreased body weight of F2 pups was observed in the highest dose group (-17% in males and –21% in females). In females dosed at and above 350 ppm a dose-related decreased thymus weight was observed.

Based on the observed effects in the lowest dose group in the F0 and F1 animals, a NOAEL for systemic toxicity could not be established in this study, and the LOAEL for systemic toxicity is 70 ppm, equal to 5.2 mg/kg bw/day.

Based on the decreased spermatogenesis in the F1 males the NOAEL for reproduction is 350 ppm, equal to 26.2 mg/kg bw/d.

4.11.1.2 Human information

No information available.

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

STUDY 1

Characteristics

reference/notifier	:	B. Holzum (1998)	Exposure	:	Day 6 to 28 p.c.
type of study	:	Developmental toxicity study.	Doses[1]	:	0, 100, 300, 1000 mg/kg bw/day
year of execution	:	1997/1998	vehicle	:	0.5% carboxymethylcellulose
test substance	:	BAJ 2740 (spirodiclofen, purity 97.9% to 98.5%)	GLP statement	:	Yes
route	:	Oral (gavage)	guideline	:	In accordance with OECD 414
species	:	Rabbit (Himalayan, CHBB:HM)	acceptability	:	acceptable
group size	:	22 females/dose	**NOAEL**mat	:	100 mg/kg bw/day
			NOAELdev		300 mg/kg bw/day

1 dose levels were based on the results of a pilot developmental toxicity study.

Study design

The study was performed in accordance with OECD guideline 414. Day 0 of gestation was the day on which copulation was observed.

Results

The results of the study are presented in tables 54-55.

Table 54 Overview of the results of the rabbit developmental toxicity study (Holzum, 1998)

Dose (mg/kg bw)	**0**	**100**	**300**	**1000**	**dr**
Maternal effects					
Mortality	none				
Clinical signs (days 6-29)					
- killed because of abortion				1/22	
- cold to touch (ears)			2/22	3/22	
- alopecia	2/22	2/22	0/22	5/22	
- reduced feces	8/22	8/22	14/22	17/22	f
- discolored urination (light yellow)	1/22	3/22	3/22	7/22	f
- light colored feces				14/22	
Pregnant animals	No toxicologically relevant effects				
Abortions				1/22	
Body weight (gain)			ds[1]	ds[1]	
Food consumption			ds[1]	ds[2]	

Dose (mg/kg bw)	**0**	**100**	**300**	**1000**	**dr**
Water consumption	No toxicologically relevant effects				
Pathology					
- distinct liver lobulation				1/22[3]	
Litter response					
Live fetuses	No toxicologically relevant effects				
Fetal weight	No toxicologically relevant effects				
Post implantation loss	No toxicologically relevant effects				
Sex ratio	No toxicologically relevant effects				
Examination of the fetuses					
Fetal malformations	No toxicologically relevant effects				
Fetal external and visceral deviations					
Total:					
- Number of foetuses per group	138	137	136	139	
- Number of foetuses with deviations	6	0[ds]	4	21[is]	
- Foetuses with deviations per group (%)	4.4	0.0	2.9	15.1	
- Number of litters per group	21	22	20	20	
- Number of litters with deviations	3	0	2	5	
- Litters with deviations per group (%)	14.3	0.0	10.0	25.0	
Distinct liver lobulation					
- Number of foetuses with distinct liver lobulation	3	0	1	14 is	
- Foetuses with distinct liver lobulation per group (%)	0.7	0	0.7	10	
- Number of litters with distinct liver lobulation	2	0	1	3	
- Litters with deviations per group (%)	9.5	0	5	15	

dr dose related
ds/is statistically significantly decreased/increased compared to the controls
a/r absolute/relative organ weight
1 days 6-9 p.c.
2 days 6-15 p.c.
3 observed on day 20 in the female that aborted
( ) number of litters affected

Table 55 Overview of body weight gain of the females with viable fetuses (Holzum, 1998)

Dose	Mean body weight gain (g)			
(mg/kg bw/d)	day 6-9 p.c.	day 6-29 p.c.	day 0-29 p.c.	corrected day 0-29 p.c.
0	-23.7	177.2	202.4	-147.7
100	-28.3	197.6	184.7	-163.7
300	-55.8*	229.1	269.7	-91.7
1000	-72.7**	144.1	143.8	-223.2

* statistically significant difference to control p<0.05
** statistically significant difference to control p<0.01

Acceptability

The study was considered acceptable.

Conclusions

Maternal body weight was significantly decreased at and above 300 mg/kg bw/day on days 6-9 p.c. (i.e. the first days of exposure). At later time periods, body weight gain in these experimental groups was even higher than controls.

In these dose groups also reduced food consumption and reduced feces were observed. The study author concluded that it cannot be excluded that the observed distinct lobulation of the liver, observed in de female that aborted on day 20, is treatment-related. Consequently, it cannot be excluded either that the observed significantly increased incidence in liver lobulation in fetuses of the highest dose group is treatment-related. Moreover, the increased incidence in fetal liver lobulation was observed in foetuses (fetal incidence: 10%) of 3 different litters (litter incidence: 15%) in the high dose group. Additional information from the study report on historical controls for this type of effect in this species/strain showed that the fetal incidence was outside the range of historical controls (fetal incidence-historical controls: 0-7.1 %) though the litter incidence was within the range of historical controls (litter incidence- historical controls: 0-33.3 %). The total number of deviations was significantly increased in the high dose group.

Based on the observed decreased body weight at and above 300 mg/kg bw, the NOAEL for maternal toxicity in this study is 100 mg/kg bw. Based on the observed increased incidence in liver lobulation in fetuses of the highest dose group, the NOAEL for fetal toxicity in this study is 300 mg/kg bw.

STUDY 2

Characteristics

reference/notifier	:	A.M. Klaus (2000)	exposure	:	Day 6-19 p.c.
type of study	:	Developmental toxicity study.	doses	:	0, 100, 300, 1000 mg/kg bw/day
year of execution	:	1998	vehicle	:	0.5% aqueous carboxy methylcellulose
test substance	:	BAJ 2740 (spirodiclofen, purity 97.9%)	GLP statement	:	yes
route	:	Oral (gavage)	guideline	:	According to OECD 414
species	:	Wistar rat (Hsd Cpb:WU)	acceptability	:	acceptable
group size	:	28 females/dose	**NOAEL**mat	:	1000 mg/kg bw/day
			NOAELdev		1000 mg/kg bw/day

Study design

The study was performed in accordance with OECD guideline 414 (deviations: no data on water consumption and excretory products were included). Day 0 of gestation was the day on which detection of sperm in the vaginal smear on the morning following mating was observed.

Results

The results of the study are presented in Table 56.

Table 56 Overview of the results of the rat developmental toxicity study (Klaus, 2000)

Dose (mg/kg bw)	**0**	**100**	**300**	**1000**	**dr**
Maternal effects					
Mortality	none				
Clinical signs[1]	No toxicologically relevant effects				
Pregnant animals	No toxicologically relevant effects				
Abortions	none				
Preimplantation loss*	44/24	52/25	62/25	60/26	
Body weight	No toxicologically relevant effects				
Food consumption	No toxicologically relevant effects				
Water consumption	No data				
Pathology					
macroscopy	No toxicologically relevant effects				
Litter response					
Live fetuses	No toxicologically relevant effects				
Fetal weight	No toxicologically relevant effects				
Post implantation loss	No toxicologically relevant effects				
Sex ratio	No toxicologically relevant effects				
Examination of the fetuses **Fetal malformations**	No toxicologically relevant effects				
Fetal external and visceral deviations	No toxicologically relevant effects[1]				
Fetal skeletal deviations	No toxicologically relevant effects[1]				

dr dose related

1 several minor changes were observed (dilation of renal pelvis, incompletely ossified proximal phalanx digits, wavy ribs, asymmetrical sternebrae) which are considered not substance related.

* It is assumed that these numbers correspond to the number of losses/number of litters. However, no information was available in the DAR

Acceptability

The study was considered acceptable.

Conclusions

An increased incidence in preimplantation loss was observed at and above 300 mg/kg bw/day, which is however considered not toxicologically relevant, given the treatment period. The NOAEL for maternal toxicity in this study is 1000 mg/kg bw/day, the highest dose tested.

In fetuses, several minor visceral and skeletal deviations were observed (dilation of renal pelvis, incompletely ossified proximal phalanx digits, wavy ribs, asymmetrical sternebrae), which were considered not toxicologically relevant. The NOAEL for fetal toxicity in this study is 1000 mg/kg bw/day, the highest dose tested.

4.11.2.2 Human information

No information available.

4.11.3 Other relevant information

No information available.

4.11.4 Summary and discussion of reproductive toxicity

In the 2-generation study with rats, F0 male animals showed in all dose groups decreased body weight, increased brain weight and decreased liver weight. In females effects were observed in the mid and high dose groups and included decreased body weight and increased severity of adrenal vacuolation. F1 pups showed in the mid and high dose groups decreased body weight at birth and during lactation and changed weights of brain and spleen. In the F1 animals in all dose groups dose related decreases in cholesterol and triglycerides were observed. Further, weights of brain, adrenals, liver, kidneys, ovaries and uterus in the F1 animals had changed. In the high dose group, decreases were observed in the number of spermatids in the testes and in the number of sperms in the epididymis. Testes atrophy was observed with incidences of 0/1/1/4 for the control, low, mid and high dose group. Testes atrophy was only observed in the F1-males and not in the F0-males indicating that this observed effect might not be the result of exposure of adult animals (i.e. an effect on sexual function and fertility). In stead, the observed effects might rather be the result of an exposure during the prenatal or pre-adult period (i.e. developmental effect). Nevertheless, the findings of testes atrophy in the high dose groups were within the ranges for historical controls. Further, the testes effects were observed in presence of significant general toxic effects (i.e. significantly reduced body weight in the animals with testes atrophy). The testis effects are therefore considered not relevant for classification. Other effects included decreased bw at birth and decreased thymus weight (f) in F2 pups in the mid and high dose groups.

In addition, effects on the reproductive organs were observed in the repeated dose toxicity and carcinogenicity studies (as described in paragraph 4.7 and 4.10). Table 57 presents an overview of these (non-neoplastic) effects. Effects on testes were observed in all studied species (i.e. mouse, rat, dog), though most pronounced in dogs. These effects included increased testis weight (absolute + relative), hyperplasia, hypertrophy and vacuolisation of testis, but also oligo- and aspermia (in 4- and 14-week dog studies, 18-month mouse study). Further, changes of weight of uterus/oviduct and ovaries were observed in female animals.

The mechanistic studies (paragraph 4.12.1.3) showed that spirodiclofen clearly interferes with steroid hormone synthesis in the adrenals and gonads. Further, it was noted that the effect on

steroidogenesis is probably mediated by effects on general biochemical pathways (interference with the formation of mitochondrial and cytoplasmatic NADPH, which is an important co-substrate in several steps of the biosynthesis of steroid hormones), and that no androgenic, antiandrogenic, estrogenic or anti-estrogenic effects were noted in mechanistic studies. Further, a direct effect of spirodiclofen on enzymes involved in the synthesis of steroidhormones in the testes (microsomal hydrogenases) could not be excluded. The observed effects on testes (enlargement, hypertrophy, hyperplasia), uterus (enlargement) but also adrenals (enlargement, vacuolisation) might therefore be an adaptive response in order to keep the steroidogenesis on a adequate level. However, at the high dose levels this adaptation probably failed wich resulted in clear effects on spermatogenesis (i.e. oligospermia, aspermia). Aspermia was also observed in the 18-month mouse study, though at much higher dose levels. The dog was shown to be the most sensitive species. No information is available which indicate that the effects observed in dogs (including the underlying mechanisms) are not relevant for humans.

In the teratogenicity study with rabbits, maternal body weight was decreased at and above 300 mg/kg bw/day on days 6-9 p.c. (i.e. the first days of exposure). At later time periods, body weight gain in these experimental groups was however higher than controls. In the high dose group, one female showed liver lobulation. In fetuses of the highest dose group, an increased incidence in liver lobulation was observed. The fetal incidence for liver lobulation is outside the range of historical controls, whereas the litter incidence for this type of effects is within the range of historical controls. It is therefore not fully clear whether the observed effect of liver lobulation is treatment-related. Further, no information on the severity of liver lobulation was presented in the study report.

Two neurodevelopmental toxicity studies in rats were presented (see section 4.12.1). The first study showed negative results with the exception of some equivocal results for the water maze test. In a follow-up study in which parts of the developmental neurotoxicity study were repeated (and included two types of water maze tests) no neurotoxic effects were observed.

In the teratogenicity study with rats, no toxicologically relevant effects were observed.

Reproductive toxicity of impurity N,N-dimethylacetamide: Spirodiclofen contains the impurity N,N-dimethylacetamide (≤ 0.4%) (see paragraph 1.2). N,N-dimethylacetamide has a harmonised classification for reproductive toxicity as Repro. 1B (H360D: May damage the unborn child) with an SCL of 5%. A CLH proposal for N,N-dimethylacetamide (prepared by NL) to remove the current SCL of 5% was recently accepted by RAC (RAC opinion, d.d. 12-09-2014). The GCL of 0.3% for Repro 1B is then applicable. This could result in an additional classification of spirodiclofen with Repro 1B - H360D (May damage the unborn child) over time depending on the actual concentration of DMAC in each batch.

Table 57. Overview of effects on reproductive organs in available repeated dose toxicity studies with spirodiclofen (paragraph 4.7)

		Observed effects reproductive organs	
		males	**females**
Leser, Romeike (1998)	13-wk oral mouse	≥ 163.8 mg/kg bw/d: Increased (dose-related) testes weights (absolute+relative) Hypertrophy/activation of Leydig cells (testes) ≥1629.9 mg/kg bw/d: Vacuolization of Leydig cells (testes)	≥ 2685.2 mg/kg bw/d: Increased weight ovaries (relative)
Wahle (2000)	18-month mouse	≥ 610 mg/kg bw/d: Increased testis weight (absolute+relative) Hypertrophy/hyperplasia interstitial cells testis ≥1216 mg/kg bw/d aspermia	-
Krötlinger, Geiß (2000)	4-wk oral rat	-	≥ 569.3 mg/kg bw/d: Decreased weight ovaries (absolute+relative)
Wirnitzer, Romeike (1998)	14-week oral rat	≥ 851.4 mg/kg bw/d: Increased testes weights (relative)	-
Wirnitzer (2000)	108-week rat	≥110.14 mg/kg bw/d: Increased testis weight (relative) Focal Leydig cell hyperplasia	≥19.88 mg/kg bw/d: Increased ovaries (absolute+relative)
Wetzig, Romeike, Sander (2001)	4-week oral dog	≥ 65.5 mg/kg bw/d: Leydig cell vacuolization ≥ 284.5 mg/kg bw/d: Leydig cell hypertrophy/activation: Immature testes/prostate Massive oligo-/aspermia, slight spermic debris	≥ 65.5 mg/kg bw/d: Increased weight uterus (absolute + relative) ≥ 284.5 mg/kg bw/d: Increased weight ovaries (absolute + relative)
Wetzig, Hartmann (2001b)	8-week oral dog	≥ 2.9 mg/kg bw/d: Decreased weight prostate (absolute + relative) Degeneration germinal epithelium ≥ 55.9 mg/kg bw/d: Hypertrophy and vacuolization of Leydig cells	-
Wetzig, Hartmann (2001a)	14-week oral dog	≥ 8.0 mg/kg bw/d: Decreased prostate weight (relative) ≥ 27.3 mg/kg bw/d: Vacuolization Leydig cells Hypertrophy Leydig cells Aspermia Oligospermia	≥ 8.0 mg/kg bw/d: Decreased uterus weight (relative)

		Immature prostate <u>≥ 82.8 mg/kg bw/d</u> Degeneration germinal epithelium	
Wetzig, Ruhl-Fehlert (2001)	52-week oral dog	<u>≥0.57 mg/kg bw/d:</u> Increased testes weight (absolute + relative) <u>≥1.45 mg/kg bw/d:</u> Increased epididymis weight (absolute + relative) <u>≥4.54 mg/kg bw/d:</u> Focal tubular degeneration testes <u>≥16.9 mg/kg bw/d:</u> Increased prostrate weight (absolute + relative) Vacuolization Leydig cells Hypertrophy Leydig cells	<u>≥16.9 mg/kg bw/d:</u> decreased uterus/oviduct weight (absolute + relative)
Krottlinger, Sander (1999)	4-wk dermal rat	-	-

4.11.5 Comparison with criteria

Substances are classified in Category 1 for reproductive toxicity when they are known to have produced an adverse effect on sexual function and fertility, or on development in humans or when there is evidence from animal studies, possibly supplemented with other information, to provide a strong presumption that the substance has the capacity to interfere with reproduction in humans. The classification of a substance is further distinguished on the basis of whether the evidence for classification is primarily from human data (Category 1A) or from animal data (Category 1B).

- *Category 1A*: Known human reproductive toxicant. The classification of a substance in this Category 1A is largely based on evidence from humans.
- *Category 1B*: Presumed human reproductive toxicant. The classification of a substance in this Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate

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

Fertility effects:

Classification in category 1A is not considered for spirodiclofen as there are no human data regarding reproductive toxicity (fertility).

No effects on fertility were observed in the 2-generation study in rats. Effects on sexual function parameters observed in the rat 2-generation study included decreased number of spermatids in the testes, testes atrophy and decreased number of sperms in the epididymis. These effects were significant in the highest dose group of the F1 male animals (1750 ppm, 177.6 mg/kg bw/day; 4 rats). However, body weight was significantly reduced in the four rats with testes atrophy, which might have contributed to the findings in the male sexual organs. Furthermore, the findings of testes atrophy in the high dose groups were within the ranges for historical controls. Also, in the repeated dose toxicity studies in rats, no such effects were observed and at high dose levels an increase in testes weight was observed. The observed effects on sexual function in the 2-generation rat study are therefore considered not relevant for classification.

The available repeated dose toxicity and carcinogenicity studies showed clear effect on the reproductive organs in all tested species (mouse, rat, dog), though the effects were most pronounced in dogs at lower dose levels as compared to rat and mouse. Observed effects included changes of weight of uterus/oviduct and ovaries in female animals, however without histopathological changes.

Further, in male animals effects such as increased testis weight (absolute + relative), hyperplasia, hypertrophy and vacuolisation of testis cells, but also oligospermia and aspermia (in 4- and 14-week dog studies, 18-month mouse study) were observed. These latter effects are considered relevant for classification.

The mechanistic studies showed that spirodiclofen has a direct effect on steroid hormone synthesis, which is probably mediated by effects on general pathways (interference with formation of NADPH). Further, it was shown that spirodiclofen might have a direct effect on the enzymes involved in the steroidogenesis in testis. These data indicate that the observed effects are not secondary to general toxic effects, but rather a direct effect of spirodiclofen.

The dog was shown to be the most sensitive species for the effects on the reproductive organs which occurred at relevant dose levels. No oligo/aspermia was observed in rat. Further, these adverse effects (aspermia) were observed in mice, although only at very high dose levels. These data indicate that species-differences in toxicodynamics occur, which might be related to potential species-differences in kinetics. Based on the available data it is not clear whether or not the dog is the most relevant species for evaluating potential classification of spirodiclofen for effects on sexual function and fertility for humans. Given the observations that the effects were only observed at high dose levels in mice and the effects were not observed in rat, might indicate that the dog should not be regarded as the most relevant species for evaluating potential fertility effects of spirodiclofen in humans. However, clear effects on sexual function were observed, and as it cannot be excluded that the observed effects in dogs (including the underlying mechanism) are relevant for evaluating potential fertility effects of spirodiclofen in humans, the observed effects should be taken into account for potential classification for effects on sexual function and fertility.

It can be concluded there is some evidence for reproductive effects of spirodiclofen, and spirodiclofen can be considered as a suspected human reproductive toxicant. Classification for effects on sexual function and fertility as Repro 2 (H361f: Suspected of damaging fertility) is proposed.

Classification in category 1B is not considered for spirodiclofen, as 1) fertility effects on reproductive organs were observed in dogs (a single species), 2) effects on reproductive organs and on fertility were not observed in rats, 3) in mice, these effects (aspermia) were observed at very high dose levels, 4) the observed accompanying testis enlargement and vacuolisation are considered adapative.

Developmental toxicity: In the rabbit developmental study, in fetuses of the highest dose group, an increased incidence in liver lobulation was observed. The fetal incidence for liver lobulation was outside the range of historical controls, whereas the litter incidence for this type of effects was within the range of historical controls. It is therefore not fully clear whether the observed effect of liver lobulation is treatment-related. Further, no information on the severity of liver lobulation was presented in the study report. Also the severity of this type of effect (variation or malformation) is not clear but seen the historical control incidence in fetusses and liters it is more likely that the severity of this type of effect is limited. Based on the available data, the observed effect of liver lobulation are considered not relevant for classification. In the rat developmental toxicity study, no relevant substance-related effects were observed in the foetuses. Classification for developmental toxicity is not required.

Effects on or via lactation: Classification for effects on or via lactation is not proposed, due to lack of data on the concentration of spirodiclofen in milk and whether the effects observed during lactation are likely to be due to the transfer of spirodiclofen to offspring. According to Table 3.7.1b of CLP-regulation, classification for effects on or via lactation can be assigned on the:

a) human evidence indicating a hazard to babies during the lactation period; and/or
b) results of one or two generation studies in animals which provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk; and/or
c) absorption, metabolism, distribution and excretion studies that indicate the likelihood that the substance is present in potentially toxic levels in breast milk.).

Based on currently available data, these are not applicable to spirodiclofen. Classification for effects on or via lactation is not necessary.

4.11.6 Conclusions on classification and labelling

Classification of spirodiclofen for reproductive toxicity is required under the CLP regulation. Spirodiclofen should be classified for effects on sexual function and fertility - Repro 2 (H361f: Suspected of damaging fertility).

Spirodiclofen contains the impurity N,N-dimethylacetamide (≤ 0.4%). N,N-dimethylacetamide has a harmonised classification for reproductive toxicity as Repro. 1B (H360D: May damage the unborn child) with an SCL of 5%. A CLH proposal for N,N-dimethylacetamide (prepared by NL) to remove the current SCL of 5% was recently accepted by RAC (RAC opinion, d.d. 12-09-2014). The GCL of 0.3% for Repro 1B is then applicable. This could result in an additional classification of spirodiclofen with Repro 1B - H360D (May damage the unborn child) over time depending on the actual concentration of DMAC in each batch.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

The evaluation of reproductive toxicity of spirodiclofen by the DS was based on a two-generation study on Wistar rats (OECD TG 416, GLP compliant), a developmental toxicity study on Himalayan rabbits and one in Wistar rats (OECD TG 414, GLP compliant). In addition, several repeated dose toxicity studies were used for the effects on reproductive organs.

In the 2-generation study with rats, the F0 male animals showed in all dose groups decreased bodyweight, increased brain weight and decreased liver weight. In females, effects were observed in the mid and high dose groups and included decreased body weight and increased severity of adrenal vacuolation. F1 pups showed in the mid and high dose groups decreased body weight at birth and during lactation and at the same dose groups changed relative and absolute weights of brain and spleen. In the F1 animals in clinical chemistry observations in all dose groups, dose related decreases in cholesterol and triglycerides were seen. Furthermore, in the high dose group, F1 animals showed changes in weights of brain, adrenals, liver, kidneys, ovaries and uterus. In the high dose group, decreases were also observed in the number of spermatids in the testes and in the number of sperms in the epididymis. Testes atrophy was observed with incidences of 0/1/1/4 for the control, low, mid and high dose group. Since testes atrophy was only observed in the F1-

males and not in the F0-males it is probable that this observed effect may not be the result of exposure of adult animals (an effect on sexual function and fertility) but instead it may be the result of an exposure during the prenatal or pre-adult period (developmental effect). However, the findings of testes atrophy in the high dose groups were within the ranges for historical controls. In the repeated dose toxicity studies in rats and at similar doses, no such effects were observed and at the high dose an increase in testes weight was observed which which might have been due to compensation effect. Thus, in the 2-generation study no effects on sexual function are considered for classification.

In the repeated dose toxicity and carcinogenicity studies changes on reproductive organs and on sexual function/fertility were observed. Effects on testes were observed in all studied species (mouse, rat, dog), though most pronounced in dogs since effects occurred at lower dose levels. These included increased testis weight (absolute and relative), hyperplasia, hypertrophy and vacuolisation of testis, but also oligo- and aspermia (in 4-and 14-week dog studies, 18-month mouse study). Further, changes of weight of uterus/oviduct and ovaries were observed in female animals as summarised in the table below.

