CLH report

Proposal for Harmonised Classification and Labelling

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

Substance Name: Amisulbrom

EC Number: **Not allocated**

CAS Number: **348635-87-0**

Index Number: **Not available**

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	**Amisulbrom**
EC number:	**Not allocated**
CAS number:	**348635-87-0**
Annex VI Index number:	**Not available**
Degree of purity:	**985 g/kg**
Impurities:	**None relevant to classification and labelling. Full information is provided in the technical dossier.**

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	**Not in Annex VI**
Current proposal for consideration by RAC	**Eye Irritant 2; H319 - Causes serious eye irritation** **Carc. 2; H351 - Suspected of causing cancer** **Aquatic Acute 1; H400 - Very toxic to aquatic life** **M = 10** **Aquatic Chronic 1; H410 - Very toxic to aquatic life with long lasting effects** **M = 10**
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	**Eye Irritant 2; H319 - Causes serious eye irritation** **Carc. 2; H351 - Suspected of causing cancer** **Aquatic Acute 1; H400 - Very toxic to aquatic life** **M = 10** **Aquatic Chronic 1; H410 - Very toxic to aquatic life with long lasting effects** **M = 10**

1.3 Proposed harmonised classification and labelling

Table 3: Proposed classification

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	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.2.	Flammable gases	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.3.	Flammable aerosols	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.4.	Oxidising gases	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.5.	Gases under pressure	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.6.	Flammable liquids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.7.	Flammable solids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.8.	Self-reactive substances and mixtures	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.9.	Pyrophoric liquids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.10.	Pyrophoric solids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.11.	Self-heating substances and mixtures	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.12.	Substances and mixtures which in contact with water emit flammable gases	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.13.	Oxidising liquids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.14.	Oxidising solids	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification

2.15.	Organic peroxides	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
2.16.	Substance and mixtures corrosive to metals	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.1.	Acute toxicity - oral	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
	Acute toxicity - dermal	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
	Acute toxicity - inhalation	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.2.	Skin corrosion / irritation	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.3.	Serious eye damage / eye irritation	**Eye Irritant 2; H319 - Causes serious eye irritation**	Not applicable	Not classified	
3.4.	Respiratory sensitisation	Not classified	Not applicable	Not classified	Data lacking
3.4.	Skin sensitisation	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.5.	Germ cell mutagenicity	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.6.	Carcinogenicity	**Carc 2; H351 - Suspected of causing cancer**	Not applicable	Not classified	
3.7.	Reproductive toxicity	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.8.	Specific target organ toxicity –single exposure	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.9.	Specific target organ toxicity – repeated exposure	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
3.10.	Aspiration hazard	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification
4.1.	Hazardous to the aquatic environment	**Aquatic Acute 1; H400 - Very toxic to aquatic life**	**Acute M = 10**	Not classified	

		Aquatic Chronic 1; H410 - Very toxic to aquatic life with long lasting effects	**Chronic M = 10**		
5.1.	Hazardous to the ozone layer	Not classified	Not applicable	Not classified	conclusive but not sufficient for classification

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

Labelling:

Pictogram(s): **GHS07, GHS08, GHS09**

Signal word: **Warning**
Hazard statements: **H319; Causes serious eye irritation**
H351; Suspected of causing cancer
H410; Very toxic to aquatic life with long lasting effects

Precautionary statements: **Not required**

Proposed notes assigned to an entry: **None**

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

Amisulbrom is a pesticidal active substance in the scope of Directive 91/414/EEC (and now Regulation 1107/2009). There are no existing entries in Annex VI of CLP for amisulbrom and the classification and labelling has not been considered at the EU level before. In accordance with Article 36(2) of CLP, it is now subject to the harmonised classification and labelling procedure.

At the time of submission the substance is not registered under REACH.

2.2 Short summary of the scientific justification for the CLH proposal

Amisulbrom is an active substance in the scope of Dir 91/414/EEC (Regulation 1107/2009). In July 2014, EFSA published a conclusion on the peer review of the risk assessment for the active substance. This highlighted a concern for serious eye damage, reproductive toxicity (fertility and development), carcinogenicity and Aquatic acute and chronic toxicity.

Classification for Eye Irritant 2; H319 – Causes severe eye irritation is considered appropriate based on a standard eye irritation study in which mild conjunctival erythema (grade 1) was observed in treated rabbits. It should be noted that effects were observed at the end of the observation period (i.e. 21 days) and a simple argument for classification in Category 1 could be made. However, the results are confounded by inconsistent responses in the individual animals and Category 2 is therefore considered more appropriate (refer to section 4.4.2 of the CLH report).

An increase in benign liver tumours was observed in male rats and mice. Mechanistic data have been provided to investigate the mode of action (MoA) for these tumours and suggest the most likely mode of action involves enzyme induction, hypertrophy and increased cell proliferation. However, whilst reassuring, these data are not considered to be sufficient to exclude human relevance and, consequently, classification with Carc 2; H351 – Suspected of causing cancer is proposed (refer to section 4.10 of the CLH report).

A female-mediated reduction in fertility was observed following exposure to amisulbrom. However the reduced fertility was only observed at doses associated with severely impaired bodyweight development, reduced ovarian weight and function with associated histopathology. Food restriction in untreated animals during gestation, lactation and weaning up to PND 40 caused similar effects. As such, it is concluded that the effects on fertility are the secondary consequence of impaired nutrition and growth during the early stages of ovary development and no classification is proposed (refer to section 4.11 of the CLH report).

A low incidence of cleft palate/chondrodystrophy, was observed in rats in the absence of maternal toxicity. However, the pattern of the finding suggests a spontaneous (genetic) aetiology rather than an effect of treatment with amisulbrom and no classification is proposed (refer to section 4.11 of the CLH report).

On the basis of reliable degradation and bioaccumulation data, amisulbrom is considered to be 'not rapidly degradeable'. Acute and chronic aquatic toxicity data on amisulbrom are available for fish, invertebrates and algae. The most acutely sensitive trophic group is fish with a 96-hr LC_{50} value for *Cyprinus carpio* of 0.0229 mg/l. As the acute fish endpoint is in the range 0.01 mg/l $< L(E)C_{50} \leq 0.1$ mg/l, amisulbrom should be classified for acute environmental (aquatic) hazard as Aquatic Acute category 1 with an acute M-factor of 10.

The lowest chronic aquatic endopoint available for amisulbrom is for algae. However, due to the acute fish endpoint being lower than the chronic fish endpoint there is uncertainty regarding the adequacy of the chronic data set. Amisulbrom has therefore been classified using the surrogate acute approach. As the acute fish endpoint is in the range 0.01 mg/l $<L(E)C_{50} \leq 0.1$ mg/l and , amisulbrom is 'not rapidly degradable', it should be classified as Aquatic Chronic Category 1 with a chronic M-factor of 10. Refer to section 5 of the CLH report.

2.3 Current harmonised classification and labelling

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

There is currently no entry in Annex VI for amisulbrom.

2.4 Current self-classification and labelling

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

The following entries exist on the C&L Inventory at the time of submission

Classification	Labelling	Number of notifiers
Aquatic Acute 1; H400	H400	19
Acute Tox. 4; H332 Aquatic Acute 1; H400 Aquatic Chronic 1; H410	Warning H332, H410	23
Acute Tox 4; H332 Aquatic Chronic 1; H410	Warning GHS07, GHS09 H332, H410	1

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

Amisulbrom is a new pesticidal active substance in the scope of directive 91/414/EEC. As such, it is subject to the harmonised classification and labelling process in accordance with Article 36 (2) of CLP.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

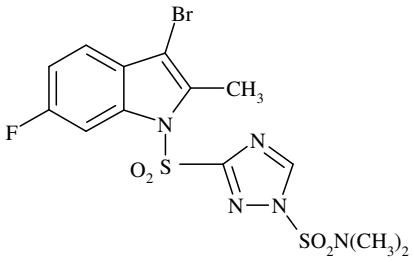
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 4: Substance identity

EC number:	Not listed
EC name:	Not listed
CAS number (EC inventory):	Not listed
CAS number:	348635-87-0
CAS name:	1H-1,2,4-Triazole-1-sulfonamide, 3-[(3-bromo-6-fluoro-2-methyl-1H-indol-1-yl)sulfonyl]-N,N-dimethyl-
IUPAC name:	3-[(3-bromo-6-fluoro-2-methyl-1H-indol-1-yl)sulfonyl]-N,N-dimethyl-1H-1,2,4-triazole-1-sulfonamide
CLP Annex VI Index number:	Not listed
Molecular formula:	$C_{13}H_{13}BrFN_5O_4S$
Molecular weight:	466.3 g/mol

Structural formula:



1.2 Composition of the substance

Table 5: Constituents (non-confidential information)

Constituent	Typical concentration	Concentration range	Remarks
≥ 98.5%			

Current Annex VI entry: None

Table 6: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
Confidential			

There are a number of process impurities identified in the substance all of which are present below 0.2%. These have been taken into consideration and are not considered to impact on the classification proposed in this dossier. Further information on the impurities is considered to be confidential but full details are provided in the technical dossier.

Current Annex VI entry: None

Table 7: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
None				

Current Annex VI entry: Not applicable

1.2.1 Composition of test material

The batches used in the relevant studies were considered equivalent to the substance as described above.

1.3 Physico-chemical properties

All studies were completed to an acceptable standard and the results were considered valid in the review of the active substance. References are taken from the Draft Assessment Report Volume 3, Annex B.2; Physical and Chemical Properties – February 2012. The relevant section references are provided.

Table 8: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Solid (Pale yellow crystalline)	Ogi, N. 2003(a) (DAR B.2.1.7 & 2.1.8)	99.8%
Melting/freezing point	128.6 – 130 °C	Takehara, K. 2003(a) (DAR B.2.1.1)	OECD 102 (Capillary/liquid bath method) 99.8%
Boiling point	After melting, the substance decomposed at 242 -303 °C. No boiling point was observed.	Iijima, K. 2003 (DAR B.2.1.2 & 2.1.3)	OECD 113 (DTA and TGA analysis) 99.8%
Relative density	1.72 1.61	Comb, A. 2003(a) Comb, A. 2003(b) (DAR B.2.1.4)	EEC Method A3 (pycnometer) 99.8% 99.1%
Vapour pressure	1.8 x 10-8 Pa at 25 °C	Comb, A. 2003(c) (DAR B.2.1.5)	EEC Method A4 (Effusion method:vapour pressure balance) 99.8%
Surface tension	Not tested, solubility is < 1 mg/l	(DAR B.2.1.24)	
Water solubility	0.11 mg/L at 20 °C and pH 7 (Does not contain any groups that ionise between pH 4-6 and 8-10. Solubility is not affected by pH).	Takehara, K. 2003(b) (DAR B.2.1.11)	EEC Method A6 (column elution method) 99.8%
Partition coefficient n-octanol/water	Log Pow = 4.4 at 25 °C pH 6.4	Ogi, N. 2003(d) (DAR B.2.1.13)	EEC Method A8 (HPLC) 99.8%
Flash point	Not applicable (solid with melting point > 40 °C)	(DAR B.2.1.21)	
Flammability	The test substance melted and burned but did not sustain combustion on removal of the ignition source. Experience in handling and use indicate that the substance is not pyrophoric and does not emit flammable gases	Comb, A. 2003(e) (DAR B.2.1.20)	EEC Method A10 99.1%

	on contact with water.		
Explosive properties	The substance was not sensitive to the effects of flame, shock or friction.	Comb, A. 2003(g) (DAR B.2.1.22)	EEC Method A14 99.1%
Self-ignition temperature	The substance did not self ignite prior to melting at 130 ^{o}C	Comb, A. 2003(f) (DAR B.2.1.20)	EEC Method A16 99.1%
Oxidising properties	The test substance did not sustain a flame on removal of the ignition source	Comb, A. 2003(h) (DAR B.2.1.23)	EEC Method A17 99.1%
Granulometry	No data		
Dissociation constant	Not measured. Does not contain any groups that ionise between pH 4-6 and 8-10.	(DAR B.2.1.18)	
Viscosity	Not relevant		

2 MANUFACTURE AND USES

2.1 Manufacture

Amisulbrom is manufactured outside of the EU.

2.2 Identified uses

Amisulbrom is currently used as a fungicide on grapes and potatoes within the EU.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Table 9: Summary table for relevant physico-chemical studies

Method	Results	Remarks	Reference
Refer to table 8			

3.1 *Physico-chemical properties*

3.1.1 Summary and discussion

In a standard study (EEC A10, Comb, A. 2003(e) DAR B.2.1.20), Amisulbrom melted and burned but did not sustain combustion on removal of the ignition source. Consequently, it does not meet the criteria for classification as a flammable solid. In addition, experience in handling and use indicates that the substance is not pyrophoric and does not emit flammable gases on contact with water.

In a standard study (EEC A14, Comb, A. 2003(g) DAR B.2.1.22), Amisulbrom was not found to be sensitive to the effects of flame, shock or friction. Consequently, it does not meet the criteria for classification as an explosive substance.

In a standard study (EEC A17, Comb, A. 2003(h) DAR B.2.1.23), Amisulbrom did not sustain combustion on removal of the ignition source. Consequently, it does not meet the criteria for classification as an oxidising solid.

3.1.2 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4 HUMAN HEALTH HAZARD ASSESSMENT

References are taken from the Draft Assessment Report – Volume 3. Annex B6: Toxicology and Metabolism – February 2012. The relevant section references are provided.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

The toxicokinetics of amisulbrom following oral administration have been investigated in a number of studies in rats. These studies are detailed in the DAR. Only an overall summary of the toxicokinetics of amisulbrom (as described in the DAR) is provided here as this information is not critical to the hazard classification of the substance.

4.1.1 Non-human information

4.1.2 Human information

4.1.3 Summary and discussion on toxicokinetics

Absorption

Amisulbrom was rapidly absorbed following the administration of single low or repeated low doses. Maximum plasma concentrations were attained within 2-6 hours. Absorption was less rapid following the administration of a single high dose; maximum plasma concentrations were attained within 12-24 hours of administration. A comparison of C_{max} and AUC values indicates less extensive oral absorption at the high dose level; using figures for biliary and urinary excretion of radioactivity, oral absorption was estimated to be ~50% at the low dose level and ~5% at the high dose level. Profiles of blood and plasma radioactivity indicate the absorption of biliary metabolites (enterohepatic circulation).

Distribution

Amisulbrom was found to be rapidly and relatively evenly distributed. Highest tissue levels of radioactivity were noted in the liver and kidneys, consistent with the excretion profile. Radioactivity levels in blood and plasma indicate persistent binding of a T (triazole group)-labelled metabolite of amisulbrom to the cellular fraction. With this exception, distribution of amisulbrom was found to be comparable in all groups. Residual levels of radioactivity at 120 hours were very low or non-detectable in the majority of tissues. Low levels of radioactivity in fat do not indicate any potential for bioaccumulation.

Metabolism

Absorbed amisulbrom was found to be completely metabolised; whereas unchanged amisulbrom accounted for nearly all of the faecal radioactivity. The metabolism of amisulbrom in the rat was found to involve a number of steps. The initial steps are proposed to be cleavage of the sulphonylamino sidechain on the triazole ring to form IT-4, oxidative debromination to form IT-11 or hydroxylation on the indole ring to form IT-2. The initial metabolites are excreted or further metabolised by debromination, cleavage of the sulphonylamino side chain, opening of the indole ring (to form I-2) and glucuronide conjugation. There was also limited cleavage of the sulphonyl bridge to liberate 1,2,4-triazole (T-4).

Excretion

Excretion of administered radioactivity was relatively rapid (largely within 24 hours) and predominantly (~80%) in the faeces. Excretion profiles were broadly comparable in all groups of non-cannulated animals. Urinary excretion by cannulated animals was comparable to that seen in non-cannulated animals (~15%), however the extent of biliary excretion (~40%) indicates that approximately half of the faecal radioactivity in non-cannulated animals is of biliary origin. There is also some evidence from plasma and blood radioactivity profiles of enterohepatic circulation. Total residual levels of radioactivity at 120 hours indicate almost complete excretion of amisulbrom and its metabolites.

(Refer to DAR – Volume 3. Annex B6: Toxicology and Metabolism – February 2012, section B.6.1)

4.2 Acute toxicity

Table 10: Summary table of relevant acute toxicity studies

Acute Oral		
Method	**LD_{50}**	**Observations and remarks**
Rat, Sprague-Dawley, 3/sex 5000 mg/kg bw 1 % methylcellulose (aq.) OECD TG 423 GLP Purity 99.1 % 2003a (DAR B.6.2.1)	LD_{50} > 5000 mg/kg bw	No deaths occurred and no signs of toxicity were observed during the study period of 14 days. No treatment-related findings during gross necropsy
Acute Inhalation		
Method	**LC50**	**Observations and remarks**
Rat, Sprague-Dawley, 5/sex 2.85 mg/L (4 h) (nose-only exposure, MMAD 4.3 ± 1.2 µm) OECD TG 403 (1981) GLP Purity 99.1 % 2003 (DAR B.6.2.2)	LD_{50} > 2.85 mg/L	No deaths occurred during the study period. Signs of toxicity included exaggerated breathing during exposure. Wet fur and brown facial staining were observed on days 1 and 2 only. Reduced weight gain in females during week 1 No treatment-related findings during gross necropsy
Acute Dermal		
Method	**LD50**	**Observations and remarks**
Rat, Sprague-Dawley, 5/sex 5000 mg/kg bw 80 % in 1 % methylcellulose (aq.) OECD TG 402 GLP Purity 99.1 % 2003a (DAR B.6.2.3)	LD_{50} > 5000 mg/kg bw	No deaths occurred and no signs of systemic toxicity during the study period of 14 days. Grade 1 -2 erythema observed in all animals on days 2 -3 Reduced weight gain in all females No treatment-related findings during gross necropsy

4.2.1 Non-human information

4.2.1.1 Acute toxicity: oral

In a single guideline study, amisulbrom was administered to fasted male and female rats by oral gavage. The LD_{50} was > 5000 mg/kg bw.

4.2.1.2 Acute toxicity: inhalation

In a single guideline study, male and female rats were exposed to amisulbrom (nose-only) at a concentration of 2.85 mg/L (the maximum achievable concentration achievable in this study) for a period of 4 hours. The 4h-LC_{50} was > 2.85 mg/L.

4.2.1.3 Acute toxicity: dermal

In a single guideline study, amisulbrom (formulated as an 80 % concentration in 1 % methylcellulose aq.) was applied to the shorn dorsal skin of male and female rats for 24 hours, before washing the application site with water. The LD_{50} was > 5000 mg/kg bw.

4.2.1.4 Acute toxicity: other routes

No information is available.

4.2.2 Human information

There is no relevant human information available

4.2.3 Summary and discussion of acute toxicity

Three guideline studies were performed to investigate the acute toxicity of amisulbrom in rats via oral, inhalation and dermal routes. No deaths occurred during any of the studies at the limit dose administered. In the inhalation study, rats experienced exaggerated breathing during exposure only and some minor toxicity for 2 days after exposure. These effects were not deemed significant and were no longer present on day 3.

4.2.4 Comparison with criteria

Classification for Acute Tox 4 (oral) is applicable where $300 < LD_{50} \leq 2000$ mg/kg bw. The oral LD_{50} for amisulbrom was > 5000 mg/kg bw – therefore no classification is warranted.

Classification for Acute Tox 4 (inhalation of dusts and mists) is applicable where $1 <$ 4h-$LC_{50} \leq 5$ mg/L. Amisulbrom was tested at the maximum achievable concentration of 2.85 mg/L at which no deaths occurred. No classification is warranted.

Classification for Acute Tox 4 (dermal) is applicable where $1000 < LD_{50} \leq 2000$ mg/kg bw. The dermal LD_{50} for amisulbrom was > 5000 mg/kg bw – therefore no classification is warranted.

4.2.5 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

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

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

There were few signs of toxicity in the acute studies of amisulbrom in rats. Reduced weight gain in females was seen in all studies, however all animals gained weight overall. In the acute inhalation study, exaggerated breathing was noted during the 4 h exposure period only. On days 1 and 2 following this, animals exhibited wet fur and brown facial staining. These effects were no longer observed on day 3. All these effects were considered mild and non-specific signs of general toxicity. Gross necropsy did not reveal any treatment-related findings.

4.3.2 Comparison with criteria

No significant specific target effects on a target organ or tissue were observed after oral, dermal or inhalation single exposure with amisulbrom in rats. Therefore, amisulbrom does not meet the classification criteria for STOT SE.

4.3.3 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4.4 Irritation

4.4.1 Skin irritation

Table 11: Summary table of relevant skin irritation studies

Method	Results	Remarks	Reference
Rabbit, New Zealand White, 3 male 0.5 g (4 h) OECD TG 404 (1992) GLP Purity 99.1 %	No signs of dermal irritation observed in any animal at any timepoint	Non-irritant	2003a (DAR B.6.2.4)

4.4.1.1 Non-human information

The skin irritation potential of amisulbrom has been investigated in a single standard study in rabbits. No signs of irritation were observed in any animal at any timepoint.

4.4.1.2 Human information

There is no relevant information available.

4.4.1.3 Summary and discussion of skin irritation

Amisulbrom did not cause any signs of irritation in a guideline study in rabbits.

4.4.1.4 Comparison with criteria

No irritation was observed.

4.4.1.5 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4.4.2 Eye irritation

Table 12: Summary table of relevant eye irritation studies

Method	Results	Remarks	Reference
Rabbit, New Zealand White, 6 male (3 washed, 3 unwashed) 0.1 g OECD TG 405 (1992) GLP Purity 99.1 %	Individual animal scores over 24 – 72 h (unwashed eyes) Corneal opacity: 0, 0, 0 Iris lesion: 0, 0, 0 Conjunctivae erythema: 0.66, 0.66, 0.33 Conjunctivae oedema: 0, 0, 0	Conjunctivae erythema was still present in two rabbits at day 21 (grade 1) Data for washed eyes have not been included in the evaluation.	2003(b) DAR B.6.2.5)

4.4.2.1 Non-human information

In a single guideline study, 100 mg of amisulbrom was instilled into one eye of six rabbits. The eyes of three of the rabbits were washed with physiological saline approximately 30 seconds following instillation. The three remaining rabbits did not have their eyes washed. Ocular reactions were assessed up to 21 days following instillation, using the Draize scale (Table 13).

Table 13: Ocular reactions following installation of amisulbrom to eyes of rabbits

Observation		1 h	24 h	48 h	72 h	Mean (24 - 72 h)	7 d	14 d	21 d
Unwashed Eyes									
Cornea	**Opacity**	0	0	0	0	**0**	0	0	0
Iris	**Lesion**	0	0	0	0	**0**	0	0	0
Conjunctivae	**Erythema**	2, 1, 1	1, 0, 1	0, 1, 1	0, 1, 0	**0.56**	1, 1, 0	1, 1, 0	1, 1, 0
	Oedema	0	0	0	0	**0**	0	0	0
Washed Eyes									
Cornea	**Opacity**	0	0	0	0	**0**	0	0	0
Iris	**Lesion**	0	0	0	0	**0**	0	0	0
Conjunctivae	**Erythema**	1, 2, 2	1, 1, 1	1, 0, 0	0, 1, 0	**0.56**	0, 0, 0	0, 1, 0	0, 1, 0
	Oedema	0	0	0	0	**0**	0	0	0

4.4.2.2 Human information

There is no relevant information available.

4.4.2.3 Summary and discussion of eye irritation

Mild conjunctival erythema (grade 1) was observed in the unwashed eyes of rabbits after treatment with amisulbrom. After 7 days, and lasting until the end of the study period of 21 days, two rabbits still exhibited grade 1 conjunctival erythema. However, for rabbit 1 this was noted to have resolved by 48 h, but at 7 days grade 1 redness was observed again. The conjunctival erythema of rabbit two appeared to have resolved by 24 h, however at 48 h, grade 1 redness was noted lasting for the duration of the study. The results of the washed rabbits are not to be included in the classification for eye irritancy, as they do not follow the study guidelines, however, a similar effect was noted whereby one rabbit exhibited no conjunctival erythema at 48 h but a return to grade 1 was observed at 72 h. For the same rabbit, at 7 days there was an absence of erythema but at 14 days and beyond grade 1 erythema was observed.

Whilst the aetiology of the reoccurrence of the conjunctival injection was unknown, it was not considered by the applicants to be amisulbrom-related. It was rationalised that the redness could not be due to a delayed systemic effect either as the untreated eye would be expected to show evidence of redness also, and this was not the case. The applicant suggested that the cause of the conjunctival injection in question could be due to hairs entering the eye liberated through grooming. However, again, there is no indication that there was any erythema occurring at any stage of the experiment in untreated eyes, as might be expected, if this were the case.

4.4.2.4 Comparison with criteria

A single guideline study of amisulbrom applied to the eyes of New Zealand white rabbits resulted in the presence of grade 1 conjunctival erythema in the eyes of two rabbits from 7 days to 21 days post-treatment (but not from 3 days to 6 days post-treatment). According to the CLP criteria, if, when applied to the eye of an animal, a substance produces in at least one animal effects that have not fully reversed within an observation period of 21 days, then it may be classified as Eye Damage 1 – irreversible effects on the eye. The applicant suggested that the cause of the reoccurrence of conjunctival erythema could be due to hairs liberated through grooming entering the eye. However, it is important to note that this effect was not present in the untreated eyes and is not an affect observed generally in eye irritation experiments in rabbits. With these points in mind, a simple interpretation of the criteria could lead to classification as Eye Damage 1, or no classification at all. However, on the basis that there was irritation up until day 21 of the study but that the effects observed were mild (grade 1) and inconsistent throughout the study, classification as Eye Irritant Category 2 is considered appropriate.

4.4.2.5 Conclusions on classification and labelling

Eye Irritant Category 2 - H319 Causes serious eye irritation

4.4.3 Respiratory tract irritation

4.4.3.1 Non-human information

There are currently no validated animal tests that deal specifically with respiratory tract irritation but a single guideline study to investigate acute toxicity via inhalation has been presented (Section 4.2). During 4 hours of nose-only exposure to amisulbrom (2.85 mg/L, 4.3 µm) rats experienced exaggerated breathing. This effect was not observed at the end of the exposure time. Toxic effects

noted were wet fur and brown facial staining for up to 2 days post-exposure. By day 3, no toxic effects were observed. Gross necropsy did not reveal any treatment-related findings.

4.4.3.2 Human information

There is no relevant information available.

4.4.3.3 Summary and discussion of respiratory tract irritation

Based on the data available from the acute inhalation toxicity study in rats treated to a single exposure of amisulbrom there was no clinical signs of respiratory tract irritation. Gross necropsy revealed no treatment-related findings.

4.4.3.4 Comparison with criteria

Amisulbrom did not show any clinical or histopathological signs of respiratory tract irritancy and therefore does not meet the criteria for a respiratory tract irritant.

4.4.3.5 Conclusions on classification and labelling

Not classified – Data lacking

4.5 Corrosivity

Table 14: Summary table of relevant corrosivity studies

Method	Results	Remarks	Reference
Not relevant			

4.5.1 Non-human information

No signs of corrosivity were observed in the available irritation studies conducted with amisulbrom (see Section 4.4).

4.5.2 Human information

No relevant information is available.

4.5.3 Summary and discussion of corrosivity

As no signs of corrosion were observed in the available irritation studies amisulbrom is not considered corrosive.

4.5.4 Comparison with criteria

Amisulbrom did not show any corrosivity in the irritation studies nor does it have a pH ≤ 2 or ≥ 11.5. Therefore, it does not meet the criteria for classification as a corrosive substance.

4.5.5 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4.6 Sensitisation

4.6.1 Skin sensitisation

Table 15: Summary table of relevant skin sensitisation studies

Species/Method	Doses	No. sensitised/total no.	Result	Reference
Guinea Pig, Dunkin-Hartley, 20 females/test group, 10 females/ control group Maximisation Study OECD TG 406 GLP Purity 99.1 %	*Induction*: Intradermal: 1 % w/v in Alembicol D Topical: 100 % w/v in Alembicol D (0.5 mL) *Challenge:* 50 % and 100 % w/v in Alembicol D	Test: No dermal reactions were observed in test animals. Negative Control: No dermal reactions occurred in control animals An acceptable positive control study was reported	Negative	2002 (DAR B.6.2.6)

4.6.1.1 Non-human information

In a single guideline guinea pig maximisation study amisulbrom was assessed for its potential to cause skin sensitisation. Guinea pigs were intradermally induced by injection with 1 % test material in Alembicol D, FCA and FCA/test material. Topical application was performed after 7 days, using 48 hour occlusive applications of amisulbrom (100 % w/v in Alembicol D, 0.5 mL). Irritation had previously been induced at the application site by topical application of SDS (10 % in petrolatum, 0.5 mL). Control animals were similarly treated during the induction phase, with vehicle in place of the test material. After a further two weeks, all animals were challenged using a 24 hour occlusive application of amisulbrom (50 % and 100 % w/v in Alembicol D). Dermal reactions were then assessed at 24 hours and 48 hours following removal of the dressing. Signs of irritation were observed in test and control animals following intradermal induction; no signs of irritation were observed following topical induction. No dermal reactions were observed in test or control animals following the challenge exposure.

4.6.1.2 Human information

There is no relevant information available.

4.6.1.3 Summary and discussion of skin sensitisation

Amisulbrom did not demonstrate any signs of skin sensitisation in this study.

4.6.1.4 Comparison with criteria

No evidence of skin sensitisation was observed therefore amisulbrom does not meet the criteria for classification.

4.6.1.5 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4.6.2 Respiratory sensitisation

Table 16: Summary table of relevant respiratory sensitisation studies

Method	Results	Remarks	Reference
Not applicable			

4.6.2.1 Non-human information

No data are available.

4.6.2.2 Human information

No data are available.

4.6.2.3 Summary and discussion of respiratory sensitisation

No data are available.

4.6.2.4 Comparison with criteria

No data are available.

4.6.2.5 Conclusions on classification and labelling

Not classified - data lacking

4.7 Repeated dose toxicity

The repeated dose toxicity of amisulbrom has been investigated by the oral route in rats (28-day study, 90-day study and combined chronic/carcinogenicity study), mice (28-day study, 90-day study and carcinogenicity study) and dogs (90-day study and 1-yr study). A 21-day dermal study in the rat is also available. The combined chronic/carcinogenicity study in the rat and the mouse carcinogenicity study are described in the carcinogenicity section (4.10).

Table 17: Summary table of relevant repeated dose toxicity studies

Method	Dose Levels	Observations and Remarks	Reference
28-day study SD rats (5/sex/group) Dietary administration EU B7 method Not GLP Amisulbrom 99% pure	0, 2500, 5000, 10000, 20000 ppm (0, 206, 424, 833, 1699 mg/kg bw/d in males; 0, 224, 459, 875, 1816 mg/kg bw/d in females) Dose level relevant for classification (guidance value for subacute rat study) ≤ 300 mg/kg bw/d	**20000 ppm (1699/1816 mg/kg bw/d in M/F):** Transient ↓fc-food consumption (first 3 days) in M-males and F-females; ↓MCV (M)*, Hb (M)* and MCH (M & F)*; ↑PLT (M)*; ↑rel-relative liver wt-weight (M & F)*; Hepatocyte hypertrophy (M & F)*; **10000 ppm (833/875 mg/kg bw/d in M/F):** Transient ↓fc (first 3 days) in females; ↓MCV (M)* and MCH (M & F)*; ↑rel liver wt (M)*; Hepatocyte hypertrophy (M & F)*; **5000 ppm (424/459 mg/kg bw/d in M/F):** ↑rel liver wt* (M); Hepatocyte hypertrophy (M)*; **2500 ppm (206/224 mg/kg bw/d in M/F):** No significant effects; **NOAEL (M) = 2500 ppm (206 mg/kg bw/d)[$]** **NOAEL (F) = 5000 ppm (459 mg/kg bw/d)[$]**	2000(a) (DAR B.6.3.1(a))
90-day study Han Wistar rats (10/sex/group) Dietary administration EU B26 method GLP Amisulbrom 98% pure	0, 2000, 6300, 20000 ppm (0, 170, 525, 1716 mg/kg bw/d in males; 0, 187, 587, 1880 mg/kg bw/d in females) Dose levels relevant for classification (guidance value for subchronic rat study) ≤ 100 mg/kg bw/d	**20000 ppm (1716/1880 mg/kg bw/d in M/F):** ↓bwg-body weight gain (31% M; 20% F)* during wk-weeks 0-13; ↓fc (18% M; 10% F) during wk 0-13; Ghost vessels in the eyes of 4 M and 1 F; ↑platelet count (13% M*; 7% F); ↑AP (14% M)*, ↑AAT (14% M)*, ↑γGT (300% M)* ↓Total triglycerides (28% F)*; ↑urea (29% M)*; ↓total protein (4%)*; ↑Phosphorous (64% M; 47% F)*; ↑urinary pH (17% M; 18% F)*; ↑incidence of minimal/slight hepatocyte hypertrophy (M); ↑incidence of sinus erythrocytosis in mandibular and mesenteric lymph nodes (M); **6300 ppm (525/587 mg/kg bw/d in M/F):** ↓bwg (21% M)* during wk 0-13; ↓fc (15% M) during wk 0-13; ↑urinary pH (11% F)*; **2000 ppm (170/187 mg/kg bw/d in M/F):**	2003(a) (DAR B.6.3.1(b))

		No treatment-related effects; **NOAEL (M) = 2000 ppm (170 mg/kg bw/d)$** **NOAEL (F) = 6300 ppm (587 mg/kg bw/d)$**	
28-day study CD-1 mice (5/sex/group) Dietary administration EU B7 method Not GLP Amisulbrom 99.7% pure	0, 1250, 2500, 5000, 10000 ppm (90, 221, 470, 904, 1860 mg/kg bw/d in males; 0, 296, 542, 1076, 2192 mg/kg bw/d in females) Dose levels relevant for classification (guidance value for subacute rat study) ≤ 300 mg/kg bw/d	**1250, 2500, 5000 and 10000 ppm (1860/2192 mg/kg bw/d in M/F):** No treatment-related effects seen;	2001 (DAR B.6.3.2(a))
90-day study CD-1 mice (10/sex/group) Dietary administration Range-finding study for carcinogenicity study (Functional observations, motor activity, ophthalmoscopy, clotting time, urinalysis or microscopic pathology were not conducted) GLP Amisulbrom 98.7% pure	0, 800, 2500, 8000 ppm (0, 119, 400, 1280 mg/kg bw/d in males; 163, 505, 1638 mg/kg bw/d in females) Dose levels relevant for classification (guidance value for subchronic rat study) ≤ 100 mg/kg bw/d	**8000 ppm (1280/1638 mg/kg bw/d in M/F):** ↓bwg (39% F); ↑platelet count (18% M; 14% F)*; ↓neutrophil count (36% M; 29% F)*; ↓monocyte count (33% M)*; ↓total cholesterol (22% F)*; ↑rel liver wt (28% M; 16% F)*; ↑abs-absolute liver wt (13% M)*; **2500 ppm (400/505 mg/kg bw/d in M/F):** ↓bwg (24% F); ↑platelet count (11% F)*; ↓neutrophil count (32% M)*; ↓total cholesterol (25% F)*; ↑rel liver wt (13% M)*; **800 ppm (118/163 mg/kg bw/d in M/F):** No treatment-related effects; **NOAEL = 800 ppm (118-163 mg/kg bw/d)$**	2003(b) (DAR B.6.3.2(b))
90-day study Beagle dog (4/sex/group) Capsule administration EU B27 method GLP Amisulbrom 98.7% pure	0, 100, 300, 1000 mg/kg bw/d Dose levels relevant for classification (guidance value for subchronic rat study) ≤ 100 mg/kg bw/d	**1000 mg/kg bw/d:** ↓ bwg (25% M; 37% F) in wk-0-3; ↓ fc (15% M; 16% F*) in wk 0-4; ↑ Alkaline phosphatase (49% F)* at wk 13; **100 and 300 mg/kg bw/d:** No treatment-related effects; **NOAEL = 300 mg/kg bw/d$**	2003 DAR B.6.3.3(a))

1-yr study Beagle dog (4/sex/group) Capsule administration EU B30 method GLP Amisulbrom 99.1% pure	0, 10, 100, 300, 1000 mg/kg bw/d Dose levels relevant for classification (guidance value for subchronic rat study) ≤ 100 mg/kg bw/d	**1000 mg/kg bw/d:** Liquid faeces in both sexes; ↓ bwg (61% M*; 73% F*) in wk 0-4; ↓ bwg (32% M*; 42% F*) in wk 0-52; ↓ fc (14% M; 25% F*) in wk 0-4; ↓ total plasma protein level (11% M*); ↑ rel adrenal wt (31% M*); Adrenal cortical hypertrophy in males; Centrilobular hepatocyte hypertrophy in 2 males; Slight-minimal thymus atrophy in some animals; Decreased cellularity of red pulp in the spleen in some animals; **300 mg/kg bw/d :** Intermittent frequency of liquid faeces in males ; ↑ rel adrenal wt (31% M*); Adrenal cortical hypertrophy in males; Decreased cellularity of red pulp in the spleen in some animals; **10 and 100 mg/kg bw/d:** No adverse or treatment-related effects; **NOAEL = 100 mg/kg bw/d$**	2005 (DAR B.6.3.3(b))
21-day dermal study SD Rat (10/sex/group) Topical 6hr application under occlusive dressing for 21 days EU B9 method GLP Amisulbrom 99.01% pure	0, 100, 300, 1000 mg/kg bw/d Dose levels relevant for classification (guidance value for subacute rat study) ≤ 300 mg/kg bw/d	**1000 mg/kg bw/d:** ↓ bwg (24% M); ↓ cholesterol (18% M*); ↓ triglycerides (27% M*); **100 and 300 mg/kg bw/d:** No treatment-related effects; **NOAEL = 300 mg/kg bw/d$**	2004 (DAR B.6.3.4)

Statistically significant; $ = As given in the DAR

↓ = decrease ↑ = increase

4.7.1 Non-human information

The following text has been extracted from the DAR.

4.7.1.1 Repeated dose toxicity: oral

Rat

28-day study

In a guideline 28-day study, groups of 5 male and 5 female Sprague-Dawley CD rats were fed dietary concentrations of 0, 2500, 5000, 10000 or 20000 ppm amisulbrom for 4 weeks. The overall mean achieved daily intakes were 205.6, 423.5, 832.7 and 1699.1 mg/kg bw/d in males and 223.7, 458.6, 875.4 and 1816.1 mg/kg bw/d in females at 2500, 5000, 10000 and 20000 ppm, respectively. All tissues from the control and highest dose level (20000 ppm) were examined microscopically following histopathological processing. The liver, kidneys and spleen from the low and intermediate dose levels (2500, 5000 and 10000 ppm) were also examined.

There were no mortalities and no clinical signs of toxicity. There were no treatment-related effects on bodyweights. There were some decreases in food consumption observed during the first three days of administration, at 20000 ppm in male rats and at 10000 and 20000 ppm in female rats, although this effect was transient in nature. There were some transient reductions in feed efficiency observed in female rats.

In male rats, significant reductions in MCV (Mean Corpuscular Volume) and MCH (Mean Corpuscular Hemoglobin) were observed at 10000 ppm, and at 20000 ppm significant reductions in Hb (hemoglobin), MCV and MCH and significant increases in PLT were noted. In female rats, significant reductions in MCH were observed at doses of ≥10000 ppm.

In male rats, significant increases in Phospholipids (PL) were observed at 10000 ppm. In female rats, significant reductions in Aldolase (ALD) were observed at 2500 ppm. Significant reductions in Glutamic-Pyruvic Transaminase (GPT), Creatinine (CRE) and Fe, and significant increases in Unsaturated Iron-binding capacity (UIBC) were noted at 10000 ppm. At 20000 ppm, there were significant reductions in GPT, LAP (Leucine Aminopeptidase), CRE, and Fe, and significant increases in UIBC, Na and K. As all of these values were within the historical control ranges, they were not considered to be of toxicological significance.

Relative liver weights were increased in males fed doses ≥5000 ppm and in females given 20000 ppm. A trace to slight hepatocyte hypertrophy was seen at doses ≥2500 ppm. The increased incidence was statistically significant at doses ≥5000 ppm in males and at doses ≥10000 ppm in females.

Overall, dietary administration of amisulbrom to rats for 28 days caused haematology changes in males and females from a dose of 10000 ppm (833-875 mg/kg bw/d) and increased liver weight accompanied by hepatocyte hypertrophy from a dose of 5000 ppm (424 mg/kg bw/d) in males and from a dose of 10000 ppm (875 mg/kg bw/d) in females.

90-day study

In a guideline 90-day study, groups of 10 male and 10 female Han Wistar rats were given dietary concentrations of 0, 2000, 6300 or 20000 ppm amisulbrom for 13 weeks. The mean achieved test material intakes were 0, 170.6, 525.0 and 1715.8 mg/kg bw/d in males and 0, 186.6, 587.2 and 1880.4 mg/kg bw/d in females, respectively.

The appearance and behaviour of the animals was considered unaffected by treatment and no animals died. There were no treatment-related observations in the standard arena. Sensory reactivity, grip strength and motor activity recorded during Week 12 were unaffected by treatment.

Bodyweight gain in males fed 6300 ppm and in both sexes given 20000 ppm was reduced during Weeks 1 and 2 compared with the control values. This reduced weight gain continued in Weeks 3 and 4 for males at 20000 ppm but thereafter reductions in these groups were less. Overall bodyweight gain in males was 79% of controls at 6300 ppm and was 69% and 80% of controls in males and females respectively at 20000 ppm. Bodyweight gain was unaffected at 2000 ppm and in females fed 6300 ppm. At 6300 and 20000 ppm, total food consumption by males was significantly lower than controls (85 and 82% respectively). It was also slightly low in females fed 20000 ppm (90% of controls). Food scatter during the first two weeks of treatment was increased in males and females at 6300 and 20000 ppm. The study authors considered this was indicative of initial unpalatability. However as the overall food conversion efficiency was reduced in males given 6300 ppm and in both sexes fed 20000 ppm, this showed that the reduced weight gain was not solely due to reduced food intake and there was an underlying toxic response.

Ophthalmic examination during Week 13 of treatment revealed ghost vessels in four males and one female receiving 20000 ppm, compared with none in controls. There were no treatment-related ophthalmoscopic findings in animals receiving 2000 or 6300ppm. It was noted that there were no significant histopathological changes in the eye.

The haematological examination in Week 13 indicated slightly high platelet counts in males and females receiving 20000 ppm, though only in males was the difference statistically significant.

Biochemical examination of the blood plasma in Week 13 indicated slightly high alkaline phosphatase, aspartate amino-transferase and gamma glutamyl transpeptidase activities in males receiving 20000 ppm. There was no similar change in the females. At 20000 ppm there was an increase in plasma urea concentration in both sexes, though only in males was the difference statistically significant ($p<0.01$). Total plasma triglyceride concentrations were slightly low in females receiving 20000 ppm; males were unaffected. Total protein concentrations were slightly low in males receiving 20000 ppm. In addition, plasma phosphorus concentrations were high in males receiving 20000 ppm.

Urinary pH at Week 13 was slightly high in both sexes at 20000 ppm and in females given 6300 ppm. This was considered to be due to the presence of amisulbrom and/or metabolite(s) in urine.

Microscopic changes attributed to treatment with amisulbrom were seen in the liver, where there was minimal or slight centrilobular hepatocyte hypertrophy in males that received 20000 ppm. Females were unaffected. An equivocal increased incidence of sinus erythrocytosis/erythrophagocytosis was also seen in the mandibular and mesenteric lymph nodes of males at 20000 ppm.

Overall, dietary administration of 6300 ppm (525 mg/kg bw/d) and 20000 ppm (1715-1880 mg/kg bw/d) amisulbrom to Han Wistar rats for 13 weeks induced non-specific toxicity, including reductions in overall bodyweight gain and food consumption and food conversion efficiency in males at 6300 ppm and in both sexes at the higher dose level. Additionally at 20000 ppm, there was the presence of ghost vessels in the eye, slightly increased platelet counts, clinical chemistry changes indicative of effects on liver function and, in males, increased incidence of centrilobular hepatocyte hypertrophy and sinus erythrocytosis/erythrophagocytosis was also noted. The NOAEL was 2000 ppm in males (corresponding to 170.6 mg/kg bw/d) and 6300 ppm in females (corresponding to 587.2 mg/kg bw/d).

Mouse

28-day study

In a guideline 28-day study, groups of 5 male and 5 female CD-1 mice were fed dietary concentrations of 0, 1250, 2500, 5000 or 10000 ppm of amisulbrom for 4 weeks. The mean achieved test material intakes were 0, 221.26, 470.16, 904.04 and 1860.24 mg/kg bw/d in males and 0, 296.42, 542.60, 1076.62 and 2192.36 mg/kg bw/d in females, respectively.

No mortality occurred. There were no treatment-related clinical signs of toxicity. Bodyweights and food consumption were unaffected. No treatment-related effects were noted in the haematology investigations. Although slight to mild centrilobular hypertrophy of hepatocytes was observed in a few male and female mice dosed at levels ≥2500 ppm, these findings were not dose-related.

Overall, no significant toxicity was detected following repeated dietary administration of amisulbrom at doses up to 10000 ppm (1860-2192 mg/kg bw/d) for four weeks in mice.

90-day study

In a 90-day range-finding study, groups of 10 male and 10 female CD-1 mice were given dietary concentrations of 0, 800, 2500 or 8000 ppm amisulbrom for 13 weeks. The group mean achieved intakes of amisulbrom were 118.8, 400.3 and 1280.2 mg/kg bw/d in males and 163.4, 505.6 and 1638.4 mg/kg bw/d in females at 800, 2500 and 8000 ppm, respectively. No histopathological examinations were conducted in this study.

There were no treatment-related deaths. The appearance and behaviour of the animals was unaffected by treatment. The overall bodyweight gain of females receiving 2500 or 8000 ppm was lower than that of the controls. In males, lower gains were observed from 800 ppm, but due to the lack of a dose-response relationship and inconsistencies between animals and time points, these were not considered to be treatment-related. Food consumption was unaffected by treatment.

The haematological investigations in Week 13 indicated high platelet counts, compared with the controls, for animals receiving 8000 ppm and for females receiving 2500 ppm. Slightly low neutrophil counts, with a concomitant decrease in total leukocyte count, were seen in animals receiving 8000 ppm and in males receiving 2500 ppm. In addition, monocyte counts were slightly low at 8000 ppm in males.

Biochemical examination of the blood plasma after 13 weeks of treatment indicated that total cholesterol concentrations were slightly low, compared with the controls, for females receiving 2500 or 8000 ppm.

After 13 weeks of treatment, high relative liver weights were recorded for animals treated at 2500 or 8000 ppm, although the mean value for the females treated at 2500 ppm did not attain statistical significance. In addition, the absolute liver weights of males treated at 8000 ppm were higher than those of the controls. There were no macroscopic changes after 13 weeks that were attributable to treatment with amisulbrom.

Overall, in a range-finding study, dietary administration of 2500 (400-505 mg/kg bw/d) and 8000 ppm (1280-1638 mg/kg bw/d) amisulbrom to mice for 90 days induced reductions in body weight gain, slight haematology changes and findings indicative of liver effects including increased liver weight and slightly decreased plasma cholesterol. The NOAEL was 800 ppm, corresponding to 118.8 and 163.4 mg/kg bw/d in males and females respectively.

Dog

90-day study

In a guideline study, groups of 4 male and 4 female Beagle dogs received a single daily oral dose of 0, 100, 300 or 1000 mg/kg bw/d amisulbrom in gelatine capsules for 13 consecutive weeks.

There were no clinical signs of toxicity and no animals died during the treatment period. Slightly lower bodyweight gain was recorded up to Week 2 in males and up to Week 3 in females receiving 1000 mg/kg bw/d. Slightly reduced food consumption was recorded during the first four weeks of treatment for males and females receiving 1000 mg/kg bw/d, attaining statistical significance in females. There were no treatment-related ophthalmic, haematology or urinalysis findings. In Week 13 there was a statistically significant increase in plasma alkaline phosphatase activity in females receiving 1000 mg/kg bw/d; males were unaffected. This difference was not evident at Week 6. Changes in other clinical-chemistry parameters were not considered to be treatment-related. Organ weights were unaffected after 13 weeks of treatment. There were no treatment-related macroscopic or microscopic changes.

Overall, capsule administration of amisulbrom to Beagle dogs for 13 weeks at dosages up to 1000 mg/kg bw/d was well-tolerated, producing a slight non-specific toxicity during the early weeks of the treatment period in both sexes and an increase in plasma alkaline phosphatase in females at the highest dose. A NOAEL of 300 mg/kg bw/d was identified from this study.

1-year study

In a guideline study, groups of 4 male and 4 female Beagle dogs received a single daily oral dose of 0, 10, 100, 300 or 1000 mg/kg bw/d amisulbrom in gelatine capsules for 52 consecutive weeks.

There were no premature deaths during the course of this study. A gradual increase in the incidence of liquid faeces up to approximately Week 36 was apparent for the animals receiving 1000 mg/kg bw/d and persisted until the end of the treatment period. The males receiving 300 mg/kg bw/d also showed an intermittent but variable frequency of liquid faeces. Lower bodyweight gain was recorded during the first four weeks of treatment for males and females receiving 1000 mg/kg bw/d; it persisted until the end of the study. Bodyweight gain was also reduced during the first four weeks of treatment in males at 100 and 300 mg/kg bw/d. However, as these reductions were not dose-related, were driven by the weight gains of one or two animals and were accentuated by the higher weight gains of the control animals, they were not considered to be treatment-related. Slightly reduced food consumption was recorded during the first four weeks of treatment for males and females receiving 1000 mg/kg bw/d. There were no treatment-related ophthalmic, haematology or urinalysis findings. Clinical-chemistry investigations showed a reduced total protein concentration in Week 13 and 26 in males receiving 1000 mg/kg bw/d. Relative adrenal weight was increased in males at 300 and 1000 mg/kg bw/d. This increase was associated with cortical hypertrophy. In the liver, minimal centrilobular hepatocyte hypertrophy was seen in 2 males given 1000 mg/kg bw/d. Histopathological findings were also noted in some animals in the thymus (involution/atrophy) at 1000 mg/kg bw/d and spleen (decreased cellularity of red pulp activity) at 300 and 1000 mg/kg bw/d. The study authors considered this as a typical physiological response to stress.

Overall, administration of amisulbrom to Beagle dogs for 52-weeks at dosages up to 1000 mg/kg bw/d was well-tolerated, producing initial effects on bodyweight gain and food consumption and effects on the liver (histopathology and clinical chemistry) at the top dose. Liquid faeces were observed in males at 300 and 1000 mg/kg bw/d and stress-related effects on the adrenals, thymus and spleen were seen in some animals at 300 and 1000 mg/kg bw/d. A NOAEL of 100 mg/kg bw/d was identified from this study.

4.7.1.2 Repeated dose toxicity: inhalation

No data are available.

4.7.1.3 Repeated dose toxicity: dermal

A guideline short-term (21-day) dermal toxicity study is available in the rat. In this study, groups of 10 male and 10 female Sprague-Dawley CD rats were given a daily 6-hour topical application for 21 consecutive days of 0, 100, 300 or 1000 mg/kg bw/d amisulbrom to the shaved dorsal skin (about 6 x 6 cm) under an occlusive dressing. Ophthalmoscopic, haematology, clinical-chemistry and urinalysis investigations were included in the study.

There were no treatment-related deaths, clinical signs of toxicity and no evidence of dermal irritation. At 1000 mg/kg bw/d, male body weight gain was slightly reduced and food conversion efficiency was slightly lower. There were no treatment-related ophthalmic, haematology or urinalysis findings. Clinical-chemistry investigations showed reduced plasma levels of cholesterol and triglycerides in males at 1000 mg/kg bw/d. There were no treatment-related effects on organ weights, macroscopic or microscopic findings.

Overall, dermal application of amisulbrom to SD rats for 21 days up to 1000 mg/kg bw/d was well-tolerated, causing effects on body weight gain and clinical chemistry parameters in males at the top dose. A NOAEL of 300 mg/kg bw/d was identified from the study.

4.7.1.4 Repeated dose toxicity: other routes

No data are available.

4.7.1.5 Human information

No data are available.

4.7.1.6 Other relevant information

No data are available.

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

The repeated dose toxicity of amisulbrom has been investigated by the oral route in rats (28-day study, 90-day study and combined chronic/carcinogenicity study), mice (28-day study, 90-day study and carcinogenicity study) and dogs (90-day study and 1-yr study). A 21-day dermal study in the rat is also available.

In the rat, specific target organ toxicity was seen in the liver (clinical chemistry changes and hypertrophy), blood (increased platelet counts) and mesenteric lymph nodes (sinus erythrocytosis) at the very high dose of 1700 (M)/1880 (F) mg/kg bw/d for 90 days. Similar target organs of toxicity were identified in the 2-year study, with the liver (clinical chemistry changes and hypertrophy), biliary system (bile duct hyperplasia) and kidneys (cortical tubular pigment) affected from a dose of 96 (M)/112 (F) mg/kg bw/d; and with the caecum (submucosal oedema), duodenum

(epithelial hyperplasia), forestomach (inflammation, ulceration, hyperkeratosis, hyperplasia) and mesenteric lymph nodes (sinus erythrocytosis and mastocytosis) affected from a dose of 568 (M)/946 (F) mg/kg bw/d. No specific target organ of toxicity was identified in the 21-day dermal study up to the limit dose of 1000 mg/kg bw/d.

In the mouse, specific target organ toxicity was seen in the liver (clinical chemistry changes and increased weight) and blood (increased platelet and neutrophils count) from a dose of 400 (M)/505 (F) mg/kg bw/d for 90 days. In addition, the liver (increased weight), kidney (lymphoid aggregation), caecum (pigmentation of submucosal venules) and adrenal glands (cortical hypertrophy) were identified as target organs of toxicity from a dose of 98 (M)/121 (F) mg/kg bw/d in the 18-month carcinogenicity study.

In the dog, the main target organ of toxicity was the liver (clinical chemistry changes and hypertrophy), with effects occurring at the high dose of 1000 mg/kg bw/d in both the 90-day and 1-year studies. A stress response, with effects on the adrenal glands, spleen and thymus was also seen from a dose of 300 mg/kg bw/d in the 1-year study.

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

The repeated dose toxicity of amisulbrom has been investigated by the oral route in rats (28-day study, 90-day study and combined chronic/carcinogenicity study), mice (28-day study, 90-day study and carcinogenicity study) and dogs (90-day study and 1-yr study). A 21-day dermal study in the rat is also available.

Overall, as described in section 4.8.1 above, significant and specific target organ toxicity occurs with amisulbrom mainly in the rat and mouse but at relatively high dose levels, well in excess of the guidance values for classification with STOT-RE Category 2 (100 mg/kg bw/d for rat oral 90-day study). Therefore, classification of amisulbrom for STOT-RE is not required.