Table: Overview of effects on sexual function/fertility parameters and reproductive organs in available repeated dose toxicity, carcinogenicity and reproductive toxicity studies

Study		**males**	**females**
Leser, Romeike (1998)	**13-wk oral mouse repeated dose toxicity study** 0, 100, 1000, 10000 ppm	**≥ 1000 ppm** slight ↓bw, 8 % ↑ r (dose-related) testes weights Hypertrophy/activation of Leydig cells (testes) 1/10, 1/10, **9/10**, 10/10 Average Severity (1) **≥ 10000 ppm** 12% ↑ r testes weights Hypertrophy/activation of Leydig cells (testes) 1/10, 1/10, 9/10, **10/10** Average Severity (2.3) Vacuolation of Leydig cells 7/10 Average Severity (1.1)	**≥ 1000 ppm** no effects **≥ 10000 ppm** slight ↓bw, 10% ↑ weight ovaries
Wahle (2000)	**18-month mouse carcinogenicity study** 0, 25, 3500, 7000 ppm	**≥ 3500 ppm** no mortality, ↓ bw (statistically not consistent) ↑ food consumption ↑ar testis weight Hypertrophy/hyperplasia interstitial cells testis **≥ 7000 ppm** **Epididymides** Aspermia: 15/50, 15/50, 15/50, 26/50, ↑ s average severity: 4.3, 4.2, 4.8, 4.7 **Testes** 23% ↑r testes weight Hypertrophy/hyperplasia of interstitial cells	**≥ 3500 ppm** no mortality, ↓ bw (statistically not consistent) terminal body weight ↓ significantly **≥ 7000 ppm** no mortality, ↓ weight (statistically not consistent) body weight, terminal body weight was significantly ↓ **Ovaries** 38% ↓ r ovaries wt

		6/50, 6/50, 26/50, 31/50 Average Severity: 1.2, 1.3, 1.8, 2.5	
Krottlinger, GeiB (2000)	**4-wk oral rat** f, 0,100, 500, 5000 ppm	–	**≥ 5000 ppm** no mortality, no bw change, ↓17% r weight ovaries
Wirnitzer, Romeike - 1998	**14-week oral rat** 0, 100, 500, 2500, 12500 ppm	**≥ 12500 ppm:** 10% ↑r testes weight, no mortality, no clinical signs, ↓ s bw m, ↓ water consumption ↓ s food consumption	**≥ 12500 ppm:** ↓ s bw f
Wirnitzer 2000	**108-week rat carcinogenicity study** 0, 50, 100, 350, 2500 ppm	**≥ 350 ppm:** no effects **≥ 2500 ppm:** no mortalities, no clinical signs ↓s bw, ↑ food consumption ↑[r] testis weight Focal Leydig cell hyperplasia 4/31, 4/30, 4/36, 6/31, 19/41 ↑[s]	**≥ 350 ppm:** 33% ↑ar ovaries weight **≥ 2500 ppm:** no mortalities, no clinical signs, ↓s bw, ↑ food consumption
Wetzig, Romeike, Sander (2001)	**4-week oral dog** 0, 400, 2000, 10000 ppm	**≥ 2000 ppm**: no general toxicity effects Leydig cell vacuolation 2/2 (1,1) **≥ 10000 ppm:** no general toxicity effects Leydig cell vacuolation 2/2 (3,1) Leydig cell hypertrophy/activation 1/2 (3) Immature testes/prostate, 1/2 (2) Massive oligospermia, slight spermic debris 1/2 (5)	**≥ 2000 ppm:** no general toxicity effects 33% ↑ar weight uterus **≥ 10000 ppm:** no general toxicity effects 43 % ↑ar weight ovaries 18 % ↑ar weight uterus
Wetzig, Hartmann (2001b)	**8-week oral dog** 0, 100, 2000 ppm	**≥ 100 ppm:** no general toxicity effects ↓ar wt prostate (dr), 13 % ↓r wt prostate Degeneration germinal epithelium 1/5 (2) **≥ 2000 ppm:** no general toxicity effects Hypertrophy and vacuolization of Leydig cells (testes) 5/5 (3,2,3,2,2) Degeneration germinal epithelium 4/5 (2,1,1,1)	-
Wetzig, Hartmann (2001a)	**14-week oral dog** 0, 200, 630, 2000 ppm	**≥ 200 ppm**: 52% ↓r weight prostate **≥ 630 ppm:** ↓ bw Testes Vacuolization Leydig cells, 2/4 (2,3) Hypertrophy Leydig cells, 2/4 (2,2) Epididymides Aspermia, 1/4 Oligospermia, 2/4 (2,2) Immature prostate, 1/4 (4)	**≥ 200 ppm**: ↓ r weight uterus **≥ 630 ppm:** ↓ bw ↓ r weight uterus

		≥ 2000 ppm: ↓ bw Testes Degeneration germinal epithelium, 2/4 Vacuolization Leydig cells, 4/4 (3,2,2,3) Hypertrophy Leydig cells, 3/4 (3,3,4) Epididymides Aspermia, 2/4 Immature prostate, 4/4 (4,3,3,4)	**≥ 2000 ppm:** ↓ bw 48% ↓ r weight uterus 15% ↓ r weight ovaries
Wetzig, Ruh-Fehlert (2001)	**52-week oral dog** 0, 20, 50, 150, 600 ppm	**≥ 20 ppm:** no general toxicity effects ↑ ar testes weight **≥ 50 ppm:** no general toxicity effects ↑ ar testes weight, ↑ ar epididymis weight **≥ 150 ppm:** no general toxicity effects ↑ ar testes weight, ↑ ar epididymis weight Focal tubular degeneration testes, 1/4 (1) **≥ 600 ppm:** no general toxicity effects 30% ↑ r testes wt, 17% ↑ r epididymis wt 19% ↑ ar prostate weight Vacuolization Leydig cells, 4/4 (1,2,1,1) Hypertrophy Leydig cells, 1/4 (2) Focal tubular degeneration testes, 1/4 (2)	**≥ 20 ppm:** no general toxicity effects ↓ ar uterus/oviduct weight 29% ↓ r uterus/oviduct weight
Krottlinger, Sander (1999)	4-wk dermal rat	–	–
Eiben (2000)	**2-generation study rat** 0, 70, 350 & 1750 ppm	**F0:** ↓ bw dose related **≥ 70 ppm:** ↓ bw ↑sr prostate weight ↓srepididymides weight, ↓sr seminal vesicles **≥ 350 ppm:** ↓ s bw ↑sr prostate weight ↓sr epididymides weight, ↓sr seminal vesicles **≥ 1750 ppm:**↓ s bw ↑sr testes weight **Testes** (diminished in size) 0/25, 1/25, 1/25, **4/25** **Epididymides** (diminished in size)	**F0:** ↓ bw dose related **≥ 70 ppm:** – **≥ 350 ppm:** bw **≥ 1750 ppm:**↓ s bw –

		0/25, 1/25, 1/25, **4/25** **F1:**↓ bw dose related **≥ 350 ppm:** ↓ bw **≥ 1750 ppm:** ↓ s bw, ↑s food consumption **Mating/fertility/gestation*** spermatids per mg testis: -23% sperms per mg epididymides: -18% **Testes*** atrophy, diffuse: 0/25, 1/25, 1/25, 4/25** **Epididymides*** Oligospermia: 0/25, 1/25, 1/25, 4/25 Atrophy: 0/25, 1/25, 1/25, 4/25 **Prostate*** Atrophy: 0/25, 0/25, 0/25, 3/25	**F1** **≥ 350 ppm:** – **≥ 1750 ppm:** ↓ s bw ↑ ar uterus & ovaries weight

* Effects were observed in four specific animals where there was a sever decrease in body weight.
** The testes atrophy was within the HCD range.
a: absolute, r: relative, s: statistically significant, bw: body weight

The observed effects on testes (enlargement, hypertrophy, hyperplasia), uterus (enlargement) but also adrenals (enlargement, vacuolation) may be secondary to the effects or spirodiclofen on steroidogenesis i.e. an adaptive response. However, at the high dose levels clear effects on spermatogenesis (oligospermia, aspermia) were observed. Thus, the DS proposed that the adaptative effects (enlargement, hypertrophy, hyperplasia and vacuolation) should not be considered for classification in contrast to oligospermia and aspermia.

In the developmental toxicity studies on rabbits, in the high dose group, one female showed liver lobulation. In foetuses of the highest dose group, an increased incidence in liver lobulation was observed which was outside the range of historical controls, whereas the litter incidence for this type of effects was within the range of historical controls. It could therefore not fully excluded that the observed effect of liver lobulation was treatment related. However, no information on the severity of liver lobulation was presented in the study report.

Two neurodevelopmental toxicity studies in rats were presented. The first study showed negative results with the exception of some equivocal results for the water maze test. In a follow-up study in which parts of the developmental neurotoxicity study were repeated (and included two types of water maze tests) no neurotoxic effects were observed.

In the teratogenicity study with rats, no toxicologically relevant effects were observed.

Comments received during public consultation

Two MSCA's commented and agreed with the proposal by the DS for the classification of spirodiclofen for the effects on sexual function and fertility – Repr.2(H361f: Suspected of damaging fertility).

The second MSCA added the following reasoning: in the 2-generation study with rats, weights

of adrenals, ovaries and uterus in the F1 animals had changed. In the high dose group, decreases were observed in the number of spermatids in the testes and in the number of sperms in the epididymidis. In addition, effects on the reproductive organs were observed in the repeated dose toxicity and carcinogenicity studies. Effects on testes were observed in all studied species (i.e. mouse, rat, dog), though most pronounced in dogs. These effects included increased testis weight (absolute + relative), hyperplasia, hypertrophy and vacuolation of testis, but also oligo- and aspermia (in 4- and 14-week dog studies, 18-month mouse study). Further, changes of weight of uterus/oviduct and ovaries were observed in female animals.

Assessment and comparison with the classification criteria

Effects on fertility and sexual function

In the 2-generation study (Eiben, 2000) in rats there were effects on sexual function parameters such as decreased number of spermatids in testes, testes atrophy and decreased number of sperms in the epididymis. The effects were seen at doses with general toxicity and the testes atrophy was within HCD (Table 53 of the CLH report) excluding the evidence of clear substance related effects. Moreover, in the 14 weeks toxicity study in rats at high dose levels an increase in testes weight was observed, which could be a compensation effect for the spirodiclofen induced hormonal disruption/depletion.

RAC notes that in the repeated dose toxicity and carcinogenicity studies there were clear effects on the sexual function/fertility parameters as well as on the reproductive organs in all tested species although more pronounced in dogs since the effects were observed at lower doses than the ones in mice and rats and clearly at levels where there is no general toxicity. RAC concluded that although it could be argued that some of the observed effects are adaptive, the oligospermia and aspermia seen in dogs are **not** secondary effects and could be relevant for the evaluation of possible fertility effects of spirodiclofen in humans. It is noted that the aspermia observed in the 4 week dog study was massive but occurred in one dog with relative testes weight 4.6% less than the average of the control and testing animals and with a relative prostate weight reduction of 56%. Morever, the aspermia observed in the 18 month carcinogenicity study in mice increased significantly both in frequency and severity but only at the high dose. Therefore, aspermia was observed in two species but the significance of the incidence is limited. In addition, the Leydig cell and degeneration of the germinal epithelium effects as well as the uterus/oviduct/ovaries effects observed in dogs are consistent and not in general toxicity doses and should also be considered for classification purposes. Thus, the afore mentioned observed effects in dogs should be taken into account for potential classification for effects on sexual function and fertility. Similarly, leydig cell hypertrophy/activation was observed at the mid dose in mice where there were no signs of general toxicity effects. At the high dose, leydig cell vacuolation (Leser 1998) and hypertrophy/hyperplasia of interstitial cells (Wahle 2000) were seen. In rats, at the high dose in the carcinogenicity study focal leydig cell hyperplasia was observed (Wirnitzer 2000). A table summarising the sexual function/fertility/reproductive organ effects is presented hereafter:

Effect/target reproductive organ	Mouse	Rat	Dog
Aspermia/Oligospermia	+	-	++
Testes	+	+	++

Epididymides	+	-	++
Prostate	-	-	++
Ovaries/corpora lutea	+	+	++
Uterus	-	++	++

+: slight/moderate; ++: pronounced; -: not observed

Therefore, RAC concludes that although there is only one adverse effect observed (aspermia/oligospermia), in combination with the effects on reproductive organs (more pronounced in dogs) there is enough evidence for classification as Repr.2; H361f: Suspected of damaging fertility.

Developmental toxicity

RAC considers the results of the rabbit study as being equivocal. In the highest dose group foetuses show incidence for liver lobulation outside the HCD for the specific strain but in the litter the incidences lie within the HCD. In addition there is no data regarding the severity of the effect but based on the HCD for foetuses and litters it is more likely that the severity of this type of effect is low. Thus, it is not clear whether the effect of liver lobulation is treatment related and therefore RAC believes that it should not be considered for classification. In the rat developmental toxicity study, no substance related effects were observed and therefore RAC agrees that no classification is required for developmental toxicity.

RAC also agrees with the DS that no classification is required regarding effects on or via lactation. There is lack of data on the concentration of spirodiclofen in milk and therefore no conclusion can be drawn whether the effects observed during lactation are due to the transfer of spirodiclofen to the offspring via milk.

Conclusions on classification and labelling

In conclusion, RAC agrees with the DS, that spirodiclofen should be classified for effects on sexual function and fertility as **Repr. 2 (H361f: Suspected of damaging fertility)**.

Spirodiclofen may also contain the impurity N,N-dimethylacetamide. DMAC has a harmonised classification for reproductive toxicity as Repr.1B (H360D: May damage the unborn child) with a generic concentration limit (GCL) of 0.3%. Therefore, the presence of DMAC above the GCL could result in an additional classification of spirodiclofen for developmental toxicity as Repr.1B (H360D: May damage the unborn child)

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

STUDY 1

Characteristics

reference	:	L.P. Sheets, R.G. Gilmore, B.P Stuart (2000)	exposure	:	Single dose, sacrifice 15 days after exposure

type of study	:	Acute oral neurotoxicity study.	Doses[1]	:	0, 200, 500, 2000 mg/kg bw (actual doaes: 0, 230, 563, 2000 mg/kg bw)
year of execution	:	1998	vehicle	:	0.5% methylcellulose / 0.4% Tween 80 in deionized water
test substance	:	BAJ 2740 (spirodiclofen, purity 97.7%, 97.9%)	GLP statement	:	yes
route	:	Oral (gavage)	guideline	:	In accordance with OPPTS , U.S. EPA, Health Effects Test Guideline 870.6200, Neurotoxicity Screening Battery (1998).
species	:	Rat (Wistar Crl:WI(HAN)BR)	acceptability	:	acceptable
group size	:	12/sex/dose	**NOAEL** (neurotoxicity)	:	2000 mg/kg bw

1 doses were selected on the basis of results from an acute oral dose-range finding study.

Study design

The following observations and measurements were included in the study: clinical observations, mortality check, body weight, automated measurements of activity (figure-eight maze), a functional observational battery, brain weight, gross necropsy, and microscopical examination (controls and high dose group, 6/sex/group) of skeletal muscle, peripheral nerves, eyes (with optic nerves) and tissues from the central nervous system for lesions. Motor and locomotor activity were examined for 90-minute sessions and during each ten-minute intervals. Motor activity was measured as the number of beam interruptions that occured during the test session. Locomotor activity was measured by eliminating consecutive counts for a given beam. Habituation was evaluated as a decrement in activity during the test session.

Based on the estimated time of peak of neurobehavioral effects, the FOB started at approximately four hours (minimum) following administration of the dose and the automated measurements of activity at approximately seven hours following treatment and were determined again after 7 and 14 days following treatment.

The study was performed following U.S. EPA pesticide guidelines. However, evaluation is done on the basis of OECD guidelines. The study was performed in accordance with OECD guideline 424 (deviation: room temperature was 18 - 26°C instead of 22 ± 3°C).

Results

The results of the study are presented in Table 58.

Table 58 Overview of the results of the rat acute oral neural toxicity study (Sheets, 2000)

Dose (mg/kg bw)	**0**		**200**		**500**		**2000**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none								
Clinical signs	No toxicologically relevant effects								
Body weight (gain)	No toxicologically relevant effects								
Functional observational battery	No toxicologically relevant effects								
Motor/locomotor activity measurements	No toxicologically relevant effects								

Dose (mg/kg bw)	0		200		500		2000		dr
	m	f	m	f	m	f	m	f	
Gross pathology	No toxicologically relevant effects								
Brain weight	No toxicologically relevant effects								
Neuropathology	No toxicologically relevant effects								

dr dose related

Acceptability

The study was considered acceptable.

Conclusions

No compound-related effects were observed in the present acute neurotoxicity study. Therefore, the NOAEL for acute neurotoxicity in this study is 2000 mg/kg bw, the highest dose tested.

STUDY 2

Characteristics

Reference	:	Sheets, L.P., R.G. Gilmore , B.P. Stuart (2001)	exposure	:	13 weeks
type of study	:	Subchronic neurotoxicity screening study.	Doses[1]	:	Nominal: 0, 100, 1000, 12500 ppm; (0, 7.2, 70.3, 1088.8 mg/kg bw/day in males and 0, 9.1, 87.3, 1306.6 mg/kg bw/day in females)
year of execution	:	1999	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 97.8%, 97.4%)	GLP statement	:	yes
Route	:	Oral (diet, with corn oil (1% w/w); acetone as solvent: evaporated)	guideline	:	OPPTS, U.S. EPA, Health Effects Test Guideline 870.6200, Neurotoxicity Screening Battery (August 1998).
Species	:	Rat, Wistar Crl:WI (HAN)Br	acceptability	:	acceptable
group size	:	12/sex/dose (neurobehavioral evaluation); 6/sex/dose (micropathology)	**NOAEL** (neurotoxicity)	:	70 mg/kg bw/dag

1 doses are based on a subchronic (14-week) oral toxicity study with rats.

Study design

Groups of 12 rats/sex were given feed containing spirodiclofen 7 days per week for up to 13 weeks for evaluation of potential neurotoxicity. All rats completed a functional observational battery (FOB), landing foot splay and grip strength, automated measurements of motor activity (figure-eight maze) (weeks 0, 4, 8 and 13). Motor and locomotor activity were examined for 90-minute sessions and during each ten-minute intervals. Motor activity was measured as the number of beams interruptions that occurred during the test session. Locomotor activity was measured by eliminating consecutive counts for a given beam. Habituation was evaluated as a decrement in activity during

the test session. In addition to these observations, animals were examined for clinical signs, mortality, body weight, food consumption, ophthalmic changes, brain weight, gross necropsy and microscopic changes (skeletal muscle, peripheral nerves, eyes, with optic nerves and tissue of the central nervous system).

Neuropathologic evaluations were completed on 6 rats/sex/dose at 13 weeks. The study was performed following U.S. EPA pesticide guidelines. However, evaluation is done on the basis of OECD guidelines. The study was performed in accordance with OECD guideline 424.

Results

The results of the study are presented in Table 59.

Table 59 Overview of the results of the rat subchronic neurotoxicity study (Sheets, 2001)

Dose (ppm)	**0**		**100**		**1000**		**12500**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none								
Clinical signs									
- urine stain							9/12	12/12	
- oral stain								3/12	
- red-tinged stains (various locations: paws, snout, forelimbs, ears)[1]								4/12	
Body weight (gain)							ds	ds	
Food consumption							ds	ds	
Ophthalmology	No toxicologically relevant effects								
Functional observational battery									
- urine stain (yellow)[2]							11/12	11/12	
- red tinged paws[2]							3/12	2/12	
- decreased foot splay							d	d	
- forelimb/hindlimb grip strength							d	d	
Motor activity measurements									
- motor activity, week 4								-29%	
- locomotor activity, week 4								-28%	
- summary interval motor activity, week 4, all intervals								d	
- summary interval locomotor activity, week 4, intervals 3-9								d	
Habituation[3]	No toxicologically relevant effects								
Brain weight									
- relative brain weight							is	is	
Gross pathology neural tissue									
- ventrum wet/stained							7/7	12/12	
Histopathology neural tissue[4]	No toxicologically relevant effects								

dr dose related

1 stains at various locations observed in 1 to 4 females.

2 Observed at all time points. In males, the number increased with increaseing exposure time, whereas in females the number did not increase.
3 According to the study author, habituation was not affected. However, this conclusion was not supported by data (time-measured ANOVA).
4 Control and high dose animals only.

Acceptability

The study is considered acceptable.

Conclusions

Compound-related effects were observed in the high-dose animals only, and included significantly decreased body weight (18-25% in males and 8-15% in females) and food consumption, urine stain, red tinged paws, stained ventrum and significantly increased relative brain weights.

In the highest dose group decreased foot splay was observed (20-22% in males and 12-16% in females) and decreased forelimb/hindlimb grip strength (1-17% in males and 2-15% in females). The study author ascribes the decreased foot splay and decreased grip strength to the lower body weight. However, a compound-related effect cannot be excluded.

Based on the observed decreased body weights and neurobehavioral effects in the high dose group, the NOAEL in this study is, in accordance with the opinion of the study author, set at 1000 ppm, equal to 70 mg/kg bw/day.

Remark: The study author concluded that the findings are expected to be reversible following the discontinuation of exposure. No data were included which supported this conclusion, however.

STUDY 3

Characteristics

reference/notifier	:	Wirnitzer, U., U. Bach, E. Hartmann (2000)	exposure	:	77 weeks
type of study	:	Functional observation battery (in: combined study on chronic toxicity and carcinogenicity in Wistar rats. Dietary administration over 2 years).	doses	:	0, 50, 100, 350, 2500 ppm (2.04, 4.11, 14.72, 110.14 mg/kg bw/day in males and 2.87, 5.93, 19.88, 152.90 mg/kg bw/day in females)
year of execution	:	1997-1999	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen,purity 98.6%, 98.5%, 97.9%, 97.8%, 97.6%, 97.8%)	GLP statement	:	yes
route	:	Oral (diet, with 1% peanut oil)	guideline	:	In accordance with OECD 453
species	:	Wistar rat (Hsd Cpb:WU)	acceptability	:	acceptable
group size	:	50/sex/dose	**NOAEL** (neurotoxicity)	:	110.14 mg/kg bw/day

Study design

This study was part of of a combined study on chronic toxicity and carcinogenicity (see section B.6.5.1). Neurotoxicity was tested on week 77 (Functional Observational Battery, FOB) with the observer aware of the animal's treatment assignment and the test runs were done in the same order (control-50 ppm- 100 ppm- 350 ppm- 2500 ppm).

The FOB included home cage and open field observations, neuromuscular and sensorimotor tests involving handling of the rat.

Results

The results of the study are summarised in Table 60.

Table 60 Overview of the results

Dose (ppm)	0		50		100		350		2500		dr
	m	f	m	f	m	f	m	f	m	f	
Mortality	No toxicologically relevant effects										
Clinical signs	No toxicologically relevant effects										
Body weight (gain)[1]									ds	ds	
Functional observational battery	No toxicologically relevant effects										
Motor activity measurements	No toxicologically relevant effects										
Neuropathology	No toxicologically relevant effects										

dr dose related

[1] The difference was significant nearly throughout the whole treatment period in males (up to 11%) and from week up to and including week 53 in females (up to 8%).

Acceptability

In spite of the awareness of the observer of the animal's treatment and the test runs performed in the same order (increasing concentrations), the study was considered acceptable.

Conclusions

The FOB showed no toxicologically relevant signs or symptoms indicating evidence for neurotoxic potential in rats exposed to spirodiclofen for 77 weeks. Based on these results the NOAEL for neurotoxicity in this study is 2500 ppm, equal to 110 mg/kg bw/day, the highest dose tested.

STUDY 4

Characteristics

Reference	:	Sheets *et al.,* 2004	Route	:	oral, diet
Year of execution	:	2002	Group size	:	30 females/dose
Guideline	:	OPPTS 870.6300 (EPA, 1998)	Exposure	:	gestation day (GD) 0 to lactation day (LD) 21 (dams) to post-natal day 21 (offspring)

GLP	:	yes	Doses	:	0, 70, 350, 1500 mg/kg food[1]
Test substance	:	Spirodiclofen, batch no. 06480/0002; beige powder	Vehicle	:	diet
Purity	:	97%	Acceptable	:	yes
Species	:	rat, Wistar Hannover Crl:WI (Glx/BRL/Han) IGS BR	**NOAEL**mat	:	350 mg/kg food (32 mg/kg bw/d)
			NOAELdev	:	350 mg/kg food (32 mg/kg bw/d)
			neurotoxic effects		not observed

[1] equal to 0, 6.5, 32, 136 mg/kg bw/d (gestation) and 0, 14, 70 and 274 mg/kg bw/d (lactation)

Study design

The study was performed in accordance with EPA guideline OPPTS 870.6300. In short, the study was executed as follows:

Animals were exposed as described above. On post-natal day (PND) 4, litters with a minimum of eight pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing at least 20 litters per level, were subjected to evaluation using the following observations and measurements: preputial separation or vaginal patency, body weight, food consumption, a functional observational battery (FOB), automated measures of activity (figure-eight maze), acoustic startle habituation, learning and memory (passive avoidance after weaning and a water maze task on PND 60) and an ophthalmic examination. Serum cholesterol was measured in the dams (LD 21) and offspring (PND 4 and 21) and neural tissues were collected from 10 rats/sex/dietary level (representing approximately 20 litters) on PND 21 (brain only) and at study termination (approximately 75 days of age) for microscopic examination and morphometry.

The following modifications of the guideline were implemented:

- Increased number of offspring for morphometric and neuropathological evaluation (10/sex/dose instead of 6)
- Extended exposure (GD 0 to PND21 instead of GD 6 to PND 10).
- Animals were perfused before collecting brains at the end of exposure on PND 21 and 75.

The first two changes increased the sensitivity of the study, while the last increased the quality of the samples. Therefore, these changes with respect to the guidelines are considered acceptable.

Results

The results are presented in Table 61, 62 and 63.

Table 61 Overview of results for dams and litter data

Dose (mg/kg food)	**0**	**70**	**350**	**1500**	**dr**
F-0 dams					
Mortality	0/30	1/30	0/30	0/30	

Dose (mg/kg food)	**0**	**70**	**350**	**1500**	**dr**
Clinical signs	no treatment related findings				
Pregnant animals	29	29	29	30	
Body weight					
GD 0-20	no treatment related findings				
LD 0-14	no treatment related findings				
LD 14-21			d (4%)	dc (5%)	
Food consumption					
GD 0-6				dc (16%)	
GD 6-20	no treatment related findings				
LD 0-7	no treatment related findings				
LD 7-14			d (6%)	dc (8%)	
LD 14-21	no treatment related findings				
FOB	no treatment related findings				
Organ weight					
Pathology					
macroscopy	no treatment related findings				
Litter data					
Life foetuses	no treatment related findings				
Viability index	no treatment related findings				
Lactation index	no treatment related findings				
Pup weight					
PND 0-11	no treatment related findings				
PND 17			d (5%)	dc (5%)	
PND 21			d (4%)	dc (8%)	yes
Pup weight gain			d (5%)	dc (9%)	yes

dr dose related
dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
a/r absolute/relative organ weight

Table 62 Overview of results for F-1

Dose (mg/kg food)	**0**		**70**		**350**		**1500**		**dr**
	m	f	m	f	m	f	m	f	
F-1 animals									
Clinical signs	no treatment related findings								
Body weight	no treatment related findings								

Dose (mg/kg food)	**0** m	f	**70** m	f	**350** m	f	**1500** m	f	**dr**
Food consumption	no treatment related findings								
Sexual maturation	no treatment related findings								
Pupil constriction (PND 21)	no treatment related findings								
Ophthalmoscopy	no treatment related findings								
FOB	no treatment related findings								
Motor and locomotor activities	no treatment related findings								
Acoustic startle	no treatment related findings								
Passive avoidance	no treatment related findings								
Water maze									
Learning phase	no treatment related findings								
Retention phase - trials to criterion[1]				ic (47%)		ic (26%)		i (53%)	
Clinical chemistry	no treatment related findings								
Brain weight	no treatment related findings								
Pathology									
<u>macroscopy</u>	no treatment related findings								
<u>microscopic measurements</u>	m	f	m	f	m	f	m	f	
- caudate putamen size PND-21			ne	ne	ne	ne		dc (3%)	
- caudate putamen PND-75			ne	ne	ne	ne		ic (3%)	
- parietal cortex PND-75			ne	ne	ne	ne	dc (6%)		
<u>histopathology</u>	no treatment related findings								

dr dose related
dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
a/r absolute/relative organ weight
ne not examined
[1] tested for significant differences between control group and individual treated groups with Fisher's exact test

Table 63 Trials to criterion in water maze test for female rats in retention phase

Dose (mg/kg food)	**0**	**70**	**350**	**1500**
Number of Animals	16	16	16	16
Trials to Criterion (Mean + S.D.)	5.8 ± 1.9	8.5 ± 3.7*	7.3 ± 2.3*	8.9 ± 4.5

$p < 0.05$ in with Fisher's exact test

Acceptable

The study is considered acceptable.