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

Not classified – Conclusive but not sufficient for classification

4.9 Germ cell mutagenicity (Mutagenicity)

Table 18: Summary table of relevant in vitro and in vivo mutagenicity studies

In Vitro Data			
Method	**Organism/strain**	**Concentrations tested**	**Result**
Ames – bacterial reverse mutation test (n = 3) OECD TG 471 GLP Purity 99.1 % May K (2002) (DAR B.6.4.1(a))	*S. typhimurium:* TA98, TA100, TA1535 and TA1537 *E-coli :* WP2*uvrA*	5 – 5000 µg/plate (5 concentrations)	**Negative ± S9** No evidence of cytotoxicity at 5000 µg/plate but precipitation at this concentration was observed.
Mammalian cell mutation (n = 2) OECD TG 476 GLP Purity 99.1 % Lloyd M (2004) (DAR B.6.4.1(b))	Mouse lymphoma L5178Y cells	0 – 80 µg/mL	**Negative ± S9** Slight cytotoxicity (> 70 % relative survival) was observed at all concentrations and precipitation was observed at concentrations ≥ 20 µg/mL (-S9) and ≥ 60 µg/mL (+S9)
Chromosomal aberration assay (n = 3) OECD TG 473 GLP Purity 99.1 % Kumaravel TS (2004) (DAR B.6.4.1(c))	Human peripheral blood lymphocytes	0 -240 µg/mL	**Negative ± S9** An increase in the proportion of aberrant cells was observed at an intermediate concentration in one experiment (-S9). This was not seen in the initial experiment or in a confirmatory assay. Precipitation of amisulbrom was observed at concentrations ≥ 98.3 µg/mL
In vivo Data			
Method	**Organism/strain**	**Concentrations tested**	**Result**
Micronucleus test Oral gavage Vehicle: 0.5 % methylcellulose aq. OECD TG 474 GLP Purity 99.1 % 2003 (DAR B.6.4.2(a))	Mice, CD-1 (males, n = 7/group)	0, 500, 1000, 2000 mg/kg bw Mice were sacrificed at 24 h (all dose levels) or 48 h (controls and 2000 mg/kg)	**Negative** There was a slight (statistically insignificant) increase in the proportion of micronucleated cells (MnPCEs) observed at 24 h – this was not dose-related and within the laboratory historical control range: MnPCEs (/2000) Historical 0.54 Vehicle Control 0.1 500 mg/kg 0.7 1000 mg/kg 0.7 2000 mg/kg 0.3 Positive Control 28.0**

			There was a slight (statistically insignificant) decrease in the proportion of PCEs at 48 h only. *Toxicological findings:* No deaths occurred. Piloerection, hypoactivity, rales, hunching, ptosis and irregular breathing observed in animals dosed with 1000 and 2000 mg/kg at 1 - 3 h post dosing
Micronucleus test Intraperitoneal (ip) (two injections 24 h apart) Vehicle: olive oil OECD TG 474 GLP Purity 99.1 % 2007 (DAR B.6.4.2(g))	Mice, CD-1 (males, n = 6/group)	0, 250, 500, 1000 mg/kg bw Mice were sacrificed at 24 h post administration of second dose	**Negative** No increase in the frequency of micronucleated cells was observed: MnPCEs (/2000) Vehicle Control 1.2 250 mg/kg 0.8 500 mg/kg 1.0 1000 mg/kg 1.2 Positive Control 64 There was a substantial and significantly decreased in the proportions of polychromatic erythrocytes at all dose levels indicating toxicity to bone marrow cells. *Toxicological findings:* No deaths occurred. Piloerection, hypoactivity, hunching, ptosis, irregular breathing, elevated gait, thin build, swollen abdomen and body weight loss 4 – 12 % observed in animals dosed with amisulbrom.
Unscheduled DNA synthesis test Oral gavage Vehicle: 0.5 % methylcellulose aq. OECD TG 486 GLP Purity 99.1 % 2005 (DAR B.6.4.2(b))	Rats, F344 (males, n = 3/group)	0, 400, 2000 mg/kg bw Rats were sacrificed at 2 or 16 h post dosing	**Negative** No evidence of unscheduled DNA synthesis in the rat liver. No increase in the net number of nuclear grains. No increase in the proportion of cells in repair. *Toxicological findings:* No deaths occurred. No signs of toxicity observed in any group.

4.9.1 Non-human information

4.9.1.1 In vitro data

There are three guideline studies investigating the in vitro genotoxicity of amisulbrom. When tested in the bacterial reverse mutation assay, a mouse cell lymphoma mutation assay and a

chromosomal aberration test using human peripheral blood lymphocytes, amisulbrom was negative in the presence and absence of S9.

4.9.1.2 In vivo data

Two studies in mice have been evaluated to determine the potential of amisulbrom to cause cytogenic damage and DNA damage and repair in vivo. In a micronucleus test (2003) male CD-1 mice received an oral dose of amisulbrom (0, 500, 1000 or 2000 mg/kg bw) and were sacrificed at either 24 h or 48 h (controls and 2000 mg/kg only). For the animals sacrificed at 24 h only, there was a slight and statistically insignificant increase in the proportion of micronucleated cells. This was not dose-related and was within the contemporary historical control range. At 48 h the proportion of micronucleated cells was similar to the 24 h results but in line with the vehicle control. Concerns were raised that oral absorption of amisulbrom at the top dose level might be low (~ 5 %) and so systemic exposure might have been limited. In order to address these issues a second test was carried out by the same laboratory, administering amisulbrom interperitonealy. Male CD-1 mice received a dose of 0, 250, 500 or 1000 mg/kg bw/day for two days. The proportion of micronucleated cells were then determined at 24 or 48 h. The results of this study showed no increase in the frequency of micronucleated polychromatic erythrocytes in any of the amisulbrom-treated groups compared with the vehicle controls. There were substantial decreases in the proportion of polychromatic erythrocytes at all dose levels of amisulbrom indicating exposure to the bone marrow.

An unscheduled DNA synthesis test in rats was perfomed following test guidelines and to GLP. The results of this test were negative with no evidence of unscheduled DNA synthesis in the rat liver, no increase in the net number of nuclear grains and no increase in the proportion of cells in repair.

4.9.2 Human information

There is no relevant information.

4.9.3 Other relevant information

Four none standard studies have been carried out in order to further understand the genotoxic potential and mechanism of adenoma formation in the carcinogenicity studies of amisulbrom. The studies include a liver micronucleus assay in rats, a rat liver comet assay, a mouse liver comet assay and a rat stomach/forestomach comet assay.

Table 19:

Method	Organism/strain	Concentrations tested	Result
Rat liver micronucleus assay Micronucleus formation in hepatocytes after 3, 4 and 5 days Oral Gavage Purity 99.1 %	Rat/F344/4 f/group (amisulbrom) Rat/F344/4 f/group (DEN)	0, 500, 2000 mg/kg bw amisulbrom (single dose) 50 mg/kg bw DEN (single dose)	**Negative** for MN in hepatocytes at any time point; ↓ 65% Mitotic index on Day 3* at 2000 mg/kg bw amisulbrom ↑ MN in hepatocytes at all time points with DEN

2004 (DAR B.6.4.2(c))			
Rat liver comet assay DNA damage in hepatocytes by comet assay Oral Gavage Purity 99.1 % 2005(a) (DAR B.6.4.2(d))	Rat/Han Wistar/4 f/group (amisulbrom) Rat/Han Wistar/4 f/group (DEN and MMS)	0, 500, 2000 mg/kg bw amisulbrom (single dose) 160 mg/kg bw DEN (single dose) 80 mg/kg bw MMS (single dose)	**Negative** No increase in hepatocyte nuclei tail moment with amisulbrom Positive response with DEN or MMS
Mouse liver comet assay DNA damage in hepatocytes by comet assay Oral Gavage Purity 99.1 % 2005(b) (DAR B.6.4.2(e))	Mouse/CD-1/4 m/group (amisulbrom) Mouse/CD-1/4 m/group (DEN and MMS)	0, 500, 2000 mg/kg bw amisulbrom (single dose) 160 mg/kg bw DEN (single dose) 80 mg/kg bw MMS (single dose)	**Negative** No increase in hepatocyte nuclei tail moment with amisulbrom Positive response with DEN or MMS
Rat stomach/forestomach comet assay DNA damage in stomach/forestomach mucosal cells by comet assay Oral Gavage Purity 99.1 % 2005(e) (DAR B.6.4.2(f))	Rat/Han Wistar/4 f/group (amisulbrom) Rat/Han Wistar/4 f/group (DEN and MMS)	0, 500, 2000 mg/kg bw amisulbrom (single dose) 160 mg/kg bw DEN (single dose) 80 mg/kg bw MMS (single dose)	**Inconclusive response**: Slight increase in nuclei tail moment with amisulbrom only at 3 hr in stomach (0.68, 0.98, 1.19 at 0, 500 and 2000 mg/kg bw) Small increases with MMS at 3 hours in stomach (3.03 vs 0.68 in controls)* and forestomach (1.67 vs 0.73 in controls) Small increases with DEN at 24 hours in stomach (1.4 vs 0.64 in controls)* and forestomach (2.97 vs 1.04 in controls) *

* statistically significant

Rat liver micronucleus assay

Groups of four female F344 rats (4 weeks old) were gavaged with a single dose of amisulbrom (suspended in 0.5% aqueous methylcellulose) at dose levels of 500 or 2000 mg/kg bw (2004). Positive control groups were administered diethylnitrosamine (50 mg/kg bw). Animals were sacrificed on days 3, 4 or 5; hepatocytes were obtained by collagenase perfusion and stained with acridine orange/DAPI. At least 2000 hepatocytes per animal were examined using fluorescence microscopy and scored for the presence of micronuclei. The mitotic index was also calculated from 1000 hepatocytes.

No deaths occurred and no signs of toxicity were observed during the study period; weight gain was comparable in all groups. Administration of amisulbrom did not significantly increase the proportion of micronucleated hepatocytes at any time point, but the mitotic index was decreased at 2000 mg/kg bw on day 3. Marked (and statistically significant) increases in the proportions of micronucleated hepatocytes were observed at all time points in the positive control animals.

Overall, amisulbrom did not induce the formation of micronuclei in the liver of female F344 rats up to a dose (2000 mg/kg bw) causing cytotoxicity.

Rat liver comet assay

Groups of four female Han Wistar rats were gavaged with a single dose of amisulbrom (suspended in 0.5% aqueous methylcellulose) at dose levels of 0, 500 or 2000 mg/kg bw (2005a). Positive control groups of four female rats were administered diethylnitrosamine (DEN; 160 mg/kg bw) or methylmethanesulphonate (MMS; 80 mg/kg bw). Groups were sacrificed at 3 hours or 24 hours following administration. Liver samples were homogenised and suspensions of hepatocyte nuclei were prepared by centrifugation. Nuclei were lysed in an agarose gel, subjected to electrophoresis and stained with ethidium bromide. Fifty hepatocyte nuclei from each animal were randomly chosen for the assessment of tail moment.

No deaths occurred and no signs of toxicity were observed during the study period; weight gain was comparable in all groups. Mean hepatocyte nuclei tail moment was comparable in controls and amisulbrom-treated animals. Tail moment in hepatocyte nuclei from the positive control groups was significantly greater, demonstrating the sensitivity of the assay.

Overall, amisulbrom did not cause DNA damage in the liver of female Han Wistar rats up to the high dose of 2000 mg/kg bw.

Mouse liver comet assay

Male CD-1 mice (four/group) were gavaged with a single dose of amisulbrom (suspended in 0.5% aqueous methylcellulose) at dose levels of 0, 500 or 2000 mg/kg bw (2005b). Positive control groups of four male mice were administered diethylnitrosamine (DEN; 160 mg/kg bw) or methylmethanesulphonate (MMS; 80 mg/kg bw). Animals were sacrificed at 3 hours or 24 hours following administration. Liver samples were homogenised and a suspension of hepatocyte nuclei prepared by centrifugation. Nuclei were lysed in an agarose gel, subjected to electrophoresis and stained with ethidium bromide. Fifty hepatocyte nuclei from each animal were randomly chosen for the assessment of tail moment.

No deaths occurred and no signs of toxicity were observed during the study period; weight gain was comparable in all groups. Mean hepatocyte nuclei tail moment was comparable in controls and amisulbrom-treated animals. Tail moment in hepatocyte nuclei from the positive control groups was significantly greater demonstrating the sensitivity of the assay.

Overall, amisulbrom did not cause DNA damage in the liver of male CD-1 mice up to the high dose of 2000 mg/kg bw.

Rat stomach/forestomach comet assay

Female Han Wistar rats (four/group) were gavaged with a single dose of amisulbrom (suspended in 0.5% aqueous methylcellulose) at dose levels of 0, 500 or 2000 mg/kg bw (2005e). Positive control groups of four female rats were administered diethylnitrosamine (DEN; 160 mg/kg bw) or methylmethanesulphonate (MMS; 80 mg/kg bw). Animals were sacrificed at 3 hours or 24 hours following administration. Samples of glandular stomach and forestomach mucosa were homogenised and a suspension of mucosal cell nuclei prepared by centrifugation. Nuclei were lysed

in an agarose gel, subjected to electrophoresis and stained with ethidium bromide. Fifty mucosal cell nuclei from each animal were randomly chosen for the assessment of tail moment.

No deaths occurred and no signs of toxicity were observed during the study period; weight gain was comparable in all groups. Mean tail moment from glandular stomach mucosal cell nuclei was increased in a dose-related fashion in rats administered amisulbrom at three hours; similar findings were not seen at 24 hours or in forestomach cell nuclei. The significance of these isolated increases at one time point and in the stomach only is doubtful. Tail moment in nuclei from the positive control groups was significantly greater in most cases (not at 3 hours with MMS in the forestomach). The lack of statistical significance for the 3-hour forestomach MMS group, the low magnitude of the positive control values and the overlapping negative and positive control ranges indicate that the assay in the forestomach/stomach tissues is not particularly sensitive.

Overall, no conclusions can be drawn about the genotoxic potential of amisulbrom in the stomach and forestomach of female Han Wistar rats.

4.9.4 Summary and discussion of mutagenicity

The genotoxicity of amisulbrom was investigated in a battery of studies in vitro and in vivo. No evidence of mutagenicity was seen in an Ames test or a mouse lymphoma assay, although the highest concentration of amisulbrom in the mouse lymphoma assay was limited by solubility. A non-reproducible increase in the proportion of cells with chromosome aberrations was observed at an intermediate concentration in a study using human lymphocytes.

No evidence of genotoxicity was observed in vivo in a mouse bone marrow micronucleus assay. There was a slight increase in the proportion of micronucleated cells seen in amisulbrom-treated mice at 24 h. This was not considered of any biological relevance, as not only was it slight and statistically insignificant but it was associated with a low concurrent control value. There were concerns that systemic exposure in this study was low due to low oral absorption so a second study was conducted employing the intraperitoneal route of exposure. The results of this study showed no statistical increases in the frequency of micronucleated polychromatic erythrocytes (MnPCEs) in any of the amisulbrom-treated groups compared with vehicle controls. However, there were substantial decreases in the proportion of PCEs at all dose levels indicating toxicity to the bone marrow cells.

No evidence of genotoxicity was observed in a rat liver UDS assay or in a micronucleus assay. None-guideline comet assays performed in vivo on rat and mouse hepatocytes did not show any evidence of genotoxicity. The results of a comet assay on rat gastric mucosal cells were less clear due to the relative insensitivity of the assay to one of the positive controls. Comet assays are not routinely performed for regulatory submissions and so the applicants did not consider the shortcomings of the gastric mucosal assay to be of relevance to the overall classification.

Whilst the available studies do not show any evidence that amisulbrom was genotoxic, it was believed that investigations of mutagenicity in vitro and in vivo might have been limited by poor solubility and low oral absorption respectively.

4.9.5 Comparison with criteria

There was no evidence of mutagenicity in vitro or in vivo; therefore, amisulbrom does not meet the criteria for classification as a germ cell mutagen.

4.9.6 Conclusions on classification and labelling

Not classified – Conclusive but not sufficient for classification

4.10 Carcinogenicity

The carcinogenicity of amisulbrom has been investigated in rats and mice by the oral route.

Table 20: Summary table of relevant carcinogenicity studies

Method	Dose levels	Observations and remarks (effects of major toxicological significance)
2-yr chronic toxicity/ carcinogenicity study EC B33 GLP Dietary Rat, Han Wistar Chronic toxicity phase: 20/sex/group for 52 weeks Carcinogenicity phase: 50/sex/group for 104 weeks Amisulbrom: 99.1% pure 2005(a) (DAR B.6.5.1)	Chronic phase: 0, 200, 2000, 10000, 20000 ppm (0, 11, 112, 568, 1160 mg/kg bw/d in males; 0, 14, 147, 753, 1503 mg/kg bw/d in females) for 1-yr Carcinogenicity phase: 0, 2000, 10000, 20000 ppm (0, 96, 496, 1008 mg/kg bw/d in males; 0, 129, 697, 1436 mg/kg bw/d in females) for 2-yr	**20000 ppm (1008/1436 mg/kg bw/d in M/F):** Mortality: 64% vs 32% in controls (F)* Clinical signs of toxicity: thin build, piloerection, hunched posture, excessive chewing and pale teeth (F); hair loss on the ventral body surface (M) ↓ bw gain 32% (F)*; 23% (M)* (chronic phase) ↓ bw gain 53% (F)*; 22% (M)* (carcinogenicity phase) ↓ food consumption 8% (F); 8% (M) (chronic phase) ↓ food consumption 9% (F); 6% (M)* (carcinogenicity phase) ↑ γGTP (M &F)* ↑ urine pH 12% (F)*; 8% (M)* ↑ urine protein 260% (F)* ↑ rel liver wt 93% (F)*; 46% (M)* ↑ rel kidney wt 47% (F)* Non-neoplastic findings in <u>liver</u> (M&F), <u>biliary system</u> (M), <u>kidney</u> (M&F), <u>mesenteric lymph nodes</u> (M&F) (chronic phase) Non-neoplastic findings (carcinogenicity phase): <u>Adrenals</u> (cortical vacuolation) in F* <u>Caecum</u> (submucosal oedema) in F* <u>Duodenum</u> (epithelial hyperplasia) in F* <u>Eyes</u> (keratisis) in F* <u>Kidney</u> (cortical tubular pigment and chronic progressive nephropathy) in M and F* <u>Liver</u> (centrilobular hepatocyte hypertrophy, bile duct hyperplasia in M and F; midzonal hepatocyte vacuolation in F; cystic degeneration in M; reduced basophilic foci in M and F)* <u>Mesenteric lymph nodes</u> (sinus erythrocytosis and mastocytosis in M and F; sinus histiocytosis in F)* <u>Forestomach</u> (hyperplasia, hyperkeratosis, ulceration, submucosal inflammation and oedema, peritonitis) in F* <u>Thymus</u> (involution/atrophy) in F* <u>Thyroids</u> (follicular cell hypertrophy in M and F*, cystic follicular cell hyperplasia in F*) <u>Uterus</u> (myometrial atrophy and fibrosis)* <u>Vagina</u> (reduced mucification)* **10000 ppm (496/697 mg/kg bw/d in M/F):** Mortality: 46% vs 32% in controls (F) Clinical signs of toxicity: thin build, piloerection, hunched posture, excessive chewing and pale teeth (F); hair loss on the ventral body surface (M) ↓ bw gain 30% (F)*; 16% (M)* (chronic phase)

↓ bw gain 47% (F)*; 17% (M)* (carcinogenicity phase)
↓ food consumption 6% (F); 5% (M) (chronic phase)
↓ food consumption 8% (F); 5% (M) (carcinogenicity phase)
↑ γGTP (M &F)*
↑ urine pH 10% (F)*; 5% (M)*
↑ urine protein 153% (F)*
↑ rel liver wt 78% (F)*; 35% (M)*
↑ rel kidney wt 36% (F)*
Non-neoplastic findings in liver (M&F), biliary system (M), kidney (F), mesenteric lymph nodes (M&F) (chronic phase)
Non-neoplastic findings (carcinogenicity phase):
Caecum (submucosal oedema) in F
Duodenum (epithelial hyperplasia) in F*
Eyes (keratisis) in F*
Kidney (cortical tubular pigment and chronic progressive nephropathy) in M and F*
Liver (centrilobular hepatocyte hypertrophy, bile duct hyperplasia in M and F; midzonal hepatocyte vacuolation in F; cystic degeneration in M; reduced basophilic foci in M and F)*
Mesenteric lymph nodes (sinus erythrocytosis and mastocytosis in M and F; sinus histiocytosis in F)*
Forestomach (hyperplasia, hyperkeratosis, ulceration, submucosal inflammation and oedema, peritonitis) in F*
Thymus (involution/atrophy) in F*
Thyroids (follicular cell hypertrophy) in F
Uterus (myometrial atrophy and fibrosis)

2000 ppm (96/129 mg/kg bw/d in M/F):

↓ bw gain 16% (F)*; 9% (F)* (carcinogenicity phase)
↑ γGTP (M)*
↑ urine pH 6% (M)*
↑ rel liver wt 16% (M)*
Non-neoplastic findings in liver (M&F), biliary system (M), kidney (F) (chronic phase)
Non-neoplastic findings (carcinogenicity phase):
Kidney (cortical tubular pigment) in F*
Liver (centrilobular hepatocyte hypertrophy, bile duct hyperplasia) in M* and F

200 ppm (11.1/14.3 mg/kg bw/d in M/F):

no treatment-related effects

Tumours

Liver

Microscopic findings in the liver at necropsy (all animals)

Parameter	Dose level (ppm)			
	Males			
	0	2000	10000	20000
No. of rats examined	50	50	50	50
hepatocellular carcinoma	-	-	1 (2%)	-
hepatocellular adenoma	-	2 (4%)	10 (20%)**	13 (26%)**
	Females			
	0	2000	10000	20000

		<table><tr><td>No. of rats examined</td><td>50</td><td>50</td><td>50</td><td>50</td></tr><tr><td>hepatocellular carcinoma</td><td>-</td><td>-</td><td>2 (4%)</td><td>1 (2%)</td></tr><tr><td>hepatocellular adenoma</td><td>-</td><td>1 (2%)</td><td>24 (48%)**</td><td>28 (56%)**</td></tr></table> Adenoma laboratory HCD: 0 – 6% (M); 0 – 6% (F) **<u>Stomach</u>** Microscopic findings in the forestomach at necropsy (all animals) <table><tr><th rowspan="2">Parameter</th><th colspan="4">Dose Level (ppm)</th></tr><tr><th colspan="4">Males</th></tr><tr><td></td><td>0</td><td>2000</td><td>10000</td><td>20000</td></tr><tr><td>No. of rats examined</td><td>50</td><td>50</td><td>50</td><td>50</td></tr><tr><td>- squamous cell carcinoma</td><td>-</td><td>-</td><td>-</td><td>-</td></tr><tr><td>- squamous cell papilloma</td><td>-</td><td>-</td><td>-</td><td>-</td></tr><tr><td></td><td colspan="4">Females</td></tr><tr><td></td><td>0</td><td>2000</td><td>10000</td><td>20000</td></tr><tr><td>No. of rats examined</td><td>50</td><td>50</td><td>50</td><td>50</td></tr><tr><td>- squamous cell carcinoma</td><td>-</td><td>-</td><td>-</td><td>1 (2%)</td></tr><tr><td>- squamous cell papilloma</td><td>-</td><td>-</td><td>1 (2%)</td><td>2 (4%)</td></tr></table> **NOAEL (toxicity) = 200 ppm (11.1/14.3 mg/kg bw/d in M/F)$** **NOAEL (carcinogenicity) = 2000 ppm (96/129 mg/kg bw/d in M/F)$**
Carcinogenicity study EC B33 GLP Dietary Mouse, CD-1 50/sex/group for 784 weeks Amisulbrom: 99.1% pure 2005(b) (DAR B.6.5.2)	0, 100, 800, 4000, 8000 ppm (11.6, 97.8, 494, 1035 mg/kg bw/d in males; 13.5, 121, 594, 1255 mg/kg bw/d in females)	**8000 ppm (1035/1255 mg/kg bw/d in M/F):** ↓ bw gain 13% (F); 37% (M)* ↑ rel liver wt 31% (F)*; 34% (M)* Non-neoplastic findings <u>Adrenals</u> (cortical hypertrophy) in M <u>Caecum</u> (pigmentation of submucosal venules) in M and F* <u>Kidney</u> (cortical tubular basophilia in F, lymphoid aggregation in M and F) <u>Liver</u> (focal hepatocyte necrosis in M)* **4000 ppm (494/594 mg/kg bw/d in M/F):** ↓ bw gain 23% (M)* ↑ rel liver wt 11% (F)*; 33% (M)* Non-neoplastic findings <u>Adrenals</u> (cortical hypertrophy) in M <u>Caecum</u> (pigmentation of submucosal venules) in M and F* <u>Kidney</u> (cortical tubular basophilia in F*, lymphoid aggregation in F) **800 ppm (97.8/121 mg/kg bw/d in M/F):** ↓ bw gain 14% (M) ↑ rel liver wt 17% (M)* Non-neoplastic findings <u>Adrenals</u> (cortical hypertrophy) in M <u>Caecum</u> (pigmentation of submucosal venules) in M and F <u>Kidney</u> (lymphoid aggregation in F)* **100 ppm (11.6/13.5 mg/kg bw/d in M/F):**

No treatment-related effects

Tumours:

Liver

Macroscopic findings in the liver at necropsy (all animals)

Parameter	Dose Level (ppm)				
	Males				
	0	100	800	4000	8000
No. mice examined	50	50	50	50	50
- Hepatocellular carcinoma	2(4%)	3(6%)	4(8%)	4(8%)	2(4%)
- Hepatocellular adenoma	8(16%)	12(24%)	17(34%)*a	23(46%)**	18(36%)*
	Females				
	0	100	800	4000	8000
No. mice examined	50	50	50	50	50
- Hepatocellular carcinoma	-	-	-	-	
- Hepatocellular adenoma	1(2%)	-	-	2(4%)	-

a: Not significant when time to tumour formation taken into account (Peto analysis)
Adenoma laboratory HCD: 7.8 – 30.8% (M) from 14 studies conducted between 1993 and 2002

NOAEL (toxicity) = 100 ppm (11.6/13.5 mg/kg bw/d in M/F)$

NOAEL (carcinogenicity) = 100 ppm (11.6/13.5 mg/kg bw/d in M/F)$

* = statistically significant, $ = As given in the DAR

↓ = decrease ↑ = increase

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Rat

In a guideline chronic toxicity/cancinogenicity bioassay (2005a), groups of 20 male and 20 female Han Wistar rats were given dietary concentrations of 0, 200, 2000, 10000 or 20000 ppm amisulbrom for 52 weeks. These rats comprised the chronic toxicity phase of the study. In addition, groups of 50 males and 50 females were given dietary concentrations of 0, 2000, 10000 or 20000 ppm amisulbrom for 104 weeks in order to assess its carcinogenic potential. The mean achieved test material concentrations in the chronic toxicity phase were 0, 11.1, 112, 568 and 1160 mg/kg bw/d in males and 0, 14.3, 147, 753 and 1503 mg/kg bw/d in females at 0, 200, 2000, 10000 and 20000 ppm respectively.
Corresponding values for the carcinogenicity phase were 0, 96, 496 and 1008 mg/kg bw/d in males and 0, 129, 697 and 1436 mg/kg bw/d in females at 0, 2000, 10000 and 20000 ppm respectively.

There were 125 unscheduled deaths amongst animals assigned to the carcinogenicity phase. Statistical analysis of mortality revealed that amongst females receiving 20000 ppm the number of deaths was significantly higher than controls (64% mortality vs 32% in controls). A high incidence of females treated at 10000 or 20000 ppm were killed or found dead from Week 92. The females in

these groups had low body weight gain during the study and the condition of the animals killed or found dead deteriorated rapidly during this final part of the study. The percentage survival in high dose females dipped below 50% between weeks 97-98; therefore, the length of exposure in this group of animals was sufficient to assess carcinogenicity.

Clinical signs recorded for animals assigned to the carcinogenicity phase included high incidences of thin build, piloerection, hunched posture, excessive chewing and pale teeth for females receiving doses ≥10000 ppm. Slightly increased incidences of hair loss on the ventral body surface, when compared with the controls, were recorded for males receiving doses ≥10000 ppm.

In the chronic toxicity phase of the study, there was a dose-related reduction in bodyweight gain during the 52 week treatment at doses ≥2000 ppm. It was marked in 20000 ppm males (77% of controls) and in females at both 10000 and 20000 ppm (70% and 68% of controls respectively). At doses ≥10000 ppm, overall food consumption was slightly reduced. During the first 2 weeks of treatment the reduction in food intake was particularly marked at 20000 ppm. Food scatter was also increased at doses ≥10000 ppm during Week 1 and suggested initial unpalatability of the test diets. Overall food conversion efficiency was slightly low during the first 16 weeks of treatment, particularly in males given doses ≥10000 ppm. The effect was particularly marked in Week 1 in males fed 20000 ppm. This indicated that reduced bodyweight gain was not entirely due to low food consumption and indicated an underlying toxic effect.

In the carcinogenicity phase of the study, there was a dose-related reduction in bodyweight gain at doses ≥10000 ppm. Bodyweight gain to 104 weeks was markedly reduced at 10000 and 20000 ppm (males: 83% and 78% of control; females: 53% and 47% of control both respectively). During the second year of treatment, female mean bodyweight at 20000 ppm showed little change i.e. was static, whilst at 10000 ppm the depression of weight gain compared to controls was 88%. Overall food consumption was slightly reduced in females fed 2000 ppm and in both sexes given doses ≥10000 ppm. Food scatter was also increased at doses ≥10000 ppm during Week 1. Overall food conversion efficiency was slightly low during the first 16 weeks of treatment, particularly in Week 1 in males given doses ≥10000 ppm.

Treatment-related and statistically significantly high gamma-glutamyl transpeptidase activities were seen in Week 26 in animals treated at ≥10000 ppm and in males in Week 52 at doses ≥2000 ppm. Several individual values in these groups exceeded the historical control range.

Urinalysis investigations during Week 12 indicated slightly high pH in males receiving 2000 ppm and in males and females receiving doses ≥10000 ppm. In Week 51 there was a slightly high pH in females receiving 20000 ppm. In addition, in Week 51, females receiving doses ≥10000 ppm had slightly high urinary protein concentration, when compared with the controls.

In the toxicity phase, there were dose-related increases in relative liver and kidney weights at doses ≥2000 ppm. In the carcinogenicity phase, relative liver weight was slightly increased in males at 2000 ppm. At doses ≥10000 ppm, there were increases in both absolute and relative liver weights of both sexes, particularly females, and in relative kidney weights in females.

In the carcinogenicity phase, macroscopic findings occurred in the duodenum, kidneys, lachrymal glands, liver, mesenteric lymph nodes and stomach. At 2000 ppm, treatment-related changes were confined to females which exhibited pale areas in the liver. Females given doses ≥10000 ppm generally appeared thinner with distension and thickening of the duodenum; depressions and thickening in the stomach; pallor and granular appearance of the kidneys; pale and dark areas,

accentuated lobular pattern and an increased incidence of masses and enlargement of the liver; darkened lachrymal glands; congestion of the mesenteric lymph nodes. Males given doses ≥10000 ppm showed an increase in pale areas of the liver and slightly increased congestion of the mesenteric lymph nodes. At 20000 ppm, cysts were increased in the liver.

With regard to microscopic non-neoplastic findings at 52 weeks (toxicity phase), changes were seen in the liver, biliary system, kidney and mesenteric lymph nodes. At doses ≥2000 ppm, there was an increased incidence in minimal or slight midzonal hepatocyte vacuolation in both sexes. At doses ≥10000 ppm, minimal centrilobular hepatocyte hypertrophy was found in a few males. At doses ≥2000 ppm, there was a dose-related increase in the incidence and severity of bile duct hyperplasia in males. A marginal non-statistically significant increase of bile duct hyperplasia was seen in females given doses ≥10000 ppm. At 10000 ppm, there was a multilocular biliary cyst in 1 female and at 20000 ppm, 1 male exhibited a simple biliary cyst. At 20000 ppm, the incidence of portal inflammation in males was significantly increased and extrahepatic bile duct dilatation seen in both sexes at necropsy was confirmed microscopically. In females given doses ≥2000 ppm, and in males fed 20000 ppm, there was an increased incidence of minimal or slight cortical tubular pigment, identified as lipofuscin. At doses ≥10000 ppm, the incidence of minimal or slight cortical tubular basophilia was increased in females. There was a dose-related increase in the incidence and severity of sinus erythrocytosis/erythrophagocytosis and of mastocytosis although statistical significance was only achieved at doses ≥10000 ppm. In dietary studies, the mesenteric lymph node is usually the affected lymph node. The underlying mechanism of mastocytosis is unclear. The Applicant considered that the findings in the lymph nodes were probably due to local disturbance in the node in response to exposure to high levels of xenobiotic in the GI tract.

With regard to microscopic non-neoplastic findings at 104 weeks (carcinogenicity phase), treatment-related findings occurred in the adrenals, caecum, duodenum, eyes, kidneys, liver, mesenteric lymph nodes, mammary glands, forestomach, thymus, thyroids, uterus and vagina. At 20000 ppm, there was an increased incidence of cortical vacuolation of the adrenal in females which was considered by the Applicant to be associated with stress. In addition, a reduction in the frequency of cortical cystic/haemorrhagic degeneration and cortical hypertrophy was found in these animals, which was attributed to reduced bodyweight and poor nutritional status. At doses ≥10000 ppm, sub-mucosal oedema of the caecum was found in females. It was considered to be associated with the poor condition (or a local effect due to loads of unabsorbed material) of these animals and was not seen in either the controls or at 2000 ppm. Epithelial hyperplasia of the duodenum was found in females fed doses ≥10000 ppm and in 1 male given 20000 ppm. Its aetiology was considered to be similar to the changes in the stomach. Increased incidence of keratitis of the eyes was seen in females at doses ≥10000 ppm; it was considered by the Applicant to be due to their poor condition, possibly arising from reduced grooming. An increased frequency and severity of cortical tubular pigment of the kidney was found in both sexes at doses ≥10000 ppm and in females given 2000 ppm. Also, at doses ≥10000 ppm the incidence of chronic progressive nephropathy was increased, particularly in females.

In the liver, incidences of centrilobular hepatocyte hypertrophy and bile duct hyperplasia were increased in both sexes at doses ≥2000 ppm. In addition, in females given doses ≥10000 ppm, there was a significant increase in midzonal hepatocyte vacuolation. Periportal hepatocyte hypertrophy was found in 10 females fed 2000 ppm. At doses ≥10000 ppm, the incidences of basophilic foci were significantly reduced in both sexes, and cystic degeneration was increased in males. Midzonal hepatocyte vacuolation is suggestive of an effect on hepatic fat metabolism. Changes in the biliary system (including bile duct hyperplasia, cystic degeneration, extrahepatic bile duct dilatation and portal inflammation seen in the chronic toxicity phase) are indicative of hepatotoxicity and may

have been associated with excretion of amisulbrom and/or its metabolites in the bile. In the mesenteric lymph nodes, the frequency of sinus erythrocytosis/erythrophagocytosis and mastocytosis was increased in both sexes at doses ≥10000 ppm. In addition, sinus histiocytosis was increased in females at doses ≥10000 ppm. In the mammary gland, the incidences of secretory activity and acinar hyperplasia in females were reduced at the top dose. This was attributed to the reduced bodyweight gain observed in these animals. In the forestomach, incidences of epithelial hyperplasia, hyperkeratosis, ulceration, submucosal inflammation and submucosal oedema were increased in females at doses ≥10000 ppm. Peritonitis was also slightly increased in these animals. The study authors postulate that the inflammatory and hyperplastic changes in the keratinised region of the stomach (forestomach) may be due to a direct irritant effect of the test material.

Thymic involution/atrophy was increased in females fed doses ≥10000 ppm. This was attributed to stress. Increased incidences of follicular cell hypertrophy of the thyroid were found in both sexes at 20000 ppm and in females given 10000 ppm. Cystic follicular cell hyperplasia was significantly increased in females at 20000 ppm. Thyroid follicular cell hypertrophy and cystic follicular cell hyperplasia were considered to be secondary to the hypertrophic change in the liver. In the uterus, there were increased incidences of myometrial atrophy and myometrial fibrosis at 10000 and 20000 ppm. These findings were attributed to stress and the poor condition of these animals. At 20000 ppm, the incidence of epithelial mucification of the vagina was reduced. This was also considered to be due to stress and the poor condition of the animals.

Treatment-related neoplastic findings were found in the liver and stomach.

In the liver, hepatocellular carcinomas were found in 1 male and 2 females given 10000 ppm and in 1 female fed 20000 ppm. The increases in carcinomas were very small compared to the incidences of adenomas at the same dose levels and not dose-related. They were therefore considered to be unlikely to be related to treatment. At 10000 and 20000 ppm, there was an increased incidence of hepatocellular adenomas, particularly in females. Although 2 males and 1 female at 2000 ppm also exhibited this neoplasm, the incidences (4.0% and 2.0%) were within the historical control range (males: mean = 1.64%; range = 0.0-6.0%; n = 365; females: mean = 1.92%; range = 0.0-6.0%; n= 365). The study authors consider that the increased incidences of hepatocellular tumours at doses ≥10000 ppm were a consequence of persistent hypertrophic change. Overall, there was a statistically significant increase in the incidence of liver adenoma at 10000 and 20000 ppm in males and females. In females, the increase in liver adenoma was observed at dose levels causing excessive toxicity (increased mortality, clinical signs of toxicity and 53% reduction in body weight gain at 20000 ppm; increased mortality, clinical signs of toxicity and 47% reduction in body weight gain at 10000 ppm). In males, the increase in liver adenoma occurred at dose levels causing a moderate level of toxicity (clinical signs of toxicity and 22% reduction in body weight gain at 20000 ppm; clinical signs of toxicity and 17% reduction in body weight gain at 10000 ppm).

Table 20(a): Summary of neoplastic findings in the liver

Parameter	Dose level (ppm)							
	Males				Females			
	0	2000	10000	20000	0	2000	10000	20000
Microscopic findings at necropsy (all animals)								
Liver								
Number of rats examined	50	50	50	50	50	50	50	50
- hepatocellular carcinoma	-	-	1 (2%)	-	-	-	2 (4%)	1 (2%)
- hepatocellular adenoma	-	2 (4%)	10 (20%)**	13 (26%)**	-	1 (2%)	24 (48%)**	28 (56%)**

Adenoma laboratory HCD: 0 – 6% (M); 0 – 6% (F)

In the forestomach, a squamous cell carcinoma was found at 20000 ppm in 1 female. Squamous cell papillomas were seen in 1 female given 10000 ppm and in 2 females fed 20000 ppm. The incidences were not statistically significant by Fisher's Exact test. However, when data for 20000 ppm were included, a trend test for the combined incidence of benign squamous cell papilloma and malignant squamous cell carcinoma was significant ($p<0.0183$). Overall, there was an increase in the incidence of papilloma of the forestomach in females at 10000 and 20000 ppm and an increase of the incidence of carcinoma of the forestomach in the 20000 ppm females. These tumours were considered to be due to chronic inflammation resulting from a local irritant effect. It is noted that the package of genetic toxicity tests with amisulbrom was negative. It is well-established that the rat forestomach acts as storage reservoir releasing undigested food into the glandular stomach in response to energy demand. Hence, the rat forestomach mucosa may be exposed to the test material in the diet for far longer periods than elsewhere in the gastrointestinal (GI) tract. Forestomach tumours in rats are known to occur following inflammation, hyperplasia and hyperkeratosis by food additives such as butylated hydroxyanisole. However, exposure of the human stomach to such food additives does not result in tumour formation (Greaves, 2000). Overall, it is concluded that the forestomach tumours associated with amisulbrom exposure in female rats are not relevant to humans because in humans persistently high local concentrations of amisulbrom in the GI tract cannot be achieved.

Table 20(b): Summary of neoplastic findings in the stomach

Parameter	Dose level (ppm)							
	Males				Females			
	0	2000	10000	20000	0	2000	10000	20000
Microscopic findings at necropsy (all animals)								
Forestomach								
Number of rats examined	50	50	50	50	50	50	50	50
- squamous cell carcinoma	-	-	-	-	-	-	-	1(2%)
- squamous cell papilloma	-	-	-	-	-	-	1(2%)	2(4%)

Overall, during the first 12 months dietary administration of amisulbrom at doses ≥2000 ppm to Han Wistar rats caused non-specific toxicity evidenced as reductions in bodyweight gain and food consumption. The target organs were the liver and kidneys. Evidence of effects on the liver were increased γ-glutamyl transpeptidase activity, increased liver weights, and histopathological changes such as hepatocellular hypertrophy/midzonal hepatocyte vacuolation. Kidney weights were increased and there was an increased incidence of minimal or slight cortical tubular basophilia. In addition to these changes there was a dose-related increase in the incidence and severity of sinus erythrocytosis/erythrophagocytosis and of mastocytosis in the mesenteric lymph nodes (statistical significant at doses ≥10000 ppm).

In the carcinogenicity element of the study it was evident that the two higher dose levels (10000 and 20000 ppm) caused excessive toxicity in females (increased mortality and a marked reduction in bodyweight gain). Hepatocellular adenoma was seen at doses ≥10000 ppm in males and females. In addition there was a low frequency of squamous cell carcinoma and papillomas in the forestomach at doses ≥10000 ppm in females only.

Mouse

In a guideline cancer bioassay, groups of 50 male and 50 female CD-1 mice were given dietary concentrations of 0, 100, 800, 4000 or 8000 ppm amisulbrom for 78 weeks (2005b). The overall mean achieved test material intakes were 11.6, 97.8, 494 and 1035 mg/kg bw/d in males and 13.5, 121, 594 and 1255 mg/kg bw/d in females.

Mortality was not adversely affected by treatment. There were no treatment-related clinical signs. Overall bodyweight gain was low in males fed 4000 ppm and in both sexes at 8000 ppm. In males, bodyweight gains were 77% and 63% of controls at 4000 and 8000 ppm respectively, and 87% of controls in females fed 8000 ppm. There was also a non-statistically significant decrease in body weight gain (86% of controls) in males given 800 ppm. At 4000 and 8000 ppm, food consumption was slightly reduced in Week 1. Overall food consumption was unaffected. At 8000 ppm, male food conversion efficiency was low during the first 3 weeks of treatment and was low overall for Weeks 1 – 14. There were no toxicologically significant effects on blood parameters or in blood smears. At termination, there was a dose-related increase in relative liver weight in males fed 800 ppm and in both sexes given doses $\geq$4000 ppm.

Treatment-related non-neoplastic changes were seen in the adrenals, caecum, kidneys and liver. There was a slight increase in the incidence of cortical hypertrophy of the adrenal in males given 800 ppm or more. At 8000 ppm, the severity was slightly increased. However, none of these findings were statistically significant. In the caecum, intracellular pigment deposition was seen in the mucosal and sub-mucosal regions and in the walls of the venules at dose levels of $\geq$800 ppm. There was a general dose-related trend, although the incidences at 4000 and 8000 ppm were similar. Although the identity of the pigment was not established, it was considered that it probably was a bile pigment. In the kidney, the incidence of cortical tubular basophilia was increased in females at dose levels of $\geq$4000 ppm. Incidences of perivascular lymphoid aggregations were also slightly increased in females fed 800 ppm or more, and in males given 8000 ppm. In the liver, there was an increased incidence of focal hepatocyte necrosis in males at 8000 ppm.

The only treatment-related neoplastic change was an increase in hepatocellular adenomas in males at doses $\geq$800 ppm (34%, 46% and 36% at 800, 4000 and 8000 ppm respectively vs 16% in controls). The mean numbers of tumours per mouse was increased as well as the numbers of animals affected. The higher incidence of hepatocellular adenomas at 100 ppm (24%) was within the laboratory historical control range (mean 17.1%; range: 7.8 - 30.8% from 14 studies conducted between 1993 and 2002). This was confirmed by data generated from a recently completed study (2005) in which the mean control incidence was 26% (n=50). Incidences of hepatocellular carcinomas were unaffected. Analysis for trend for male data was significant when all groups were included and if data for 8000 ppm were excluded. However, when data for the 4000 ppm group were excluded, the trend was no longer statistically significant. A trend test for the combined incidences of hepatocellular adenomas and carcinomas was not significant. Overall, there was a slight increase (above laboratory historical control ranges) in the incidence of liver adenoma in males from a dose of 800 ppm. The increase in liver adenoma at 8000 ppm (36%) occurred at a dose level causing excessive toxicity (37% reduction in body weight gain).

Table 20 (c): Summary of neoplastic findings in the liver

Parameter	**Dose level (ppm)**									
	Males					**Females**				
	0	**100**	**800**	**4000**	**8000**	**0**	**100**	**800**	**4000**	**8000**
Macroscopic findings at necropsy (all animals)										
Liver										
No. of mice examined	50	50	50	50	50	50	50	50	50	50

Hepatocellular carcinoma	2(4%)	3(6%)	4(8%)	4(8%)	2(4%)	-	-	-	-	-
Hepatocellular adenoma	8(16%)	12(24%)	17(34%)*[a]	23(46%)**	18(36%)*	1(2%)	-	-	2(4%)	-

a: Not significant when time to tumour formation taken into account (Peto analysis)
Adenoma laboratory HCD: 7.8 – 30.8% (M) from 14 studies conducted between 1993 and 2002

In conclusion, dietary administration of amisulbrom to CD-1 mice for 78 weeks produced significant non-specific toxicity at doses ≥4000 ppm (reduced bodyweight gain and food efficiency and an initial reduction in food consumption) and treatment-related changes in the adrenal glands, kidneys and caecum at ≥800 ppm. From 800 ppm there was an increased incidence of hepatocellular adenoma in males. At 8000 ppm, incidence of focal hepatocyte necrosis was increased in males.

4.10.1.2 Carcinogenicity: inhalation

No data are available.

4.10.1.3 Carcinogenicity: dermal

No data are available.

4.10.2 Human information

No data are available.

4.10.3 Other relevant information

Numerous mechanistic studies (including *in vivo* genotoxicity investigations) on the liver carcinogenicity of amisulbrom are available. A comet assay in the rat stomach/forestomach is also available. The *in vivo* genotoxicity studies have been reported in the mutagenicity section.

Table 21: Summary table of relevant carcinogenicity mechanistic studies

<table>
<tr><th>Method</th><th>Dose levels</th><th>Observations and remarks</th></tr>
<tr>
<td><u>Medium-term liver carcinogenesis bioassay (Ito model)</u>
Rat/F-344/males/10-20/group
Mechanistic study to investigate promotional activity of amisulbrom by measuring GST-P positive foci in the liver
Amisulbrom</td>
<td>
<table>
<tr><th>Group number</th><th>DEN[a]</th><th>Compound</th><th>Dose level (ppm)[c]</th><th>Number of rats</th></tr>
<tr><td>1</td><td>+</td><td rowspan="4">amisulbrom</td><td>0</td><td>20</td></tr>
<tr><td>2</td><td>+</td><td>200</td><td>20</td></tr>
<tr><td>3</td><td>+</td><td>2000</td><td>20</td></tr>
<tr><td>4</td><td>+</td><td>20000</td><td>20</td></tr>
<tr><td>5</td><td>+</td><td>PB</td><td>500</td><td>20</td></tr>
<tr><td>6</td><td>-[b]</td><td rowspan="2">amisulbrom</td><td>0</td><td>10</td></tr>
<tr><td>7</td><td>-[b]</td><td>20000</td><td>10</td></tr>
</table>
[a]:Given a single intraperitoneal injection of 200 mg/kg bw of *N*-nitrosodimethylamine
</td>
<td>20000 ppm (1448 mg/kg bw/d) (initiated):
↓11% bw*
↑rel liver wt 52%*
↑no/cm^2 GST-P positive foci (6.7 vs 2.9 in controls)*

2000 ppm (120 mg/kg bw/d) (initiated):
↑rel liver wt 22%*
↑no/cm^2 GST-P positive foci (4.8 vs 2.9 in controls)*

200 ppm (12 mg/kg bw/d) (initiated):
↑rel liver wt 2.5%*</td>
</tr>
</table>

(99.1%) 2005 (DAR B.6.8.1(a))	[b]:Given a single intraperitoneal injection of saline [c] In the initiated groups fed amisulbrom, overall mean achieved test material intakes were 12.04, 120.01 and 1448.38 mg/kg bw/d at 200, 2000 and 20000 ppm respectively. In the uninitiated group fed 20000 ppm, it was 1795.67 mg/kg bw/d. The overall mean intake in the group fed 500 ppm of PB was 31.13 mg/kg bw/d	**20000 ppm (1795 mg/kg bw/d) (uninitiated):** ↓10% bw* ↑rel liver wt 54%* **500 ppm (31 mg/kg bw/d) PB:** ↑rel liver wt 43%* ↑no/cm^2 GST-P positive foci (7.8 vs 2.9 in controls)*
<u>*Enzyme induction in rat liver*</u> Rat/Han Wistar (m and f) EROD, PROD, MFCOD, and T-OH activity measured in liver S9 fractions Amisulbrom (99.1%) in the diet PB by gavage 2005(c) (DAR B.6.8.1(b)) EROD: Ethoxyresorufin-O-deethylation PROD: Pentoxyresorufin-O-deethylation MFCOD: 7-Methoxy-4-trifluoromethylcoumarin-O-deethylation T-OH: Testosterone-6B-hydroxylation	0, 200 (21 and 20 mg/kg bw/d in M and F), 20000 ppm (1946 and 2083 mg/kg bw/d in M and F) amisulbrom for 7 days 50 mg/kg bw/d PB for 7 days	**20000 ppm (1946/2083 mg/kg bw/d in M/F):** ↓6% bw (day 3) in males* ↓22% bwg in males*; 26% in females ↓22% fc in males*; 27% in females* ↑rel liver wt 10% (M)*; 18% (F)* ↑ EROD: 3.2x(M)*; 4.8x(F)* ↑ PROD: 14.8x(M)*; 13x(F)* ↑ MFCOD: 3.2x(M)*; 3.5x(F)* ↑ T-OH: 1.5x(M)*; 3.7x(F)* **200 ppm (21/20 mg/kg bw/d in M/F):** No effects **PB:** ↑rel liver wt 15%(M)*; 21%(F)* ↑ EROD: 3.1x(M)*; 1.2x(F)* ↑ PROD: 39x(M)*; 32x(F)* ↑ MFCOD: 5.7x(M)*; 5.9x(F)* ↑ T-OH: 2.8x(M)*; 5.8x(F)*
<u>*Enzyme induction in mouse liver*</u> CD-1 Mouse (m and f) EROD, PROD and T-OH activity measured in liver S9 fractions Amisulbrom (99.1%) in the diet PB by gavage	0, 100 (14 and 17 mg/kg bw/d in M and F), 8000 ppm (1079 and 1310 mg/kg bw/d in M and F) amisulbrom for 7 days 50 mg/kg bw/d PB for 7 days	**8000 ppm (1079/1310 mg/kg bw/d in M/F):** ↓(Day 3) fc 23% (M)*; 21%(F)* ↑rel liver wt 11% (M)*; 11% (F)* ↑EROD: 1.33x(M)*; 1.52x(F)* ↑PROD:1.87x(M)*; 1.64x(F)* ↑T-OH: 1.33x(M); **100 ppm (14/17 mg/kg bw/d in M/F):** No effects

2005(d) (DAR B.6.8.1(c))		**PB:** ↓9% bw in F* ↓bwg in M* and F* ↓26% fc (Day 3) in F* ↑rel liver wt 9% (M)*; 19% (F)* ↑EROD: 1.84x(M)*; 2.23x(F)* ↑PROD:14.2x(M)*; 7.71x(F)* ↑T-OH: 1.64x(M)*; 1.24x(F)
Replicative DNA synthesis - male rat liver (single dose) Rat/Han Wistar/12 m/group (amisulbrom) Rat/Han Wistar/4 m/group (PB) Amisulbrom (99.1%) by gavage PB by gavage Replicative DNA synthesis measured in the liver 2005(a) DAR B.6.8.1(d))	0, 1000 and 2000 mg/kg bw amisulbrom (in 0.5% methylcellulose) 50 mg/kg bw PB	**2000 mg/kg bw:** ↑ rel liver wt 11% (39 hr)* ↑ RDS 2.9x(39 hr)*; 4.4x(48 hr) **1000 mg/kg bw:** ↑ RDS 2.5x(24 hr); 3.7x(39 hr)*; 3.8x(48 hr) **PB:** ↑ rel liver wt 13% (39 hr)*; 10% (48 hr)* ↑ RDS 2.7x(24 hr); 5.9x(39 hr)*; 6.2x(48 hr)*
Replicative DNA synthesis - female rat liver (single dose) Rat/Han Wistar/12 f/group (amisulbrom) Rat/Han Wistar/4 f/group (PB) Amisulbrom (99.1%) by gavage PB by gavage Replicative DNA synthesis measured in the liver	0, 1000 and 2000 mg/kg bw amisulbrom (in 0.5% methylcellulose) 50 mg/kg bw PB	**2000 mg/kg bw:** ↑ RDS 1.7x(39 hr); 2.4x(48 hr)* **1000 mg/kg bw:** ↑ RDS 2.1x(24 hr); 2.8x(39 hr); 3.4x(48 hr)* **PB:** ↑ rel liver wt 6% (48 hr)* ↑ RDS 5.4x(24 hr); 1.6x(39 hr); 1.7x(48 hr)

2005(b) (DAR B.6.8.1(e))		
Replicative DNA synthesis - male rat liver (repeat dose) Rat/Han Wistar/8 m/group (amisulbrom) Rat/Han Wistar/8 m/group (PB) Amisulbrom (99.1%) in the diet PB by gavage Replicative DNA synthesis measured in the liver 2005(c) (DAR B.6.8.1(f))	0, 200, 2000 and 10000 ppm (0, 15, 136, 572 mg/kg bw/d) amisulbrom for 7 days 50 mg/kg bw PB for 3 or 7 days	**10000 ppm (572 mg/kg bw/d)**: ↓bwg (Day 3) 5%* ↓fc (Day 3) 34%*; fc (Day 7) 14%* ↑ RDS 3x(Day 3) **2000 ppm (136 mg/kg bw/d)**: ↓fc (Day 3) 9%* ↑ RDS 1.4x(Day 3) **200 ppm (15 mg/kg bw/d):** No effects **PB**: ↑ abs liver wt (Day 3) 11%* ↑ rel liver wt (Day 3) 12%*; (Day 7) 17%* ↑ RDS 3.9x(Day 3)*
Replicative DNA synthesis - female rat liver (repeat dose) Rat/Han Wistar/8 f/group (amisulbrom) Rat/Han Wistar/8 f/group (PB) Amisulbrom (99.1%) in the diet PB by gavage Replicative DNA synthesis measured in the liver 2005(d) (DAR B.6.8.1(g))	0, 200, 2000 and 10000 ppm (0, 17, 150, 656 mg/kg bw/d) amisulbrom for 7 days 50 mg/kg bw PB for 3 or 7 days	**10000 ppm (656 mg/kg bw/d)**: ↓ bw (Day 3) 3% ↓ fc (Day 3) 28%* ↑ RDS 4.8x(Day 3)* **2000 ppm (150 mg/kg bw/d)**: ↑ RDS 4x(Day 3)* **200 ppm (17 mg/kg bw/d)**: No effects **PB**: ↑ abs liver wt (Day 3) 20%* ↑ rel liver wt (Day 3) 15%*; (Day 7) 18%* ↑ RDS 5.9x(Day 3)*
Replicative DNA synthesis - male	0, 100 and 8000 ppm (0, 15, 1021 mg/kg bw/d) amisulbrom for 7 days	**8000 ppm (1021 mg/kg bw/d)**:

mouse liver (repeat dose) Mouse/CD-1/8 m/group (amisulbrom) Mouse/CD-1/8 m/group (PB) Amisulbrom (99.1%) in the diet PB by gavage Replicative DNA synthesis measured in the liver 2005(e) (DAR B.6.8.1(h)	50 mg/kg bw PB for 3 or 7 days	↓fc (Day 3) 20%* ↑abs liver wt (Day 3) 13%; (Day 7) 16% ↑ RDS 5.1x(Day 3); 5.3x(Day 7)* **100 ppm (15 mg/kg bw/d)**: No effects **PB**: ↓ bw (Day 3) 4%; (Day 7) 11% ↑abs liver wt (Day 3) 16%* ↑rel liver wt (Day 3) 15%*; (Day 7) 19% ↑ RDS 4.1x(Day 3); 4.2x(Day 7)
Replicative DNA synthesis - female mouse liver (repeat dose) Mouse/CD-1/8 f/group (amisulbrom) Mouse/CD-1/8 f/group (PB) Amisulbrom (99.1%) in the diet PB by gavage Replicative DNA synthesis measured in the liver 2005(f) (DAR B.6.8.1(i))	0, 100 and 8000 ppm (0, 17, 1234 mg/kg bw/d) amisulbrom for 7 days 50 mg/kg bw PB for 3 or 7 days	**8000 ppm (1234 mg/kg bw/d)**: ↓fc (Day 3) 17%* ↑rel liver wt (Day 7) 20%* **100 ppm (17 mg/kg bw/d)**: No effects **PB**: ↓ bw (Day 3) 6%; (Day 7) 6% ↑rel liver wt (Day 7) 15%* ↑ RDS 5.1x(Day 3); 5.4x(Day 7)

* statistically significant

↓ = decrease ↑ = increase

Medium-term liver carcinogenesis (Ito) rat bioassay

A medium-term liver carcinogenisis (Ito model) bioassay was conducted in the rat (2005). This study was conducted to investigate any promotion potential of amisulbrom on liver carcinogenesis by investigating the development of glutathione S-transferase placental form (GST-P) positive foci

as end-point lesions. Male F344 rats (at 6 weeks of age) were given a single intraperitoneal injection of 200 mg/kg bw N-nitrosodiethylamine (DEN) to initiate hepatocarcinogenesis. Two weeks later, they received the diet containing amisulbrom at 0 (control), 200, 2000 and 20000 ppm for 6 weeks. The positive control group was administered the diet containing 500 ppm of phenobarbital sodium salt (PB) as a promoter. Groups without DEN initiation also received 0 (for control) or 20000 ppm of the test material. After one week of the treatment period, at the end of the experimental week 3, all animals were subjected to two-thirds partial hepatectomy, and all surviving animals were sacrificed at the end of week 8 (the end of test material treatment period) and quantitative analysis of the numbers and areas of GST-P positive foci in the livers was performed.