Conclusions

The water maze test showed a significantly decreased memory performance for low and mid dose females. In high dose females, this performance was also less than in the control group, but the difference was not statistically significant. No clear dose relation was observed and the dispersion of the measurements was quite high, varying between ca. 30 to 50%, increasing the likelihood of false positive results. Therefore, it is doubtful that the test substance had a negative influence on memory performance in the water maze test. Furthermore, according to a re-analysis of the study results made by a third party (de Raat and Verhaar (2005)), not linked to the laboratory that executed the study) in assignment of the notifier the number of trials to criterion is an inadequate measure of learning and retention, as it disregards the amount of errors made before reaching the criterion (five consecutive errorless trials). Instead de Raat and Verhaar performed analyses of covariance using the variables "number of errors to criterion", "dose" and "sex". The outcome of these analyses showed that treatment did not influence the number of errors made, be it in the learning or retention phase of male or female rats.

Microscopic brain sections of high dose and control groups of the offspring were measured at PND-21 and PND-75. At PND-21, in females, the caudate putamen was decreased in size, while at PND-75 it was increased. At PND-75 the parietal cortex size was decreased in males. All these changes were relatively small (between 3 and 6%). Furthermore, no histopathological changes were observed in the brain areas concerned. Therefore, these changes in brain measurements are not considered of toxicological significance.

Only in the high dose group (1500 mg/kg food), statistically significant, but minor, effects were observed both in the dams as in the offspring. In the dams these effects were limited to occasional decreases in food consumption in the first days of gestation and midway lactation, which only in the last week of lactation led to decreased body weight, be it minor (5%). In the offspring, pup weight (gain) was reduced. Based on these effects, the NOAEL for maternal and developmental effects is set at 350 mg/kg food (equal to 32 mg/kg bw/d; LOAEL 136 mg/kg bw/d).

STUDY 5

Characteristics

Reference	:	Gilmore *et al.*, 2007	Route	:	oral, diet
Year of execution	:	2006	Group size	:	30 females/dose
Guideline	:	OPPTS 870.6300 (EPA, 1998)	Exposure	:	gestation day (GD) 0 to lactation

					day (LD) 21 (dams) to post-natal day 21 (offspring)
GLP	:	yes	Doses	:	0, 70, 350, 1500 mg/kg food[1]
Test substance	:	Spirodiclofen, batch no. 06480/0002; beige powder	Vehicle	:	diet
Purity	:	98.3%	Acceptable	:	yes, as supplementary study
Species	:	rat, Wistar Hannover Crl:WI	**NOAEL**mat	:	n/a (1500 mg/kg food (119 mg/kg bw/d)[2]
			NOAELdev	:	n/a (350 mg/kg food (29 mg/kg bw/d)[2]
			neurotoxic effects	:	not observed

[1] equal to 0, 5.3, 29, 119 mg/kg bw/d (gestation) and 0, 13, 66 and 263 mg/kg bw/d (lactation)

[2] due to the limited number of parameters investigated no NOAELs to be used in risk assessment may be derived based only on this study

Study design

The study was set-up to complement an earlier study by Sheets *et al*. (2004), which had produced equivocal results with the M-maze test. Like the previous study, it was performed in accordance with EPA guideline OPPTS 870.6300, but with a restricted number of parameters being observed and reported. In comparison with the earlier study, one type of measurement procedure for memory effects was added: the Cincinnati water maze. Whereas the M-maze focuses on associative learning and memory, primarily involving the hippocampus, the labyrinth Cincinnati Water maze does not allow spatial navigation and is a test of path integration ability, requiring an intact entorhinal cortex where information from the hippocampus is integrated.

In short, the study was executed as follows:

Animals were exposed as described above. On post-natal day (PND) 4, litters with a minimum of seven pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing at least 18-20 litters per level, were subjected to evaluation using the following observations and measurements: detailed clinical observations, body weight, tests of spatial learning and memory (i.e., M- and Cincinnati Water Mazes) beginning on PND 60 (± 2 or 4 days, respectively) and an ophthalmic examination. Whole-brain tissue was collected from 10/sex/dietary level (representing 20 litters) on PND 21 and at study termination (approximately 75 days of age) for morphometry.

The following modifications of the guideline were implemented:

- Increased number of offspring for morphometric and neuropathological evaluation (10/sex/dose instead of 6)
- Extended exposure (GD 0 to PND21 instead of GD 6 to PND 10).
- Animals were perfused before collecting brains at the end of exposure on PND 21 and 75.

The first two changes increased the sensitivity of the study, while the last increased the quality of the samples. Therefore, these changes with respect to the guidelines are considered acceptable.

Results

The achieved intakes of spirodiclofen in this study were lower than those in the earlier study by Sheets *et al*. (2004): 18, 9, and 13% less at low, mid and high dose, respectively, during gestation and 7, 6 and 4% less at low, mid and high dose, respectively, during lactation. As the disputed maze findings of Sheets *et al*. were observed at all dose levels, the lower intakes in this study do not

hamper a good re-evaluation of these effects.

Performance in the M-maze test was evaluated on the basis of the number of trials to criterion and the number of errors to criterion. Using these criteria, no treatment related differences were observed between treated and control groups.

An overview of the results is presented in Tables 64-67.

Table 64 Overview of results for dams and litter data

Dose (mg/kg food)	**0**	**70**	**350**	**1500**	**dr**
F-0 dams					
Mortality	0/30	0/30	0/30	0/30	
Clinical signs	no treatment related findings				
Pregnant animals	28	27	29	30	
Body weight					
GD 0-20	no treatment related findings				
LD 0-21	no treatment related findings				
Food consumption					
GD 0-20	no treatment related findings				
LD 0-7				d (5%)	
LD 7-14				d (4%)	
LD 14-21				d (5%)	
FOB	not included in assessment				
Organ weight	not included in assessment				
Pathology					
macroscopy	not included in assessment				
Litter data					
Life foetuses	no treatment related findings				
Viability index	no treatment related findings				
Lactation index	no treatment related findings				
Pup weight					
PND 0-11	no treatment related findings				
PND 17				d (4%)	
PND 21				dc (8%)	
Pup weight gain				dc (9%)	

dr dose related

dc statistically significantly decreased compared to the controls

d decreased, but not statistically significantly compared to the controls

Table 65 overview of results for f-1

Dose (mg/kg food)	**0** m f	**70** m f	**350** m f	**1500** m f	**dr**
F-1 animals					
Clinical signs	no treatment related findings				
Body weight (post-weaning)	no treatment related findings				
Food consumption	no treatment related findings				
Sexual maturation	not included in assessment				
Pupil constriction (PND 21)	not included in assessment				
Ophthalmoscopy	no treatment related findings				
FOB	not included in assessment				
Motor and locomotor activities	not included in assessment				
Acoustic startle	not included in assessment				
Passive avoidance	not included in assessment				
Water mazes					
M-maze					
Learning phase	no treatment related findings				
Retention phase	no treatment related findings				
Cincinnati maze					
Learning phase	no treatment related findings				
Retention phase	no treatment related findings				
Clinical chemistry	not included in assessment				
Brain weight	no treatment related findings				
Pathology					
macroscopy (only brain examined)	no treatment related findings				
microscopic measurements	no treatment related findings				
histopathology	not included in assessment				

dr dose related

Table 66 Summary of results m-maze test (mean ± standard deviation) (errors are presented as numbers)

Session	Parameter	Dose (mg/kg food)			
		0	70	350	1500
Males					
Learning Phase	Number of animals	16	16	16	15
	Trials to criterion	6.4±1.4	6.3±2.5	7.9±2.4	7.6±2.1
	Trial 1 - Errors	0.6±0.6	0.6±0.9	0.6±0.8	1.2±1.1
	Trial 2- Errors	0.4±0.5	0.3±0.4	0.7±0.8	1.0±1.0
Retention Phase	Number of animals	16	15	16	15
	Trials to criterion	5.4±1.0	6.5±2.4	5.6±1.4	6.7±2.6
	Trial 1 - Errors	0.2±0.4	0.3±0.5	0.3±0.7	0.1±0.3
	Trial 2 - Errors	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Females					
Learning Phase	Number of animals	16	16	15	16
	Trials to criterion	7.6±2.6	7.3±2.1	8.1±3.1	7.3±2.9
	Trial 1 - Errors	0.8±1.0	0.8±1.1	0.9±1.2	0.4±0.8
	Trial 2 - Errors	0.7±1.1	0.9±1.1	0.7±1.3	0.5±1.0
Retention Phase	Number of animals	15	16	14	15
	Trials to criterion	7.9±3.6	8.4±3.9	8.0±3.8	8.9±4.2
	Trial 1 - Errors	0.0±0.0	0.4±0.9	0.4±0.6	0.1±0.4
	Trial 2 - Errors	0.2±0.6	0.3±0.6	0.1±0.5	0.1±0.4

Table 67 Summary of results cincinnati maze test (mean ± standard deviation) (latency in seconds; errors are presented as numbers)

Session	Parameter	Dose (mg/kg food)							
		0		70		350		1500	
Males									
Learning Phase	Number of animals	10		10		10		10	
		Latency	**Errors**	**Latency**	**Errors**	**Latency**	**Errors**	**Latency**	**Errors**
	Day 1	300±0	18.5±3.1	286±35	17.3±3.8	300±0	17.8±4.2	289±28	16.6±3.9
	Day 2	241±84	17.6±7.8	227±94	15.8±5.8	217±85	16.0±6.4	269±31	16.7±3.9
	Day 3	175±117	9.9±7.6	118±109	6.0±5.1	104±60	6.5±4.9	171±100	9.7±5.7
	Day 4	80±60	4.8±5.5	100±105	4.8±7.2	48±19	1.8±1.8	96±96	4.1±5.6
	Day 5	52±37	2.0±3.3	63±86	2.1±4.2	48±37	0.9±1.5	44±25	1.1±1.1
Retention Phase	Number of animals	10		9		10		10	
	Day 12	42±19	1.0±1.1	40±26	1.9±3.3	40±16	0.4±0.5	35±21	0.5±0.9
Females									
Learning Phase	Number of animals	10		10		10		10	
		Latency	**Errors**	**Latency**	**Errors**	**Latency**	**Errors**	**Latency**	**Errors**
	Day 1	278±68	20.2±6.4	280±34	22.3±4.0	296±14	21.7±5.9	282±57	21.5±5.8
	Day 2	242±82	17.9±6.0	218±83	16.7±6.3	224±85	17.5±8.2	178±102	16.1±9.4
	Day 3	154±99	11.2±7.6	112±73	8.0±7.0	110±108	5.8±6.1	85±59	6.1±5.3
	Day 4	81±66	4.5±5.5	42±18	2.0±1.4	66±86	2.8±4.7	52±45	2.3±2.8
	Day 5	41±30	1.4±1.9	24±6.3	0.5±0.7	57±87	1.4±2.0	35±19	1.7±1.2
Retention Phase	Number of animals	10		10		9		10	
	Day 12	72±69	4.9±7.4	57±50	1.8±2.9	49±46	2.2±3.6	89±85	5.0±5.8

Acceptability

The study is considered acceptable as a supplementary study to the study performed by Sheets *et al*. (2004).

Conclusions

Both the M-maze and the Cincinnati maze test demonstrated no significant differences between control and treated groups at any of the dose levels investigated. This confirms the interpretation of the equivocal results of the M-maze test reported by Sheets *et al*. (2004) that there is no effect of spirodiclofen on the performance of the offspring of treated rats in this type of learning and memory test.

Only in the high dose group (1500 mg/kg food), minor effects were observed both in the dams and in the offspring. In the dams these effects were limited to decreases of ca. 5% in food consumption during the lactation period, which were not statically significant and did not lead to decreased body

weight and are therefore not considered toxicologically relevant. In the offspring, pup weight (gain) was reduced by ca. 8 to 9% (statistically significant). Based on these effects, the NOAEL for maternal and developmental effects in this study is considered to be respectively 1500 mg/kg food (equal to 119 mg/kg bw/day) and 350 mg/kg food (equal to 29 mg/kg bw/d). The changes in pup weight were reversible, as after weaning no significant differences in body weight were observed between treated and control animals in the F-1 generation. No neurodevelopmental effects were observed in this study. The effects observed in this study are identical to those observed in the earlier study by Sheets *et al*. (2004), and occurred at the same food level of spirodiclofen.

4.12.1.2 Immunotoxicity

STUDY 1

Characteristics

reference/notifier	:	Wirnitzer, U., A. Romeike (1998)	exposure	:	4 weeks for immunotox (main study 14 weeks)
type of study	:	14 week oral toxicity study with 4 week recovery phase (see B.6.3.4 study 2)	Doses[1]	:	0, 100, 500, 2500 and 12500 ppm (0, 8.6, 44.7, 232.4, 1284.9 mg/kg bw/day for males and 0, 9.3, 45.0, 237.6, 1466.1 mg/kg bw/day for females)
year of execution	:	1996	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 99.1%)	GLP statement	:	yes
route	:	Oral (diet, with 1% peanut oil)	guideline	:	Not completely in accordance with OECD 408
species	:	Rat, Wistar (Hsd Cpb:WU)	acceptability	:	acceptable
group size	:	5/sex/dose for immunotox (main study 10/sex/dose)	**NOAEL**	:	45 mg/kg bw/day

[1] doses are based on results of a subacute study with female rats (0 to 5000 ppm).

Study design

The main study (14 weeks oral toxicity study) was performed in accordance with OECD guideline 408, with the following deviations: sensory reactivity to stimuli of different types was not investigated. For immunotoxicological investigations satellite groups of only 5 animals/sex/dose were orally exposed to spirodiclofen only for 4 weeks. The main study is described in section 4.7.1.1.

The following investigations were carried out: cell counts in the spleen, lymph nodes and bone marrow, FACScan analyses for determination of subpopulations in the spleen cells and lymph node cells (pooled samples for each dose group) with surface markers of B-cells (PanB; RLN-9D3), antigen presenting cells (I-a; OX3), T-helper cells (CD4; OX-35), lymphocytes (CD45; OX-22), T-cells (CD2), interleukin-2 receptor expressing cells (CD25; OX-39) and double labeling was done with CD4/CD45R (15-17) and CD2/CD25 , macrophage activity in the spleen and the mesenteric lymph nodes, sera antibody titers (IgG, IgM and IgA), 5 animals/dose were immunized i.v. with sheep erythrocyres (SRBC) for performing the Plaque-Forming Cell Assay (PFCA), and organs and/or sera were examined.

Results

The results of the study are summarized in Table 68

Table 68 Overview of the results of the rat 14-week study for immunotoxicity (Wirnitzer, 1998)

Dose (ppm)	**0**		**100**		**500**		**2500**		**12500**		**dr**
	m	**f**	**m**	**f**	**m**	**f**	**m**	**f**	**m**	**f**	
Mortality	none										
Clinical signs	No toxicologically relevant effects										
Body weight (gain)							d	d	ds	ds	m, f
Food consumption	No toxicologically relevant effects										
Water consumption									d	d	
Ophthalmoscopy	Not performed										
Urinalysis	Not performed										
Haematology	Not performed										
Clinical chemistry	Not performed										
Organ weights	Not performed										
Pathology											
macroscopy	Not performed										
microscopy	Not performed										
Immunology											
Cell counts[1]											
-spleen							d		d		m
-lymph node								d		d	
Subpopulation composition (FACScan)[2,3]											
-spleen T-helper cells (CD4/CD45^{low})							d		ds	d	m
-spleen lymphocytes (CD45)							d		ds	ds	m
-spleen T-cell marker (CD2)					ds		d		d		
-spleen B-cells (PanB)										d	
Macrophage activity[4]											
Lymph node, after PMA stimulation			i						d		
Plaque forming cell assay (anti-SRBC)											
-IgM										d	
-IgG										d	
Serum antibody titer											
-IgM					is						
-IgA								ds		ds	

dr dose related
dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased.
[1] individual data were not included
[2] remark of the study author: since the marker expression on cells was performed in pooled samples, the statement "significant" is made on the basis of the range of variance of our "historical data ' of control animals. A difference of more than 1 SD from mean or a distinct dose dependency is called 'tendency" while a difference of more than 2 SD is called "significant". However, historical data are not included.
[3] due to cell destruction, evaluation of marker expression on lymph node cells was not possible
[4] remark of the study author: due to a machine error the samples of the females have not been stimulated and a reasonable analysis of the macrophage activity of the females was not possible.

Acceptability

The study was considered acceptable for immunotoxicologal investigation after 4 weeks oral exposure study, although only 5 animals/sex/dose were examined and for FACScan determination pooled samples per dose were used and compared to historical data, which were not not included.

Conclusions

The observed increased PMA stimulated macrophage activity in males of the lowest dose group and serum IgM titer in males dosed 500 ppm were not observed in males of the higher dose groups, and these effects are considered not toxicologically relevant. The decrease in spleen T-cell marker in males dosed 500 ppm was also observed in males of the higher dose groups. Since this was the only effect observed in this dose group and the effect was not dose-related, the NOAEL was not based on this effect only. Based on the observed dose-related decreased bw in the two highest dose groups, and the observed effects on immunological parameters in the two highest dose groups, the NOAEL in this study is 500 ppm, equal to 45 mg/kg bw/day.

4.12.1.3 Specific investigations: other studies

STUDY 1

Characteristics

reference/notifier	:	U. Schmidt (2001a)	exposure	:	8 weeks
type of study	:	Testicular toxicity study in dogs.	doses	:	0, 100, 2000 ppm
year of execution	:	1998	vehicle	:	unknown
test substance	:	BAJ 2740 (spirodiclofen, purity unknown)	GLP statement	:	no
Route	:	Oral (diet)	guideline	:	unknown
Species	:	Beagle dogs	acceptability	:	acceptable
group size	:	5m/dose		:	

Study design

This study was performed to investigate a possible mechanism involved in the observed cholesterol-reducing potential of spirodiclofen, which was obvious in many toxicological studies in several species. In order to investigate involvement of inhibition of HMG-CoA reductase in the observed spirodiclofen induced decreased cholesterol levels, besides cholesterol and triglycerides also concentrations of α-tocopherol and ubiquinone in plasma and liver were determined as well as concentrations of dolichols and dolicholphosphates (chain length of 17-22 isoprene units) in the target organ testes were investigated (dolichols and dolicholphosphates have a function as membrane components and for N-glycosylation of proteins also in the Leydig cells). In liver tissue, GSH concentration was measured.

Results

In this study, plasma cholesterol and triglycerides were reduced in the high dose group to 70% and 88% respectively. Plasma ubiquinone (Q10) was significantly reduced (to 64%) and also the

dolichols and dolicholphosphates were (not-significantly) decreased in the testes (up to about 80%, dose-related).

In plasma and liver tissue, α-tocopherol was significantly reduced (to 42% and 46% respectively).

In liver tissue, GSH concentration was increased (about 120%) in treated groups.

Acceptability

The study was considered acceptable.

Conclusions

According to the study author, an effect on the HMG-CoA reductase as an inhibiting step in the cholesterol biosynthesis cannot be excluded, as indicated by the observed decrease in ubiquinone and dolichol levels.

According to the study author, the observed reduced α-tocopherol levels in plasma and liver tissue could be correlated to reduced lipid transport capacity.

STUDY 2

Characteristics

reference/notifier	:	A. Freyberger (2001a)	exposure	:	
type of study	:	In vitro mechanistic study.	Doses[1]	:	BAJ 2740 (spirodiclofen): 1.5, 5, 15, 25, 50 100 µM BAJ 2510: 1, 10, 100, 300, 550, 1000 µM 4-OH BAJ 2740: 1, 10, 100, 1000 µM 3-OH BAJ 2740: 1, 10, 100, 1000 µM
year of execution	:	1998-2000	vehicle	:	0.5% DMSO
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%); BAJ 2510 (purity 98%); 3-OH BAJ 2510 (purity 99.9%); 4-OH BAJ 2510 (purity 98.7%)	GLP statement	:	no
route	:	In vitro (testes-slices)	guideline	:	unknown
species	:	rat	acceptability	:	acceptable
group size	:			:	

[1] 50 µM spirodiclofen resulted in opaque medium, 100 µM BAJ 2740 resulted in precipitation.

Study design

Spirodiclofen induced histological changes were observed in adrenals and testes of mice, rats and dogs. In addition, testosterone-sensitive tissues were affected as wel. In order to investigate a mechanism interfering with testosterone synthesis, potential interaction of spirodiclofen and its main metabolites BAJ 2510, 3-OH-BAJ 2510 and 4-OH-BAJ 2510 was tested in a steroidogenesis assay using rat testicular fragments. Testosterone secretion into the medium by testicular fragment in dynamic organ culture was assayed by means of a radioimmunoassay and was used as a measure for testosterone biosynthesis. Testosterone secretion was determined in the presence of hCG, dibuteryl-cAMP and hCG-stimulated tissue supplemented with 25-hydroxycholesterol (25-OH

cholesterol: to circumvent inhibitory effects on cholesterol esterase and on cholesterol transport into mitochondria (availability of cholesterol to mitochondria)).

Results

Testosterone secretion into the medium was not statistically significantly inhibited in all tests performed by BAJ 2740(spirodiclofen), 3-OH-BAJ 2510 and 4-OH-BAJ 2510, and without a clear dose-response in the dose range tested. BAJ 2510 (dose-dependently) reduced testosterone secretion into the medium, with statistical significant inhibition in two experiments of ≥10 and ≥100 μM respectively.

Dibuteryl-cAMP stimulated testosterone synthesis was significantly decreased (50%) by BAJ 2740 (spirodiclofen, 50 μM), BAJ 2510 (10 μM, 60% inhibition), 3-OH-BAJ 2510 and 4-OH-BAJ 2510 (1000 μM, 70% inhibition).

In hCG-stimulated rat testicular tissue, supplemented BAJ 2510 (100 and 300 μM) inhibited testosterone secretion by 86%, and similar inhibition observed in the presence of 25-hydroxycholesterol (2.5 μM and 25 μM).

Acceptability

The study was considered acceptable.

Conclusions

BAJ 2510 decreased testosterone secretion in medium of cultured rat testicular cells. This decrease was observed hCG-stimulated, dibuteryl-cAMP-stimulated and in hCG-stimulated + 25-hydroxycholesterol supplemented tissue. BAJ 2740 (spirodiclofen) 3-OH-BAJ 2510 and 4-OH-BAJ 2510 also decreased testosterone secretion in medium of cultured rat testicular cells (stimulated with hCG or dibuteryl-cAMP), but to a lesser extend compared to BAJ 2510.

STUDY 3

Characteristics

reference/notifier	:	G. Schmuck (1999)	exposure	:	24h incubation
type of study	:	In vitro mechanistic study.	doses	:	10^{-11} – 10^{-5} M
year of execution	:		vehicle	:	
test substance	:	BAJ 2740 (spirodiclofen), BAJ 2510, hydroxy-BAJ 2510	GLP statement	:	no
route	:	In vitro	guideline	:	-
species	:	Human MCF 7 breast cancer cell line and a prostate PC-3 cell line (MVLN cell line = estrogenic; PALM cell line = androgenic)	acceptability	:	acceptable
group size	:			:	

Study design

A human MCF 7 breast cancer cell line and a prostate PC-3 cell line transfected with the response element coupled to luciferase gen (MLNV cell line = estrogenic; PALM cell line = androgenic) were used. Incubations were performed with BAJ 2740 (spirodiclofen) BAJ 2510 and hydroxy-BAJ 2510 in a concentration range of 10^{-11} – 10^{-5} M. Results of this reporter gen assay were verified by receptor binding studies. The affinity of BAJ 2740 (spirodiclofen) , BAJ 2510 and hydroxy-BAJ 2510 to the human estrogen receptors α and β was measured in a competitive binding assay with a fluorescent labeled estrogen.

Results

Spirodiclofen and BAJ 2510 were not cytotoxic to either of the cell cultures, whereas hydroxy-BAJ 2510 had a slight cytotoxic potential to MLNV cells (10^{-6}M).

Up to and including a concentration of 10^{-5} M spirodiclofen (BAJ 2740), BAJ 2510 and hydroxy-BAJ 2510 showed no androgenic or antiandrogenic activity.

Up to and including a concentration of 10^{-5} M spirodiclofen (BAJ 2740) and hydroxy-BAJ 2510 showed no estrogenic or antiestrogenic activity, whereas BAJ 2510 showed contradictory results (marked effects to no effects on both endpoint in 5 single experiments). Additional testing showed, that estrogenic and more pronounced antiestrogenic potency of BAJ 2510 was pH dependent, and effects were only observed at pH 6.

Binding studies with the human α- and β-estrogen receptor showed no binding activity at pH 7.5 of spirodiclofen (BAJ 2740), BAJ 2510 and hydroxy-BAJ 2510. At pH 6-6.5, BAJ 2510 showed slight binding activity.

Acceptability

The study was considered acceptable.

Conclusions

Under physiological pH values, spirodiclofen, BAJ 2510 and hydroxy-BAJ 2510 had no receptor mediated hormonal activity (neither estrogenic or antiestrogenic nor androgenic or antiandrogenic activity up to a concentration of 10 μM).

STUDY 4

Characteristics

reference/notifier	:	P. Andrews (2001)	exposure	:	19 weeks (followed by 11 weeks of recovery)
type of study	:	Special subchronic toxicity study on hormone levels in female ats	Doses[1]	:	0, 2500, 10000 ppm (0, 242.4, 1209.6 mg/kg bw/day)
year of execution	:	2000	vehicle	:	-
test substance	:	BAJ 2740 (spirodiclofen, purity 97.8%, 97.5%)	GLP statement	:	Yes[2]
route	:	Oral (diet, with 1% peanut oil)	guideline	:	Not in accordance with OECD 408
species	:	Wistar rat (Hsd Cpb:WU)	acceptability	:	acceptable
group size	:	15 females/dose	**NOAEL**	:	< 242 mg/kg bw/day

1 Doses were based on a subchronic rat feeding study.

2 Exception: stability of the test article in the feed mix was only ascertained analytically for 7 days, however, the feed mixture was fed to the animals from day 2 to 8 after preparation.

Study design

The study was not performed according to OECD 408, as stated by the study author. In the presented study, spirodiclofen was orally administered to female rats (15/dose) in the feed in concentrations of 0, 2500 or 10000 ppm during 19 weeks. The animals were kept without treatment for another 11 weeks. Clinical examination was performed daily, body weight and feed intake were determined weekly, blood samples for hormone determinations were taken in the morning of rats in the diestrus stage (for determination of testosterone, estradiol, luteinizing hormone (LH) and progesterone, in weeks 7, 9, 11, 13, 17 and 19 of exposure and after 2 and 6 weeks without exposure). At necropsy, liver, adrenals, ovaries and uterus were weighed and fixed in formalin solution.

Results

Results are summarised in Table 69.

Table 69 Overview of the results

Dose (ppm)	**0**	**2500**	**10000**	**dr**
	f	**f**	**f**	
Mortality	No toxicologicalle relevant effects			
Clinical signs - discolored feces		7/15	15/15	f
Body weight		d	ds	
Food consumption		is	is	f
Ophthalmoscopy	Not performed			
Haematology	Not performed			
Clinical chemistry	Not performed			
Urinalysis	Not performed			
Organ weights				

Dose (ppm)	0	2500	10000	dr
	f	f	f	
- adrenals		i[r]	is[r]	f
- ovaries			i[r]	
Gross pathology[1]				
- increased size of the uterus			4/14	
Hormone determinations				
- estradiol		d	ds	
- LH			d	
- progesterone			ds	
- ratio estradiol/progesterone		i	is	
- testosterone[2]				

dr dose related
i/d increased/decreased
a/r absolute/relative organ weight
[1] necropsy report was not included
[2] The study author describes in the result section that testosterone levels showed a statistical decrease whith increasing doses of spirodiclofen. However, all testosterone values presented are around the limit of quantification for testosterone, and no increase could be observed in the pesented data.

Acceptability

The study was considered acceptable.

Conclusions

Body weights were decreased in the low and high dose animals by –7.1% and –24.1% respectively, and at the end of the recovery period, bw of the high dose animals were still lower compared to controls (-11.6%). Mean feed consumption was increased by 10% in low-dose animals and by 37% in high-dose animals. In week 2 of the recovery period, feed consumption was no longer different from controls.

Gross pathology at the end of the 11 week recovery period showed increased uterine size in 4/14 rats in the high dose. The study author considered this observation not treatment-related, as the increase was not substantiated by uterus weight measurements. However, the observed increase in relative uterus weight (+13.6%) was considered as relevant by the dossier submitter, and this increased weight is substantiated by the observed increased uterus size.

In the high dosed females, both estradiol and progesterone levels were decreased,. Since progesterone levels were stronger decreased than estradiol levels, an increased estradiol: progesterone ratio was observed, which was statistically significant in weeks 13 and 17 of treatment. After 2 and 6 weeks without treatment, progesterone levels were no longer decreased, whereas estradiol levels were still decreased, though not statistically significantly (-13%). Consequently, a decreased estradiol/progesterone ratio was observed after 2 and 6 weeks without treatment. Since testosterone levels were about the LOQ, no conclusions can be taken from the presented results on testosterone.

Substrate-related effects on body weight, adrenal weight, estradiol and on the ratio estradiol/progesterone were observed already at the lowest dose group. Therefore, a NOAEL in this study could not be established and the LOAEL is 242 mg/kg bw/day.

STUDY 5

Characteristics

reference/notifier	:	A. Freyberger (2001b)	exposure	:	
type of study	:	In vitro mechanistic study.	doses	:	50 µM BAJ 2740[1] (spirodiclofen); 300 µM BAJ 2510
year of execution	:	2000	vehicle	:	DMSO
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%), BAJ 2510 (purity 98%)	GLP statement	:	no
route	:	In vitro	guideline	:	unknown
species	:	Rat testicular microsomes	acceptability	:	acceptable
group size	:	Duplicate incubations		:	

1 maximally employable concentration as indicated by some precipitation of test compound

Study design

In order to study potential effects on cytochrome-P450-dependent microsomal monooxygenases involved in steroid hormone synthesis, the effects of spirodiclofen and its metabolite BAJ 2510 on steroid 17α-monooxygenase and C-17, 20-lyase were studied in vitro. Rat testicular microsomes served as enzyme source (from deep-frozen testicular tissue). Microsomes were incubated with precursor steroid in the presence of test compounds and resulant products were quantified by UV spectroscopy following HPLC separation from educts. Ketoconazole was included as reference inhibitor.

Results

In metabolically competent incubations, neither 50 µM BAJ 2740 (spirodiclofen) nor 300 µM BAJ 3510 affected steroid 17α-monooxygenase, whereas ketokonazole concentration-dependently inhibited the enzyme.

HPLC analysis of incubates demonstrated small amount of androstenedione and testosterone, indicating that formation of 17α-hydroxyprogesterone may not ideally reflect 17α-monooxygenase activity. Overall formation of 4-ene-3-ketosteroids (17α-hydroxyprogesterone, androstenedione and testosterone) was used to assay steroid 17α-monooxygenase activity. Neither 50 µM BAJ 2740 (spirodiclofen) nor 300 µM BAJ 3510 affected the formation of 4-ene-3-ketosteroids, whereas ketokonazole concentration-dependently inhibited the enzyme.

C-17, 20-lyase was not affected by 50 µM BAJ 2740 and 300 µM BAJ 3510, whereas 10 µM ketokonazole the enzyme inhibited.

Acceptability

The study was considered acceptable.

Conclusions

In this in vitro study with rat testicular microsomes, steroid 17α-monooxygenase and C-17, 20-lyase were neither affected by 50 µM spirodiclofen nor 300 µM BAJ 2510.

STUDY 6

Characteristics

reference/notifier	:	A. Freyberger (2001c)	exposure	:	20 minutes
type of study	:	In vitro mechanistic study.	doses	:	25, 50[1] µM BAJ 2740 (spirodiclofen), 100, 300 µM BAJ 2510, 100, 1000 µM 3-OH BAJ 2510, 100, 1000 µM 4-OH BAJ 2510
year of execution	:	2000	vehicle	:	DMSO
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%); BAJ 2510 (purity 98%); 3-OH BAJ 2510 (purity 99.9%); 4-OH BAJ 2510 (purity 98.7%)	GLP statement	:	no
route	:	In vitro	guideline	:	unknown
species	:		acceptability	:	acceptable
group size	:			:	

1 maximally employable concentration as indicated by some precipitation of test compound.

Study design

The study is a mechanistic study to evaluate potential effects on microsomal dehydrogenases involved in steroid hormone synthesis. The effects of spirodiclofen and its metabolites BAJ 2510, 3-OH-BAJ 2510 and 4-OH BAJ 2510 on 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase and 17β-hydroxysteroid dehydrogenase were studied in vitro. Rat testicular microsomes (deep-frozen) served as the enzyme source. Microsomes were incubated with (radiolabeled) precursor steroids in the presence of test compounds and resultant products were quantified by UV spectroscopy or liquid scintillation counting following HPLC separation from educts. Reference inhibitor 17α-OH-pregnenolone, a competing substrate, and coumesterol were used throughout the studies.

Results

3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase was inhibited in the presence of 25 and 50 µM BAJ 2740 (spirodiclofen) by 20% and 22% respectively (measured as conversion of the substrate pregenenolone into progesterone), whereas BAJ 2510 (up to 300 µM), 3-OH BAJ 2510 (100 µM) and 4-OH BAJ 2510 (100 µM) did not inhibit the enzyme. For the reference inhibitor 17α-OH-pregnenolone, an IC_{50} value of 50 µM was established.

17β-hydroxysteroid dehydrogenase (measured as the conversion of the substrate androstenedione into testosterone) was not inhibited in the presence of 50 µM BAJ 2740 (spirodiclofen), 100 µM BAJ 2510, 1000 µM 3-OH BAJ 2510 or 1000 µM 4-OH BAJ 2510, whereas the IC 50 value for the reference inhibitor coumesterol was 4 µM.

Acceptability

The study was considered acceptable.

Conclusions

In the present study, an inhibitory effect of spirodiclofen was observed on 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase in vitro. The observed inhibition by spirodiclofen of 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase in vitro may contribute to the observed reduction of testosterone synthesis in cultured, spirodiclofen-treated testicular tissue.

STUDY 7

Characteristics

reference/notifier	:	A. Freyberger (2000)	Exposure	:	
type of study	:	In vitro mechanistic study.	doses	:	5 µM, 50 µM BAJ 2740 (spirodiclofen)
year of execution	:	2000	vehicle	:	ethanol
test substance	:	BAJ 2740 (spirodiclofen, purity 98.6%), BAJ 2510 (purity 98%)	GLP statement	:	no
route	:	In vitro	guideline	:	unknown
species	:	-	acceptability	:	acceptable
group size	:			:	

Study design

The effects of spirodiclofen and BAJ 2510 on cholesterol esterase were studied in vitro using a radiometric assay. In order to investigate whether spirodiclofen could act as competing substrate for cholesterol esterase, spirodiclofen was incubated with the enzyme in the absence of cholesteryl oleate and potential conversion of spirodiclofen by the enzyme was tested. A commercially available pancreatic cholesterol esterase was used as a model esterase for cholesterol esterase present in steroid hormone producing tissues. Reference inhibitors of cholesterol esterase were included throughout these studies.

Results

Inhibition of cholesterol esterase by spirodiclofen was observed in two independent experiments, with IC_{50} values of 12 µM and 43 µM respectively. BAJ 2510 inhibited the enzyme only at 1000 µM.

Spirodiclofen did not act as a substrate for cholesterol esterase.

Acceptability

The study was considered acceptable.

Conclusions

In two independent experiments, spirodiclofen inhibited pancreatic cholesterol esterase, whereas a mild inhibitory potential of BAJ 2510 could only be demonstrated at 1 mM.

Spirodiclofen did not act as a competing substrate for cholesterol esterase.

STUDY 8

Characteristics

reference/notifier	:	A. Freyberger (2001d)	exposure	:	10 minutes
type of study	:	Mechanistic study to evaluate effects of BAJ 2510 on steroidogenesis.	doses	:	10, 30, 100, 300 µM BAJ 2510
year of execution	:	2000	vehicle	:	DMSO
test substance	:	BAJ 2510 (purity 98%)	GLP statement	:	no
route	:	In vitro	guideline	:	unknown
species	:	rat testicular mitochondria	acceptability	:	acceptable
group size	:			:	

Study design

The study was performed to investigate the effects of BAJ 2510 on steroidogenesis and identificate malate dehydrogenase isoenzymes as molecular target. Interactions with mitochondrial cholesterol side chain cleavage was studied in freshly isolated rat testicular mitochondria. Pregnenolone formed by side chain cleavage and released from mitochondria was enzymatically converted into progesterone and determined by HPLC. Effects on enzymes involved in the generation of reducing equivalents for the side chain cleavage enzyme (cytochrome P-450_{scc}) were studied using cultured rat testicular tissue as well as commercially available purified enzymes. The effects of BAJ 2510 on the availability of cytoplasmic reducing equivalents were also studied.

Results

In the presence of 25-OH-cholesterol and malate, incubation of freshly isolated rat testicular mitochondria with 100 µM BAJ 2510 resulted in decreased (-29%) side chain cleavage (decreased progesterone formation). This inhibition could be reversed by increasing malate concentrations or by replacing malate by citrate. No interaction with the side chain cleavage was observed in the presence of 50 µM BAJ 2740 (spirodiclofen), 100 µM 3-OH BAJ 2510 or 100 µM 4-OH BAJ 2510.

Incubation of commercially available purified enzymes resulted in BAJ 2510-induced inhibition of mitochondrial malate dehydrogenase (MD), whereas malic enzyme was not affected.

In cultured rat testicular tissue, BAJ 2510 decreased the hCG-stimulated synthesis of progesterone used as measure for side chain cleavage (50% inhibition at 300 µM BAJ 2510). This decrease was lower than the formation of testosterone (90% inhibition at 100 µM BAJ 2510). Similar findings were observed for ketoconazole, which is known to interfere with mitochondrial and microsomal target enzymes.

Acceptability

The study was considered acceptable.

Conclusions

BAJ 2510 is able to interfere with cholesterol side chain cleavage in malate-supplemented mitochondria in the concentration range up to and including 100 µM. BAJ 2510 effectively suppresssed side chain cleavage in the presence of malate but not in the presence of citrate.

Compared to testosterone synthesis, progesterone synthesis in cultured rat testicular tissue was only moderately reduced by BAJ 2510.

In vitro incubation of BAJ 2510 with commercially available purified malic enzyme and mitochondrial malate dehydrogenase resulted in inhibition of mitochondrial malate dehydrogenase, but not malic enzyme.

STUDY 9

Characteristics

reference/notifier	:	U. Schmidt (2000)	exposure	:	82 weeks
type of study	:	Determination of BAJ 2740 and BAJ 2510 in plasma.	doses	:	0, 50, 100, 350, 2500 ppm BAJ 2740 (spirodiclofen)
year of execution[1]	:	1997-1999	vehicle	:	
test substance	:	BAJ 2740 (spirodiclofen); BAJ 2510	GLP statement	:	no
route	:	Oral (diet)	guideline	:	unknown
species	:	Wistar rat (HsdCpb:Wu)	acceptability	:	acceptable
group size	:	10/sex/dose		:	

1 The animal study was performed under study no T 7061640.

Study design

In a chronic toxicity study, rats were orally exposed to 0, 50, 100, 350 or 2500 ppm spirodiclofen in their feed. Blood samples were taken at week 82 from 10 rats/sex/dose for determination of spirodiclofen and BAJ 2510 in plasma. Plasma samples were extracted and analysed by means of HPLC. The limit of quantification (LOQ) in plasma was 5 µmol/L for both spirodiclofen and BAJ 2510.

Results

The concentration of spirodiclofen was in all plasma samples below the limit of quantification. The concentration of BAJ 2510 in males was below the LOQ and increased dose-dependently to mean concentrations of 1.4, 5 and 45 nmol/ml in dose groups 100, 350 and 2500 ppm respectively. The concentrations in plasma of females was higher than in male rats, and was dose-dependently increased to mean concentrations of 0.4, 1.4, 5.5, 64.2 nmol/L in dose groups 50, 100, 350 and 2500 ppm respectively. Recovery of BAJ 2510 from plasma was about 70%, whereas recovery in acidified samples increased to about 90%. The presented plasma concentrations were not corrected by the recovery.

Acceptability

The study was considered acceptable.

Conclusions

In rats orally exposed to 0, 50, 100, 350 or 2500 ppm spirodiclofen in their feed during 82 weeks, BAJ 2510 was dose-dependently increased in plasma, with higher plasma concentrations of BAJ 2510 in female rats than in male rats.

STUDY 10

Characteristics

reference/notifier	:	U Schmidt (2001b)	exposure	:	20 weeks (plasma samples); 28 weeks (urine samples)
type of study	:	Determination of BAJ 2740 and BAJ 2510 in plasma and urine.	doses	:	0, 20, 50, 150, 500/600 ppm
year of execution[1]	:	1998-1999	vehicle	:	
test substance	:	BAJ 2740 (spirodiclofen)	GLP statement	:	no
route	:	Oral (diet)	guideline	:	unknown
species	:	Beagle dog	acceptability	:	
group size	:	Plasma samples: 4/sex of the high dose group Urine sample: 1f control and 3f/1m of the high dose group.		:	

Study design

In a chronic toxicity study, dogs were orally exposed to 0, 20, 50, 150 or 500/600 ppm spirodiclofen in their feed. Blood samples were taken at week 20 at 0, 2, 4, 7 and 24 h from 4 dogs/sex of the high dose group for determination of spirodiclofen and BAJ 2510 in plasma.

Plasma samples were extracted and analysed by means of HPLC. The limit of quantification (LOQ) in plasma was 5 µmol/L for both spirodiclofen and BAJ 2510.

Urine samples were taken at week 28 from 3 female and 1 male animal of the high dose group and from 1 female control animal for determination of spirodiclofen and BAJ 2510 in urine. No information was provided on recovery and methods used by determination of BAJ 2510 in urine samples, and no LOQ in urine was given.

Results

The concentration of spirodiclofen was in all plasma samples below the limit of quantification.

The concentration of BAJ 2510 in plasma before feeding was 24.8 and 26.8 nmol/ml in males and females respectively, and was at the same level as 7h and 24h after feeding. At 2 h and 4 h after feeding, plasma concentrations of BAJ 2510 were lower (about 18 and 16 nmol/L in males and females respectively).

The volume of the urine samples ranged from 19 ml (m) to 305 ml (f). An absolute quantification of the renal elimination was not given. The concentration of BAJ 2510 ranged from 0.12 to 0.46 µmol/ml in females and was 0.05 µmol/ml in the male.

Acceptability

The study was considered acceptable as additional study. No information was included on determination of spirodiclofen and BAJ 2510 in urine samples. It is not clear what the concentration of BAJ 2510 in plasma samples is, since in the result section a concentration of 248 nmol/ml (m) and 268 nmmol/ml (f) is given, whereas in the tables mean concentrations of 24.8 nmol/ml (m) and 26.8 nmol/ml (f) are described.

Conclusions

In plasma samples of dogs fed 500/600 ppm spirodiclofen, concentrations of spirodiclofen were below the LOQ. Plasma concentrations of BAJ 2510 were lower 2h and 4h after feeding, whereas 7h and 24h after feeding, plasma concentrations were at the same level as before feeding.

STUDY 11

Characteristics

reference/notifier	:	A. Freyberger (2002)	exposure	:	
type of study	:	Effects of BAJ 2510 on rat testicular mitochondrial NADH and NADPH levels.	doses	:	300 µM
year of execution[1]	:	2000 and 2002	vehicle	:	
test substance	:	BAJ 2510 (purity 98%, 99.9%)	GLP statement	:	no
route	:	In vitro	guideline	:	unknown
species	:	Rat testicular mitochondria	acceptability	:	acceptable
group size	:			:	

Study design

Development of a methodology to measure intramitochondrial NADH and NADPH and effects of BAJ 2510 on NADH and NADPH concentrations in rat testicular mitochondria are described. Freshly isolated rat testicular mitochondria, 25-hydroxycholesterol were incubated with the test compound. Cholesterol side chain cleavage was initiated by adding of malate for 10 minutes. For quantification, samples were analysed by HPLC.

Results

BAJ 2510 (300 µM) statistically significantly decreased NADH concentrations by 26% - 47%, 44% - 47% and 13% - 19% in the presence of 0.05mM, 0.5 mM or 5 mM malate, respectively. NADPH concentrations were decreased by by 26% - 41%, 25% - 31% and marginally in the presence of 0.05mM, 0.5 mM and 5 mM malate respectively. Tartronate (5 mM), a known inhibitor of the NADPH generating mitochondrial malic enzyme, decreased NADH concentrations by 0 – 24%, 31% - 32% and 29% - 38% in the presence of 0.05 mM, 0.5 mM and 5 mM malate, respectively. In the presence of tartronate (5 mM), NADPH levels were decreased by 19% - 30%, 25% - 28% and 24% - 27% in the presence of 0.05 mM, 0.5 mM and 5 mM malate, respectively.

Acceptability

The study was considered acceptable.

Conclusions

In the present study, BAJ 2510 (300 µM) significantly decreased intramitochondrial NADH levels in the presence of 0.05mM, 0.5 mM and 5 mM malate. NADPH levels were significantly reduced in the presence of 0.05 mM and 0.5 mM malate, whereas in the presence of 5 mM malate NADPH levels were not significantly reduced. These results support the hypothesis of inhibition of malate dehydrogenase by BAJ 2510.

STUDY 12

Characteristics

reference/notifier	:	A. Freyberger (2003)	exposure	:	
type of study	:	Mechanistic study to evaluate the potential effects of BAJ 2510 on intramitochondrial NADH and NADPH levels of rat testicular mitochondria.	doses	:	100, 200, 300 µM BAJ 2510
year of execution[1]	:	2002	vehicle	:	
test substance	:	BAJ 2510 (purity99.9%)	GLP statement	:	No
route	:	In vitro	guideline	:	unknown
species	:	Rat testicular mitochondria	acceptability	:	acceptable
group size	:			:	

Study design

The concentration dependency of BAJ 2510-induced decrease of intramitochondrial reducing equivalents under conditions known to be associated with effective side chain cleavage was studied.

Freshly prepared rat testicular mitochondrial were incubated with 100, 200 or 300 µM BAJ 2510 and 0.5 mM malate. Intramitochondrial reducing equivalents (NADH + NADPH) were determined by HPLC. Tartronic acid was included as positive control.

Results

BAJ 2510 concentration-dependently decreased the overall amount of reducing equivalents (NADH + NADPH, statistically significant at ≥100 µM BAJ 2510), NADH levels (statistically significant at ≥200 µM BAJ 2510) and NADPH levels (statistically significant at ≥100 µM BAJ 2510). In the presence of 5 mM tartronic acid, the overall amount of NADH+NADPH and NADPH concentrations were statistically significantly reduced by 30% and 34% respectively, and NADH levels were non-significantly decreased by 28%.

Acceptability

The study was considered acceptable.

Conclusions

Under conditions known to be associated with effective side chain cleavage, BAJ 2510 concentration-dependently decreased the overall amount of reducing equivalents and of levels of NADH and NADPH in mitochondria.

STUDY 13

Position paper by Sittert N.J. et al (2002).

Summary:

As the mechanism of observed effects of spirodiclofen in adrenals, testis and uterus, the notifier proposed that the compound indirectly interferes with steroid hormone synthesis via an effect on the generation of co-substrate NADPH (so called 'reducing equivalents').

The authors of the report indicated that cholesterol is the precursor for both gonadal and adrenal steroid hormone biosynthesis. The bulk of the cholesterol is derived by uptake from plasma cholesterol (esterified) rather than by intracellular synthesis. The first step in the steroidogenesis involves cleavage of the terminal six carbons of the side chain of cholesterol, resulting in the formation of pregnenolone. From pregnenolone a number of pathways lead to the formation of metabolic intermediates that give ultimately rise to the synthesis of androgenic and estrogenic sex hormones.

Conversion of cholesterol to pregnenolone occurs within the mitochondria. Enzymes involved in this step are collectively known as cholesterol side-chain cleavage cytochrome P-450 complex, comprising various NADPH-dependent hydroxylases. Pregnenolone is then released from the mitochondria in the smooth endoplasmatic reticulum and biotransformed in progesterone, by the enzyme 38-hydroxysteroid dehydrogenase-A4'5 -isomerase. Another dehydrogenase involved in synthesis of testosterone is 17β-hydroxysteroid dehydrogenase. Microsomal cytochrome P-450-dependent monooxygenases involved in testosterone synthesis are NADPH-dependent 17a-hydroxylase (or steroid-17α-monooxygenase) and C-17,20-lyase (17 α-hydroxyprogesterone aldolase). Inhibition by spirodiclofen or its metabolites of one or more of these enzymes would be a critical event leading to decreased levels of testosterone, progesterone or 17β-estradiol.

To function, several of these enzymes need co-substrate NADPH. Thus interference with the formation of mitochondrial and cytoplasmatic NADPH by spirodiclofen or its metabolites would also have consequences for steroid hormone biosynthesis. Cytoplasmatic NADPH is also required for cholesterol and fatty acid synthesis and inhibition may result in lower blood levels of these substances.

The authors evaluated essentially the same studies as described in the DAR and addendum. Important target organs following subchronic and chronic dosing of spirodiclofen to mice, rats and dogs are the adrenal glands and the testes. It was shown that spirodiclofen produces:

• generalised enlargement of these organs in mice, rats and dogs;

• vacuolation of adrenal cortex in mice, rats and dogs;

• increased size of testicular cells (hypertrophy) in mice, rats and dogs;

• increase in testicular cell number (hyperplasia) in mice and rats;

• testicular and uterine tumours in the rat.

As both the adrenal glands and the gonads produce and release steroid hormones in response to adrenocorticotrophic and gonadotrophic hormones released from the pituitary, it is expected that the effects of spirodiclofen on adrenal glands and gonads are produced through an endocrine-mediated mechanism.

The authors concluded that the two-generation reproduction study in rats and developmental toxicity studies in rats and rabbits did not show effects on fertility and reproduction parameters, such as estrus cycle, insemination index, fertility, sperm count and production, livebirth index, viability index, lactation index and uterus weight. Neither were developmental effects observed. The NOAEL for reproductive toxicity was based on reduced parental and pup body weights. The results thus indicated that the influence of spirodiclofen on the endocrine tissues, if any, was apparently weak. In agreement with the notifier, the authors concluded that according to EPA criteria, spirodiclofen has no androgenic or estrogenic properties in mammals.