At termination (Week 8), all animals, including decedents, were necropsied and the main organs and tissues within the thoracic and abdominal cavities examined. The liver was excised, weighed and fixed in 10% buffered formalin. For rats surviving to termination, slices of liver (about 5 mm) from the right lateral lobe (cranial and caudal aspects) and caudate lobe (caudal aspect) were fixed, routinely processed and embedded in paraffin wax and then stained immunohistochemically for GST-P (ABC method). The numbers and areas of each GST-P positive focus >0.2 mm in diameter and the total area of the liver section (/cm^2) were determined using a colour image processor. Remaining parts of the liver were stored.

There was no treatment-related mortality or clinical signs of toxicity. At 20000 ppm amisulbrom, bodyweight was significantly reduced during the treatment period in both the initiated (by 11%) and uninitiated animals (by 10%). In initiated rats, relative liver weight was slightly but significantly increased in those fed 200 ppm amisulbrom. Both absolute and relative liver weights were significantly increased in initiated animals given 2000 or 20000 ppm. In the uninitiated group fed 20000 ppm amisulbrom, absolute and relative liver weight was also significantly increased compared with the corresponding controls. Similarly, both absolute and relative liver weights of initiated rats fed 500 ppm of PB were significantly increased compared with controls.

Liver immunohistochemical staining showed that the numbers and areas of GST-P positive foci were significantly increased in initiated rats fed 2000 or 20000 ppm amisulbrom. The numbers and areas in those fed 200 ppm were unaffected. Therefore amisulbrom acted as a promoter of liver carcinogenicity at 2000 and 20000 ppm but not at 200 ppm. The numbers and areas of GST-P positive foci in the PB treated group were also significantly increased compared with the controls. PB is a known liver tumour promoter and this finding confirms the sensitivity of the assay for detecting such activity. No GST-P positive foci were found in uninitiated groups or in the controls.

Overall, dietary administration of 2000 and 20000 ppm amisulbrom (equivalent to 120 and 1448 mg/kg bw/d) to DEN-initiated male Fisher 344 rats for 6 weeks exerted promotional tumourigenic activity in the liver. No such activity was demonstrated at 200 ppm.

Enzyme induction in rat liver

Changes in drug-metabolizing enzymes in rat liver following administration of amisulbrom in diet at doses of 200 or 20000 ppm for seven days to male and female rats were investigated (2005c). Overall mean achieved intakes of amisulbrom were 21.12 and 1946.24 mg/kg bw/d in males. Corresponding intakes in females were 20.64 and 2083.02 mg/kg bw/d, respectively. Control groups were untreated and positive control groups received phenobarbital by gavage at a dose of 50 mg/kg bw. After preparing S9 fractions from livers harvested from each group, activities of EROD, PROD, MFCOD, and T-OH were measured in the presence of NADPH.

At 20000 ppm amisulbrom, male bodyweight was significantly decreased on Day 3 and overall bodyweight gain was reduced. Female bodyweight was unaffected at 20000 ppm but food consumption of both sexes was significantly reduced on Day 3. At 20000 ppm amisulbrom and in phenobarbital-treated rats, relative liver weight was significantly increased.

At 200 ppm amisulbrom, there was no effect on the degree of activity on any of the enzymes. At 20000 ppm amisulbrom, there was a significant increase in EROD activity in the S9 fractions (3.17-fold in males; 4.76-fold in females). Activity was also increased in the liver S9 fractions from phenobarbital-treated males (3.11 fold in males). A slight increase was seen in females (1.71-fold). At 20000 ppm amisulbrom, there was a significant increase in PROD activity in the S9 fractions (14.8-fold in males; 13.0-fold in females). Activity was also increased in the S9 fractions from both sexes of phenobarbital-treated rats (39.1-fold in males; 31.9-fold in females). At 20000 ppm amisulbrom, there was a significant increase in MFCOD activity of the S9 fractions of both sexes (3.15-fold in males; 3.50-fold in females). Activity was also increased in the S9 fractions of both male and female rats given phenobarbital sodium salt (5.66-fold in males; 5.88-fold in females). At 20000 ppm amisulbrom, there was a significant increase in T-OH activity in the S9 fractions of both sexes (1.45-fold in males; 3.67-fold in females). Activity was also increased in the S9 fractions of both males and females given phenobarbital sodium salt (2.76-fold in males; 5.75-fold in females).

Overall, administration of amisulbrom at a dose of 20000 ppm (1900-2000 mg/kg bw/d) increased EROD, PROD, MFCOD, and T-OH activities in both male and female rats by 3.2-4.8, 13-15, 3.2-3.5, and 1.5-3.7 fold, respectively, as compared with control groups. There was a marked increase in PROD activity, in particular, suggesting superior induction of CYP2B. This enzyme induction pattern was similar to that seen with phenobarbital. Administration of amisulbrom at a dose of 200 ppm did not significantly affect hepatic drug metabolizing enzyme activities.

Enzyme induction in mouse liver

Changes in drug-metabolizing enzymes in mouse liver following administration of amisulbrom in diet at doses of 100 or 8000 ppm for 7 days to male and female mice were investigated (2005d). Overall mean achieved intakes of amisulbrom were 13.88 and 1079.08 mg/kg bw/d in males. Corresponding intakes in females were 16.94 and 1310.34 mg/kg bw/d, respectively. Control groups were untreated and positive control groups received phenobarbital by gavage at a dose of 50 mg/kg bw. After preparing S9 from livers harvested from each group, activities of EROD, PROD, and T-OH were measured in the presence of NADPH.

Female bodyweight was significantly reduced in mice dosed with phenobarbital. Bodyweight gain was significantly reduced in both sexes of these animals. At 8000 ppm amisulbrom, food consumption of both sexes was significantly reduced on Day 3. Food consumption of females given phenobarbital was also reduced on Day 3. Relative liver weights were significantly increased in both sexes of mice fed 8000 ppm amisulbrom or given phenobarbital.

At 8000 ppm amisulbrom, there was a significant increase in EROD activity in the S9 fractions (1.33-fold in males; 1.52-fold in females). Activity was also increased in the liver S9 fractions from phenobarbital-treated mice (1.84-fold in males; 2.23-fold in females). At 8000 ppm amisulbrom, there was a significant increase in PROD activity in the S9 fractions (1.87-fold in males; 1.64-fold in females). Activity was also increased in the S9 fractions from both sexes of phenobarbital-treated mice (14.2-fold in males; 7.71-fold in females). At 8000 ppm amisulbrom, there was a slight increase in T-OH activity in the S9 fractions of males but not females (1.33-fold in males; 0.95-fold

in females). In phenobarbital-treated mice, activity was significantly increased in the S9 fractions of males but only slightly increased in females (1.64-fold in males; 1.24-fold in females).

Overall, administration of amisulbrom at a dose of 8000 ppm (1000-1300 mg/kg bw/d) significantly increased EROD and PROD activities in both male and female mice as compared with control groups. No significant increase in T-OH activity was noted in male or female mice. Administration of phenobarbital significantly increased EROD and PROD activities by 1.8-2.2 and 7.7-14.2 fold, respectively, while it significantly enhanced T-OH activity by 1.6 fold only in male mice. This study indicates that dietary administration of amisulbrom at 8000 ppm produced a liver enzyme induction pattern (mainly PROD activity) similar to that of phenobarbital administration, although the extent of induction is different between the two substances. Administration of amisulbrom at a dose of 100 ppm did not affect hepatic drug metabolizing enzyme activities.

Replicative DNA synthesis - male rat liver (single dose)

Three groups of 12 male Han Wistar rats were given a single oral dose, by gavage, of 0, 1000 or 2000 mg/kg bw amisulbrom in 0.5% methylcellulose (2005a). Another group of 4 males was similarly dosed with 50 mg/kg bw of phenobarbital in water. Groups of 4 males from each of the amisulbrom and phenobarbital-treated groups were killed 24, 39 or 48 hours after dosing. Two hours prior to termination they were given a single intraperitoneal injection of 10 mg/100 g bw of 5-bromo 2'-deoxyuridine (BrdU) in physiological saline. At termination, each rat was killed and the liver excised, examined macroscopically, weighed and fixed in 10% neutral buffered formalin. After 24 hours in this fixative, each liver was processed and stained immunohistochemically for BrdU. For each liver, the number of hepatocyte nuclei and those that stained positive for BrdU in 15 fields were scored using an image analyser. The induction of replicative DNA synthesis (RDS) in each treated group (percentage of BrdU-positive nuclei) was compared with control values. The criterion for an increased response in treated groups was a value in excess of 3x-standard error of the mean (+3xSEM) of the control group.

At 2000 mg/kg of amisulbrom, both absolute and relative liver weights were significantly increased in the animals killed 39 hours post-dosing. In the phenobarbital-treated group, absolute and relative liver weights were increased 39 and 48 hours post-treatment.

At 1000 mg/kg bw of amisulbrom, the incidence of RDS in hepatocytes was increased at all time points i.e. at 24, 39 and 48 hours post-dosing. At 2000 mg/kg bw, it was increased at both 39 and 48 hours post-treatment. In both groups, values were statistically significant at 39 hours. Therefore, a single oral dose by gavage of 1000 or 2000 mg/kg bw of amisulbrom induced RDS activity in male rats. In the phenobarbital-treated group, the incidence was increased at all time points and was significantly higher at both 39 and 48 hours.

Overall, a single oral administration, by gavage, of 1000 or 2000 mg/kg bw of amisulbrom to male Han Wistar rats increased hepatic replicative DNA synthesis.

Replicative DNA synthesis - female rat liver (single dose)

Three groups of 12 female Han Wistar rats were given a single oral dose, by gavage, of 0, 1000 or 2000 mg/kg bw amisulbrom in 0.5% methylcellulose (2005b). Investigations were the same as reported for the previous study in male rats.

In the phenobarbital-treated group, relative liver weight was significantly increased 48 hours post-dosing. At 1000 mg/kg bw of amisulbrom, the incidence of RDS in hepatocytes was increased at all time points i.e. at 24, 39 and 48 hours post-dosing. At 2000 mg/kg bw, it was increased 39 and 48 hours post-treatment, with values being statistically significant at 48 hours post-dosing. Therefore, a single oral dose by gavage of 1000 or 2000 mg/kg bw/d amilsulbrom induced RDS activity in female rats. In the phenobarbital-treated group, the incidence was increased at all time points.

Overall, a single oral administration, by gavage, of 1000 or 2000 mg/kg bw of amisulbrom to female Han Wistar rats increased hepatic replicative DNA synthesis.

Replicative DNA synthesis - male rat liver (repeat dose)

Four groups of 8 male Han Wistar rats were given dietary concentrations of 0, 200, 2000 or 10000 ppm amisulbrom (equivalent to 15, 136 and 572 mg/kg bw/d) for 7 days (2005c). Another group of 8 males was given 3 or 7 daily oral doses, by gavage, of 50 mg/kg bw of phenobarbital in water. Groups of 4 males from each of the amisulbrom and phenobarbital-treated groups were killed after 3 or 7 days of dosing. Two hours prior to termination they were given a single intraperitoneal injection of 10 mg/100 g bw of 5-bromo 2'-deoxyuridine (BrdU) in physiological saline. At termination, each rat was killed and the liver excised, examined macroscopically, weighed and fixed in 10% neutral buffered formalin. After 24 hours in this fixative, each liver was processed and stained immunohistochemically for BrdU. For each liver, the number of hepatocyte nuclei and those that stained positive for BrdU in 15 fields were scored using an image analyser. The induction of replicative DNA synthesis (RDS) in each treated group (percentage of BrdU-positive nuclei) was compared with control values. The criterion for an increased response in treated groups was a value in excess of 3 x standard error of the mean (+3 x Standard Error of the Mean) of the control group.

There were no mortalities or clinical signs of toxicity. At 10000 ppm amisulbrom, bodyweight gain was significantly reduced by Day 3. In the phenobarbital-treated group, absolute and relative liver weights were significantly increased on Day 3 whilst relative liver weight was increased on Day 7. At 2000 and 10000 ppm amisulbrom, the incidence of RDS was increased on Day 3.

Therefore, repeated (for 7 days) dietary treatment of amisulbrom from a dose of 136 mg/kg bw/d induced RDS activity in male rats, with a transient peak on Day 3.

Replicative DNA synthesis - female rat liver (repeat dose)

Four groups of 8 female Han Wistar rats were given dietary concentrations of 0, 200, 2000 or 10000 ppm amisulbrom (equivalent to 17, 150 and 656 mg/kg bw/d) for 7 days (2005d). Another group of 8 females was given 3 or 7 daily oral doses, by gavage, of 50 mg/kg bw of phenobarbital in water. Investigations were the same as reported for the previous study in male rats.

There were no mortalities or clinical signs of toxicity. At 10000 ppm amisulbrom, there was a transient reduction in bodyweight on Day 3. In the phenobarbital-treated group, absolute and bodyweight-relative liver weights were significantly increased on Day 3 whilst bodyweight-relative weight was increased on Day 7. At 2000 and 10000 ppm amisulbrom, the incidence of RDS was increased on Day 3.

Therefore dietary administration of amisulbrom from a dose of 150 mg/kg bw/d to female Han Wistar rats for 1 week increased hepatic replicative DNA synthesis with a transient peak on Day 3.

Replicative DNA synthesis - male mouse liver (repeat dose)

Three groups of 8 male CD-1 mice were given dietary concentrations of 0, 100 or 8000 ppm amisulbrom (equivalent to 15 and 1021 mg/kg bw/d) for 7 days (2005e). Another group of 8 males was given 3 or 7 daily oral doses, by gavage, of 50 mg/kg bw of phenobarbital in water. Four males from each of the amisulbrom and phenobarbital-treated groups were killed after 3 or 7 days of dosing. Two hours prior to termination they were given a single intraperitoneal injection of 10 mg/100 g bw of 5-bromo 2'-deoxyuridine (BrdU) in physiological saline. At termination, each mouse was killed and the liver excised, examined macroscopically, weighed and fixed in 10% neutral buffered formalin. After 24 hours in this fixative, each liver was processed and stained immunohistochemically for BrdU. For each liver, the number of hepatocyte nuclei and those that stained positive for BrdU in 15 fields were scored using an image analyser. The induction of replicative DNA synthesis (RDS) in each treated group (percentage of BrdU-positive nuclei) was compared with control values. The criterion for an increased response in treated groups was a value in excess of 3 x standard error of the mean (+3xSEM) of the control group.

There were no mortalities or clinical signs of toxicity. In the phenobarbital-treated group, bodyweight was reduced on Days 3 and 7. At 8000 ppm amisulbrom, food consumption was significantly reduced on Day 3. At 8000 ppm amisulbrom, the incidence of RDS was increased on Days 3 and 7, with values on Day 7 being statistically significantly elevated. In the phenobarbital-treated group, there were increases on Days 3 and 7 but they were not statistically significant.

Overall, dietary administration of 1021 mg/kg bw/d amisulbrom to male CD-1 mice for 1 week increased hepatic replicative DNA synthesis.

Replicative DNA synthesis - female mouse liver (repeat dose)

Three groups of 8 female CD-1 mice were given dietary concentrations of 0, 100 or 8000 ppm amisulbrom (equivalent to 17 and 1234 mg/kg bw/d) for 7 days (2005f). Another group of 8 females was given 3 or 7 daily oral doses, by gavage, of 50 mg/kg bw of phenobarbital in water. Investigations were the same as reported for the previous study in male mice.

In the phenobarbital-treated group, bodyweight was reduced on Days 3 and 7. At 8000 ppm amisulbrom, food consumption was significantly reduced on Day 3. The increased incidence of RDS seen on Day 7 at 8000 ppm amisulbrom was due to a single female. In the phenobarbital-treated group, there were increases on Days 3 and 7.

Overall, no conclusions can be drawn on whether or not dietary administration of 1234 mg/kg bw/d amisulbrom to female CD-1 mice for 1 week induced replicative DNA synthesis in the liver.

Conclusion of mechanistic studies

Liver

The available mechanistic studies show that amisulbrom is not genotoxic in rat and mouse liver. Amisulbrom has promotional tumourigenic activity in the liver of male Fisher 344 rats. In addition, amisulbrom at high doses causes liver enzyme induction in males and females of Han Wistar rats and CD-1 mice. The pattern of enzyme induction is similar to that caused by phenobarbital, with a marked increase in PROD activity (CYP2B). Furthermore, amisulbrom, similarly to phenobarbital,

produces increases in replicative DNA synthesis in the liver of male and female Han Wistar rats and in the liver of male CD-1 mice, with a transient peak on Day 3.

Stomach/forestomach

A comet assay in the rat stomach/forestomach is available (see mutagenicity section). Due to the low sensitivity of the test in these tissues, no conclusions could be drawn about the genotoxic potential of amisulbrom in the stomach and forestomach of female Han Wistar rats.

4.10.4 Summary and discussion of carcinogenicity

The carcinogenic potential of amisulbrom has been investigated by the oral route in Han Wistar rats and CD-1 mice. Liver tumours were increased in rats and mice and forestomach tumours were increased in female rats.

Liver tumours

In the rat study, there was a statistically significant increase in the incidence of liver adenoma at 496/697 (m/f) and 1008/1436 (m/f) mg/kg bw/d in males (20% and 26% respectively vs 0% in controls) and females (48% and 56% respectively vs 0% in controls). In females, the increase in liver adenoma was observed at dose levels causing excessive toxicity (increased mortality, clinical signs of toxicity and 53% reduction in body weight gain at 1436 mg/kg bw/d; increased mortality, clinical signs of toxicity and 47% reduction in body weight gain at 697 mg/kg bw/d). Therefore, the increase in liver adenoma observed in females in this study is highly confounded by the presence of excessive toxicity and it is therefore considered to be of questionable toxicological significance for cancer hazard assessment. In males, the increase in liver adenoma occurred at dose levels causing moderate toxicity (clinical signs of toxicity and 22% reduction in body weight gain at 1008 mg/kg bw/d; clinical signs of toxicity and 17% reduction in body weight gain at 496 mg/kg bw/d). Therefore, the carcinogenic response seen in males was not compromised by excessive toxicity. Overall, in the rat, there was a reliable and biologically significant increase in benign liver tumours in males from a dose of 496 mg/kg bw/d.

In the mouse study, there was a slight but statistically significant increase (above the laboratory historical control ranges) in the incidence of liver adenoma in males from a dose of 98 mg/kg bw/d (34%, 46% and 36% at 98, 494 and 1035 mg/kg bw/d vs 16% in controls). The increase in liver adenoma at the top dose occurred in the presence of excessive toxicity (37% reduction in body weight gain). This reduces the toxicological significance of the carcinogenic response seen at the top dose of 1035 mg/kg bw/d. The increase in liver adenoma observed at 98 mg/kg bw/d (34%) was small when considering that the concurrent controls had already a 16% incidence of these tumours and that the laboratory historical controls presented up to a 31% incidence of this tumour type. However, at the dose of 494 mg/kg bw/d, there was a clear increase (46%) above concurrent controls (16%) and historical control ranges (31%) in liver adenoma, which was not confounded by the presence of excessive toxicity (23% reduction in body weight gain). Overall, therefore, in the mouse, there was a biologically significant but modest carcinogenic response in males (benign liver tumours) at a dose of 494 mg/kg bw/d.

Extensive mechanistic data have shown that the most likely MoA for these liver tumours in rats and mice is via enzyme induction, hypertrophy and increased cell proliferation. This MoA has been shown to be similar to that via which phenobarbital causes liver tumours in rodents.

It is now well accepted that there are uncertainties in this MoA, in particular in relation to its relevance/non-relevance to humans. There is insufficient evidence on which key event in the MoA does not occur in humans. In addition, the epidemiological data on the relationship between liver cancer and phenobarbital exposure in epileptic patients are not conclusive.

Furthermore, other MoAs might be responsible for the observed liver tumours. It is noted that there were signs of liver toxicity in the 2-year rat study at the same dose levels at which the tumours occurred (increased γGTP, bile duct hyperplasia, hepatocyte vacuolation, cystic degeneration and decreased basophilic foci) which may have contributed to the induction of the tumours.

Overall, therefore, although reassuring, these mechanistic data are insufficient to exclude the relevance to humans of the liver tumours seen in rats and mice with amisulbrom.

Forestomach tumours

In the rat study only, there was a small increase in the incidence of papilloma of the forestomach in females at 697 (2% vs 0% in controls) and 1436 mg/kg bw/d (4% vs 0% in controls) and an increase in the incidence of carcinoma of the forestomach in the 1436 mg/kg bw/d females (2% vs 0% in controls). These tumours were considered to be due to chronic inflammation resulting from a local irritant effect. It is well-established that the rat forestomach acts as storage reservoir releasing undigested food into the glandular stomach in response to energy demand. Hence, the rat forestomach mucosa is exposed to the test material in the diet for far longer periods than elsewhere in the gastrointestinal (GI) tract. However, the human stomach is not a storage organ. Overall, therefore, the forestomach tumours associated with amisulbrom exposure in female rats are not relevant to humans because in humans persistently high local concentrations of amisulbrom in the GI tract cannot be achieved.

4.10.5 Comparison with criteria

The carcinogenic potential of amisulbrom has been investigated by the oral route in Han Wistar rats and CD-1 mice. Liver tumours were increased in rats and mice and forestomach tumours were increased in female rats.

Forestomach tumours
As explained above, the forestomach tumours associated with amisulbrom exposure in female rats are considered to be not relevant to humans because persistently high local concentrations of amisulbrom in the GI tract cannot be achieved in humans. Therefore, in accordance with the criteria, no classification is required on the basis of these forestomach tumour findings.

Liver tumours
With regard to the liver tumours, it is concluded that in the rat, there was a reliable and biologically significant increase in benign liver tumours in males from a dose of 496 mg/kg bw/d. It is also concluded that in the mouse, there was a biologically significant but modest carcinogenic response in males (benign liver tumours) at a dose of 494 mg/kg bw/d.

With regard to these liver tumours, extensive cancer mechanistic data have been provided. These data, although reassuring, are insufficient to exclude the relevance to humans of these tumours.

When comparing the liver tumour findings with the criteria, the following conclusions can be drawn:

Category 1A (known to have carcinogenic potential for humans) is not appropriate as *there is no human evidence establishing a causal relationship* between exposure to amisulbrom and the development of liver (and/or other) cancer.

Category 1B (presumed to have carcinogenic potential for humans) is also not appropriate as *there is not sufficient evidence of carcinogenicity in experimental animals*. A causal relationship between treatment with amisulbrom and an increased incidence of malignant tumours or of an appropriate combination of benign and malignant tumours in two or more species has not been established. The liver tumours noted with amisulbrom were only benign and, although seen in two species (rats and mice), they occurred reliably in one sex only (males). Therefore, the benign nature and the questionable significance in female cast serious doubts on amisulbrom being considered a presumed human liver carcinogen.

Category 2 (suspected to have carcinogenic potential for humans) might be appropriate because it could be concluded that *there is limited evidence of carcinogenicity in experimental animals* (amisulbrom increases the incidence of benign liver neoplasms in male rats and mice). However, consideration of additional factors (those described in the criteria) leads to a decrease of the level of concern such that "*no-classification"* might also be appropriate.

A detailed consideration of these additional factors and their impact on the level of concern is presented below:

a) Tumour type and background incidence – amisulbrom only causes liver adenomas (benign neoplasms) in rats and mice, with mice having already a high background incidence of this tumour type, as shown by the concurrent controls and laboratory historical control data. (↓ concern).

b) Multi-site responses – only the liver was affected. No other organs showed a relevant carcinogenic response after treatment with amisulbrom. (↓ concern).

c) Progression of lesions to malignancy – amisulbrom only causes liver benign adenomas which did not progress to malignant lesions. (↓ concern).

d) Reduced tumour latency – tumour latency was not investigated. (neutral).

e) Whether responses are in single or both sexes –a reliable, biologically significant carcinogenic response occurred only in males. (↓ concern).

f) Whether responses are in a single species or several species - although liver adenomas were seen in two species (rats and mice), the response was sex-specific in both species and rather modest above background in the mouse. (neutral).

g) Structural similarity to a substance(s) for which there is good evidence of carcinogenicity – amisulbrom has no structural similarity to substance(s) for which there is good evidence of carcinogenicity. (↓ concern).

h) Routes of exposure – with amisulbrom, tumours were seen by the oral route. Other routes were not investigated. The oral route is likely to have maximized the potential to detect tumours because by this route absorption of amisulbrom is significant. (neutral).

i) Comparison of ADME between test animals and humans – a comparison is not possible for amisulbrom. ADME behaviour of amisulbrom in test animals and humans is assumed to be similar. (neutral).

j) The possibility of a confounding effect of excessive toxicity at test doses – in the rat, the increase in benign liver tumours in females occurred in the presence of excessive toxicity. The biological significance of this response has already been questioned and dismissed. (neutral).

k) Modes of action and its relevance for humans - there are extensive liver cancer mechanistic data for amisulbrom which, although reassuring, are insufficient to exclude the relevance to humans of the observed liver tumours. (neutral).

Overall, amisulbrom causes relevant, reliable and biologically significant increases in benign liver tumours in male rats and mice. These findings are sufficient to justify classification of amisulbrom in Category 2. However, when taking into account the benign nature of the tumours in both species, the confounded results in females and the modest increase above background in the mouse, the level of concern is decreased. Therefore, it can be concluded that the case for Category 2 classification is weak and that the evidence is borderline between Category 2 and no classification. The dossier submitter deems that Category 2 classification is more appropriate than no classification because although some factors decrease the level of concern, other elements (response in two species and uncertainties on whether the excessive toxicity seen in female rats is sufficient to disregard the biological significance of the female rat carcinogenic response) act in the opposite direction.

4.10.6 Conclusions on classification and labelling

Carc Cat 2; H351 – Suspected of causing cancer

4.11 Toxicity for reproduction

4.11.1 Effects on fertility

The potential effects of amisulbrom on fertility and reproductive performance have been investigated in a guideline multigeneration study in the rat.