> **NOTE**: *in the DAR and addendum, the NOAEL for reproductive toxicity is based on the decreased spermatogenesis in the high dose F1 males (NOAEL 26.2 mg/kg bw/d, LOAEL 134.8 mg/kg bw/d).*

Indications of endocrine-mediated effects were increased LH levels in plasma of dogs and decreased estradiol and progesterone levels in plasma of rats in subchronic studies. Based on the data base, including the mechanistic studies, the authors support the notifier's conclusion that spirodiclofen interferes with the generation of NADPH, which is an important co-substrate in several steps of the biosynthesis of steroid hormones. However, several assumptions had to be made which were not experimentally verified, e.g. quantitative data of reduced NADPH levels were not available.

In summary, according to the authors, the adrenal, testicular and uterine effects are the result of the following mechanism. A high dietary level of spirodiclofen increases the release of adrenocorticotrophins and gonadotrophins from the pituitary as a result of spirodiclofen-induced inhibition of steroid hormone biosynthesis. The increase in gonadotrophins then results in chronic stimulation of testicular Leydig cells and endometrial uterine cells, resulting in hypertrophy, hyperplasia and tumour formation. Spirodiclofen was not mutagenic/genotoxic or clastogenic in *in vitro* and *in vivo* test systems, which confirms that the spirodiclofen-induced carcinogenic response in rats is through a hormone-mediated non-genotoxic mechanism for which a threshold dose exists, below which no carcinogenic effects occur.

Spirodiclofen-induced testicular and uterine carcinogenicity could not be demonstrated in mice and dogs, which suggests a species-specificity for the rat. It is unlikely that this rat-specificity results from species differences in spirodiclofen-inhibition of steroid hormone synthesis, because there is no reason to assume that BAJ 2510-inhibition of malate dehydrogenase is qualitatively and quantitatively different among species. Species differences may, therefore, result from differences in gonadotropin-mediated effects.

With regard to the hepatocellular adenomas and carcinomas found in mice in the high-dose groups, the authors regard it to be unlikely that these tumours were induced through an endocrine-mediated mechanism. In analogy with observations in dogs, at high dose levels spirodiclofen may induce certain drug metabolising cytochrome P450-dependent enzymes in mouse liver. Liver enzyme induction in the mouse might subsequently result in hypertrophy, hyperplasia and tumour formation, as also shown for organochlorine pesticides. Tumour induction by organochlorine pesticides was not observed in other species, including humans. Spirodiclofen-induced liver tumours in mice are, therefore, deemed as mouse-specific and not of relevance to humans.

The question whether or not spirodiclofen should be considered as an endocrine active compound (EAC) cannot be answered with a simple yes or no. Endocrine active compounds (also referred to as endocrine disrupters) can be defined as chemical substances that modify the normal functioning of human hormone systems or that alter hormonal regulation. Biological endpoints to be associated with EAC's are, for example, reproduction and developmental parameters, steroid receptor binding or inhibition, altered hormone levels, altered cell proliferation and cell differentiation, etc. The authors feel that the nature and severity of the hormonal effect should also be taken into account for classification of chemicals such as EAC.

Hormone-mediated effects of spirodiclofen include vacuolation, hypertrophy, hyperplasia, testicular and uterine cancer. Furthermore, spirodiclofen induced an increase in LH release from the pituitary observed in male dogs and a decrease in levels of progesterone and estradiol in female rats, which are also seen as hormonal effects. On the other hand, spirodiclofen does not act via an androgen or estrogen-receptor mediated mechanism and spirodiclofen or its metabolite BAJ 2510 do not interact with enzymes involved in hormone steroid biosynthesis. Most importantly, the two-generation toxicity study in rats and developmental toxicity studies in rats and rabbits did not show effects on fertility, reproduction and developmental parameters at levels which did not cause parental toxicity. The primary effect of spirodiclofen is disturbance of NADPH generation in mitochondria and cytoplasma through inhibition of malate dehydrogenase. This triggers a cascade of hormone-mediated events. The authors feel that, according to the above definition, spirodiclofen is an endocrine active compound. However, it should be placed in the same category as, for instance, ethanol, which compound acts through a similar mechanism *(e.g.* through NADPH depletion).

4.12.2 Human information

No information available.

4.12.3 Summary and discussion

Neurotoxicity studies

In an acute oral neurotoxicity study, no evidence of neurotoxicological effects was observed, and the NOAEL or neurotoxicity in this study was found to be 2000 mg/kg bw, the highest dose tested.

In a 13 week subchronic oral neurotoxicity study, in the highest dose group of 1089 mg/kg bw/day, decreased foot splay and decreased forelimb/hindlimb grip strength were observed. Although body weight was decreased in the highest dose group which may have contributed to the observed effect, a compound-related effect cannot be excluded. The NOAEL for neurotoxicity in this subchronic study was 70 mg/kg bw/day.

In a 77 week chronic oral neurotoxicity study, no evidence of neurotoxicological effects was observed, and the NOAEL for neurotoxicity in this study was found to be 110 mg/kg bw, the highest dose tested.

Two neurodevelopmental toxicity studies in rats were presented. The first study showed negative results with the exception of some equivocal results for the water maze test. In a follow-up study in which parts of the developmental neurotoxicity study were repeated (and included two types of water maze tests) no neurotoxic effects were observed.

Immunotoxicity studies

In a 14 week oral toxicity study, immunotoxicological investigation with satelite groups was performed after 4 weeks of exposure. At and above 232.4 mg/kg bw/day, decreased body weight was observed and effects on immunological parameters.

Mechanistic studies

In an 8 week oral toxicity study with male dogs, in addition to reduced plasma cholesterol and triglycerides, plasma ubiquinone (Q10), dolichols and dolicholphosphates in the testes were decreased. These observation indicates that an effect on the HMG-CoA reductase as as inhibiting step in the cholesterol biosynthesis cannot be excluded. Reduced α-tocopherol in plasma and liver may be related to reduced lipid transport capacity.

An in vitro mechanistic study with testes-slices showed decreased (stimulated) testosterone secretion into the medium in the presence of all test substances, with BAJ 2510 as the most potent inhibitor.

In a receptor binding study with human cell lines, no receptor-mediated hormonal activity (estrogenic, antiestrogenic, androgenic or antiandrogenic) was observed under physiological pH values in the presence of the test substances.

In a subchronic 19 weeks oral toxicity study on hormone levels in female rats besides decreased bw increased adrenal weight and ratio estradiol/progesterone were observed in all dosed groups. In addition to these observations, in the highest dose group increased ovary weight, increased uterus size and decreased LH were observed. Since testosterone levels were at about the LOQ, no conclusions on testosterone levels can be drawn from the study. A NOAEL in this study could not be established and the LOAEL is 242.4 mg/kg bw/day.

An in vitro mechanistic study with rat testicular microsomes on potential effects of spirodiclofen and BAJ 2510 on cytochrome P450-dependent microsomal monooxygenases involved in steroid hormone synthesis showed no effect on 17α-monooxygenase and C-17, 20-lyase.

An in vitro mechanistic study with rat testicular microsomes was performed to evaluate potential effects of spirodiclofen and its metabolites on microsomal dehydrogenases involved in steroid hormone synthesis. Only spirodiclofen showed an inhibitory effect on 3β-hydroxysteroid dehydrogenase-$\Delta^{4,5}$-isomerase in vitro. It cannot be excluded that this effect may contribute to reduction of testosterone synthesis in spirodiclofen treated testicular tissue. 17β-hydroxysteroid dehydrogenase was not inhibited in the presence of the test substances.

In a study which investigated whether spirodiclofen or BAJ 2510 could act as competing substrate for cholesterol esterase it was shown that spirodiclofen inhibited pancreatic cholesterol esterase but

did not act as a competing substrate for cholesterol esterase BAJ 2510 showed a mild inhibitory potential on cholesterol esterase.

An in vitro mechanistic study was performed to investigate effects of BAJ 2510 on steroidogenesis and identify malate dehydrogenase isoenzymes as molecular target. Interactions with mitochondrial cholesterol side chain cleavage was studied in rat testicular mitochondria. It was shown that BAJ 2510 interfered with cholesterol side chain cleavage in malate-supplemented mitochondria, but not in the presence of citrate. Compared to testosterone synthesis, progesterone synthesis in cultured rat testicular tissue was only moderately reduced by BAJ 2510. In vitro incubation of BAJ 2510 with commercially available purified malic enzyme and mitochondrial malate dehydrogenase resulted in inhibition of mitochondrial malate dehydrogenase, but not malic enzyme.

In a chronic oral toxicity study with rats, blood samples after 82 weeks of exposure were taken for determination of spirodiclofen and BAJ 2510 in plasma. In all samples, spirodiclofen concentrations were below the LOQ. BAJ 2510 was dose-dependently increased in plasma, with higher concentrations in females compared to males.

In a chronic oral toxicity study with dogs, samples were taken after 20 weeks and 28 weeks of blood and urine respectively for determination of spirodiclofen and BAJ 2510. In all samples, spirodiclofen concentrations were below the LOQ. Plasma concentrations of BAJ 2510 were lower 2h and 4h after feeding, whereas 7h and 24h after feeding, plasma concentrations were at the same level as before feeding. Urine samples were only determined from 3f and 1m, and the measured concentration BAJ 2510 is only indicative for the presence of the metabolite of spirodiclofen.

In an in vitro study with rat testicular mitochondria, BAJ 2510 (300 µM) significantly decreased intramitochondrial NADH levels in the presence of 0.05mM, 0.5 mM and 5 mM malate. NADPH levels were significantly reduced in the presence of 0.05 mM and 0.5 mM malate, whereas in the presence of 5 mM malate NADPH levels were not significantly reduced. These results support the hypothesis of inhibition of malate dehydrogenase by BAJ 2510.

In an in vitro study with rat testicular mitochondria under conditions known to be associated with effective side chain cleavage, BAJ 2510 concentration-dependently decreased the overall amount of reducing equivalents and of levels of NADH and NADPH in mitochondria.

An overview of the immunotoxicity and mechanistic studies can be found in Table 70.

Table 70 Overview of the immunotoxicity and mechanistic studies

Test substance	Duration, route	Species	NOAEL (mg/kg bw/day)	LOAEL (mg/kg bw/day)	Critical effects	Reference/ Notifier
(spirodiclofen) BAJ 2740	4 weeks, oral	rat	45	232	Decreased BW, effects on immunological parameters (decreased cell counts spleen and lymph node, decreased spleen T-helper cells, T-cell marker and lymphocytes and decreased serum IgA titer)	U. Wirnitzer, A. Romeike (1998)
(spirodiclofen) BAJ 2740	8 weeks, oral	dog	Not applicable, the study was performed to investigate the involvement of HMG-CoA reductase in spirodiclofen-induced decrease in cholesterol levels.			U. Schmidt (2001a)

Spirodiclofen (BAJ 2740), BAJ 2510, 3-OH BAJ 2510, 4-OH BAJ 2510	In vitro	rat (testes slices)	Not applicable, the study was performed to investigate a mechanism interfering with testosterone synthesis.			A. Freyberger (2001a)
Spirodiclofen (BAJ 2740), BAJ 2510, hydroxy-BAJ 2510	In vitro	Human cell lines	Not applicable, this receptor binding study was performed to investigate receptor-mediated hormonal activities of the test compounds.			G. Schmuck (1999)
Spirodiclofen (BAJ 2740)	19 weeks, oral	rat	< 242	242	Decreased bw, increased adrenal weight, decreased estradiol concentration and increased estradiol/progesterone ratio	P. Andrews (2001)
Spirodiclofen (BAJ 2740), BAJ 2510	In vitro	Rat (testicular microsomes)	Not applicable, the study was performed to investigate potential effects of spirodiclofen and BAJ 2510 on cytochrome-P450-dependent microsomal monooxygenases involved in steroid hormone synthesis.			A. Freyberger (2001b)
Spirodiclofen (BAJ 2740), BAJ 2510, 3-OH BAJ 2510, 4-OH BAJ 2510	In vitro	Rat (testicular microsomes)	Not applicable, the study was performed to investigate potential effects of the test substances on microsomal dehydrognases involved in steroid hormone synthesis.			A. Freyberger (2001c)
Spirodiclofen (BAJ 2740), BAJ 2510	In vitro	Pancreatic cholesterol esterase (commercial available)	Not applicable, the study was performed to investigate the effects of the test sustances on cholesterol esterase.			A. Freyberger (2000)
BAJ 2510	In vitro	Rat (testicular mitochondria)	Not applicable, the study was performed to investigate the effects of BAJ 2510 on steroidogenesis and identificate malate dehydrogenase isoenzymes as molecular target.			A. Freyberger (2001d)
Spirodiclofen (BAJ 2740)	82 weeks, oral	rat	Not applicable, the study was performed to determine spirodiclofen and BAJ 2510 in plasma samples.			U. Schmidt (2000)
Spirodiclofen (BAJ 2740)	20/28 weeks	dog	Not applicable, the study was performed to determine spirodiclofen and BAJ 2510 in plasma and urine samples.			U. Schmidt (2001b)
BAJ 2510	In vitro	Rat (testicular mitochondria)	Not applicable, the study was performed to measure intramitochondrial NADH and NADPH and to study effects of BAJ 2510 on NADH and NADPH concentrations in rat testicular mitochondria.			A. Freyberger (2002)
BAJ 2510	In vitro	Rat (testicular mitochondria)	Not applicable, the study was performed to measure intramitochondrial NADH and NADPH and to study effects of BAJ 2510 on NADH and NADPH concentrations in rat testicular mitochondria.			A. Freyberger (2002)

4.12.4 Comparison with criteria

Neurotoxicity effects

In the acute and 77-week exposure neurotoxicity studies, no neurotoxicological effects were observed. Classification for STOT SE is therefore not needed.

In a neurodevelopmental study in rats, no neurotoxic effects were observed except for some equivocal results in a water maze test. In a consecutive study in which parts of the previous developmental neurotoxicity study were repeated (and included two types of water maze tests) no neurotoxic effects were observed. In the 13-week rat study, some potential neurotoxic effects were seen. These effects included decreased foot splay and decreased forelimb/hindlimb grip strength seen in the 12500 ppm dose group (1088.8 and 1306.6 mg/kg bw/day for male and female animals respectively). It cannot be excluded that these effects are compound-related. However, as these effects are observed above the upper limit for STOT RE 2 classification (100 mg/kg bw/day for a 90-d study), classification for STOT RE is not needed.

Immunotoxicological effects

In the 4-week immunotoxicity study, some effects on immunological parameters were observed. These effects included decreased cell counts (spleen and lymph node), decreased splenic T-helper cells (CD4/CD45), spleen lymphocytes (CD45), spleen T-cell marker observed at the two highest dose levels (2500 and 125000 ppm; i.e. 232.4 and 1284.9 mg/kg bw/day for males and 237.6 and 1466.1 mg/kg bw/day for female animals).

However, as most of these effects are observed above the upper limit for STOT RE 2 classification (300 mg/kg bw/day for a 28-day study), and the type and severity does not fulfill the classification criteris for STOT RE, classification for STOT RE is not needed.

4.12.5 Conclusions on classification and labelling

Based on the available neurotoxicity and immunotoxicity studies, classification of spirodiclofen for STOT SE/RE is not required.

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

No classification is proposed by the DS due to lack of data.

Comments received during public consultation

There was one comment received from a MSCA during public consultation supporting the DS's proposal not to classify spirodiclofen with regard to aspiration toxicity/hazard.

Assessment and comparison with the classification criteria

No assessment and comparison with the classification criteria is possible due to lack of data.

5 ENVIRONMENTAL HAZARD ASSESSMENT

The environmental hazards of spirodiclofen were assessed in the Draft Assessment Report, addenda and Proposed Decision of the Netherlands prepared in the context of the possible inclusion of spirodiclofen in Annex I of Council Directive 91/414/EEC (Draft Assessment Report, April 2004 and updated September 2006 concerning the placing of plant protection product on the market. The DAR is publicly available via the EFSA web site (http://dar.efsa.europa.eu/dar-web/provision).

The summaries included in this proposal are copied from the DAR (and its addenda and assessment reports when these contain updated information). For an overview of the hazard property being evaluated, all reliable information relating to that property has been summarised in a table. Detailed information is included for the key studies used to derive the classification. For more details the reader is referred to the DAR and its addenda.

5.1 Degradation

Table 71: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Spirodiclofen			

EPA guideline and SETAC GLP study	*DT_{50} for hydrolysis at 20 ºC pH 4: 124 days pH 7: 53.4 days pH 9: 2.2 days	Hydrolytically unstable Major hydrolysis product: Spirodiclofen-enol	Babczinsky, (2000)[a]
UBA (1992) guideline GLP study	DT_{50} at 50 ºN 80 days (no clouds). DT_{50} at 50 ºN 110 days (cloudiness) in June	Photodegradation not expected	Hellpointer, (1998)[a]
EPA guideline GLP study	DT_{50} at 40 ºN 123 days (midsummer)	Photodegradation not expected	Stupp and Brumhard, (2000)[a]
BBA IV and SETAC guideline GLP study Water/sediment aerobic	DT_{50s} for Hönniger pond system Water[(A)]: 0.3 day Sediment: 4.4 days System: 4.2 days DT_{50s} Anglerweiher pit system Water: 1.1 day Sediment: 2.5 days System: 2.3 days	(A) Calculated using measured values in the water on day 0 and 1, assuming first order decline.	Riegner, (1999)[a]
EPA (Pesticide Assessment Guidelines Subdivision N, Series 162-3) guideline GLP study Water/sediment anaerobic	DT_{50} Water: -- Sediment: 9.8 days System: 10 days		Wujcik CE et al., (2000)[a]
Metabolite of Spirodiclofen			
Spirodiclofen-enol (BAJ 2740-enol) EPA guideline; GLP study	DT_{50} at 25 ºC 7.6 hours	Photodegradation is expected	Babczinski, (2000)[a]
BBA IV and SETAC guideline GLP study Water/sediment aerobic	DT_{50s} for Hönniger pond system Water: 186 days Sediment: -- System: 393 days DT_{50s} Anglerweiher pit system Water: -- Sediment: -- System: --		Riegner, (1999)[a]
EPA (Pesticide Assessment Guidelines Subdivision N, Series 162-3) guideline GLP study Water/sediment anaerobic	DT_{50} Sediment: 175 days	Extrapolated value	Wujcik CE et al., (2000)[a]

*DT_{50} values at 20 ºC at pHs 4, 7 and 9 were calculated by the RMS;
[a] As summarised in the DAR vol 3-B8, April 2004.

5.1.1 Stability

A hydrolysis study (Babczinsky, 2000) with radiolabelled spirodiclofen (radiochemical purity 99.1%) was conducted according to EPA guideline and SETAC and in compliance with GLP. Dihydrofuranone-3-^{14}C-spirodiclofen was incubated in sterile buffer solutions at three pH levels (pH 4, 7, and 9). Test solutions contained 1% (v/v) acetonitrile. Incubation was in the dark at either 50ºC for up to 7 days (pH 4 and 7) or 16 days (pH 9) or 25ºC for up to 30 days. Samples were taken

at minimum of seven time points after application. Radioactivity was quantified by LSC and compound identification was performed by co-chromatography with the unlabelled reference standards of spirodiclofen and the metabolite BAJ 2740-enol. The identity of the hydrolysis product BAJ 2740-enol was confirmed by LC-MS-MS.

The recovery of radioactivity was 97.4 – 106.7% (range for all samples). Spirodiclofen accounted for 95 – 101% of applied radioactivity (AR) at day 0 and decreased to 70.3, 51.0, and 1.1% AR after 30 days at pH 4, 7, and 9, respectively with first-order half-lives of 63.6, 30.8 and 1.9 days at 25ºC. The major hydrolysis product was BAJ 2740-enol. All other fractions were < 2% AR. At 50ºC, DT_{50}-values were 3.1, 2.5, and 0.4 days at pH 4, 7, and 9, respectively. DT_{50} values at 20ºC were determined from an Arrhenius plot and were 119.6, 52.1 and 2.5 days at pH 4, 7, and 9, respectively.

Comment by RMS
The rapporteur has recalculated the DT_{50} values at 20ºC using the original rate constants, resulting in DT_{50} values of 124, 53.4,and 2.2 days at pH 4, 7, and 9, respectively.

Photodegradation in water

Study 1

In a photodegradation study (Hellpointer, 1998) the absorption of UV light at 295 – 310 nm of spirodiclofen (chemical purity 97.5%) in acetonitrile/water (1/1) solution was measured according to UBA guideline in compliance with GLP. UV spectrum of spirodiclofen at 5.11 mg as/L and 25ºC for up to 9 hours was recorded under continuous irradiation. The concentration of spirodiclofen was analysed by HPLC-UV. The light intensity was determined and the absorbed light was calculated using the $\varepsilon(\lambda)$ from the UV spectrum of spirodiclofen.

Spirodiclofen degraded with DT_{50} values of 47.01 and 48.05 hours (duplicate values). The absorbed light intensity was 0.0144 (mean value). This mean absorbed light intensity was used to calculate photolysis half lives in pure water (0-5 cm depth) using GC solar, which does not consider clouds, and according to Frank and Klőpffer (cloudiness taken into account). At 30, 40, 50, and 60 ºN, GC solar estimated DT_{50s} during summer were estimated to be 54, 64, 80, and 110 days, respectively. The minimum DT_{50} at 50 ºN predicted by Frank and Klőpffer was 110 days for June.

Comment by RMS
(1) The report presented no data (e.g., record of temperature measurements) to confirm that the stated value of 25°C was maintained.

(2) The lack of a dark control is considered to be without a significant effect on the quantum yield (DT50 for hydrolysis of Spirodiclofen was 30.8 days at 25°C; see B.8.4.1).

(3) Mercury lamps are not recommended for photolysis studies, since the light intensity distribution deviates from that of natural sunlight. They can be acceptable for the determination of the quantum yield, however, since this parameter is considered to be independent of the intensity of the incident light and, within the same absorption band, also of wavelength.

(4) No justification was provided for the high level of co-solvent (50%) in the test medium. The solubility of Spirodiclofen in buffer pH 4 (0.05 mg/L at 20°C) may have been be too low for sufficient analytical accuracy with the methods of the study. In water/acetonitrile (1/1) a test concentration of 5.11 mg/L could be achieved. Moreover, in the next study (Stupp & Brumhard, 2000) it was shown that 20% acetonitrile as co-solvent was required to avoid sorption to glass. The environmental DT_{50}s for photolytic degradation calculated in this study and the next are of

comparable magnitude. This indicates that, within the range of 20-50%, the level of acetonitrile had no important impact on the conclusions.

Study 2

A second photolysis of spirodiclofen (Stupp and Brumhard, 2000) was studied according to EPA guideline and with GLP. Radio labelled [dihydrofuranone-3-^{14}C]-spirodiclofen and [cyclohexyl-1-^{14}C]-spirodiclofen (radiochemical purity > 98%, at 0.025 mg/L) were incubated in a sterile buffer solution of sodium acetate with 20% acetonitrile to avoid sorption to glass. Incubation was at 25 ºC for up to 19 days under continuous irradiation (290 nm). A dark control was included. The spectral distribution of the light was comparable to natural midday midsummer sunlight. Samples were analysed by LSC and TLC, compound identification was by co-chromatography with reference standards. Identity of spirodiclofen was confirmed by LC-MS/MS. Recovery of the radioactivity was 94 – 106% AR.

In the main test with [dihydrofuranone-3-^{14}C]-spirodiclofen organic volatiles amounted to 3.7% AR after 19 days. CO2 was the main degradants (21.8% AR after 19 days). The DT_{50} value of 28.8 days was found in irradiated samples. In the dark control, a complete degradation was found because of hydrolysis due to pH shift. In the additional test, the pH was relatively stable and Spirodiclofen degraded in irritated samples with a DT_{50} of 23.4 days.

In the supplementary test, with [cyclohexyl-1-^{14}C]-spirodiclofen levels of organic volatiles and CO2 were lower than for the other label (0.5 and 2.3% AR respectively after 17 days). The pH in the test solution was stable and DT_{50} value of 99.4 days was calculated, corresponding with an environmental DT_{50} of 123 days under midday midsummer conditions at 40 ºN.

Comment by RMS
In the test with the cyclohexyl-label, the pH of irradiated and dark samples was comparable. In the tests with the dihydrofuranone-label, the pH in irradiated samples was higher than in the dark samples of the additional test. Therefore, the DT_{50} determined in the supplementary test with the cyclohexyl-label is considered to be the better estimate for the rate of photolytic degradation.

Photodegradation in water spirodiclofen-enol (BAJ 2740-enol); metabolite of spirodiclofen

The photodegradation of BAJ 2740-enol in Rhine water was determined according to EPA guideline with GLP (Babczinsky, 2000). A solution of [dihydrofuranone-3-^{14}C]-BAJ 2740-enol (radiochemical purity 98.5%, 0.176 mg/L) in Rhine water (7 mg/L TOC, 4.2 mg/L nitrate, pH 7.4) containing 0.1% methanol was tested. Vessels were incubated at 25 ºC for up to 24 hours under continuous irradiation (< 290 nm). A dark control was included. Analysis was by LSC and TLC, compound identification by co-chromatography with reference standards. Identity of BAJ 2740-enol in the stock solution was confirmed by LC-MS/MS. Recovery of radioactivity was 96 – 102% AR. Besides the unidentified components (M3: 25.6% AR and M4: 11.0% AR after 24 h), up to 9 other unidentified fractions (none > 7.9% AR) were determined. BAJ 2740-enol degraded with a DT_{50} of 7.6 hours. No degradation in the dark control. The metabolite M3 was identified as BAJ2740-dioxoketone (spirodiclofen-dioxoketone).

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

5.1.2.2 Screening tests

A ready biodegradability test is not available.

5.1.2.3 Simulation tests

Biodegradation in water/sediment systems

Aerobic water/sediment

The study comprised two trials, both performed with the same systems. An aerobic water/sediment study with [dihydrofuranone-3-^{14}C]-spirodiclofen (radiochemical purity > 99 %) was performed according to BBA IV (section 5-1, 1991) and SETAC (1995) guidelines, in compliance with GLP (Riegner, 1999). One system was collected from a pond (Hőnniger, Wipperfurth with sandy loamy silt sediment; pH water and sediment: 6.5 and 5.8, respectively) in Germany. The second from a gravel pit (Anglerweiher, Leverkusen with silty sand sediment; pH water and sediment: 7.6 and 7.1, respectively) in Germany.

Sediment samples were unsieved and dry weight was determined, water was filtered. Vessels were filled with 50 g dry weight sediment and 450 mL water. Vessels were equilibrated for 5 weeks prior to treatment. Radio labelled [dihydrofuranone-3-^{14}C]-spirodiclofen was added in acetonitrile / water to the water in the vessels at 0.05 mg a.s./L. Vessels were incubated in the dark at 20 ºC for up to 110 days. Volatiles were trapped in soda lime. Duplicate samples were taken between 0.5 and 2 h after application and at 9 time points up to 110 days. Sediment and water were separated by decantation. Radioactivity in water was analysed by LSC after acidification with formic acid and metabolites were extracted and analysed by TLC. Sediment was acidified with formic acid and extracted with acetonitrile buffer (pH 5). Analysis of sediment by LSC (radioactivity) and TLC. The identity of BAJ 2740-enol was confirmed by HPLC-MS/MS.

In the first trial, erratic low levels of the metabolite BAJ 2740-enol were found in the water phase of certain samples, together with increased levels of unknown degradates, but the sum of BAJ 2407-enol and unknown degradates was as expected. . Direct chromatography of these water phases (without extraction) showed no or only very low levels of unknown degradate. This indicated that the formation of unknown degradates from BAJ 2740-enol was an artifact of sample processing. To confirm this, a second trial was performed using the same systems and procedures, with the following modifications: only two vessels per system were set-up without traps for volatiles, and at each sampling interval (after 0, 14, 29, 60 and 99 days) two aliquots of 2 mL of the water were removed, diluted with acetonitrile and analysed by direct TLC.

In the second trial, parent Spirodiclofen represented >99% of the radioactivity in the water on day 0 and was not detectable afterwards. The metabolite BAJ 240-enol represented 0.4% of the radioactivity in the water on day 0, and >98% on day 14-99. At any sampling, ≤1.7% of the radioactivity in the water consisted of unidentified compounds. This confirmed that the high levels of unidentifieds found during the first trial in certain replicates was an artifact of sample processing. Therefore, the unidentifieds in the water phase, which during trial 1 emanated as artifacts of sample processing, were included in the total level for the metabolite BAJ 240-enol. This approach was supported by the RMS.

In trial 1, one day-110 replicate Anglerweiher sample, which had a mass balance of 120.2%, was excluded from the data by the RMS. The total recoveries of both systems were 96 – 103% AR. The distribution of the radioactivity in both systems was similar. A maximum of 78.7% AR was found in the sediment of the pond system at day 1 compared to 64.5% AR of the pit system. Levels of non-extractable radioactivity were low 6.8% and 1.5% AR after 110 days in pond and pit system, respectively. Organic volatiles were < 0.02% AR and CO_2 levels (mineralisation) by day 110 were also low (2.1 – 2.6% AR maximum).

Spirodiclofen was rapidly lost from the water phase of both systems, with maximum levels occurring in sediment of 58 – 68% AR on day 1 and declining thereafter. In the water of the pond system, the major metabolite BAJ 2740-enol increased from <1% AR to a maximum level of 73.8% AR on day 37, declining afterwards, and in the water of the pit system to 83-84% AR between day 14 and 110. In the sediment of pond and pit system, maximum levels of metabolite BAJ 2740-enol (29.6 and 14.0% AR) were measured after 110 days. The total level of unidentified degradates in any system, at any time point, were ≤5.0% AR.

First order DT_{50s} and DT_{90s} of Spirodiclofen, calculated by the authors are shown in Table 72. For the calculation of DT_{50} system, values <<10% AR were not included (diffusion may be come rate-limiting and/or analytical inaccuracy). DT_{50s} for BAJ 2740-enol were based on few data points late in the study, when microbial activity was low and sediment not anaerobic.

Table 72: DT_{50} values for the water/sediment systems for spirodiclofen and the metabolite BAJ 2740-enol.

Compound	**Phase**	**Hőnniger pond system DT_{50}**	**Anglerweiher pit system DT_{50}**
Spirodiclofen	Water Sediment system	0.3 d 4.4 d 4.2 d	1.1 d 2.5 d 2.3 d
BAJ 2740-enol	Water Sediment system	186 d -- 393 d	-- -- --

d=day; -- values could not be determined

Comment by RMS

Disappearance of Spirodiclofen from the water was in fact faster than indicated by the above DT_{50s} (water), since already at t=0 (0.5-2 hours after treatment) about 50% of the added Spirodiclofen had partitioned into sediment. Rapid partitioning of Spirodiclofen into sediment may have been enhanced by the limited water solubility of the substance (50 µg/L, equal to the test concentration).

Anaerobic water/sediment

An anaerobic water/sediment study was conducted in one system according to EPA guideline under GLP conditions (Wujcik et al., 2000). The system was collected from a lake (loamy sand sediment) in California. Water (unfiltered, 150 mL) and sediment (unsieved, 50 g dry weight) were amended with glucose and equilibrated in sealed flasks under nitrogen for 61 days to establish anaerobic conditions. Thereafter, [dihydrofuranone-3-^{14}C]-spirodiclofen (radiochemical purity 98.4%) was added in acetonitrile at 0.0125 mg a.s./L. Flasks were purged with nitrogen, sealed and incubated in the dark at 20 ºC for up to 365 days. The redox potential was -152 to -298 mV and dissolved O_2 ≤0.3 mg/L, showing an anaerobic environment. Duplicate samples were taken immediately after

application and at 11 time points up to 365 days. At each sampling date, flasks were purged with nitrogen and volatiles were trapped. Sediment and water were separated by centrifugation. Radioactivity in water was analysed by LSC and filtered water was extracted by C18 disk or ethyl acetate. Sediment was extracted by acetonitrile or ethyl acetate and analysed by LSC and HPLC. Non-extractable compounds were quantified by combustion LSC. Compound identification was performed by comparison to reference standards. The identity of spirodiclofen and the metabolite BAJ 2740-enol was confirmed by LC-MS.

Mean recoveries were 100 – 104% AR. The radioactivity in the water increased from 5.1% AR on day 0 to 80.0% on day 56 and remained at a comparable level until day 365. The radioactivity dissipated immediately into the sediment (maximum 94.9% AR on day 0) and declined to 18.9% AR on day 365. Levels of non-extractable radioactivity (1.5% AR on day 0 increasing to 3.6% AR on day 365). CO_2 (≤1.0% AR) and organic volatiles (≤0.5% AR) were low during the study.

Spirodiclofen disappeared from the water phase at the first sampling, maximum level in sediment of 91.9% AR on day 0 and declined thereafter (40% AR on day 14 and 0.5% AR on day 295). Spirodiclofen in the water accounted for only 1.3% AR on day 0 and were at or below detection limit (0.6% AR) from day 2 and onwards.

BAJ 2740-enol was the major metabolite and reached maximum levels of 71 – 80% AR (water) and 82 – 94% AR (whole system) between day 34 and 365. In sediment, BAJ 2740-enol increased from 1.5% AR on day 0 to 15.6% AR on day 246 and declined thereafter to 9.6% AR at day 365. Up to 9 unidentified compounds were detected in water and sediment extracts. At any time point total level of degradates in the system was ≤15.5% AR. Individual compounds did not exceed < 4.8% AR.

The calculated first order DT_{50} values for spirodiclofen were 9.8 days for sediment and 10.0 days for the whole system. DT_{50} value for BAJ 2740-enol was 175 days (extrapolated value) for sediment.

5.1.3 Summary and discussion of degradation

Spirodiclofen is not susceptible to hydrolysis. Spirodiclofen hydrolyses in the pH range 4-9 in the absence of light under sterile conditions, with first-order DT_{50s} at 20°C of 119.6, 52.1 and 2.5 days at pHs 4, 7 and 9 respectively. The hydrolytic stability of Spirodiclofen decreases as temperature and pH increase. The major hydrolysis product was BAJ 2740-enol. The total amount of other products at any time point did not exceed 2% AR. BAJ 2740-enol is hydrolytically stable

Spirodiclofen is not considered to be directly photodegradable in two photodegradation studies. Environmental photolysis half lives in pure water were estimated to be 54, 64, 80 and 110 days at 30, 40, 50 and 60 °N, respectively (clouds not considered), or 110 days at 50 °N (cloudiness taken into consideration). The aqueous photolysis of BAJ 2740-enol was studied in Rhine water. When irradiated with a xenon lamp (equivalent light intensity of midsummer sunlight, 40 °N) the half-life was 7.6 hours. BAJ 2740-dioxoketone was the only photodegradate formed at >10% AR (max. 25.6% AR).

No study on ready biodegradability was submitted.

In the aerobic water/sediment system the $DT_{50,\ water}$ of spirodiclofen was 0.3 day and 1.1 days. This is indicative that spirodiclofen disappears rapidly from the water phase. The $DT_{50,\ system}$ resulted in a range of 4.2 days and 2.3 days. Mineralization was at a comparable low level in systems, 2.1% and 2.6% at 110 days. The major metabolite was BAJ 2407-enol reached maximum levels of 84% (day14-59) and 30% (day 110) in water and sediment, respectively.

The total levels of other degradates in any system were ≤5.0% AR. The DT_{50s} of 186 and 393 days was calculated for the disappearance of BAJ 2740-enol from the pond water and system, respectively. In the pit system, no degradation of BAJ 2740-enol was apparent until study end (day 110).

In the anaerobic water/sediment study, spirodiclofen was almost quantitatively lost from the water phase at the first sampling, with a maximum level occurring in sediment of 91.9% AR on day 0. Spirodiclofen dissipated from sediment and overall system with a DT_{50} of 9.8 and 10 days, respectively. Maximum levels of the major metabolite BAJ 2740-enol were 71-80% AR (water) and 82-94% AR (whole system) between day 34 and 365.Levels of non-extractable radioactivity in sediment (≤3.6% AR), production of CO_2 (≤1.0% AR) and organic volatiles (≤0.5% AR) were low throughout study.

Based on the findings from the aerobic and anaerobic water/sediment test spirodiclofen appears to be susceptible for primary degradation (DT_{50s}< 16 days) and not ultimate mineralisation (CO_2 production). The degradation product, BAJ 2740-enol is considered to fulfil the criteria for classification as hazardous to the aquatic environment. Considering the data on primary degradation and low levels of mineralisation in the simulation studies, spirodiclofen is considered not rapidly degradable (degradation of > 70% degradation within 28 days) for purposes of classification and labelling.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

Parent spirodiclofen is not stable in 0.01 M calcium chloride and no batch sorption study was performed (Hein, W 1999). The Koc value was estimated by the HPLC method to be 31037 L/kg. This value would classify Spirodiclofen as very slightly mobile in soil (Sommer, 2000).

Available adsorption coefficients of major metabolites of spirodiclofen have been determined in bath sorption studies at 20˚C and a soil/water ratio of 1.0 (BAJ 2740-enol and BAJ 274-dihydroxy) or 1.25 (2,4-dichlorobenzoic acid). Acceptable Koc values obtained were as follows: BAJ 2740-enol: 12.09-28.59 L/kg; BAJ 2740-dihydroxy: 8.9-104.7 L/kg and 2,4-dichlorobenzoic acid: 4.7-8.8 L/kg. BAJ 2740-enol is therefore considered moderately mobile, BAJ 2740-dihydroxy moderately to slightly mobile, and 2,4-dichlorobenzoic acid moderately mobile to mobile (classification according to RIVM Manual 1995).

5.2.2 Volatilisation

Spirodiclofen has vapour pressure of 3×10^{-7} Pa at 20^{0}C, according to EC A4 guideline and gas saturation method and the calculated Henry's law constant, from these vapour pressure and the water solubility, is 2×10^{-2} Pa.m^3/mole at 20 ºC (Krohn,1997). Based on this information it is concluded the volatilization of spirodiclofen into the air will be negligible.

5.2.3 Distribution modelling

No information available.

5.3 Aquatic Bioaccumulation

Table 73: Summary of relevant information on aquatic bioaccumulation

Method	Results	Remarks	Reference
BCF study with bluegill sunfish according to OECD 305 and EPA 72-6 guidelines	Low concentration BCF = 491 L/kg High concentration BCF = 484 L/kg Normalized to 5% lipid content BCF = 323 L/kg	Based on radioactivity and 7.6% lipid content.	Dorgerloh, M. et al. (2000)

5.3.1 Aquatic bioaccumulation

5.3.1.1 Bioaccumulation estimation

No information available.

5.3.1.2 Measured bioaccumulation data

In a bioaccumulation study of [dihydrofunanone-3-14C]-Spirodiclofen (chemical purity >98%, radiochemical purity >99%) in bluegill sunfish (*Lepomis macrochirus*), fish were exposed for 29 days with a depuration period of 13 days (Dorgerloh, M. et al., 2000). A continuous flow-through exposure regime was carried out at two nominal concentrations of 2.0 and 20 µg a.s./L, and a solvent control (acidified methanol). Four fish were sampled at 8 time points during exposure and five during depuration and dissected into fillets and viscera. Total radioactive residues in water and fish tissues were extracted at all sampling times using 90% acetonitrile and metabolites were identified. The extracted activity was analysed by reversed phase HPLC and normal TLC. All fractions containing ≥10% of the total radioactive residue (TRR) were identified.

The mean measured radioactivity concentrations in the water during exposure were 1.6 and 11.4 µg a.s./L for 2.0 and 20 µg a.s./L, respectively, comprising 76 – 84% parent compound. Fish showed no mortalities or abnormal behaviour during the test. Steady-state was reached within 3 days of exposure and depuration of TRR was ≥98% from all tissues within 13 days. The steady-state BCF based on radioactivity measurements was calculated to be 491 L/kg and 484 L/kg wet weight for the low and high concentration, respectively, for the whole fish. Metabolism was extensive and parent spirodiclofen accounted for only 0.33% TRR in viscera and was not detectable in fillet. The major metabolite was BAJ 2740-enol, for 62 and 28% of the TRR in fillet and viscera, respectively. Other metabolites identified in fillet and viscera were the 4-hydroxy-BAJ-enol isomers (33 and 58% TRR), the 3-hydroxy-BAj-enol isomers (1.7 and 3.1% TRR), and 2,4-dichloromandelic acid (< limit of detection and 1.6% TRR).

The mean lipid content of the fish, determined at start and end, of the study was 7.6%. When normalised for 5% lipid content the BCF value is 323 L/kg. The method of calculation of the BCF was not specified.

5.3.2 Summary and discussion of aquatic bioaccumulation

The highest BCF derived for spirodiclofen was 491 L/kg normalised to 5% lipid the BCF is 323 L/kg based on total radioactivity. Spirodiclofen therefore does not fulfill the criteria for bioaccumulation potential according to Regulation EC 1272/2008, 2nd ATP, since the BCF is < 500 L/kg.

A bioaccumulation study with the major metabolite BAJ 2740-enol is not available. The log Kow of BAJ 2740-enol is reported as 3, which is below the cut-off value of log Kow = 4. Consequently, BAJ 2740-enol is not considered to be a bioaccumulating substance.

5.4 Aquatic toxicity

Parent compound: Spirodiclofen.

Table 74: Summary of relevant information on aquatic toxicity for spirodiclofen

Method	Results	Remarks	Reference

Acute fish spirodiclofen, 96-h. OECD 203, EPA guideline	LC_{50} > 0.035 mg/L	96-h flow-through, limit test. *Oncorhynchus mykiss.* Measured concentrations.	Dorgerloh, M. (1999-a)
Acute fish spirodiclofen, 96-h. OECD 203, EPA guideline	LC_{50} > 0.0455 mg/L	96-h flow-through, limit test. *Lepomis macrochirus.* Measured concentrations.	Dorgerloh, M. (1999-b)
Acute invertebrate spirodiclofen, 48-h. OECD 202, EPA 72-2	EC_{50} > 0.0508 mg/L	48-h flow-through, limit test. *Daphnia magna.* Measured concentrations.	Heimbach, F. (1998-a)
Algae inhibition, spirodiclofen, 96-h. OECD 201, EPA 540/9-86-134.	E_rC50 > 29.2 µg/L (0.0292 mg/L) E_bC50 > 29.2 µg/L (0.0292 mg/L) NOEC ≥ 29.2 µg/L (0.0292 mg/L)	96-h static, limit test *Pseudokirchneriella subcapitata* Values based on geometrical mean measured concentrations.	Anderson, J.P.E (1998)[b].
Chronic fish, spirodiclofen, 97-days, OECD 210, FIFRA 72-4, early life stage.	NOEC = 1.95 µg as/L (0.00195 mg as/L).	97-days flow-through test, *Oncorhynchuss mykiss*, NOEC based on fish growth and measured concentration.	Dorgerloh, M. (2000)[b]
Chronic invertebrate, 21-day, spirodiclofen, OECD 202 (II), EPA 72-4.	NOEC = 24.8 µg as/L (0.0248 mg as/L)	21-d flow-through test, Daphnia magna, NOEC is based on growth and reproductive effects. Mean measured concentration.	Heimbach, F. (1998-b)[b]
Chronic invertebrate, 21-d, spirodiclofen, EPA 72-4	NOEC = 11.1 µg as/L (0.0111 mg as/L)	21-d flow-through test, Daphnia magna, NOEC is based on reproduction and mean measured concentration.	Hall and Lam, (2001)[b].

b As summarised in the DAR 08, vol.3-B9, April 2004.

<u>Metabolite of spirodiclofen: BAJ 2740-enol</u>.

Table 75: Summary of relevant information on aquatic toxicity for BAJ 2740-enol

Method	Results	Remarks	Reference

Acute fish, BAJ 2740-enol, 96-h. OECD 203, EPA 72-1 guideline	LC_{50} > 73 mg/L	96-h static, limit test. *Oncorhynchus mykiss* Mean measured concentrations.	Peither, A. (1999)
Acute invertebrate, BAJ 2740-enol, 48-h. OECD 202, EPA 72-2	EC_{50} > 95 mg/L	48-h static test, *Daphnia magna*. Mean measured concentration.	Heimbach, F. (2000)
Algae inhibition, BAJ 2740-enol, 96-h. OECD 201, EPA 123-2.	E_rC50 > 100 mg/L E_bC50 > 82.8 mg/L NOEC ≥ 6.25 mg/L	96-h static, nominal, limit test *Pseudokirchneriella subcapitata*	Seyfried, B. (2000).
Chronic fish, BAJ 2740-enol, 97-days, OECD 210, FIFRA 72-4, early life stage.	NOEC ≥ 115 µg as/L (0.115 mg as/L)	97-days flow-through test, *Oncorhynchuss mykiss*, No effects were determined. NOEC is based on measured concentration.	Dorgerloh, M. (2001)[b]
Chronic fish, BAJ 2740-enol, 115-days, FIFRA 72-5, full fish life cycle.	NOEC ≥ 190 µg as/L (0.190 mg as/L)	115 days flow-through test, *Cyprinodon variegatus*. No effects were determined. NOEC is based on mean measured concentrations.	Dionne, K. (2001)[b].
Chronic invertebrate, 21-d, BAJ 2740-enol, OECD 211, EPA 72-4	NOEC = 32 mg as/L.	21-d semi-static test, Daphnia magna, NOEC is based on reproduction and nominal concentrations.	Hendel, B. (2000)[b].

b: As summarised in the DAR 08, vol 3-B9 April 2004.

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

<u>Parent compound: Spirodiclofen</u>.

The acute toxicity of spirodiclofen to fish was tested in two different species: *Oncorhynchus mykiss* and *Lepomis macrochirus*.

In the study with rainbow trout (*Oncorhynchus mykiss)* and with bluegill sunfish (*Lepomis macrochirus)* spirodiclofen (purity 97.6%) was tested under flow-through conditions in acidified methanol test water. The tests were carried out as a limit test at or near the water solubility of

spirodiclofen in the test medium. (solubility in water 0.05 mg/L at pH 4 and 22 ºC). Water quality parameters were within acceptable levels.

Study 1

A 96-h acute toxicity flow-through limit test with technical Spirodiclofen (97.6% pure) in acidified methanol (2.5% acetic acid) test medium with *Oncorhynchus mykiss*, according to OECD 203 and EPA 72-1 guideline was conducted and in compliance with GLP (Dorgerloh, M.;1999). The limit test concentration was set at the border of the water solubility of 60.0 µg a.s./L nominal. A control and solvent control (0.1 mL/L) were included. One replicate per treatment with 30 fish (mean: 1.9 g; 5.0 cm) each. Test volume was exchanged 6 times per day. Loading of the fish was 0.24 g fish/L/d. Test water used was reconstituted water, hardness 40 – 60 as mg $CaCO_3$/L, pH: 6.5 – 7.3. Acclimatisation of the fish was at least 14 days. Observations of fish after 4 hours, and then daily for mortalities and symptoms of intoxication. Samples of test medium were taken on day 0, 2 and 4. Acetonitrile was added to the samples to avoid adsorption to glass. Analysis by HPLC with UV detection, method LOQ was 0.01 mL, LOD 10.5 µg a.s./L. Stock solution of nominal 600 mg a.i./L was also measured at 7 days before the start of the test and at day 4. Test solution was clear and colourless. The measured test concentrations were 41.1, 32.4, and 31.8 µg/L on day 0, 2, and 4, respectively, mean measured concentration 35.1 µg a.s./L (58% of nominal). The mean measured concentration in the stock solution was 491 mg a.s./L (82% of nominal).

No mortalities occurred during the test and no sublethal effects were observed at the mean measured limit test concentration of 0.035 mg a.s./L, which was at or near the limit of solubility of the compound in the test medium. No mortalities or other effects in the control and solvent control. Result, the LC_{50} > 35.1 µg a.s./L (> 0.035 mg a.s./L), is based on mean measured concentrations.

Study 2

In a 96-h acute toxicity flow-through limit test with technical Spirodiclofen (97.6% pure) in acidified methanol (2.5% acetic acid) test medium in *Lepomis macrochirus*, according to OECD 203 and EPA 72-1 guideline and in compliance with GLP (Dorgerloh, M., 1999). The limit test concentration was set at the border of the water solubility of 60.0 µg a.i./l nominal. A control and solvent control (0.1 mL/L) were included. One replicate per treatment with 30 fish (mean: 2.5 g; 4.5 cm) each. Test volume was exchanged 6 times per day. Loading of the fish was 0.31 g fish/L/d. Test water used was reconstituted water, hardness 40 – 60 as mg $CaCO_3$/L, pH: 6.6 – 7.3. Acclimatisation of the fish was at least 14 days. Observations of fish after 4 hours, and then daily for mortalities and symptoms of intoxication. Samples of test medium were taken on day 0, 2 and 4. Acetonitrile was added to the samples to avoid adsorption to glass. Analysis by HPLC with UV detection. Samples were centrifuged and injected into the HPLC, method LOQ was 0.01 mL, LOD 10.2 µg a.s./L. Stock solution of nominal 600 mg a.s./L was also measured at 7 days before the start of the test and at day 4. Test substance was observed at the surface of the vessel during the whole study. The measured test concentrations were 39.7, 43.9, and 52.9 µg/L on day 0, 2, and 4, respectively, mean measured concentration 45.5 µg/L (76% of nominal). The mean measured concentration in the stock solution was 614 mg a.i./L (102% of nominal).

No mortalities occurred during the test and no sublethal effects were observed at the mean measured limit test concentration of 0.0455 mg a.s./L, which was at or near the limit of solubility of the compound in the test medium. No mortalities or other effects in the control and solvent control. Result, the LC_{50} of > 45.5 µg a.s./L (>0.0455 mg a.s./L), is based on mean measured dissolved test concentration.

Metabolite of spirodiclofen: BAJ 2740-enol.

A 96-h static toxicity test was carried out on rainbow trout (*Oncorhynchus mykiss*) with the metabolite of spirodiclofen: BAJ 2740-enol (purity 99.9%) as a limit test according to OECD 203 guideline and in compliance with GLP (Peither, A.; 1999). Fish (mean length and weight: 5.3 cm, 1.7 g) were exposed to 100 mg a.s/L nominal. A control was included. Test concentration was based on a range-finding test. Two replicates with 15 fish each per treatment. Loading rate was 0.8 g fish wet weight/L. Acclimatisation for one week. OECD reconstituted water was used, hardness 125 mg/L as $CaCO_3$, pH 7.1. Test media were slightly aerated during the test. Test substance was mixed into test water and treated by ultrasonic treatment for 20 minutes and thereafter stirred for 3 hours. As the test concentration of 100 mg/L was at its maximum water solubility, the undissolved fraction of the test item was allowed to settle down to the bottom of the test vessels before fish were added. Observations of fish for mortality and sublethal effects after 4, 24, 48, 72 and 96 hours of exposure. Unfiltered and filtered (0.45 µm) samples of test concentration and control were taken from the freshly prepared test medium, at day 2 and day 4. Analysis was HPLC with UV/VIS detection after acetonitrile was added. Method was validated, method recovery was 101% (n=4). LC_{50} were determined directly from the raw data. Inhomogeneous dispersion of test item was observed after 4 hours, test item was visible at the surface and lying at the bottom after 24 hours and onwards.

Measured test concentration in the unfiltered test solution was 35 – 45% of nominal, 85 – 97% of nominal, and 94 – 101% of nominal at 0, 2, and 4 days, respectively. The measured concentrations in the filtered test solutions were 34 – 40% of nominal, 82 – 95% of nominal, and 91 – 97% of nominal at 0, 2, and 4 days, respectively. The mean measured test concentration in the filtered test medium was 73 mg/L.

No mortalities in the control, 2 fish died in the test solutions. No sublethal effects were observed in the test solutions and control during the whole study period. The 96-h LC_{50} is determined as > 100 mg/L, based on nominal concentration, which is the limit of the water solubility in the test water used. Based on mean measured test concentration the 96-h LC_{50} is set at >73 mg/L. Water quality parameters were within the acceptable limits. Due to visible test item during the study and the low recovery at the start, the result should be based on the mean measured filtered test concentrations. The 96-h LC_{50} is > 73 mg/L, based on mean measured concentrations.