Table 22: Summary table of relevant reproductive toxicity studies - Fertility

Method	Dose levels	Observations and remarks (effects of major toxicological significance)
2-generation reproductive toxicity study OECD 416 GLP Dietary administration Han Wistar rats form Harlan (UK) colony (28/sex/group) Amisulbrom 99.1% pure 2005 (DAR B.6.6.1)	0, 120, 600, 3000 and 15000 ppm (0, 9.8, 48.5, 240 and 1200 mg/kg bw/d in males; 0, 10.5, 53, 261, 1291 mg/kg bw/d in females)	**15000 ppm (1200/1291 mg/kg bw/d in M/F):** <u>*Parental toxicity*</u> 2 F0 dams killed for humane reasons (after weaning) due to poor physical condition; ↓bw (7% M; 9% F)* at mating in F0 adults; ↓pre-mating bwg (13% M; 19% F)* in F0 adults; ↓bw (11% F at GD0; 12% F at GD20; 14% F at LD1; 10% F at LD21)* in F0 females; ↓gestation bwg (16% F)* in F0 females; ↓fc (6% M; 9% F)* during pre-mating in F0 adults; ↓fc (9% F)* during gestation in F0 females; ↓fc (11% F) during lactation in F0 females; ↑abs liver wt (9% M; 17% F)* in F0 adults; ↓abs ovary wt (18% F)* in F0 females; ↓abs uterus wt (24% F)* in F0 females; ↓bw (42% M; 38% F)* at week 0 in F1 adults; ↓bw (30% M; 15% F)* at mating in F1 adults; ↓pre-mating bwg (27% M; 4% F)* in F1 adults; ↓fc (24% M; 14% F)* during pre-mating in F1 adults; ↑rel liver wt (32% M; 6% F)* in F1 adults; ↓abs ovary wt (75% F)* in F1 females; ↓abs uterus wt (26% F)* in F1 females; <u>*Fertility and Reproductive performance*</u> No treatment-related effects in F0 generation including sperm and histopathology of repro organs; <u>F1 adults</u>: ↓mating index (63 vs 100 in controls)*; ↓conception rate (13 vs 100 in controls)*; ↓fertility index (8 vs 100 in controls)*; extended oestrus (14 F vs 0 in controls)*; atrophic ovaries with lower follicle count (24 F vs 0 in controls)*; uterus metaplasia (8 F vs 0 in controls)*; reduced thickness of myometrium (20 F vs 0 in controls)*; vacuolation of pituitary (23 F vs 0 in controls)* <u>*Offspring toxicity*</u> ↓ pup wt (from 5% on day 1 to 31% on day 14, 40% on day 25)* in F1 females; ↓ pup wt (from 13% on day 4 to 41% on day 25)* in F1 males; ↓ pup bwg (44% M; 42% F)* during day 1-25 in F1 offspring;

		Delayed sexual maturation (46.6 d vs 34.1 d in controls)* in F1 female offspring; Delayed sexual maturation (52.8 d vs 45.9 d in controls)* in F1 male offspring; ↑rel liver wt (43% M; 58% F)* in F1 offspring; ↓rel thymus wt (31% M; 25% F)* in F1 offspring; ↓rel uterus wt (33% F)* in F1 offspring; 3 F1 pup from a single litter (dying before scheduled termination) with cleft palate out of 20 dead pups in 7 litters vs 0 in controls out of 4 dead pups in 3 litters; F2 offspring: not investigated due to the small number of litters; **3000 ppm (240/261 mg/kg bw/d in M/F):** *Parental toxicity* ↓bw (7% F at GD20; 7% F at LD1;)* in F0 females; ↓gestation bwg (12% F)* in F0 females; ↓fc (9% F)* during gestation in F0 females; ↑rel liver wt (11% M; 13% F)* in F0 adults; ↓bw (13% M; 14% F)* at week 0 in F1 adults; ↓bw (12% M; 10% F)* at mating in F1 adults; ↓pre-mating bwg (12% M; 7% F)* in F1 adults; ↓gestation bwg (10% F)* in F1 females; ↓fc (8% F)* during gestation in F1 females; ↓fc (13% F) during lactation in F1 females; ↑rel liver wt (18% M; 21% F)* in F1 adults; atrophic ovaries (4 F vs 0 in controls) and uterus metaplasia (2 F vs 0 in controls)* in F1 adults; *Fertility and Reproductive performance* No treatment-related effects in both generations; *Offspring toxicity* ↓ pup wt (from 7% on day 7 to 14% on day 25)* in F1 females; ↓ pup wt (from 9% on day 7 to 15% on day 25)* in F1 males; ↓ pup bwg (17% M; 15% F)* during day 1-25 in F1 offspring; Delayed sexual maturation (38.2 d vs 34.1 d in controls)* in F1 female offspring; ↑rel liver wt (15% M; 20% F)* in F1 offspring; ↓rel thymus wt (12% M; 9% F)* in F1 offspring; ↓rel uterus wt (30% F) in F1 offspring; 1 F1 pup (dying before scheduled termination) with cleft palate out of 6 dead pups in 6 litters vs 0 in controls out of 4 dead pups in 3 litters; ↓ pup wt (from 12% on day 7 to 24% on day 21 and to 22% on day 25)* in F2 females; ↓ pup wt (from 23% on day 14 to 27% on day 25)* in F2 males; ↓ pup bwg (30% M; 25% F)* on day 1-25 in F2 offspring;

		Delayed air righting reflex (17.5 d vs 17 d in controls)* in F2 offspring; ↑rel liver wt (15% M; 17% F)* in F2 offspring; ↓rel thymus wt (25% M; 25% F)* in F2 offspring; ↓rel uterus wt (29% F)* in F2 offspring; **600 ppm (48.5/53 mg/kg bw/d in M/F):** *Parental toxicity* No treatment-related effects in F0 or F1 adults; *Fertility and Reproductive performance* No treatment-related effects in both generations; *Offspring toxicity* ↓ pup bwg (8% M)* during day 1-14 in F1 males; **120 ppm (9.8/10.5 mg/kg bw/d in M/F):** No treatment-related effects; **NOAEL parental toxicity = 600 ppm (48.5/53 mg/kg bw/d in M/F)$** **NOAEL reproductive toxicity = 3000 ppm (240/261 mg/kg bw/d in M/F)$** **NOAEL offspring toxicity = 120 ppm (9.8/10.5 mg/kg bw/d in M/F)$**

* Statistically significant; $ = As given in the DAR

↓ = decrease ↑ = increase

4.11.1.1 Non-human information

In a guideline 2-generation study, Han Wistar rats (28/sex/group) from a Harlan (UK) colony were administered amisulbrom in the diet at concentrations of 0, 120, 600, 3000 or 15000 ppm from approximately 6 weeks of age, for ten weeks prior to mating and throughout mating (1:1), gestation and lactation of the resulting (F1) litters (2005). Selected F1 offspring (24/sex/group) were similarly administered amisulbrom in the diet for ten weeks prior to mating and throughout mating, gestation and lactation of the resulting F2 litters. F1 and F2 litters were adjusted to eight pups (4/sex where possible) at Day 4 *post partum*; the non-selected F1 pups and all F2 pups were terminated at Day 25 *post partum*.

F0 generation

Two F0 15000 ppm dams were killed for humane reasons following weaning of their litters due to poor physical condition. Mean bodyweights at pairing of both sexes at 15000 ppm were significantly lower than controls as a result of significantly reduced weight gain and food consumption during the pre-mating period. The bodyweights of 15000 ppm females remained significantly lower than controls throughout gestation and lactation; weight gain by this group was significantly reduced during gestation, but was comparable to controls during lactation. Mean bodyweights of 3000 ppm females were also significantly lower during late gestation and early lactation; weight gain was significantly lower during gestation but was significantly higher than controls during lactation. Absolute liver weights were significantly higher at the top dose level in both sexes; relative liver weights were significantly higher at ≥3000 ppm. Relative ovary weight

was slightly (but statistically significantly) lower at the top dose level; relative uterus weight was slightly (but statistically significantly) lower in this group. Gross necropsy and histopathology did not reveal any treatment-related findings. Sperm analysis in males did not reveal any treatment-related effects on morphology or motility.

Fertility was comparable in males and females of all groups; pre-coital interval was similar in all groups. Oestrus cyclicity was unaffected by treatment, with the majority of females in all groups showing evidence of regular cycles.

F1 generation

In F1 offspring, litter size and pup sex ratios were comparable in all groups at all time points. Live birth and survival indices were unaffected by treatment. Mean bodyweights of male pups at the top dose level were slightly lower than controls at birth and were significantly lower from Day 4; mean weights of male pups at 3000 ppm were significantly lower from Day 7. Bodyweight gains in males were significantly lower at ≥600 ppm. Mean bodyweights of female pups at the top dose level were lower than controls from birth; weights of 3000 ppm female pups were lower from Day 7. Weight gains of female pups were also significantly lower in these groups.

Sexual maturation of F1 offspring was significantly delayed in males at 15000 ppm and in females at ≥3000 ppm. The effects on sexual maturation in both sexes in these groups were likely to be secondary to the bodyweight effects, rather than being a direct effect of amisulbrom.

In F1 offspring, relative thymus weights were significantly lower in both sexes at ≥3000 ppm; relative uterus weight was significantly lower in females at the top dose level and slightly lower at 3000 ppm. Relative liver weights were significantly increased in males at ≥3000 ppm.

Necropsy of F1 pups dying before scheduled termination revealed incidences of cleft palate at 3000 ppm (one pup) and 15000 ppm (in three pups from a single litter). Laboratory historical control data from five 2-generation studies conducted between 2001 and 2006 show that in control F1 pups found dead before scheduled sacrifice, the incidence of cleft palate ranged from 0 – 3 pups. These data indicate that the observation of cleft palate in this study was most likely of genetic aetiology rather than related to treatment with amisulbrom. At scheduled termination, a large number of 'small' pups were noted at 15000 ppm.

In F1 adults, mean bodyweights at pairing were significantly lower in both sexes at the top dose level as a consequence of the significantly lower initial weight (in both sexes) and the significantly lower weight gain (by males) during the pre-mating period. Pre-mating bodyweights were also significantly lower at 3000 ppm due to lower initial weights and reduced weight gain in both sexes. Mean bodyweights of 3000 ppm females were significantly lower throughout gestation and lactation; weight gain in this group was slightly (but significantly) lower during gestation.

The mating performance and fertility of F1 animals was markedly impaired at 15000 ppm; only fifteen matings occurred and only two of these matings resulted in viable litters. As a result of the poor mating performance in this group, all of the 15000 ppm males were re-mated with untreated (naïve control) females: all of the males subsequently mated successfully to produce litters. Oestrus cycle analysis prior to mating revealed a significantly increased proportion of 15000 ppm F1 females with extended oestrus; four to five weeks following mating, extended oestrus was apparent in nearly all females in this group.

In F1 adults, relative liver weight was significantly increased in both sexes at 3000 and 15000 ppm, although a dose-response relationship was not apparent in females. Absolute and relative ovary

weights were markedly lower in 15000 ppm females. Mean absolute and relative uterus weights were significantly lower in 15000 ppm females.

Necropsy of the F1 adults revealed a high proportion of 15000 ppm females with treatment-related effects on the ovaries and uterus. The ovaries of females in this group were small, atrophic and cystic; with a markedly lower follicle count. Ovarian atrophy was also observed in a small number (4 vs 0 in controls) of 3000 ppm females. The incidences of uterine squamous metaplasia and reduced thickness of the myometrium were significantly higher at 15000 ppm. Uterine squamous metaplasia was also observed in a small number (2 vs 0 in controls) of 3000 ppm females. Vacuolation of the pituitary *pars distalis* was observed in a high proportion of 15000 ppm females; this finding was considered to represent 'castration cells' associated with reduced ovarian function. No treatment-related findings were apparent in males. The ovarian and uterine effects seen at 3000 ppm occurred in a small number of females from litters with relatively poor body weight performance before or after weaning. These responses did not result in impaired mating performance and fertility and hence they were not considered of relevance to the establishment of the reproductive toxicity NOAEL.

F2 generation

The offspring of the F1 parents administered 15000 ppm amisulbrom were not investigated further due to the small numbers of litters; the low fertility in this group resulted in only two live litters, one of which consisted of only one pup which was subsequently cannibalised. The remaining litter survived to weaning.

In F2 offspring, litter sizes and pup survival indices were unaffected by treatment at ≤3000 ppm. Mean pup weights at birth were slightly (but not significantly) lower at 3000 ppm, however weights were lower from Day 7 (females) or Day 14 (males) as a result of significantly lower weight gain.

Development of the air righting reflex was slightly (but significantly) delayed in 3000 ppm F2 pups; however this effect may have been secondary to the lower bodyweight in this group. The development of other reflexes was not affected by treatment.

In F2 offspring, relative liver weight was significantly increased in 3000 ppm females; absolute and relative thymus weights were significantly lower in both sexes at 3000 ppm. Absolute and relative uterus weights were significantly lower in females in this group. Gross necropsy of pups did not reveal any treatment-related findings with the exception of a marginal increase in the number of 'small' pups. In contrast to the F1 litters, no incidences of cleft palate were seen.

Overall, in this multigeneration study, administration of amisulbrom at the top dose level of 15000 ppm (1200-1300 mg/kg bw/d) had a clear and marked effect on reproduction in F1 females; a reduction in fertility at this dose level was shown to be female-mediated. Reduced fertility was associated with severely impaired bodyweight development (from 10% up to 40% reduction in body weight during gestation and lactation of F0 females and weaning and sexual maturation of F1 pups), reduced ovarian weight and function and associated histopathology.

4.11.1.2 Human information

No data are available.

4.11.2 Developmental toxicity

The developmental toxicity potential of amisulbrom has been investigated in rats (1 range-finding study and 2 guideline studies) and rabbits (1 guideline study).

Table 23: Summary table of relevant reproductive toxicity studies - Development

Method	Dose levels	Observations and remarks (effects of major toxicological significance)
Developmental toxicity range-finding study Mated female SD rats (7/group) Gavage administration on GD 6-19 Amisulbrom 99% pure 2000 (DAR B.6.6.2(a))	0, 100, 300, 1000 mg/kg bw/d	**1000 mg/kg bw/d:** *Maternal effects* ↓bwg (6%); *Foetal effects* None; **100 and 300 mg/kg bw/d:** No maternal or foetal effects were noted;
Developmental toxicity study Mated female Han Wistar rats from Harlan (UK) colony (22/group) Gavage administration on GD 6-19 OECD 414 GLP Amisulbrom 99.1% pure 2004(a) (DAR B.6.6.2(b))	0, 100, 300, 1000 mg/kg bw/d	**1000 mg/kg bw/d:** *Maternal effects* No treatment-related effects; *Foetal effects* Cleft palate in 12 foetuses in 2 litters (6 per litter) vs 0 in controls associated with other malformations (chondrodystrophy syndrome); **100 and 300 mg/kg bw/d:** No maternal or foetal effects were noted; **NOAEL maternal toxicity = 1000 mg/kg bw/d$^{\$}$** **NOAEL developmental toxicity = 300 mg/kg bw/d$^{\$}$**
Developmental toxicity study Mated female Han Wistar rats from CLEA (Japan) colony	0, 1500 mg/kg bw/d	**1500 mg/kg bw/d:** *Maternal effects* ↓bwg (7%)* during GD 6-20; ↓fc (13)* on GD 20; *Foetal effects*

(2/group) Gavage administration on GD 6-19 OECD 414 Amisulbrom 99.1% pure 2003 (DAR B.6.6.2(c))		None; **LOAEL maternal toxicity = 1500 mg/kg bw/d$** **NOAEL developmental toxicity = 1500 mg/kg bw/d$**
Developmental toxicity study Mated female NZW rabbits (24/group) Gavage administration on GD 6-28 OECD 414 GLP Amilsubrom 99.1% pure 2004(b) (DAR B.6.6.3)	0, 30, 100, 300 mg/kg bw/d	**300 mg/kg bw/d:** *Maternal effects* 1 abortion; ↓terminal bw (4%); ↓bwg (88%)* during GD 6-29; ↓fc (23-53%)* during dosing; *Foetal effects* None; **100 mg/kg bw/d:** *Maternal effects* ↓bwg (60-72%)* during GD 6-20; ↓fc (23%)* during GD 6-7; *Foetal effects* None; **30 mg/kg bw/d:** No maternal or foetal effects were noted; **NOAEL maternal toxicity = 30 mg/kg bw/d$** **NOAEL developmental toxicity = 300 mg/kg bw/d$**

*Statistically significant; $ = As given in the DAR;

↓ = decrease ↑ = increase

4.11.2.1 Non-human information

Rat

In a developmental toxicity range-finding study, mated female Crj:CD(SD)IGS rats (7/group) were gavaged with the test material (in 0.5% methylcellulose) at dose levels of 0, 100, 300 or 1000 mg/kg bw/d on Days 6-19 of gestation (2000).

No deaths occurred and no signs of toxicity were observed during the study period. Total weight gain at the top dose level was marginally (93.9% of controls) but not statistically significantly reduced. Haematology and clinical chemistry parameters were unaffected by treatment. Gross necropsy did not reveal any treatment-related findings. Litter parameters were unaffected by treatment. No treatment-related effects were noted on foetal external, visceral or skeletal parameters.

Overall, there were no signs of developmental toxicity up to the limit dose of 1000 mg/kg bw/d in this range-finding study in SD rats.

In a guideline developmental toxicity study, mated female Han Wistar rats (22/group) from a Harlan (UK) colony were gavaged with amisulbrom (suspended in 0.5% aqueous methylcellulose) on Days 6-19 of gestation at dose levels of 0, 100, 300 or 1000 mg/kg bw (2004a). Animals were terminated on Day 20 and the uterine contents investigated.

No deaths occurred during the study period. Mean bodyweight, weight gains and food consumption were unaffected by treatment. Litter parameters were unaffected by treatment; the slightly higher pre-implantation loss seen at 300 mg/kg bw/d but not at 1000 mg/kg bw/d is not considered to be attributable to the test material. The incidences of skeletal anomalies and visceral findings were unaffected by treatment.

A high incidence of cleft palate was seen in foetuses at the top dose level; similar findings were not seen in any other group. A total of 12 foetuses were affected in 2 litters (6 per litter); cleft palate was associated in the same animals with a number of other malformations (misshapen/kinked nasal septum, shortened lower jaw and constricted spinal cord at visceral examinations in 6 foetuses from 2 litters; and shortened upper/lower jaw, cervical kyphosis/lumbar lordosis, thickened/kinked ribs, distorted ribcage, bent scapula, ulna, radius & misshapen clavicle at skeletal examinations in 6 foetuses from 2 litters). This pattern of malformations constitutes the *chondrodystrophy syndrome*. The study authors noted that this strain of rats has a high background incidence of chondrodystrophy with cleft palate. They also noted that one stock male who sired a litter with cleft palate in this study was associated with cleft palate in a control litter in a subsequent study and another stock male who sired a normal litter in this study sired litters with cleft palate in two separate studies. They concluded that this evidence points towards a genetic aetiology of the finding.

Chondrodystrophy with cleft palate occurs with a relatively high frequency in Han Wistar rats from the Harlan (UK) colony (Wilby et al., 2011). In 25 developmental toxicity studies performed with this strain/colony of rat at HLS (UK) between 2002 and 2008, incidences of cleft palate/chondrodystrophy were seen in single litters in the control groups of 9 studies (with incidences of 1-5 foetuses/litter). Although the foetal and litter incidences of cleft palate in this study (6 pups/litter) just exceeded the laboratory historical control range, the pattern of the findings (seen in just 2 litters) and the association with other defects (chondrodystrophy syndrome) suggest a spontaneous (genetic) aetiology rather than a relationship to treatment with amisulbrom.

Overall, cleft palate was observed at the limit dose in this study in Han Wistar rats in the absence of maternal toxicity. Although the incidence of cleft palate in this study just exceeds the laboratory historical control range, the pattern of the finding (noted in just 2 litters) and the association with other defects (chondrodystrophy syndrome) suggest a spontaneous (genetic) aetiology rather than a relationship to treatment with amisulbrom.

In another guideline developmental toxicity study, mated female Han Wistar rats (20/group) from the CLEA (Japan) colony were gavaged with amisulbrom (suspended in 0.5% aqueous methylcellulose) on Days 6-19 of gestation at dose levels of 0 or 1500 mg/kg bw/d (2003). Animals were terminated on Day 20 and the uterine contents investigated.

No deaths occurred and no signs of toxicity were observed during the study period. The mean terminal bodyweight of treated females was slightly lower than controls due to a slightly (but significantly) reduced weight gain (by 7%) during the dosing period. Food consumption in treated females was also significantly lower (by 13%) than controls. Litter parameters were unaffected by treatment.

Litter size and foetal weight were unaffected by treatment. No malformations were observed in any foetus (including controls) in this study.

Overall, no developmental toxicity was observed in this study in Han Wistar rats from the CLEA (Japan) colony up to the high dose of 1500 mg/kg bw/d at which some maternal toxicity occurred. Cleft palate occurs very rarely in Han Wistar rats from this Japanese colony. The Applicant argues that a high frequency of cleft palate/chondrodystrophy is typical of the Harlan (UK) colony of Han Wistar rats, but that cleft palate/chondrodystrophy is not seen in the Japanese colony. This negative study therefore gives further support to the hypothesis of the genetic nature of the cleft palate/chondrodystrophy findings seen in the Harlan (UK) colony of Han Wistar rats in the other (2004a) study.

Rabbit

In a guideline developmental toxicity study, mated female New Zealand White rabbits (24/group) were gavaged with amisulbrom (suspended in 0.5% aqueous methylcellulose) on Days 6-28 of gestation at dose levels of 0, 30, 100 or 300 mg/kg bw/d (004b). Animals were terminated on Day 29 and the uterine contents investigated. All of the foetuses were investigated for external findings and for visceral findings by dissection. The dose levels used in this study were based on the results of a preliminary study (not submitted) in which abortion and effects on bodyweight and food consumption were stated to have been observed at dose levels of ≥300 mg/kg bw/d.

No deaths occurred and no signs of toxicity were observed during the study period. One animal at the top dose level aborted on Day 28; in light of the findings in the preliminary study, this finding is considered to be potentially treatment-related. Mean terminal bodyweight was lower at the top dose level as a result of significantly reduced weight gain during the dosing period. Bodyweight gains at 100 mg/kg bw/d were also slightly (but significantly) reduced at some time points. Food consumption was significantly lower at the top dose level at all time points and occasionally at 100 mg/kg bw/d. Litter parameters were unaffected by treatment.

Mean foetal weights were unaffected by treatment. The pattern of foetal malformations and variations does not indicate any relationship to treatment; total incidences were unaffected by treatment.

Overall, no evidence of developmental toxicity was seen in this study in rabbits up to a dose (300 mg/kg bw/d) causing maternal toxicity.

4.11.2.2 Human information

No data are available.

4.11.3 Other relevant information

A number of mechanistic studies have been performed in order to clarify the effects of amisulbrom on female fertility seen in the rat multi-generation study. Reduced fertility in this study was associated with histopathology indicative of adverse effects on ovarian function. These studies aimed at investigating whether the effects were mediated by an endocrine mechanism or whether they were the secondary consequence of impaired nutrition and growth.

Table 24: Summary table of mechanistic studies relevant to the amisulbrom-induced effects on female fertility

Method	Dose levels	Observations and remarks (effects of major toxicological significance)
Effects on the rat foetal ovary Ovary tissue samples taken from the rat gavage developmental toxicity study (Furukawa, 2003) 9 foetuses in controls and 11 foetuses in high dose group Analysis of primordial follicles and apoptotic bodies Han Wistar rat 2005(g) (DAR B.6.8.2(a))	0 and 1500 mg/kg bw/d	No differences were observed in the counts of ovarian primordial follicles and apoptotic bodies, but due to the small sample size, reliability is uncertain.
Effects on the ovary in young rats Han Wistar rat (8 F/group) Dietary administration from GD 0 to LD 21 Ovary histopathology Amisulbrom 99.1% pure 2005(h) (DAR B.6.8.2(b))	0 and 15000 ppm (1200 mg/kg bw/d) amisulbrom 10 mg/kg bw i.p. injection of busulphan (positive control given to 3 F)	**15000 ppm (1200 mg/kg bw/d):** ↓bw (12%)* on GD14; ↓bw (36%)* in pups on LD21; ↑rel liver wt (27%)* in pups; ↓abs ovary wt (25%)* in pups; ↑no of ovarian follicles per mm2 (25 vs 18 in controls)* in pups; **10 mg/kg bw busulphan:** ↓abs ovary wt (84%)* in pups; ↓no of ovarian follicles pwe mm2 (8 vs 18 in controls)* in pups;

Sex hormone investigations in adult rats 28-day study Han Wistar rat (8/sex/group) Dietary administration Sex hormone levels (FSH, LH, T, Prog, Prolactin) investigations in terminal blood samples Amisulbrom 99.1% pure 2005(a) (DAR B.6.8.2(c))	0, 600 and 20000 ppm (0, 48 and 1500 mg/kg bw/d in males; 0, 54 and 1760 mg/kg bw/d in females)	**20000 ppm (1500/1760 mg/kg bw/d in M/F):** ↓terminal bw (10% M; 8% F)*; ↑rel liver wt (14% M; 19% F)*; No effects on hormone levels; **600 ppm (48/54 mg/kg bw/d in M/F):** No treatment-related effects;
Uterotrophic assay in young female rats Similar to female pubertal assay (OECD…) Young (20 day-old) female Han Wistar rats (6/group) Gavage administration for 3 days Amisulbrom 99.1% pure 2005(b) (DAR B.6.8.2(d))	0, 60, 300 and 1500 mg/kg bw/d	**1500 mg/kg bw/d:** ↓bwg (40%)* No treatment-related effects on the uterus; **300 and 60 mg/kg bw/d:** No treatment-related effects
Aromatase assay in young female rats Young (27 day old) female Han Wistar rats (6/group) Gavage administration for 4 days Amisulbrom 99.1% pure Positive control:exemastane	0, 300 and 1500 mg/kg bw/d	**300 and 1500 mg/kg bw/d:** No effects on the uterus were noted;

(200 mg/kg bw/d) 2005(c) (DAR B.6.8.2(e))		
Effects on ovary development in juvenile rats (dietary dosing) Han Wistar rats (7/group) dosed from GD0 to PND40 Dietary administration Food restriction (R) in some non-treated groups Amisulbrom 99.1% pure 2006(a) (DAR B.6.8.2(f))	0, 15000 ppm (1700 mg/kg bw/d)	**15000 ppm (1700 mg/kg bw/d):** ↓bwg (22%)* during gestation; ↓bwg (95%)* on LD6; ↓fc (38%)* on GD6; ↓fc (17-35%)* during lactation; ↓milk yield (31-36%)*; ↓bwg (40-43%)* in lactating pups; Delayed eye opening (14.6 d vs 13.9 d in controls)* in lactating pups; ↓stomach weight (35%)* on PND4; No effects on ovarian follicular development in pups treated up to LD21; ↓bwg (12%)* in pups subsequently treated from PND21-40; ↓fc (12%)* in pups subsequently treated from PND21-40; Delayed vaginal opening on PND40* (1/6 vs 0/6 in controls) in pups subsequently treated from PND21-40; ↓ovary wt (40%)* in pups subsequently treated from PND21-40; ↓uterus wt (45%)* in pups subsequently treated from PND21-40; Effects on ovarian development (↑no of atretic follicles and ↓no of corpora lutea) in pups subsequently treated from PND21-40; **Food restricted animals:** ↓bw (9%)* on LD21; ↓bwg (~40%)* in lactating pups; Delayed eye opening in lactating pups; No effects on ovarian follicular development in pups on food restriction up to LD21; ↓bwg (41%)* in pups continuously on food restriction until PND40; ↓fc (44%)* in pups continuously on food restriction until PND40; ↓ovary wt (58%)* in pups continuously on food restriction until PND40; ↓uterus wt (64%)* in pups continuously on food restriction until PND40; Delayed vaginal opening on PND40* (3/6 vs 0/6 in controls) in pups continuously on food restriction until PND40; Effects on ovarian development (↑no of atretic follicles and ↓no of corpora lutea) in pups continuously on food restriction until PND40;
Effects on ovary development in juvenile rats (gavage dosing)	0, 1500 mg/kg bw/d	**1500 mg/kg bw/d:** ↓fc (20%)* on GD6 only; ↓bwg (14%)* in lactating pups during the last week of lactation only;

Han Wistar rats (7/group) dosed from GD0 to PND40 Gavage administration Amisulbrom 99.1% pure 2006(b) (DAR B.6.8.2(g))		No effects on ovaries and stomach wt on PND4; ↓bwg (6%)* on PND22-32 only in pups continuously treated until PND40; No effects on ovaries and uterus on PND40 in pups continuously treated until PND40;

*Statistically significant; ↓ = decrease ↑ = increase

Effects on the rat foetal ovary

The effects of amisulbrom on the foetal rat ovary were investigated using tissue samples taken from the developmental toxicity study (2003) performed in the Han Wistar rat. H&E-stained tissue samples from nine foetuses from the control group and eleven foetuses from the 1500 mg/kg bw/d group were investigated histopathologically for the presence of primordial follicles and apoptotic bodies (pyknotic cells).