5.4.1.2 Long-term toxicity to fish

Parent compound: Spirodiclofen.

The study was in accordance with GLP and OECD 210 and FIFRA 72-4 with no major deviations from the protocols. A 97-day fish early life stage flow-through study was undertaken with eggs, larvae and juveniles of rainbow trout (*Oncorhynchus mykiss*) (Dorgerloh, 2000). Newly fertilised eggs (1-2 hours post-fertilisation, four replicates/concentration, 35 eggs/replicate) were exposed to [dihydrofuranone-3-14C]-Spirodiclofen (chemical purity 98.1%, radiochemical purity >99%) at nominal concentrations of 0.123, 0.25, 0.49, 0.98, 1.97 and 3.93 µg a.s./L plus control and solvent control (acidified methanol). Mean measured concentrations of Spirodiclofen, based on LSC measurement, were 0.112, 0.29, 0.57, 1.09, 1.95 and 3.81 µg a.s./L, representing 91 to 118% of nominal. TLC measurements performed on day 75 or 76 and 97 showed that >99% of the radioactivity in diluter stock solutions consisted of Spirodiclofen, and 94.2 and 86.6%, respectively, of the radioactivity in the test solutions of the highest test concentration. Water quality parameters were: temperature (10 ± 1°C), dissolved oxygen (>94% of saturation) and pH (6.4 to 7.3). The total duration of the exposure period was 97 days. Alevin were thinned to 15 per replicate at day 39 to give a total of 60 individuals per concentration. Survival and growth parameters were monitored throughout the 61 day post-hatch period.

Egg hatchability, time to hatch, time to initiation of swim-up and fry survival was not affected at any test concentration when compared to the pooled control group. It was stated in the report that no test compound-related morphological and behavioural abnormalities were observed during the study.

At 61 days post-hatch, fish length was significantly reduced in the 1.09, 1.95 and 3.81 µg a.s./L treatment by 4.7, 4.3 and 6.8%, respectively, when compared to the pooled control group. At 61 days post-hatch, fish dry weight was comparable to the pooled control group at all concentrations, except at 3.81 µg a.s./L, where a reduction of 11% was recorded. This difference was not statistically significant.

The author of the report argued that the biological NOEC based on fish growth was 1.95 µg a.s./L, measured concentration, since the reductions in fish length at 1.09 and 1.95 µg a.s./L were small (≤5%), within the range of control variability and not accompanied by a simultaneous significant reduction in weight. This position is supported by RMS.

Metabolite of spirodiclofen: BAJ 2740-enol.

Study 1

The study was in accordance with GLP and OECD 210 and FIFRA 72-4 with no major deviations from the protocols. A 97-day fish early life stage flow-through study was undertaken with eggs, larvae and juveniles of rainbow trout (Oncorhynchus mykiss) (Dorgerloh, 2001). Newly fertilised eggs (2 hours post-fertilisation, four replicates/concentration, 35 eggs/replicate) were exposed to BAJ 2740-enol (purity 99.9%) at nominal concentrations of 3.00, 6.00, 12.0, 24.0, 48.0 and 95.9 µg a.s./L plus control and acetone control. Mean measured concentrations of BAJ 2740-enol were 3.28, 5.79, 12.0, 27.9, 53.3 and 115 µg a.s./L, representing 97 to 120% of nominal. Water quality parameters were: temperature (9.9 ± 0.2°C), dissolved oxygen (>90% of saturation) and pH (6.9 to 7.4). The total duration of the exposure period was 97 days. Alevin were thinned to 15 per replicate at day 39 to give a total of 60 individuals per concentration. Survival and growth parameters were monitored throughout the 61 day post-hatch period.

Egg hatchability, time to hatch, time to initiation of swim-up and fry survival was not affected at any test concentration when compared to the pooled control group. At 61 days post-hatch, no statistically significant effect on fish length and fish dry weight was observed at any test concentration when compared to the pooled control group. It was stated in the report that during the post-hatch period darkened colouration was observed sporadically, but that the lack of a dose response indicated no test item-related effects (no further data were given).

Based on the absence of test compound related effects at the highest tested concentration, the NOEC for BAJ 2740-enol is ≥115 µg/L and based on measured concentrations.

Study 2

A second study was performed as a full fish life cycle flow-through test with the estuarine species sheepshead minnow *(Cyprinodon variegatus*) (Dionne, 2001). The study was in accordance with GLP and FIFRA 72-5. Newly fertilised eggs (30 hours post-fertilisation, 200 eggs/treatment distributed over 2 aquariums with two incubation cups containing 50 eggs each) were exposed to BAJ 2740-enol (purity 99.9%) at nominal concentrations of 6.3, 13, 25, 50, 100 and 200 µg a.s./L

plus control. Mean measured concentrations of BAJ 2740-enol were 5.7, 13, 24, 48, 110 and 190 µg a.s./L, representing 91 to 110% of nominal. Water quality parameters were: temperature (mean ± SD: 28 ± <1°C), dissolved oxygen (mean ± SD: 88-89 ± 6-8% of saturation, always ≥62%), pH (7.6 to 8.2) and salinity (31-33‰). The total duration of the exposure period of the F_0 generation was 115 days. On day 4 (day 0 post-hatch), 25 of the newly-hatched fry in each incubation cup were impartially selected and transferred to growth chambers. On day 27 post-hatch, juveniles from the 2 growth chambers in each aquarium were combined and thinned to 25 per replicate aquarium to give a total of 50 individuals per concentration. At day 58-61 post-hatch, 2 F_0 spawning groups (2 males and 5 females from each aquarium) were established and kept for 14 days. Spawns were removed and counted daily. Hatching success of the F_1 was determined frequently throughout the 14-day spawning period for batches of 50 eggs. Exposure of the F_1 generation was initiated by incubating groups of 50 embryos on the day they were spawned. Following hatching, 2 groups of 25 newly hatched F_1 larvae per replicate aquarium were placed in a growth chamber for a period of 28 days post-hatch. Survival of F_0 and F_1 fish was monitored throughout the post-hatch period. Length of the F_0 fish was determined on day 27, day 58 and day 111 post-hatch, and blotted wet weight on day 111 post-hatch. Length and blotted wet weight of the F_1 fish was determined on day 28 post-hatch.

F_0 egg hatchability, fry survival at 27, 58 and 111 days post-hatch, fish length at 27, 58 and 111 days post-hatch, fish blotted wet weight at 111 days post-hatch, and fecundity were all not affected at any test concentration when compared to the control group. F_1 egg hatchability, fry survival and fish length and blotted wet weight at 28 days post-hatch were also not affected at any test concentration when compared to the control group.

Based on the absence of test compound related effects at the highest tested concentration, the NOEC for BAJ 2740-enol is ≥190 µg/L, based on mean measured concentrations.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

Parent compound: Spirodiclofen.

An acute toxicity flow-through limit test with technical Spirodiclofen (97.8% pure) in acidified methanol test medium in *Daphnia magna,* according to OECD 202 and EPA 72-2 guideline and was conducted in compliance with GLP (Heimbach, F. 1998). Daphnia magna (< 24 hours old) were exposed in a flow-through system at five nominal concentrations of 5.6, 10, 18, 32, and 56 µg a.s./L for 48 hours. The substance was tested up to the limit of its water solubility. Stock solutions were prepared with methanol with 1% volume acetic acid. A solvent control (0.1 mL methanol with 1% volume acetic acid/L) and a blank control were included. Four replicates with 10 daphnids each per treatment. Test water used was M7- Elendt medium, total hardness 196 mg/L as $CaCO_3$, pH 7.8. The stock solutions and the mixing chamber in the system were gently stirred. Twelve volume turnovers of the test solutions per day. Daphnids were not fed during the test and test solutions were not aerated. Test conditions: 20 °C, 16 : 8 hours L : D. Observations, also for sublethal effects after 24 and 48 hours of exposure. Test concentrations were analysed on day 0, 1 and 2 of the nominal concentrations of 18, 32, and 56 µg a.s./L. All stock solutions were also analysed on day 0 and day 2. Analysis by HPLC with UV detection, samples were directly injected into the HPLC, LOD of 0.1 mg/L. Stock solution samples were diluted with milli-Q water/acetonitrile (80:20 v/v). Acetonitrile and phosphoric acid were added to avoid binding to glass after sampling test concentrations. EC_{50}

was calculated by Probit analysis after the Maximum Likelihood method using the programme of Ratte. Reference substance was potassium dichromate performed three months before.

Mean measured test concentrations in the stock solutions ranged from 100.3 to 122.5% of nominal (average 109.4% of nominal). Mean measured test concentrations in the test solutions were 15.8 (88.0% of nominal), 30.7 (96.0% of nominal), and 50.8 (90.7% of nominal) µg a.s./L for nominal 18, 32, and 56 µg a.s./L, respectively.

No immobilities in the blank control and one (2.5%) in the solvent control. Immobilities in the test concentrations were 0, 0, 1 (2.5%), 1 (2.5%), and 1 (2.5%) at 5.6, 10, 18, 32, and 56 µg a.s/L nominal. No sublethal effects or behaviour was observed during the study. The 48-h EC_{50} was set at > 50.8 µg a.i./L, which is up to the water solubility and based on mean measured test concentrations. 24-h EC_{50} of the reference substance was 1.20 mg a.s./L, which lies within the required range of 0.9 – 1.9 mg a.s./L. Water quality parameters were within acceptable levels.

Metabolite of spirodiclofen: BAJ 2740-enol.

The acute toxicity of BAJ 2740-enol (purity 99.9%), a metabolite of spirodiclofen, was tested to *Daphnia magna* in a static 48-h test according to OECD 202 (part I, 1984) guideline (Heimbach, 2000). Daphnia magna (< 24 hours old) were exposed to six test concentrations and a control. The nominal test concentrations were: 3.2, 10, 18, 32, 56, and 100 mg a.s./L. OECD M7 (Elendt) test water was used, total hardness 196 mg/L as $CaCO_3$, pH 8.0. Test concentrations were prepared by stirring for 30 minutes and treated in an ultrasonic bath for 10 minutes. Three replicates per treatment with 10 daphnids each. Test solutions were not aerated and daphnids were not fed during the study. Temperature: 20 °c, 16:8 h L:D (light 700 lux). Observations for immobility and sublethal effects after 24 and 48 hours. Samples of all test concentration and control were taken at the start and end of the study. A part of the collected samples was centrifuged before analysis. Analysis of the centrifuged and non-centrifuged part by HPLC after addition of acetonitrile and pH was adjusted to 3. LOQ was 2 µg/L, LOD of 0.052 mg/L. Centrifugation was performed because in the pretest precipitation was observed at 100 mg a.s./L nominal. Reference substance tested was potassium dichromate a month before (six concentrations: 0.75, 1.00, 1.33, 1.78, 2.37, and 3.16 mg/L). EC_{50} value was calculated using probit-analysis after maximum likelihood method, programme used by Ratte.

The measured concentrations in the uncentrifuged test solutions ranged from 102 – 118% of nominal (average 107.8%) and from 104 to 119% of nominal (average 109.2%) on day 0 and 2, respectively. The measured concentrations in the centrifuged solutions ranged from 66 to 108% (average 96.0%) of nominal and from 88 to 113% of nominal (average 102.7%) on day 0 and 2, respectively.

No immobilties in the control. Immobility of 33% at 10 mg a.i./L and 43% at 100 mg a.i./L after 48 hours. No clear dose-relationship was found. Sublethal effects (hardly any movements perceivable, daphnids lying at the bottom) were observed from 10 mg a.i./L and onwards. The 48-hours EC_{50} was determined as > 100 mg a.i./L based on nominal concentrations. Based on mean measured centrifuged concentration of 100 mg/L nominal, the 48- hours EC_{50} is > 95 mg a.s./L. The 24-h EC_{50} of the reference substance was 1.59 mg/L, which was within the required range of 0.9 – 1.9 mg/L. Water quality parameters were within acceptable range. No observation of the test solutions was reported.

5.4.2.2 Long-term toxicity to aquatic invertebrates

Parent compound: Spirodiclofen.

Study 1

The chronic toxicity of [dihydrofuranone-3-14C]-Spirodiclofen (chemical purity >97.8%, radiochemical purity >99%) to *Daphnia magna* was assessed in a 21-day flow-through study (Heimbach, 1998). First instar daphnids (<24 hours old, 20 per treatment, 5 per replicate) were used to initiate the study. The nominal concentrations were 1.8, 3.2, 5.6, 10, 18, 32 and 56 µg a.s./L plus control and solvent control (acidified methanol). The mean measured concentrations, based on LSC measurement of the test solutions, were 2.59, 4.12, 7.52, 14.0, 24.8, 49.3 and 70.7 µg a.s./L, representing 126-144% of nominal. Water quality parameters were: temperature (20 ± 1°C), dissolved oxygen (≥94% of saturation) and pH (7.7 to 8.1).
Survival was not affected at any test concentration when compared to the pooled control group. Mean length was significantly reduced at mean measured concentrations of 49.3 and 70.7 mg a.s./L after 21 days exposure when compared to the solvent control group. At 24.8 µg a.s.L, mean length was also significantly reduced, but the author of the report considered this difference as being not biologically relevant, since the difference in length with the solvent control (4.59 mm versus 4.66 mm) was very slight. This position is supported. There was no adverse effect on mean weight at any of the tested concentrations when compared to the solvent control group.

A delay in time to first brood was not observed at any tested concentration. Reproductive success, as measured by the mean no. of young per adult per reproduction day, was significantly reduced in the 49.3 and 70.7 µg a.s./L group when compared to the pooled control group. No dead offspring or aborted eggs were found during the study, and no abnormal behaviour of adults or juveniles was observed.

Based on the absence of significant survival, growth or reproductive effects, the NOEC was identified as 24.8 µg a.s./L, mean measured concentration. Based on significant growth and reproductive effects, the LOEC was identified as 49.3 µg a.s./L.

Comment by RMS
The study was in accordance with GLP and OECD 202 (II) and EPA 72-4 with a few deviations from the protocols.

- The mean measured concentrations of spirodiclofen were based on LSC measurements of the test solutions, with a correction for the ratio of radiolabelled and unlabeled a.s., the latter value being taken from the measurement of the stock solution by HPLC/UV. It was not confirmed (e.g., by radio-TLC) that the radioactivity in the test solutions consisted of unchanged spirodiclofen only. Indirect evidence is however available from the acute study on the toxicity of spirodiclofen to Daphnia magna (Heimbach, 1998) which was also performed under flow-through conditions at comparable concentrations, using the same diluter system, and employing a single stock solution prepared with the same solvent. In this study (acute *Daphnia magna*), the concentrations of spirodiclofen measured in test solutions were 80 – 99% of nominal (mean 92%). Loss of test substance through degradation or other processes was therefore presumably insignificant during the reproduction test and the study is considered to be valid.

- OECD 202(II) recommends the use of at least 40 animals. The present study employed only 20 animals, but the replication (n=4) was according to OECD 202(II).

Study 2

Another chronic toxicity study of spirodiclofen (purity 97.8%) on survival and reproduction of *Daphnia magna* was performed according to EPA 72-4 (Hall and Lam, 2001). The animals were exposed to a negative control and a solvent control (acidified methanol, 1% HCl 0.1 mol/L, 0.1 mL/L) and concentrations of 4.6, 8.3, 15.1, 27.5 and 50 µg a.s/L for 21 day in a flow-through system (6.7 x turnover rate per day). 1 L beakers with 900 mL of test solution were used to expose 10 individuals (first instars < 24 h old) in 4 replicates. Daphnids were fed with algae.Dead organisms were counted and removed daily. Neonates were counted and removed 3 times per week. At test termination the body length of the parents and their dry weight were determined. Data were statistically analysed by ANOVA and by Dunnett's test or Bonferroni t-test using TOXSTAT. Samples of the test solution were taken on days 0, 7, 14 and 21 and analysed directly by HPLC with UV detection.

Mean measured test concentrations were 4.39 (95%), 6.65 (80%), 11.1 (73%), 20.2 (73%) and 32.7 (65%) µg/L for the 4.6, 8.3, 15.1, 27.5 and 50 µg/L nominal concentrations, respectively.The DOC ranged from 7.4 to 9.0 mg/L. pH: 8.0 – 8.2. Temperature: 19.7 – 21.3 °C. Hardness: 162 mg $CaCO_3$ /L. Photoperiod: 16 h light, 8 h dark., 623 lx. All parameters were within acceptable ranges.

Survival observed among the treated parents was 88-100% and was not lower than in the controls. Sublethal effects (pale colouration, abnormal position and unhatched neonates) were not dose related. Reproduction appeared to be the most sensitive endpoint. There were no significant differences in the time to first brood between the solvent control and the treatment groups and for this endpoint the resulting NOEC was 32.7 µg/L, measured concentration. The mean number of neonates per adult was significantly affected in the 20.2 µg/L and 32.7 µg/L (measured) concentrations. The mean number of neonates per adult in the pooled controls and in the 4.39, 6.65, 11.1, 20.2 and 32.7 µg/L concentrations were 6.15, 6.01, 5.55, 5.47, 4.47 and 2.78, respectively. For this endpoint the NOEC was therefore 11.1 µg/L, mean measured concentrations. For the effect on dry weight and length of the exposed adult Daphnids the NOEC was 20.2 µg/L, mean measured concentrations.

Metabolite of spirodiclofen: BAJ 2740-enol.

The chronic toxicity of BAJ 2740-enol (purity 99.9%) to Daphnia magna was assessed in a 21-day study under semi-static exposure conditions (Hendel, B.; 2000). The study was in accordance with GLP and OECD 211 and EPA 72-4, with no major deviations from the protocols. First instar daphnids (<24 hours old, 10 per treatment, 1 per replicate) were used to initiate the study. The nominal concentrations were 1.0, 3.2, 5.6, 10, 18, 32 and 56 mg a.s./L plus control. The concentrations were measured in fresh solutions of 3 study days and in the corresponding 72-h aged solutions and represented 88-107% of nominal. Statistical endpoints were calculated using the nominal concentrations. Water quality parameters were: temperature (20 ± 2°C), dissolved oxygen (≥87% of saturation) and pH (7.9 to 9.1).

Survival was reduced by 80% at 56 mg a.s./L but not affected at lower concentrations. Mean length was significantly reduced at 5.6, 18 and 56 mg a.s./L, but the differences at 5.6 and 18 mg a.s./L were considered unrelated to the treatment in the absence of a similar effect at higher concentrations of 10 and 32 mg a.s./L, respectively.

A delay in time to first brood was only observed at 56 mg a.s./L. Reproductive success, as measured by the total no. of young per adult, was significantly reduced at 5.6, 18 and 56 mg a.s./L, but the differences at 5.6 and 18 mg a.s./L were considered unrelated to the treatment in the absence of a similar effect at higher concentrations of 10 and 32 mg a.s./L, respectively. A reduction of the reproductive success, as measured by the mean number of young per adult per reproduction day, was only observed at 56 mg a.s./L. No dead offspring or aborted eggs were found during the study, and no abnormal behaviour of adults or juveniles was observed.
Based on the absence of significant survival, growth or reproductive effects, the NOEC was identified as 32 mg a.s./L, based on nominal concentrations. Based on significant survival, growth and reproductive effects, the LOEC was identified as 56 mg a.s./L, based on nominal concentrations.

5.4.3 Algae and aquatic plants

Parent compound: Spirodiclofen.

A static algal growth inhibition test with technical Spirodiclofen (97.8% pure) in acidified methanol test medium was conducted as a limit test (Anderson, 1998, amendment 2002). Test duration was 96-hours, green algae species was *Pseudokirchnerialla subcapitata* (formerly *Selenastrum capricornutum*). Test was performed according to OECD 201(1984) and EPA 540/9-86-134 (1986) guideline and in compliance with GLP. Three concentrations were tested: 20, 40, and 60 µg test item/L (19.56, 39.12, 58.68 µg a.s./L), the last concentration (60 µg test item/L) was at the limit of solubility. Six replicates per treatment. A control and a solvent control were included. Separate test concentrations without algae were made for analytical purposes. A stock solution was prepared with acidified methanol (2.5% acetic acid) and stirred for 5 minutes. OECD test medium, pH 8.3. Initial cell counts 1 x 10^4 cells/ml. Incubation at 23 °C, continuous light (8000 lux), stirring. Cell numbers were estimated photometrically at 578 nm using a single-beam photometer after 24, 48, 72, and 96 hours of exposure. Samples were taken from the concentrations without algae at the start and end of the study. The stock solution was also analysed. Analysis by HPLC with UV detection, after dilution with acetonitrile, LOQ 10 µg/L. Reference substance was potassium dichromate performed about one year before at six concentrations (0.10 – 1.80 mg/L).

Measured concentrations at the start were 102 – 105% of nominal, at the end of the study period the concentrations were declined to 22 – 24% of nominal. Measured test concentrations after 96 hours were: 6.30, 8.73, and 13.8 µg a.s./L for nominal 20, 40, and 60 µg test item/L.

Measured concentration of the stock solution was 77% of nominal on day 0 and 74% of nominal on day 4. Inhibition in biomass was 10.5% after 96 hours at nominal 60 µg test item/L and inhibition of growth rate was 1.4% after 96 hours at nominal 60 µg test item/L. E_bC_{50} and E_rC_{50} are reported to be > 60 µg test item/L, NOE_bC and $NOE_rC \geq 60$ µg test item/L, all based on nominal concentrations.

Remark: water quality parameters were within acceptable levels. No growth inhibition of algae was observed. However, test substance was tested up to its level of solubility. Test concentrations decreased during the study period, due to the instability of the test item under alkaline conditions.

The geometrical mean measured test concentration of the highest nominal concentration at 60 µg test item/L is calculated to be 29.2 µg a.s./L. The results EC_{50}-values should be based on geometrical mean measured concentrations, E_bC_{50} and E_rC_{50} > 29.2 µg a.s./L, NOE_bC and $NOE_rC \geq$ 29.2 µg a.s./L.

Metabolite of spirodiclofen: BAJ 2740-enol.

The toxicity of BAJ 2740-enol (purity 99.9%) to the green algae *Pseudokirchneriella subcapitata* (formerly: *Selenastrum capricornutum*) was tested according to OECD 201 guideline (1984) and in compliance with GLP under static conditions for 96 hours (Seyfried 2000). Six nominal concentrations were tested: 3.125, 6.25, 12.5, 25, 50, and 100 mg/L and a control. Additional flasks were prepared of each test concentration and control without algae. Test substance was mixed into the test medium by ultrasonic treatment for 15 minutes and stirred for 3 hours. OECD test medium was used, hardness of 24 mg/L as $CaCO_3$. Initial cell count was 1 x 10^4 cells/mL. Three replicates per test concentration and six in the control. Test solutions with algae were incubated at 22 – 23 °C, and continuously illuminated at 7300 lux and stirred. Samples from each test medium, without algae, were taken at the start and end of the study. Samples were not filtered. Analyses was by HPLC with UV/VIS detection at 250 nm after mechanical shaking (to obtain an homogeneous solution) and dilution with acetonitrile, method recovery ranged from 99 to 106%. Algae cell density was determined using an electronic particle counter. Algae cell shape was observed. EC50 values were calculated by probit analysis, NOEC by Dunnett test.

Measured test concentrations at the start and end of the study ranged from 92 to 105% and 86 to 98% of nominal, respectively. Inhomogeneous test suspension was observed, particles of the test item were found at the surface or bottom of the flasks. Cell counts in the control after 72 and 96 hours were 213 x 10^4 and 334 x 10^4 cells/mL, respectively. No difference in cell shape after 96 hours of exposure. 72-h E_rC_{50} is > 100 mg/L, 72-h E_bC_{50} of 82.8 mg/L and 72-h NOE_rC and NOE_bC of 6.25 mg/L. Values are based on nominal concentrations. Water quality parameters were within acceptable levels. Test substance was not homogeneous dissolved in the test media, although the test substance showed to be stable during the study. It is not reported which test concentrations, with algae, were inhomogeneous. According to the analytical report, the test concentrations were measured in the unfiltered test media without algae. Therefore, it is not clear at which concentrations the algae were exposed because in the concentrations with algae test substance was not homogeneous dissolved.

5.4.4 Other aquatic organisms (including sediment)

Table 76: Summary of relevant information on chronic toxicity of sediment dwelling organisms for spirodiclofen.

Method	Results	Remarks	Reference
Chronic toxicity study 28-day, midges, BBA guideline	NOEC emergence = 0.032 mg a.s./L EC_{50} emergence = 0.094 mg a.s./L	water/sediment, static test. *Chironomus riparius* Values based on nominal concentrations of overlying water.	Heimbach, F. (1999)[b]

b: As summarised in the DAR 08, vol 3-B9 April 2004.

The chronic toxicity of Spirodiclofen (purity 97.5%) to Chironomus riparius (<3 day old, 1st instar larvae) was assessed in a 28 day water/sediment system under static conditions using the BBA method and in accordance with GLP (Heimbach, 1999). Seven nominal test concentrations were used and a water and solvent control.. 25 midge larvae per replicate were exposed. Two replicates for each test concentration and three for the untreated and solvent control. The water layer was spiked with the test substance.Test solutions were analysed in the water phase. The measured test concentration in the water declined rapidly within the first 7 days. Total number of emerged midges was dose-related reduced and at higher concentrations, no emergence occurred. Emergence was the most sensitive parameter.EC50 and NOEC for emergence was reported to be 0.094 and 0.032 mg a.s./L, respectively.

Table 77: Summary of relevant information on chronic toxicity of sediment dwelling organisms for BAJ 2740-enol (metabolite of spirodiclofen).

Method	Results	Remarks	Reference
Chronic toxicity study 28-day, midges, BBA guideline	NOEC emergence = 3.2 mg a.s./L.	28-day water/sediment, static test. *Chironomus riparius* Values based on nominal concentrations of overlying water.	Heimbach, F. (2000)[b]

b: As summarised in the DAR 08, vol 3-B9 April 2004.

The chronic toxicity of BAJ 2740-enol (metabolite of spirodiclofen, purity 99.9%) to Chironomus riparius (<3 day old, 1st instar larvae) was assessed in a 28 day water/sediment system under static conditions using the BBA method and in accordance with GLP (Heimbach,2000). Four nominal test concentrations were tested and a water control.25 midge larvae per replicate were exposed..Two replicates for each test concentration and three for the untreated control. The water layer was spiked with the test substance. Test concentrations were analysed. Test concentrations were stable during the study. No toxicity effects at the lowest concentration, at higher concentrations no emergence occurred. The NOEC emergence was set at 3.2 mg a.s./L.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

Acute aquatic hazards.

Spirodiclofen is a poorly water-soluble substance, 0.05 mg/L at pH 4 and 22 °C. Acute aquatic toxicity is available for all three trophic levels. No effects on aquatic organisms were observed at test concentrations between 0.0292 mg/L and 0.0508 mg/L, which are near the limit of solubility of the spirodiclofen. The data is based on mean measured concentrations in the medium and the L(E)C50s values are above the water solubility. The available data show that the criteria for classification for acute aquatic hazard according to Annex I *Table 4.1.0 (a)* of the CLP regulation are not applicable to spirodiclofen. Therefore, we conclude not to classify the substance for acute aquatic hazards.

Aquatic Chronic hazards.

Spirodiclofen is considered not rapidly degradable in the environment and does not fulfil the criterion BCF > 500. Chronic aquatic toxicity is available for all three trophic levels. The lowest NOEC of 0.00195 mg/L was obtained in fish (*Oncorhynchus mykiss*). This value is below the classification threshold value of 1 mg/L. Spirodiclofen does therefore fulfil the criteria for

classification as chronic category 1 hazard to the aquatic environment. The available data indicates that the lowest NOEC value of 0.00195 mg/L falls within the range 0.001< NOEC ≤ 0.01 mg/L and is not rapidly degradable. Therefore spirodiclofen fulfils the criteria for classification as aquatic Chronic category 1 with an M-factor of 10.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Table 78. Conclusions on classification and labelling for environmental hazards of spirodiclofen.

	CLP regulation
Resulting harmonised classification.	Aquatic chronic 1 (H410: very toxic to aquatic life with long-lasting effects). Chronic M-factor: 10

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Degradation

A summary of the relevant studies included in the CLH report for the degradation of spirodiclofen is shown in the table below.

Table: degradation studies

Method	Results	Remarks	Reference
Spirodiclofen			
EPA guideline and SETAC, GLP study	*DT_{50} for hydrolysis at 20 °C pH 4: 124 days pH 7: 53.4 days pH 9: 2.2 days	Hydrolytically unstable Major hydrolysis product: Spirodiclofen-enol	Babczinsky (2000)[a]
UBA (1992) guideline GLP study	DT_{50} at 50 °N 80 days (no clouds) DT_{50} at 50 °N 110 days (cloudiness) in June	Photodegradation not expected	Hellpointer (1998)[a]
EPA guideline GLP study	DT_{50} at 40 °N 123 days (midsummer)	Photodegradation not expected	Stupp and Brumhard (2000)[a]
BBA IV and SETAC guideline GLP study Water/sediment aerobic	DT_{50}s for Honniger pond system Water[(A)]: 0.3 day Sediment: 4.4 days System: 4.2 days DT_{50}s for Anglerweiher pit system Water: 1.1 day Sediment: 2.5 days System: 2.3 days	(A) Calculated using measured values in the water on day 0 and 1, assuming first order decline	Riegner (1999)[a]

EPA (Pesticide Assessment Guidelines Subdivision N, Series 162-3) guideline, GLP study Water/sediment anaerobic Water/sediment anaerobic	DT_{50} Water: - Sediment: 9.8 days System: 10 days		Wujcik *et al.* (2000)[a]

*DT_{50} values at 20 °C at pHs 4, 7 and 9 were calculated by the RMS; [a] As summarised in the DAR vol. 3-B8, April 2004.

Spirodiclofen hydrolyses in the pH range 4-9 in the absence of light under sterile conditions, with first-order DT_{50}s at 20°C of 124, 53.4 and 2.2 days at pHs 4, 7 and 9 respectively as calculated by the rapporteur member state (RMS) during the pesticide review based on the original data. The hydrolytic stability of spirodiclofen decreases as temperature and pH increase. The main hydrolysis product was spirodiclofen-enol, the total amount of other products at any point did not exceed 2% of the applied radioactivity (AR). Spirodiclofen-enol is hydrolytically stable.

Spirodiclofen is not considered to be directly photodegradable in two photodegradation studies. Environmental photolysis half lives in pure water were estimated to be 54, 64, 80 and 110 days at 30, 40, 50 and 60 °N, respectively (clouds not considered), or 110 days at 50 °N (cloudiness taken into consideration).

No study on ready biodegradability was submitted.

In two aerobic water/sediment systems (pond and pit), the $DT_{50}s_{,water}$ of spirodiclofen were 0.3 and 1.1 days, respectively, while the whole system DT_{50}s resulted in 4.2 and 2.3 days. In both systems, spirodiclofen was detected in the sediments at levels of 58-68% AR on day 1. This indicates that spirodiclofen disappears rapidly from the water phase. Mineralisation was at a comparable low level in both systems, 2.1% and 2.6% after 110 days. The major metabolite was spirodiclofen-enol which reached maximum levels of 84% (days 14-59) and 30% (day 110) in water and sediment, respectively.

The total levels of other degradates in any system were < 5.0% AR. The DT_{50}s of 186 and 393 days were calculated for the disappearance of spirodiclofen-enol from the pond and pit system, respectively. In the pit system, no degradation of spirodiclofen-enol was apparent until the end of the study (day 110).

In an anaerobic water/sediment study, spirodiclofen was almost quantitatively lost from the water phase at the first sampling, with a maximum level occurring in sediment of 91.9% AR on day 0. Spirodiclofen dissipated from sediment and overall system with a DT_{50} of 9.8 and 10 days, respectively. Maximum levels of the major metabolite spirodiclofen-enol were 71-80% AR (water) and 82-94% AR (whole system) between days 34 and 365. Levels of non-extractable radioactivity in sediment (< 3.6% AR), production of CO_2 (< 1.0% AR) and organic volatiles (< 0.5% AR) were low throughout the study.

Based on the findings from the aerobic and anaerobic water/sediment test, spirodiclofen appears to be susceptible for primary degradation (DT_{50}s < 16 days) but not for ultimate mineralisation (CO_2 production). Considering the low levels of mineralisation in the simulation studies, spirodiclofen is considered not rapidly degradable (degradation of > 70% degradation within 28 days) by the DS for purposes of classification and labelling.

Bioaccumulation

The CLH report contains a BCF study with bluegill sunfish (*Lepomis macrochirus*) according to

OECD TG 305 and EPA 72-6 guidelines; the highest BCF derived for spirodiclofen was 491 L/kg for the whole fish, and 323 L/kg after 5% lipid normalisation. Spirodiclofen therefore does not fulfil the criteria for bioaccumulation according to Regulation EC 1272/2008, since the BCF is < 500 L/kg.

Aquatic Toxicity

The DS included in the CLH report aquatic toxicity studies for spirodiclofen and its major metabolite spirodiclofen-enol for all trophic levels: fish, invertebrates and algae. The studies for spirodiclofen are summarised in the table shown below. The studies for spirodiclofen-enol are included in Supplemental information - In depth analyses by RAC.

Table: Summary of the information on aquatic toxicity for spirodiclofen

Method	Results	Remarks	Reference
Acute fish spirodiclofen, 96h OECD TG 203, EPA guideline	LC_{50} ≥ 0.035 mg/L	96h flow-through, limit test *Oncorhynchus mykiss* Measured concentrations	Dorgerloh (1999-a)
Acute fish spirodiclofen, 96h OECD TG 203, EPA guideline	LC_{50} ≥ 0.0455 mg/L	96h flow-through, limit test *Lepomis macrochirus* Measured concentrations	Dorgerloh (1999-b)
Acute invertebrate spirodiclofen, 48h OECD TG 202, EPA 72-2	EC_{50} ≥ 0.0508 mg/L	48h flow-through, limit test *Daphnia magna* Measured concentrations	Heimbach (1998-a)
Algae inhibition, spirodiclofen, 96h OECD TG 201, EPA 540/9-86-134	E_rC_{50} ≥ 29.2 µg/L (0.0292 mg/L) E_bC_{50} ≥ 29.2 µg/L (0.0292 mg/L) NOEC ≥ 29.2 µg/L (0.0292 mg/L)	96h static, limit test Pseudokirchneriella subcapitata Values based on geometrical mean measured concentrations	Anderson (1998)[b]
Chronic fish, spirodiclofen, 97d OECD TG 210, FIFRA 72-4, early life stage	NOEC = 1.95 µg/L (0.00195 mg/L)	97 days flow-through test *Oncorhynchus mykiss* NOEC based on fish growth Measured concentration	Dorgerloh (2000)[b]
Chronic invertebrate, spirodiclofen, 21d OECD TG 202 (II), EPA 72-4.	NOEC = 24.8 µg/L (0.0248 mg/L)	21d flow-through test *Daphnia magna* NOEC is based on growth and reproductive effects Mean measured concentration	Heimbach (1998-b)[b]
Chronic invertebrate, spirodiclofen, 21d EPA 72-4	NOEC = 11.1 µg/L (0.0111 mg/L)	21d flow-through test *Daphnia magna* NOEC is based on reproduction Mean measured concentration	Hall and Lam (2001)[b].

The acute and chronic aquatic toxicity hazard endpoints for spirodiclofen were studied for all three trophic levels.

Fish

The acute toxicity of spirodiclofen in fish was tested in two different species: *Oncorhynchus mykiss* and *Lepomis macrochirus*. In both studies the concentration of spirodiclofen was at or near its water solubility in the test medium (solubility in water 0.05 mg/L at pH 4 and 22 °C). Water quality parameters were within acceptable levels.

Short term toxicity to fish

A 96h acute toxicity flow-through limit test with technical spirodiclofen (97.6% pure) in acidified methanol (2.5% acetic acid) test medium with rainbow trout (*Oncorhynchus mykiss*), according to OECD TG 203 and EPA 72-1 guidelines was conducted and in compliance with GLP. No mortalities occurred during the test and no sub-lethal effects were observed at the mean measured limit test concentration of 0.035 mg/L, which was at or near the limit of solubility of the compound in the test medium. No mortalities or other effects were observed in the control and solvent control. Thus, the LC_{50} ≥ 35.1 µg/L (≥ 0.035 mg/L), is based on mean measured concentrations.

In a 96h acute toxicity flow-through limit test with technical spirodiclofen (97.6% pure) in acidified methanol (2.5% acetic acid) test medium in bluegill sunfish (*Lepomis macrochirus*), according to OECD TG 203 and EPA 72-1 guidelines and in compliance with GLP. No mortalities occurred during the test and no sub-lethal effects were observed at the mean measured limit test concentration of 0.0455 mg/L, which was at or near the limit of solubility of the compound in the test medium. No mortalities or other effects were observed in the control and solvent control. The LC_{50} of ≥ 45.5 µg/L (≥ 0.0455 mg/L) is based on mean measured dissolved test concentration.

Long term toxicity to fish

A 97 days fish early life stage flow-through study was undertaken with eggs, larvae and juveniles of rainbow trout (*Oncorhynchus mykiss*). At day 61 post-hatch, fish length was significantly reduced at all tested concentrations (1.09, 1.95 and 3.81 µg/L) while dry fish weight was reduced by 11% only at the highest concentration (not statistically significant). The biological NOEC based on fish growth was 1.95 µg/L, measured concentration, since the reductions in fish length at 1.09 and 1.95 µg/L were small (< 5%) i.e. within the range of control variability and not accompanied by a simultaneous significant reduction in weight.

Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

An acute toxicity flow-through limit test with technical spirodiclofen (97.8% pure) in acidified methanol test medium in *Daphnia magna* was conducted according to OECD TG 202 and EPA 72-2 guidelines and in compliance with GLP. No immobilities in the blank control and one (2.5%) in the solvent control were observed. Immobilities in the test concentrations were 0, 0, 1 (2.5%), 1 (2.5%), and 1 (2.5%) at 5.6, 10, 18, 32, and 56 µg/L nominal. No sub-lethal effects or changes in behaviour were observed during the study. The 48h EC_{50} was set at ≥ 50.8 µg/L, i.e. the mean measured highest test concentration.

Long-term toxicity to aquatic invertebrates

The chronic toxicity of [dihydrofuranone-3-^{14}C]-spirodiclofen (chemical purity ≥ 97.8%,

radiochemical purity ≥ 99%) to *Daphnia magna* was assessed in a 21-days flow-through study. The study was in accordance with GLP and OECD TG 202 (II) and EPA 72-4 with a few deviations from the protocols which are acceptable. Based on survival, growth and reproductive effects, the NOEC was identified at 24.8 µg/L, mean measured concentrations and the LOEC at 49.3 µg/L.

A second chronic toxicity study of spirodiclofen (purity 97.8%) on survival and reproduction of *Daphnia magna* was performed according to EPA 72-4. Survival observed among the treated parents was 88-100% and was not lower than in the controls. Sub lethal effects (pale coloration, abnormal position and unhatched neonates) were not dose related. Reproduction appeared to be the most sensitive endpoint. There were no significant differences in the time to first brood between the solvent control and the treatment groups and for this endpoint the resulting NOEC was 32.7 µg/L, measured concentration. The mean number of neonates per adult was significantly affected in the 20.2 µg/L and 32.7 µg/L (measured) concentrations. The mean number of neonates per adult in the pooled controls and in the 4.39, 6.65, 11.1, 20.2 and 32.7 µg/L concentrations were 6.15, 6.01, 5.55, 5.47, 4.47 and 2.78, respectively. For this endpoint the NOEC was therefore 11.1 µg/L, mean measured concentrations. For the effect on dry weight and length of the exposed adult daphnids the NOEC was 20.2 µg/L, mean measured concentrations.

Algae and aquatic plants

A static algal growth inhibition test with technical spirodiclofen (97.8% pure) in acidified methanol test medium was conducted as a limit test (Anderson, 1998, amendment 2002). Test duration was 96 hours, the green algae species was *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*); it was performed according to OECD TG 201 (1984) and EPA 540/9-86-134 (1986) guideline and in compliance with GLP. No growth inhibition was observed. However, the test substance concentrations decreased during the study period, due to the instability of the test item under alkaline conditions. The geometrical mean measured test concentration of the highest nominal concentration at 60 µg/L was calculated to be 29.2 µg/L. Consequently, the EC_{50} and NOEC values were based on geometrical mean measured concentrations; EbC50 and ErC50 ≥ 29.2 µg/L, NOEbC and NOErC ≥ 29.2 µg/L.

Other Aquatic Organisms (including sediment)

The chronic toxicity of spirodiclofen (purity 97.5%) to *Chironomus riparius* (< 3 day old, 1 instar larvae) was assessed in a 28 day water/sediment system under static conditions using the BBA method and in accordance with GLP. Emergence was the most sensitive parameter. Total number of emerged midges was dose-related reduced and at higher concentrations, no emergence occurred. EC_{50} and NOEC for emergence were reported to be 0.094 and 0.032 mg/L, respectively.

Comments received during public consultation

Three MSCAs supported the classification proposed for environmental hazards as Aquatic Chronic 1 (H410) with an M factor of 10.

Assessment and comparison with the classification criteria

Degradation

No ready biodegradation study or BOD_5/COD data is available. Spirodiclofen hydrolyses rapidly

at pH 9 but not at pHs 4 and 7, thus not fulfilling the criteria for fast primary degradation.

In two water/sediment simulation studies, pond and pit, spirodiclofen half-lives in water were 0.3 and 1.1 days, and 4.2 and 2.3 days in the whole systems. However mineralisation was low in both systems, up to 2.6%, thus not fulfilling the criterion for rapid degradation. Furthermore, based on the information provided in the CLH report on the primary degradant spirodicolfen-enol it cannot be satisfactorily demonstrated that it does not fulfil the criteria for classification as hazardous to the aquatic environment. Overall, RAC considers spirodiclofen as not rapidly degradable for classification purposes based on the criteria in the CLP Regulation, and the indication of the CLP guidance, Annex II.4.

Acute aquatic hazards

RAC notes that spirodiclofen is an insecticide and acaricide, its target organisms being sucking insects and mites, and that no acute toxicity on these thropic levels have been included in the CLH report. RAC would have appreciated the presence of these studies as confirmation of the lack of acute effects.

Spirodiclofen is a poorly water-soluble substance, 0.05 mg/L at pH 4 and 22 °C. Information on acute aquatic toxicity is available for all three trophic levels. No effects on aquatic organisms were observed at test concentrations between 0.0292 mg/L and 0.0508 mg/L, which are near the spirodiclofen limit of water solubility at pH 4. The available data are based on mean measured concentrations in the test media and the L(E)C_{50}s values are above the water solubility at pH 4 (i.e. 0.05 mg/L). The available data show that the criteria for classification for acute aquatic hazards according to Annex I, Table 4.1.0 (a) of the CLP Regulation are not applicable to spirodiclofen. RAC notes that spirodiclofen solubility increases with increasing pH, from 0.05 mg/L at pH 4 to 0.19 mg/L at pH 7. As the tests were conducted at a pH higher than 4, spirodiclofen may have not been tested at the limit of its water solubility. However, due to the lack of effects observed at the tested concentrations and the absence of data at higher concentrations, RAC agrees with the DS's proposal not to classify the substance for acute aquatic hazards.

Aquatic Chronic hazards

Spirodiclofen is considered not rapidly degradable in the environment and does not fulfil the criterion for bioaccumulation, BCF < 500. Chronic aquatic toxicity data are available for all three trophic levels. The lowest NOEC of 0.00195 mg/L was obtained in fish (*Oncorhynchus mykiss*). This value is below the classification threshold value of 0.1 mg/L, therefore spirodiclofen does fulfil the criteria for classification as Chronic Category 1. As the lowest NOEC value of 0.00195 mg/L falls within the range 0.001< NOEC < 0.01 mg/L, an M-factor of 10 is applicable. In conclusion, RAC agrees with the DS proposal for classification of spirodiclofen as **Aquatic Chronic Category 1 (H410: Very toxic to aquatic life with long lasting effects)** with an **M-factor of 10**.

Supplemental information - In depth analyses by RAC

Environmental Hazard Assessment of Spirodiclofen-enol

Summary of the information on degradation of spirodiclofen-enol

Method	Results	Remarks	Reference
Metabolite of Spirodiclofen			
Spirodiclofen-enol (BAJ 2740- enol)	DT_{50} at 25 °C 7.6 hours	Photodegradation is expected	Babczinski (2000)[a]
BBA IV and SETAC guideline GLP study Water/sediment aerobic	DT_{50}s for Honniger pond system Water: 186 days Sediment: - System: 393 days		Riegner (1999)[a]
EPA (Pesticide Assessment Guidelines Subdivision N, Series 162-3) guideline GLP study Water/sediment anaerobic	DT_{50} Sediment: 175 days	Extrapolated value	Wujcik *et al.* (2000)[a]

Spirodiclofen-enol is hydrolytically stable. The aqueous photolysis of spirodiclofen-enol was studied in Rhine water. When irradiated with a xenon lamp (equivalent light intensity of midsummer sunlight, 40 °N) the half-life was 7.6 hours. BAJ 2740-dioxoketone was the only photodegradate formed at > 10% AR (max. 25.6% AR). The DT_{50}s of 186 and 393 days was calculated for the disappearance of spirodiclofen-enol from the pond water and system, respectively. In the pit system, no degradation of spirodiclofen-enol was apparent until study end (day 110).

A bioaccumulation study with the major metabolite spirodiclofen-enol is not available. The log Kow of spirodiclofen-enol is reported as 3, which is below the cut-off value of log Kow = 4. Consequently, spirodiclofen-enol is not considered to be a bioaccumulating substance.

Table: Summary of the information on aquatic toxicity for spirodiclofen-enol (BAJ 2740-enol)

Method	Results	Remarks	Reference
Acute fish, BAJ 2740-enol, 96h. OECD TG 203, EPA 72-1 guideline	LC_{50} ≥ 73 mg/L	96h static, limit test. *Oncorhynchus mykiss* Mean measured concentrations.	Peither (1999)
Acute invertebrate, BAJ 2740-enol, 48h. OECD TG 202, EPA 72-2	EC_{50} ≥ 95 mg/L	48h static test *Daphnia magna* Mean measured concentration.	Heimbach (2000)
Algae inhibition, BAJ 2740-enol, 96h. OECD TG 201, EPA 123-2.	E_rC50 ≥ 100 mg/L E_bC50 ≥ 82.8 mg/L NOEC ≥ 6.25	96h static, nominal, limit test Pseudokirchneriella subcapitata	Seyfried (2000).
Chronic fish, BAJ 2740-enol, 97 days, OECD TG 210, FIFRA 72-4, early life stage.	NOEC ≥ 115 µg/L (0.115 mg/L)	97-days flow- through test *Oncorhynchus mykiss* No effects were determined. Measured concentration.	Dorgerloh (2001)[b]
Chronic fish, BAJ 2740-enol, 115 days, FIFRA 72-5, full fish life cycle.	NOEC ≥ 190 µg/L (0.190 mg/L)	115 days flow- through test *Cyprinodon variegatus.* No effects were determined. Mean measured concentrations.	Dionne (2001)[b].
Chronic invertebrate, 21-d, BAJ 2740-enol, OECD TG 211, EPA 72-4	NOEC = 32 mg/L	21-d semi-static test *Daphnia magna* NOEC is based on reproduction Nominal concentrations.	Hendel (2000)[b].

Aquatic Toxicity

Short term toxicity to fish

Spirodiclofen-enol

A 96h static toxicity test was carried out on rainbow trout (*Oncorhynchus mykiss*) with the main spirodiclofen metabolite: spirodiclofen-enol (purity 99.9%) as a limit test according to OECD TG 203 guideline and in compliance with GLP. No mortalities in the control, 2 out of 30 fish died in the test solutions. No sub-lethal effects were observed in the test solutions and control during the whole study period. The 96h LC_{50} was determined as ≥ 100 mg/L, based on nominal concentrations, which is the limit of the water solubility in the test water used. Based on mean measured test concentrations the 96h LC_{50} was set at ≥ 73 mg/L. Water quality parameters were within the acceptable limits. Due to visible test item during the study and the low recovery at the start, the result should be based on the mean measured filtered test

concentrations.

Long term toxicity to fish

The study was in accordance with GLP and OECD TG 210 and FIFRA 72-4 with no major deviations from the protocols. A 97-day fish early life stage flow-through study was undertaken with eggs, larvae and juveniles of rainbow trout (*Oncorhynchus mykiss*). Based on the absence of test compound related effects at the highest tested concentration, the NOEC for spirodiclofen-enol is ≥ 115 µg/L and based on measured concentrations.

A second study was performed as a full fish life cycle flow-through test with the estuarine species sheepshead minnow (*Cyprinodon variegatus*). The study was in accordance with GLP and FIFRA 72-5. Based on the absence of test compound related effects at the highest tested concentration, the NOEC for spirodiclofen-enol is ≥ 190 µg/L, based on mean measured concentrations.

Aquatic invertebrates

Short-term toxicity to aquatic invertebrates

The acute toxicity of spirodiclofen-enol (purity 99.9%), was tested to *Daphnia magna* in a static 48h test according to OECD TG 202 (part I, 1984). No immobilties were observed in the control, while immobility of 33% at 10 mg/L and 43% at 100 mg/L were observed after 48 hours. No clear dose-relationship was found. Sub-lethal effects (hardly any movements perceivable, daphnids lying at the bottom) were observed from 10 mg/L and on wards. The 48h EC_{50} was determined as ≥ 100 mg/L based on nominal concentrations, while based on mean measured centrifuged concentrations, the 48h EC_{50} is ≥ 95 mg/L.

Long-term toxicity to aquatic invertebrates

The chronic toxicity of spirodiclofen-enol (purity 99.9%) to *Daphnia magna* was assessed in a 21 days study under semi-static exposure conditions. The study was in accordance with GLP and OECD TG 211 and EPA 72-4, with no major deviations from the protocols. Based on survival, growth and reproductive effects, the NOEC was identified at 32 mg/L and the LOEC at 56 mg/L, both based on nominal concentrations.

The toxicity of BAJ 2740-enol (purity 99.9%) to the green algae *Pseudokirchneriella subcapitata* (formerly: *Selenastrum capricornutum*) was tested according to OECD TG 201 guideline (1984) and in compliance with GLP under static conditions for 96 hours. Measured test concentrations at the start and end of the study ranged from 92 to 105% and 86 to 98% of nominal, respectively. Inhomogeneous test suspension was observed, particles of the test item were found at the surface or bottom of the flasks. The 72h E_rC_{50} is ≥ 100 mg/L, the 72h E_bC_{50} of 82.8 mg/L and 72h NOE_rC and NOE_bC of 6.25 mg/L, all nominal concentrations. Water quality parameters were within acceptable levels.

Other Aquatic Organisms (including sediment)

The chronic toxicity of spirodiclofen-enol (purity 99.9%) to *Chironomus riparius* (< 3 day old, 1 in star larvae) was assessed in a 28 day water/sediment system under static conditions using the BBA method and in accordance with GLP. No toxicity effects at the lowest concentration while at the highest concentrations no emergence occurred. The NOEC emergence was set at 3.2 mg/L.

Classification of Spirodiclofenol

In the two chronic fish studies which the **NOEC ≥ 115 µg/L** and **NOEC ≥ 190 µg/L** are derived, there were no "test compound related effects" observed. Thus, the results are rather

equivocal and it is not clear if spirodiclofen-enol fulfills the criteria for classification as hazardous to the aquatic environment as set out in point 4.1.3.3 of the CLP regulation. However, neither can it be excluded that relevant effects might occur in the range 0.1 – 1 mg/L. Thus, based on the information provided in the CLH report on the primary degradant spirodicolfen-enol it cannot be satisfactorily demonstrated that it does not fulfil the criteria for classification as hazardous to the aquatic environment.

6 OTHER INFORMATION

RAC evaluation of hazards to the ozone layer

Summary of the Dossier Submitter's proposal

No classification is proposed by the DS due to lack of data.

Comments received during public consultation

No comments were received

Assessment and comparison with the classification criteria

No data

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8 ANNEXES

A confidential annex is provided separately.