Counts of ovarian primordial follicles and apoptotic bodies were found to be comparable between the two groups. The results of this study seem to suggest that prenatal exposure to amisulbrom at a dose of 1500 mg/kg bw/d does not affect follicle formation in the rat ovary during organogenesis. However, due to the small sample size (9 foetuses in controls and 11 foetuses in the treated group), the reliability of this finding is uncertain.

Effects on the ovary in young rats

Mated female Han Wistar rats (eight/group) were administered amisulbrom in the diet at concentrations of 0 or 15000 ppm (1200 mg/kg bw/d) from Day 0 of gestation until Day 21 of lactation (2005h). Litter size was adjusted at birth to six; pups from four dams from the control and amisulbrom-treated groups were cross-fostered at birth. A positive control group of three pregnant females was administered a single intraperitoneal injection of busulphan (10 mg/kg bw) on Day 14 of gestation. The selected pups were terminated on Day 21 *post partum*; liver and ovary weights were recorded and ovaries investigated for the presence of follicles.

No maternal deaths occurred and no signs of toxicity were observed during the study. The mean bodyweights of females administered 15000 ppm amisulbrom were significantly lower than controls throughout gestation. Bodyweights were also lower during lactation but did not generally achieve statistical significance. Food consumption during gestation was also significantly reduced in this group.

Mean litter size and mean pup weights at birth were comparable in the control and amisulbrom treated groups. The weights of pups in the amisulbrom-treated groups were significantly lower than the control groups from Day 7 *post partum*; similar results were obtained for the cross-fostered groups, indicating that the bodyweight effects in pups were due to the post-natal administration of amisulbrom. Relative liver weight was significantly higher in the pups in both amisulbrom-treated groups. Mean absolute ovary weights were lower in both groups of amisulbrom-treated pups. The number of ovarian follicles per mm^2 was slightly but significantly increased in both groups of pups administered amisulbrom via lactation; the proportions of follicle types were comparable. This increase may be due to the smaller size of the ovary observed in this group. In the positive control

group, the total number of follicles was markedly reduced and there was a marked increase in the proportion of atretic follicles.

The results of this study indicate that the administration of amisulbrom at a dose level of 1200 mg/kg bw/d during gestation and lactation has no adverse effect on the development of ovarian follicles in young rats.

Sex hormone investigations in adult rats

Han Wistar rats (8/sex/group; 7 weeks old) were administered amisulbrom in the diet at dose levels of 0, 600 or 20000 ppm for four weeks (2005a). Vaginal smears were taken daily during the final week of the study and female rats were terminated in metoestrus. Animals were observed daily for clinical signs; bodyweights and food consumption were measured weekly. Gross necropsy was performed on all animals; the weights of the liver, testes, epididymides, ovaries and uterus were recorded. The vagina was investigated histopathologically at termination to confirm the oestrus cycle stage in females. Terminal blood samples were taken for the measurement of FSH and LH (in both sexes); testosterone (in males); progesterone and prolactin (in females).

No deaths occurred and no signs of toxicity were observed during the study period. Mean bodyweights of both sexes at 20000 ppm were significantly lower than controls during the study period; food consumption was also reduced in this group. Relative liver weight was significantly higher in both sexes at 20000 ppm. Gross necropsy did not reveal any treatment-related findings. Investigation of hormonal activities did not indicate any effect of treatment; however it is noted that the levels of these hormones are highly variable.

Overall, dietary administration of amisulbrom up to a dose level of 1500/1700 mg/kg bw/d for four weeks was found to have no effects on sex hormones or macroscopic effects on the reproductive organs of adult rats.

Uterotrophic assay in young female rats

Young (20 day-old) female Han Wistar rats (6/group) were gavaged with amisulbrom at dose levels of 0, 60, 300 or 1500 mg/kg bw/d (in 0.5% aqueous methylcellulose) on three consecutive days (2005b). Thirty minutes following dosing on each day, rats were gavaged with ethinylestradiol (0.1 µg/kg bw/d). Terminal investigations at 24 hours following the final dose consisted of measurement of wet and dry uterus weights; and gross and histopathological investigation of the uterus and vagina. BrdU was administered 2 hours prior to termination and the uterine mucosal proliferative activity was measured by BrdU staining.

No deaths occurred and no signs of toxicity were observed during the study period. Body weight gain by top dose females was significantly lower than controls. Mean absolute and relative uterus weights were marginally (but not statistically significantly) lower at the top dose level, however histopathology and BrdU staining did not reveal any effect of treatment on the level of uterine mucosal proliferative activity.

Overall, no evidence of a specific anti-oestrogenic effect was seen in this uterotrophic assay in young rats up to a dose level of 1500 mg/kg bw/d amisulbrom.

Aromatase assay in young female rats

Young (27 day old) female Han Wistar rats (6/group) were gavaged with amisulbrom (in aqueous methylcellulose) at dose levels of 0, 300 or 1500 mg/kg bw/d on four consecutive days (2005c). A positive control group was similarly administered the aromatase inhibitor exemastane (200 mg/kg bw/d) in physiological saline. Two hours following dosing on each day, animals were administered

subcutaneous injections of androstenedione (150 mg/kg bw/d). Uterus weights (wet and dry) were measured at necropsy at 24 hours following the final dose; the uterus was also investigated for gross and microscopic findings.

One rat administered 300 mg/kg bw/d died as a result of mis-dosing. No other deaths occurred and no signs of toxicity were observed during the study period. Bodyweights were unaffected by treatment. Mean absolute and relative wet and dry uterus weights in animals administered amisulbrom were comparable to controls. Uterus weights of the positive control groups were significantly (wet weight) lower than controls, demonstrating the anti-aromatase activity of exemastane and thus confirming the sensitivity of the assay.

Overall, amisulbrom did not demonstrate any anti-aromatase activity in young female rats up to a dose level of 1500 mg/kg bw/d, under the conditions of this study.

Effects on ovary development in juvenile rats (dietary dosing)

The purpose of this study was to assess the effects of dietary administration of amisulbrom on rat ovarian and uterine development during gestation, lactation and weaning up to 40 days of age. In addition, the effect of restricting food intake on ovarian development was assessed in untreated dams and pups (2006a). Groups of 7 pregnant female Han Wistar rats were given in the diet 0 (C) or 15000 ppm (T) amisulbrom (~ 1700 mg/kg bw/d) from day 0 of gestation to day 21 of lactation. An additional group of pregnant females were given a food-restricted diet (R). The number of suckling pups was adjusted on PND 0 to 10 animals for the control and treated groups and to 22 animals for the food-restricted group. At weaning on PND 21, females pups were assigned to eight groups of 6 rats each as follows: pups from control dams to a C/C group (no food restriction), a C/R50 group (1 fasting day every 2 days) and a C/R33 group (1 fasting day every 3 days); pups from the amisulbrom-treated dams to a T/C group (untreated diet) and a T/T group (15000 ppm amisulbrom diet); pups from food-restricted dams to a R/C group (no food restriction), a R/R50 group (1 fasting day every 2 days) and a R/R33 group (1 fasting day every 3 days). Treatment of these pups was performed from PND 21 to PND 40.

Table 24(a): Group composition and identification for dams (pregnancy and lactation)

Name of group	Dose (ppm)	No of animals	Food restriction
Control group (C)	0	7	No
Treated group (T)	15000	7	No
Food-restricted group (R)	0	7	Yes

Table 24(b): Group composition and identification for pups (PND 21 to 40)

Name of group	Dose (ppm)		Food restriction		No of animals
	Pregnancy/ lactation	After weaning	Pregnancy/ Lactation	After weaning	
C/C	0	0	No	No	6
C/R50	0	0	No	50%	6
C/R33	0	0	No	33%	6

T/C	15000	0	No	No	6
T/T	15000	15000	No	No	6
R/C	0	0	Yes	No	6
R/R50	0	0	Yes	50%	6
R/R33	0	0	Yes	33%	6

Dams were observed for clinical signs, body weight and food consumption throughout pregnancy and lactation. Milk yield was also determined on 2 occasions during lactation. Newborn rats were monitored for survival and eye opening from PND 0-21. Body weight was recorded at regular intervals throughout lactation. On PND 4, two pups per dam were killed to determine the weight of their stomach and the concentration of amisulbrom and its metabolites, IT-4 and IT-5, in the stomach contents. Histopathology of the ovary was also conducted. After weaning, the female pups were monitored for clinical signs, body weight, food consumption and vaginal opening. On PND 40 they were killed. Their ovaries and uterus were dissected, weighed and examined histopathologically.

In the treated dams there were reductions in body weight gain during gestation (by 22%) and on day 6 of lactation (by 95%) and in food consumption on day 6 of gestation (by 38%) and during lactation (by17-35%). Milk yield tended to be decreased (by 31-36%). In the food-restricted dams, body weight was lower than controls on day 21 of lactation (by 9%).

In the treated lactating pups (PND 0-21), body weight gain was reduced during lactation (by 40-43%) and eye opening was slightly delayed (14.6 days vs 13.9 days in controls). Similar findings were recorded in the food-restricted lactating pups. In the treated pups, on PND 4, stomach weight was reduced (by 35%) but ovarian follicular development was unaffected. The milk in the stomach of these pups contained detectable levels of parent amisulbrom, IT-4 and IT-5. The presence of parent amisulbrom was considered to be due to contamination from the maternal diet

In the female pups treated with amisulbrom from PND 21 to 40 (T/T group), body weight gain was reduced (by 12%) and food consumption was decreased (by 12%). In addition, vaginal opening was delayed (1 out of 6 pups on PND40 vs 0 out of 6 pups in controls), ovarian and uterine weights were reduced (by 40 and 45% respectively) and ovarian development was affected (increased number of atretic follicles and decreased number of corpora lutea). In the female pups from the food-restricted groups (R/R33 and C/R50), there were also reductions in body weight gain (by 41%), food consumption (by 44%) and ovary and uterus weights (by 58 and 64%, respectively). Vaginal opening was delayed (3 out of 6 pups on PND40 vs 0 out of 6 pups in controls) and ovarian development was also affected (increased number of atretic follicles and decreased number of corpora lutea). All pups in the R/R50 group died before vaginal opening. Vaginal opening, ovarian and uterus weights and ovarian histopathology were not affected in the female pups from the T/C group (treated until PND21, but left untreated from PND21 to PND 40) and from the R/C group (on food restriction until PND21 but no food restriction from PND21 to PND40).

Overall, in this study, dietary administration of amisulbrom at the relatively high dose of ~1700 mg/kg bw/d to pregnant female rats during gestation and lactation produced reduced maternal body weight gain and food consumption and decreased milk yield. Prenatal and postnatal exposure to amisulbrom (~1700 mg/kg bw/d) of offspring until PND 21 caused decreased body weight, but there were no effects on the ovaries and uterus. However, prenatal and postnatal exposure to amisulbrom (~1700 mg/kg bw/d) of offspring up to puberty (PND 40) induced lower body weight, decreased food consumption, reduced ovary and uterus weights and ovarian atrophy. Food

restriction in untreated animals during gestation, lactation and weaning up to PND 40 caused similar effects, with reduced body weights, decreased ovary and uterus weights and ovarian atrophy. Based on these findings, it can be concluded that the effects of prenatal and postnatal (up to puberty) exposure to high doses of amisulbrom on ovaries and uterus in rats are the secondary consequence of impaired nutrition and growth during development due to reduced food consumption.

Effects on ovary development in juvenile rats (gavage dosing)

The purpose of this study was to assess the effects of oral gavage administration of amisulbrom on rat ovarian and uterine development during gestation, lactation and weaning up to 40 days of age (2006b). Groups of 7 pregnant female Han Wistar rats were given single oral gavage doses of 0 (C) or 1500 mg/kg bw/d amisulbrom (T) in a solution of 0.5% methylcellulose from day 0 of gestation until day 21 of lactation. The number of suckling pups was adjusted on PND 0 to 10 animals. At weaning on PND 21, females pups were assigned to three groups of 6 rats each as follows: pups from the control dams to a C/C group (control) and pups from the treated dams to either a T/C group (solvent until PND40) or a T/T group (1500 mg/kg bw/d amisulbrom until PND40).

Dams were observed for clinical signs, body weight and food consumption throughout pregnancy and lactation. Milk yield was also determined on 2 occasions during lactation. Newborn rats were monitored for survival and eye opening from PND 0-21. Body weight was recorded at regular intervals throughout lactation. On PND 4, two pups per dam were killed to determine the weight of their stomach and the concentration of amisulbrom and its metabolites, IT-4 and IT-5, in the stomach contents. Histopathology of the ovary was also conducted. After weaning, the female pups were monitored for clinical signs, body weight, food consumption and vaginal opening. On PND 40 they were killed. Their ovaries and uterus were dissected, weighed and examined histopathologically.

The only treatment-related finding in the dams was a temporary reduction (by 20%) in food consumption on day 6 of pregnancy. Milk yield on days 5 and 12 was unaffected. In the offspring treated until weaning (T/C group), the only effect of treatment was a reduction (by 14%) of body weight gain during the last week of lactation. There were no effects on the ovaries and on stomach weight on PND 4. The milk in the stomach of these pups contained detectable levels of parent amisulbrom, IT-4 and IT-5.

In the offspring treated until PND 40 (T/T group), body weight gain was significantly reduced (by 6%) from PND 22-32, but the ovaries and uterus were unaffected at the end of the study (PND 40).

Overall, in this study, oral gavage administration of 1500 mg/kg bw/d amisulbrom to pregnant female rats during gestation and lactation produced no obvious maternal toxic effects. Prenatal and postnatal exposure to amisulbrom (gavage dose of 1500 mg/kg bw/d) of offspring either until PND 21 or until puberty (PND 40) caused only a temporary decrease in body weight gain and had no effect on follicular development in the ovary or on uterine weight.

The differences in outcome between the dietary study and the gavage study at similar dose levels are likely to be due to the bad palatability of the test substance. In addition, it is noted that as similar levels of parent amisulbrom and its metabolites, IT-4 and IT-5, were measured in milk in both studies, kinetic differences are unlikely to explain the differences in effects observed.

Conclusion of mechanistic studies

The results of the fertility mechanistic studies showed that amisulbrom had no specific effect on the rat ovaries during gestation and lactation. Also, no inhibitory effects were apparent on aromatase

activity in young female rats. In addition, no anti-oestrogenic effect was apparent in an uterotrophic assay in young female rats. Similarly, no effects on sex hormonal levels were observed in adult male or female rats. However, prenatal and postnatal exposure to amisulbrom (at the relatively high dose of ~1700 mg/kg bw/d) of offspring up to puberty (PND 40) induced lower body weight, decreased food consumption, reduced ovary and uterus weights and ovarian atrophy. Food restriction in untreated animals during gestation, lactation and weaning up to PND 40 caused similar effects, with reduced body weights, decreased ovary and uterus weights and ovarian atrophy. Based on these findings, it can be concluded that the effects of prenatal and postnatal (up to puberty) exposure to high doses of amisulbrom on ovaries and uterus in rats are the secondary consequence of impaired nutrition and growth during development due to reduced food consumption. Similar effects were not seen in a gavage study, indicating that the observed reduced food consumption was the consequence of the bad palatability of the test substance.

Overall, therefore, it can be concluded that the effects on fertility seen in the F1 females in the rat 2-generation study at a dose level (1200-1300 mg/kg bw/d) in excess of the limit dose are the secondary consequence of impaired nutrition and growth during the early phase of development of the ovaries. Reduced fertility was associated with severely impaired bodyweight development (from 10% up to 40% reduction in body weight during gestation and lactation of F0 females and weaning and sexual maturation of F1 pups) in this study.

4.11.4 Summary and discussion of reproductive toxicity

Fertility

The potential effects of amisulbrom on fertility and reproductive performance have been investigated in a guideline multigeneration study in the rat. In this study, administration of amisulbrom at the top dose level of 15000 ppm (1200-1300 mg/kg bw/d) had a clear and marked effect on reproduction in F1 females; a reduction in fertility at this dose level was shown to be female-mediated. Reduced fertility was associated with severely impaired bodyweight development (from 10% up to 40% reduction in body weight during gestation and lactation of F0 females and weaning and sexual maturation of F1 pups), reduced ovarian weight and function and associated histopathology.

A number of mechanistic studies have been performed in order to clarify the aetiology of these effects of amisulbrom on female fertility. These studies showed that amisulbrom had no specific effect on the rat ovaries during gestation and lactation. Also, no inhibitory effects were apparent on aromatase activity in young female rats. In addition, no anti-oestrogenic effect was apparent in an uterotrophic assay in young female rats. Similarly, no effects on sex hormonal levels were observed in adult male or female rats. However, prenatal and postnatal exposure to amisulbrom (at the relatively high dose of ~1700 mg/kg bw/d) of offspring up to puberty (PND 40) induced lower body weight, decreased food consumption, reduced ovary and uterus weights and ovarian atrophy. Food restriction in untreated animals during gestation, lactation and weaning up to PND 40 caused similar effects, with reduced body weights, decreased ovary and uterus weights and ovarian atrophy. Based on these findings, it can be concluded that the effects of prenatal and postnatal (up to puberty) exposure to high doses of amisulbrom on ovaries and uterus in rats are the secondary consequence of impaired nutrition and growth during development due to reduced food consumption. Similar effects were not seen in a gavage study, indicating that the observed reduced food consumption was the consequence of the bad palatability of the test substance.

Overall, therefore, it can be concluded that the effects on fertility seen in the F1 females in the rat 2-generation study at a dose level (1200-1300 mg/kg bw/d) in excess of the limit dose are the secondary consequence of impaired nutrition and growth during the early phase of development of

the ovaries. Reduced fertility was associated with severely impaired bodyweight development (from 10% up to 40% reduction in body weight during gestation and lactation of F0 females and weaning and sexual maturation of F1 pups) in this study.

This conclusion is corroborated by other feed-restriction studies in rats from the open literature. In a study by Chapin et al. (1993), food restriction of Sprague Dawley rats for 15 weeks up to GD 14 (resulting in a body weight reduction of 30%) increased the length of the estrous cycle and decreased the weight of the ovaries and the number of corpora lutea. In another study (Terry et al., 2005), food restriction of female Sprague Dawley rats for 4 weeks up to GD 7 (resulting in a body weight reduction of 29%) produced overt changes in estrous cyclicity, mating and fertility.

It should be noted that, compared to the findings of these published papers, in the amisulbrom 2-generation study, at the dose level at which reduced fertility was observed, there was a much more severely impaired bodyweight development (up to 40% reduction in body weight) which occurred not only throughout gestation, but also through lactation, weaning and sexual maturation.

Overall, there are sufficient mechanistic data and supporting evidence from the literature to conclude that the effects on fertility seen in the F1 females in the rat 2-generation study are the secondary consequence of impaired nutrition and growth during the early phase of development of the ovaries and do not arise from a specific action of amisulbrom on fertility.

Development

The developmental toxicity potential of amisulbrom has been investigated in rats (1 range-finding study and 2 guideline studies) and rabbits (1 guideline study).

In one of the two rat studies (in Han Wistar from the Harlan, UK colony), the incidence of cleft palate was increased (12 foetuses in 2 litters vs 0 in controls) at the limit dose of 1000 mg/kg bw/d in the absence of maternal toxicity. This finding was associated in the same animals with a number of other malformations, which constitute a typical genetic syndrome (chondrodystrophy). Although the incidence of cleft palate/chondrodystrophy in this study (6 foetuses/litter) just exceeded the laboratory historical control range (1-5 foetuses/litter), the pattern of the finding (noted in just 2 litters) and the association with other defects (chondrodystrophy syndrome) suggest a spontaneous (genetic) aetiology typical of this strain/colony rather than a relationship to treatment with amisulbrom. In addition, no evidence of cleft palate was seen in the other rat study (in Han Wistar from the CLEA, Japan colony) at a dose level of 1500 mg/kg bw/d, at which maternal toxicity (reduced weight gain and food consumption) was apparent; or in the range-finding study in SD rats up to 1000 mg/kg bw/d. These findings therefore indicate that the increased incidence of cleft palate/chondrodystrophy observed in the first rat study may not be treatment-related. Also, although a slightly increased incidence (3 foetuses in 1 litter vs 0 in controls) of cleft palate was apparent at the highest dose level (1200-1300 mg/kg bw/d) and at the next lower dose of 240-260 mg/kg bw/d (1 foetus in 1 litter) in the F1 offspring of the rat multi-generation study, the observed incidence was within the laboratory historical control range.

Overall, the low incidence of cleft palate observed in the Han Wistar rat (from the Harlan, UK colony) in the absence of maternal toxicity in a developmental toxicity study and in the presence of severe maternal toxicity in the multi-generation study is considered not to be related to treatment with amisulbrom but to have a genetic aetiology.

No evidence of developmental toxicity was seen at the top dose level of 300 mg/kg bw/d in the rabbit developmental study, at which maternal toxicity (reduced weight gain and food consumption) was apparent.

4.11.5 Comparison with criteria

Fertility

The potential effects of amisulbrom on fertility and reproductive performance have been investigated in a guideline multigeneration study in the rat. In this study, administration of amisulbrom at the top dose level of 15000 ppm (1200-1300 mg/kg bw/d) had a clear and marked effect on reproduction in F1 females; a reduction in fertility at this dose level was shown to be female-mediated. Reduced fertility was associated with severely impaired bodyweight development (from 10% up to 40% reduction in body weight during gestation and lactation of F0 females and weaning and sexual maturation of F1 pups), reduced ovarian weight and function and associated histopathology.

Mechanistic studies have shown that prenatal and postnatal exposure to amisulbrom (at the relatively high dose of ~1700 mg/kg bw/d) of offspring up to puberty (PND 40) induced lower body weight, decreased food consumption, reduced ovary and uterus weights and ovarian atrophy. Food restriction in untreated animals during gestation, lactation and weaning up to PND 40 caused similar effects, with reduced body weights, decreased ovary and uterus weights and ovarian atrophy.

Overall, therefore, it can be concluded that the effects on fertility seen in the F1 females in the rat 2-generation study at a dose level (1200-1300 mg/kg bw/d) in excess of the limit dose are the secondary consequence of impaired nutrition and growth during the early phase of development of the ovaries and do not arise from a specific action of amisulbrom on fertility.

When comparing these findings with the criteria, the following conclusions can be drawn:

Category 1A (known human reproductive toxicant) is not appropriate as *there is no human evidence establishing a causal relationship* between exposure to amisulbrom and an adverse effect on fertility.

Category 1B (presumed human reproductive toxicant) is also not appropriate as *there is no clear evidence of an adverse effect on fertility in experimental animals that is considered not to be the secondary, non-specific consequence of other toxic effects*. The effects on female fertility caused by amisulbrom are the secondary consequence of impaired nutrition and growth during the early phase of development of the ovaries and do not arise from a specific action of amisulbrom on fertility.

Category 2 (suspected human reproductive toxicant) is also not appropriate because *there is no evidence of an adverse effect on fertility in experimental animals that is considered not to be the secondary, non-specific consequence of other toxic effects*. The effects on female fertility caused by amisulbrom are the secondary consequence of impaired nutrition and growth during the early phase of development of the ovaries and do not arise from a specific action of amisulbrom on fertility.

Overall, therefore, classification of amisulbrom for fertility is not warranted.

Development

The developmental toxicity potential of amisulbrom has been investigated in rats (1 range-finding study and 2 guideline studies) and rabbits (1 guideline study).

No evidence of developmental toxicity was seen in the rabbit up to a dose causing maternal toxicity.

A low incidence of cleft palate/chondrodystrophy was observed in the absence of maternal toxicity in a developmental toxicity study and in the presence of severe maternal toxicity in the multi-generation study. The incidence of the finding just exceeded the laboratory historical control range in the teratogenicity study, but was within the laboratory historical control range in the 2-generation study. The pattern of the finding (noted in single litters and in association with other defects) suggests a spontaneous (genetic) aetiology rather than a relation to treatment with amisulbrom.

When comparing these findings with the criteria, the following conclusions can be drawn:

Category 1A (known human reproductive toxicant) is not appropriate as *there is no human evidence establishing a causal relationship* between exposure to amisulbrom and an adverse effect on development.

Category 1B (presumed human reproductive toxicant) is also not appropriate as *there is no clear evidence of an adverse effect on development in experimental animals.* The low incidence of cleft palate/chondrodystrophy observed in the rat has been shown not to be related to treatment with amisulbrom but to arise through a genetic mechanism.

Category 2 (suspected human reproductive toxicant) is also not appropriate because *there is no evidence of an adverse effect on development in experimental animals.* The low incidence of cleft palate/chondrodystrophy observed in the rat has been shown not to be related to treatment with amisulbrom but to arise through a genetic mechanism.

Therefore, classification of amisulbrom for developmental toxicity is not warranted.

4.11.6 Conclusions on classification and labelling

Not classified for fertility or development – Conclusive but not sufficient for classification

4.12 Other effects

4.12.1 Non-human information

No data are available.

4.12.1.1 Neurotoxicity

No acute, sub-acute or delayed neurotoxicity studies have been conducted with amisulbrom, as the chemical structure of this molecule has no relationship with compounds known to induce neurotoxicity or delayed neurotoxicity. However, no specific clinical signs of toxicity indicative of neurological effects have been seen in the toxicity tests in rodents or dogs.

4.12.1.2 Immunotoxicity

No data area available.

4.12.1.3 Specific investigations: other studies

A number of mechanistic studies are available. These were conducted to investigate the mode of action of the possible carcinogenic and fertility effects noted in some studies. These have been described in the carcinogenicity and reprotoxicity sections above.

4.12.1.4 Human information

Routine medical surveillance is carried out on the manufacturing plant (Onoda, Japan) personnel, including the reporting of symptoms, annual medical interview and biennial medical examination. No health effects related to amisulbrom have been reported in workers engaged in manufacture or formulation. Also, there have been no reports of adverse effects following exposure to amisulbrom during manufacture, formulation or from the use of plant protection products.

4.12.2 Summary and discussion

No other effects have been observed or reported in animals or humans with exposure to amisulbrom.

4.12.3 Comparison with criteria

No other effects have been observed or reported in animals or humans with exposure to amisulbrom. Classification is not required.

4.12.4 Conclusions on classification and labelling

None.

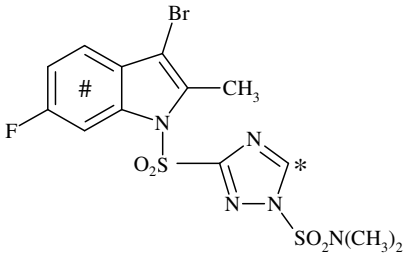
5 ENVIRONMENTAL HAZARD ASSESSMENT

Amisulbrom is a pesticidal active substance considered under Directive 91/414/EEC (subsequently Regulation 1107/2009) for representative use as a foliar fungicide against downy mildew on grapes and late blight on potatoes. Available environmental fate and ecotoxicology studies have been considered and summarised in an updated Draft Assessment Report, February 2012(Volume 3, Annex B8: Environmental Fate and Behaviour and Volume 3, Annex B9; Ecotoxicology) and subsequent DAR Addenda, 2013. The outcome of the pesticide peer review and agreed endpoints from this process are summarised in the EFSA Conclusion, 2014. The key information pertinent to determining the environmental hazard classification for amisulbrom is presented below. Unless otherwise stated, these studies were conducted in accordance with GLP and the validity criteria of the respective test guideline. They are considered reliable (Klimisch score 1 or 2) for use in hazard classification.

Amisulbrom degrades to a number of degradants in the environment, the main ones identified from a natural water/sediment study are listed (with structures) in Annex 1, Figure 1. In abiotic and biotic studies, some degradants may exceed 10% applied radioactivity (AR) and be relatively persistent. However, in natural water/sediment systems they were mainly associated with the sediment phase. Reliable GLP toxicity studies on fish, invertebrates and algae are presented for the main biotic degradants, IT-4 and IT-15, which also indicate they are less acutely and chronically toxic than the parent substance. This report therefore focuses on the classification of amisulbrom alone.

5.1 Degradation

A summary of available information on the environmental degradation of amisulbrom is presented in table 25. Radiolabel studies used either [indole-^{14}C] or [triazole-^{14}C] amisulbrom with a radiochemical purity of greater than 97% and specific activity of 4.28-4.55 MBq/mg. Indole and triazole labelling, as shown below, was used in all route of degradation studies.



[#] position of [indole-^{14}C] radiolabel
* position of [triazole-^{14}C] radiolabel

Table 25: Summary of relevant information on degradation

Method	Results	Remarks	Reference
Aqueous hydrolysis, performed according to: EU 92/69/EEC, C.7 (1992) SETAC Europe (1995) US EPA FIFRA Subdivision N,161-1 (1982) JMAFF 12 Nohsan no. 8147, 2-6-1 (2001)	Hydrolysis DT_{50}s at 25ºC in buffered solutions: pH 4: 106.1 days (first order, r^2 = 0.960, normalised to 20°C based on a Q10 value of 2.2) pH 7: 87.1 days (first order, r^2 = 0.893, normalised to 20°C based on a Q10 value of 2.2) pH 9: 7.0 days (first order, r^2 = 0.998, normalised to 20°C based on a Q10 value of 2.2)	Study performed to GLP - with no significant deviations from EU guideline; considered reliable. Study results were recalculated using non-linear first order kinetics and a mean of both label positions at each time point; hydrolytic DT50s then converted to 20ºC using Arrhenius equation	Wicks, 2004a (DAR B.8.4.1)
Aqueous photolysis, performed according to: JMAFF 12 Nohsan no. 8147, 2-6-2 (2001)	Photolysis DT_{50} in sterilised river water at pH 6.7 and 25ºC: 4.2-4.4 hours (first order, r^2 = 0.986 for indole-label and r^2 = 0.991for triazole-label respectively)	Study performed to GLP - with no significant deviations from guideline; considered reliable	Takehara, 2004 (DAR B.8.4.2)
Aqueous photolysis, performed according to: SETAC Europe (1995) US EPA FIFRA Subdivision N,161-2 (1982) JMAFF 12 Nohsan no. 8147, 2-6-2 (2001)	Photolysis DT_{50} in pH 4 buffer solution at 25ºC: 4.9 hours (first order, r^2 = 0.994) Quantum yield of direct phototransformation in water at $\Sigma > 290$ nm = 0.19 molecules/photon at pH 4 and 25ºC	Study performed to GLP - with no significant deviations from SETAC EU guideline; considered reliable. Study result recalculated using non-linear first order kinetics and a mean of both label positions	Wicks, 2004b (DAR B.8.4.2)
Ready biodegradation screening test, performed according to: EU 92/69/EEC C.4-C (1992) OECD 301B (1992) EPA OPPTS 835.3110 (1998)	No evidence of amisulbrom biodegradation by the end of the study (Day 29), conducted at 22ºC and pH 7.5-7.8. Substance considered to be not readily biodegradable under the conditions of this test	Study performed to GLP - with no significant deviations from EU guideline; considered reliable	Barnes, 2004 (DAR B.8.4.3)

Degradability and fate in simulated water/sediment systems over 120 days, performed according to: SETAC-Europe (1995) BBA VI, 5-1 (1990) OECD 308 (2002)	Wood Moss Tarn (clay loam) water/sediment system (water pH: 6.74; sediment pH: 5.9; 20ºC): Whole system dissipation DT_{50} = 64.2 days (based on slow phase rate constant, K_2 of 0.0107983) Row Pond (clay) water/sediment system (water pH: 7.99; sediment pH: 6.5; 20ºC): Whole system dissipation DT_{50} = 156.1 days (based on slow phase rate constant, K_2 of 0.00444126) Mineralization in both systems was low	Study performed to GLP - with no significant deviations from SETAC EU guideline; considered reliable. Whole system DT_{50}s recalculated using HS kinetics and a mean of both label positions (see DAR and 5.1.2.3 below for details)	Unsworth, 2004c (DAR B.8.4.4)

5.1.1 Stability

Aqueous hydrolysis

One study (Wicks, R, 2004a) was submitted on the hydrolysis of amisulbrom at 25ºC in buffered solutions of pH 4, pH 7 and pH 9 using indole and triazole labelled amisulbrom. The study followed EU guideline 92/69/EEC, C.7 (1992) (similar to OECD 111) amongst others and was conducted to GLP. For each radiolabelled form, the nominal application rate was 0.05 mg/l, which is less than half the pure water solubility of amisulbrom (i.e. 0.11 mg/l). Fortification levels achieved were 101.6% and 100.1% of the nominal application rate and for both radiolabelled forms, overall mass balance recoveries of radioactivity at pH 4, 7 and 9 from the incubated samples were in the range 91.1 - 105.9% AR (one [triazole-^{14}C] treated sample had a recovery of 88.5% AR). Samples were taken at regular interval over 20-30 days incubation and were radioassayed by LSC and analysed by HPLC. Levels of parent amisulbrom declined to between 69.9 % of applied radioactivity (AR) and 75.3% AR over the incubation period of 30 days at pH 4 and 7. At pH 9 over a 20-day incubation period, levels of amisulbrom declined to between 5.9% AR and 6.9% AR.

The rate of hydrolysis of amisulbrom was shown to be dependent on pH. For both radiolabelled forms, reported half-life values (single first order (SFO) DT_{50}) at 25ºC for the hydrolysis of amisulbrom were similar at pH 4 (DT_{50} = 78.5 days) and pH 7 (DT_{50} = 76.5 days), but were more rapid at pH 9 (DT_{50} = 5.0 days).

At pH 4 and 7, the sole degradant occurring at levels > 10% AR was IT-4, with maxima of 17.7% AR and 15.9% AR respectively. At pH 9, degradant I-1 occurred at a maximum of 70.1% AR, IT-4 occurred at a maximum of 17.8% AR and T-1 at 39.8% AR. In the DAR the UK Rapporteur Member State (RMS) for the pesticide assessment concluded that these degradants are stable to hydrolysis since they were at their maxima at study end.

The pesticide RMS under Reg 1107/2009 recalculated the Notifier's degradation values for amisulbrom using non-linear first order kinetics and a mean of both label positions at each time point. The hydrolytic degradation rates of amisulbrom when converted to 20ºC using the Arrhenius equation (DT_{50} = 106.1 days at pH 4, 87.1 days at pH 7 and 7.0 days at pH 9) were slower when compared to its dissipation from the aqueous phase of the two water/sediment systems (3.8 days and 6.1 days respectively, see Section 5.1.2.3 below). The RMS therefore concluded that degradant

formation due to hydrolysis, including that at pH9, will not be significant under field conditions, and that the degradants formed in this hydrolysis study do not need further consideration.

Aqueous Photolysis

i) A study (Takehara, K, 2004) was submitted to investigate the aquatic photolysis of amisulbrom at 25ºC in sterilised river water (pH 6.7) using indole and triazole labelled amisulbrom at a nominal concentration of 0.05 mg/l. The study followed Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) guideline 12 Nohsan no. 8147 (2000) and was conducted to GLP. The radiolabelled amisulbrom was incubated under artificial sunlight (xenon arc lamp, intensity 425 Wm^{-2}, wavelength 300-800 nm) for up to 48 hours. Samples were separated into ethyl acetate extract and aqueous residue and were analysed by liquid scintillation counting (LSC) to determine the radioactivity in each fraction. Analyses were then conducted by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC).

Overall recoveries of indole-labelled amisulbrom were in the range 91.9% to 101.1% AR. Under light-irradiated conditions, indole-labelled amisulbrom was degraded to <0.1% AR after 48 hours. Degradants I-2 (51.7% AR), IT-12 (6.8% AR) and UKI-2 (6.2% AR) were produced, along with others at lower levels. In the dark control, 91.6% AR remained as amisulbrom after 48 hours. IT-4, IT-11 and I-1 were detected.

Overall recoveries of triazole-labelled amisulbrom were in the range 98.1% to 101.8%. Under light-irradiated conditions, triazole-labelled amisulbrom was degraded to <0.1% AR after 48 hours. Degradants T-3 (50.6% AR), T-1 (22.8% AR), T-4 (15.2% AR) and IT-12 (6.7% AR) were produced, along with others at lower levels. In the dark control, 88.3% AR remained as amisulbrom after 48 hours. IT-4, IT-11 T-1 and T-3 were detected.

Aqueous photodegradation of amisulbrom was shown to follow first-order kinetics with reported photolytic DT_{50} values of 4.2 hours (r^2 = 0.986) (indole) and 4.4 hours (r^2 = 0.991) (triazole). The same results were obtained when recalculated by the RMS assuming first-order non-linear reaction kinetics. This is equivalent to approximately 0.8 days under natural sunlight at 35°N from April to June. Of the major degradants detected, IT-12, T-3, T-4 were considered stable as they were increasing or around their maxima at study end.

ii) A second study (Wicks, R, 2004b) was submitted to investigate the aquatic photolysis of amisulbrom at 25ºC in a sterile buffered solution at pH 4 - again using indole and triazole labelled amisulbrom at a nominal concentration of 0.05 mg/l. The study followed the current EU guideline (SETAC Europe, 1995) amongst others and was conducted to GLP.

The radiolabelled amisulbrom was incubated under artificial sunlight (xenon arc lamp, wavelength 290-800 nm) for up to 48 hours. This continuous irradiation of the test system was equivalent to 106 hours of natural summer sunshine at latitude 40°N (taking into account day and night phases). Fortification levels achieved were 94.8-101.0% of the nominal concentrations. Samples were taken at regular intervals and analysed by LSC to quantify radioactivity and then by HPLC and liquid chromatography-mass spectrometry (LC-MS). Mean overall mass balance recoveries from irradiated and dark control samples were in the range 91.7% to 104.3% of applied radioactivity.

Aqueous photodegradation of amisulbrom was shown to follow first-order kinetics with a reported DT_{50} value of 6.1 hours (Kp = 0.11 $hours^{-1}$, r^2 = 0.990). The reaction quantum yield (Φ) for amisulbrom was calculated in the report to be 0.19 molecules degraded/photon.

The RMS recalculated the Notifier's degradation times for amisulbrom using non-linear first order kinetics and a mean value for each time point from both label positions. This resulted in a lower photolytic DT_{50} value of 4.9 hours ($r^2 = 0.994$).

Amisulbrom was photolytically degraded to I-1, I-2, I-2-OH (I-8), I-5 dimer (I-9), IT-11, IT-12, T-1, T-2 isomer (T-7), T-3, T-4, carbon dioxide and at least six unidentified degradates which included characterised [triazole-^{14}C] polar material (approx. 10% AR). Major photolytic degradates which accounted for >10% AR were I-2, I-8, I-9, T-1 and T-7. Of these, T-1, I-2 and I-8 were all at their maxima at study end.

5.1.2 Biodegradation

5.1.2.1 Biodegradation estimation

Not submitted or required; a ready biodegradation study is available.

5.1.2.2 Screening tests

One study (Barnes, S P, 2004) was submitted to investigate the ready biodegradability of amisulbrom (98.6% pure). The study followed OECD 301B, 1992 (modified Sturm test) and was conducted to GLP.

Amisulbrom was inoculated into test vessels along with activated sewage sludge (30 mg/l solids) and a mineral salts medium to give a nominal test concentration equivalent to 10 mg Carbon/l. A reference substance, sodium benzoate, was added as an aqueous solution (1.72 g/l) to one test system treated with amisulbrom and to one test system containing mineral salts medium alone. Two negative control vessels also contained mineral salts medium and ultrapure water alone. The biodegradation of sodium benzoate in the presence of amisulbrom was determined to confirm that amisulbrom itself was not inhibitory to microbial activity.

The test vessels were continuously aerated with carbon dioxide free air for 29 days and were maintained at 22 ± 2°C and pH7.5-7.8. CO_2 production was measured in the outlet air using three consecutive traps containing a nominal 0.025 N barium hydroxide (100 ml).

The sodium benzoate reference was biodegraded by 66% of its theoretical CO_2 after 7 days, by 82% after 29 days in the absence of amisulbrom and by 61% after 6 days in its presence. This confirmed that amisulbrom was not inhibitory to the activity of the microbial inoculum. Cumulative levels of CO_2 production in the controls after 29 days (67.7 and 63.8 mg CO_2) were within an acceptable range for this type of assay system (recommended maximum = 120 mg CO_2 for a 3 litre culture). The results from the sodium benzoate incubation confirmed the validity criteria for this test.

There was no evidence of biodegradation of amisulbrom (0%) at 10 mg C/l by the end of the test on Day 29. Substances are considered to be readily biodegradable if CO_2 production is ≥60% of the theoretical carbon dioxide production value within 10 days of achieving 10% biodegradation. Amisulbrom was not, therefore, determined to be readily biodegradable under the conditions of this OECD 301B test.

5.1.2.3 Simulation tests

A study (Unsworth, R, 2004c) was submitted to investigate the fate and behaviour of radiolabelled amisulbrom in two aerobic natural water/sediment systems over 120 days in darkness at 20 ± 2°C. The study followed OECD 308, 2002 and was conducted to GLP.

Method

The clay loam sediment and overlaying water were taken from a UK parkland site (Wood Moss Tarn) and the clay sediment and water were taken from a UK woodland site (Row Pond). Details of the two test systems are given in Table 26. Aliquots of indole- or triazole-labelled amisulbrom were evenly applied to the water phase of the individual test systems at a nominal rate of 57 µg a.s./vessel (this was stated to be approximately equivalent to the amisulbrom water solubility of 0.11 mg/l). For indole- or triazole-treated systems, fortification levels were 94% and 93% of the nominal application rate respectively.

Table 26: Water/sediment system test characteristics and conditions

Phase	Parameter	Location	
		Wood Moss Tarn	**Row Pond**
Sediment	Origin	Cumbria, UK	Derbyshire, UK
	Sediment type	Clay loam	Clay
	Sand (%)	31.43	8.09
	Silt (%)	49.21	40.69
	Clay (%)	19.36	51.22
	sediment : water ratio (w/w)	1:9.5	1:4.25
	Organic carbon (%)	12.2	3.8
	Total nitrogen (mg/kg)	855.3	564.6
	Total phosphorus (mg/kg)	6202.5	2505.7
	Initial pH (1 : 5) in water	5.9	6.5
	Mean redox potential (mV)	-343.5	-358.7
	Cation exchange capacity (meq/100 g)	46.2	18.2
	Aerobic microbial biomass (mg C/100 g)		
	Initial	11.29	4.84
	Final	5.72	3.95
Water	Total phosphorous (mg/l)	0.4	0.3
	Total nitrogen (mg/l)	1.4	<0.05
	Total hardness (mg/l $CaCO_3$)	35.0	213.0
	Suspended solids (mg/l)	16.0	28.0
	Dissolved organic carbon (mg/l)	9.6	4.8
	Mean pH	5.4	8.0
	Mean redox potential (mV)	+411.8	+308.1
	Mean dissolved oxygen content (%)	78	81

Whole water/sediment samples, including 2M potassium hydroxide and 20% ethanolamine trapping solutions were sampled at regular intervals up to 120 days after fortification. Sediment and water phases were analysed separately to determine the distribution of radioactivity and levels of extractable amisulbrom and its degradation products. Key physico-chemical parameters were also measured at appropriate intervals in replicate monitoring vessels. Principal radioassay analysis of trapping solutions and each compartment was by LSC. Concentrated organo-soluble and water soluble extracts from the water phase were further analysed by reverse phase HPLC. The sediment phase was Soxhlet extracted with acetonitrile. The organo-soluble extracts and sediment and aqueous residues were radioassayed by LSC and then further analysed by reverse phase or ion-exchange HPLC or by LC-MS. Acetonitrile and ethyl acetate flask washes were included with the totals from other extracts. Sediment samples from 90 and 120 days were also characterised into humin, humic acid and fulvic acid fractions.

Results

Overall mass balance recoveries were generally >85% of applied radioactivity (AR), with most at 90-110% AR. Lower recoveries were observed from some test systems and this was attributed to experimental loss of some material on transfer of the sediment from test vessels to Soxhlet thimbles and to adherence of test material on vessel walls. However, this did not substantially affect the results and the study is considered to have been conducted in accordance with key validity criteria and to be reliable.

Radioactivity partitioned relatively quickly from the aqueous to sediment phase of both systems, and low levels of radioactivity (generally <5.0% AR) remained in the aqueous residues at Day 120. Radioactivity in the sediment phase continued to increase and by Day 120 the majority in the whole system was associated with the sediment phase (reported 74.9-85.1% AR). Extractable radioactivity from sediment decreased with a concurrent increase in bound residue (up to a reported

29.2% AR and 17.1% AR in clay loam and clay systems) over the 120 days. This was mainly associated with humin and humic acid sediment fractions. Volatile radioactivity/mineralization only accounted for maxima of 1.3% AR and 1.2% AR by Day 120 in the clay loam and clay test systems respectively. After 120 days the proportion of parent amisulbrom declined to a mean reported 16.9% AR and 39.3% AR in the whole clay loam and clay water/sediment systems respectively. DT_{50} values in the aqueous and sediment phases and for each whole system were given in the study report for amisulbrom and its main degradant (IT-4) - however, these have been substantially reanalysed by the pesticide RMS using single first order (SFO), first order multi-compartment (FOMC), double first order in parallel (DFOP) and hockey stick (HS) kinetic models according to level FOCUS guidance (2006). Further detailed discussion of the best 'fits' and results for each phase, system and model are given at B.8.4.4 in the DAR.

In summary, the RMS calculated whole system DT_{50}s for amisulbrom of 64.2 days for the clay loam system and 156.1 days for the clay system - both based on HS kinetics and, as a worst case, on the slow phase rate constant, K2.

In the sediment of the clay loam system, the degradants IT-4 and IT-15 both occurred at levels ≥5% AR for two consecutive time points in the aqueous phase. IT-15 was the only degradant occurring at >10% AR in sediment (29.1% AR). In the whole clay loam system, IT-4 occurred at a maximum 14.9% AR and IT-15 occurred at a maximum 38.9% AR. In the clay system, only IT-4 occurred at levels ≥5% AR in the aqueous phase (max. 13.0% AR). In sediment, IT-4 occurred at a maximum of 14.6% AR, and IT-15 occurred at a maximum 12.2% AR. In the whole clay system IT-4 occurred at a maximum of 21.4% AR and IT-15 occurred at a maximum 17.6% AR. In each system, levels of IT-4 had declined by the end of the study but less so in the clay system. A statistically robust kinetic fit for IT-4 could only be determined for the clay loam system. The RMS calculated dissipation DT_{50}s for IT-4 to be 28.7 days (SFO) from the aqueous phase; 116.6 days (SFO) from the sediment phase and 58.9 days (SFO) for the whole clay loam system. Due to the lack of decline phase for IT-15 in either system, the RMS did not determine DT_{50}s for this degradant. A proposed degradation pathway for amisulbrom in natural water/sediment systems is given in Figure 1 in Annex 1.

5.1.3 Summary and discussion of degradation

Abiotic degradation

In a reliable aqueous hydrolysis study (Wicks, 2004a) amisulbrom was determined to be hydrolytically stable at neutral pH (degradation DT_{50} 87.1 days at pH 7 normalised to 20°C). However, hydrolysis was pH dependant and more rapid at pH 9 (DT_{50}: 7.0 days), although this pH is less environmentally relevant and amisulbrom would still be considered moderately stable. As the hydrolysis half life is not <16 days for all relevant pH, amisulbrom screens as not rapidly degradable.

In two reliable aquatic photodegradation studies (Takehara, 2004 and Wicks, 2004b) conducted at 25°C under natural light conditions equivalent to south-central EU in spring-summer, amisulbrom was shown to be photolytically degraded with DT_{50}s in sterilised river water at pH 6.7 of 4.2-4.4 hours and in buffered pH 4 solution of 4.9 hours. It is considered that photolysis could be a major mechanism of degradation for amisulbrom in surface waters under certain conditions; however this might not be broadly applicable to turbid natural surface waters in lower insolation regions of the EU.

Biotic degradation

In a reliable OECD 301B ready biodegradation study (Barnes, 2004) conducted at 22°C and pH7.5-7.8, no substantive degradation of amisulbrom was observed over 29 days. According to the criteria requiring ≥60% of the theoretical CO_2 production within 10 days of achieving 10% biodegradation, amisulbrom is considered to be not readily biodegradable under the conditions of this test.

In a reliable OECD 308 aerobic water/sediment study (Unsworth, 2004c) conducted at 20°C for 120 days (pH range 5.4-8.0), amisulbrom was observed to dissipate from the water column to sediment in both clay loam and clay systems (dissipation DT_{50}s 6.4-7.1 days). In the sediments, some degradation of amisulbrom plus dissipation back to the water and limited mineralization was noted (volatile radioactivity/mineralization only accounted for 1.2-1.3% AR by Day 120). The pesticide RMS calculated whole system DT_{50}s for amisulbrom of 64.2 days for the clay loam system and 156.1 days for the clay system (based on biphasic hockey-stick (HS) kinetics and slow phase rate constants, K_2).

In the water/sediment systems, the only degradants occurring at levels ≥5% AR were IT-4 and IT-15. In the whole systems, IT-4 occurred at a maximum 14.9-21.4% AR and IT-15 occurred at a maximum 17.6-38.9% AR. A whole system dissipation DT_{50} for IT-4 of 58.9 days could only be determined for the clay loam system. Due to the lack of decline phase for IT-15 in either system, DT_{50}s could not be determined for this degradant.

Overall, despite evidence of photolysis under certain aqueous conditions, the available degradation information does not indicate that amisulbrom is ultimately degraded (>70%) within 28 days (equivalent to a degradation half-life of <16 days). Neither is it transformed into entirely non-classifiable degradants, as the ecotoxicology data below (Section 5.4) indicate that key degradants such as IT-4 and IT-15 do exhibit some biological activity/ecotoxicity (although less so than the parent). Consequently, amisulbrom is considered to be 'not rapidly degradable' for the purposes of classification under the CLP Regulation.

5.2 Environmental distribution

5.2.1 Adsorption/Desorption

i) A reliable study (Unsworth, R, 2004b) was submitted to investigate the adsorption/desorption behaviour and mobility of amisulbrom in five soils. The study was conducted according to OECD guideline 106 (2000) and to GLP. Full methodological details and results are given at B.8.2.1 in the DAR. The soils ranged from pH 4.3 - 7.4 and 0.5-3.2% organic carbon, which the pesticide RMS considered representative of soils likely to be encountered in Europe. The Freundlich adsorption isotherm values, adjusted for soil organic carbon content (K_{FOC}), ranged from 8156 mL/g (pH 5.3) to 44231 mL/g (pH 4.3). K_F values were fairly consistent in all tested soils, regardless of pH or organic carbon content. K_{Foc} values were more variable due to a soil with low OC content. The RMS considered the median K_{Foc} value of 10487 mL/g to be appropriate for use in the pesticide exposure assessment since this was unaffected by the variability and there was no apparent effect of pH on overall adsorption.

ii) Another reliable study (Unsworth, R, 2005 – DAR B.8.2.1)) was submitted to investigate the adsorption/desorption behaviour and mobility of IT-4 (the main soil degradant occurring at levels >10% AR) in four soils. Since the classification is focussed on the parent substance amisulbrom, this is not discussed in detail here. K_{Foc} values for IT-4 adsorption ranged from 821 mL/g (soil pH

7.4) to 11402 mL/g (soil pH 4.3). There was clear evidence of pH-dependence, with higher K_{Foc} values correlating with lower pH values.

According to the results of these studies, amisulbrom has a high potential to adsorb to soil and sediments and can be considered immobile. The degradant IT-4 can be considered immobile but may exhibit low mobility in high pH soils.

5.2.2 Volatilisation

No studies on the volatility of amisulbrom have been submitted. The Notifier cited amisulbrom's vapour pressure of 1.8 x 10^{-8} Pa at 25ºC (classified as very slightly volatile), reasoning that amisulbrom would not be expected in any significant concentration in air. The pesticide RMS considered this argument to be satisfactory and no further studies on the volatility of amisulbrom were required.

5.2.3 Distribution modelling

Not submitted and not required for classification and labelling.

5.3 Aquatic Bioaccumulation

The Log K_{ow} of amisulbrom is 4.4 (at pH 6.4 and 25°C, 99.8% pure). Its dissociation constant was not tested as amisulbrom does not contain groups that ionise between pH 4-6 and pH 8-10 and therefore Log K_{ow} and solubility in water were not expected to be pH dependent.

A Log K_{ow} >4 can influence the chronic classification and M-factor under CLP unless a reliable fish bioconcentration factor (BCF) is available. A Log K_{ow} >3 also requires that an experimental fish bioconcentration study be conducted under pesticide regulations. Such a study has been submitted and is considered below at 5.3.2.

5.3.1 Bioaccumulation estimation

As experimental data are available, estimations of bioaccumulation potential are not included.

5.3.2 Measured bioaccumulation data

One study is available on the bioconcentration of amisulbrom in bluegill sunfish (*Lepomis macrochirus*) under flow-through conditions (2005 – DAR B.9.2.1.7)). The study was carried out according to OECD 305 and US EPA OPPTS 850.1730 (draft 1996) and to GLP. It is evaluated in detail at B.9.2.1.7 in Vol. 3 of the amisulbrom DAR.

Method

Groups of fish were exposed to radiolabelled amisulbrom for 14 days under flow-through conditions at nominal concentrations of 0.05 and 0.5 µg [triazole-^{14}C]amisulbrom/l (48 and 88 fish, respectively) and 0.5 µg [indole-^{14}C]amisulbrom/l (56 fish). Each had a radiochemical purity of >95%. Stock solutions were prepared in a dimethylformamide (DMF) and methanol solvent vehicle and were added directly into the dilution water flow. A solvent control group was also included.

After 14 days of exposure the remaining fish were transferred to untreated dilution water for a further 28 days under flow-through conditions to determine the depuration of the test substance and

metabolites from fish tissues. Observations of mortality and adverse effects were made daily and test media samples were taken daily during the uptake phase from Day -1 to 14 to determine total radioactivity. The concentration of amisulbrom in the water was also determined during the test.

Total radioactive residue (TRR), the concentration of amisulbrom and, where possible, its metabolites in fish were determined during the uptake and depuration phase for each treatment group. On each sampling occasion 4 fish were taken from each test vessel. On Day 14 of the uptake phase, 40 fish from both 0.5 µg radiolabelled treatment groups were sampled for the identification and quantification of metabolites. On Day 28 of the depuration phase (study termination) the remaining fish were weighed divided into edible and non-edible parts for analysis of residues.

The TRR of test media was determined by LSC and the radiopurity of samples was determined by TLC or HPLC. Sampled fish fractions were homogenised and rinsed with acetonitrile and the eventual filtrate was radioassayed by LSC. During the uptake and depuration phase, fish extracts were also analysed by HPLC. The presence of metabolites in fish samples was determined by HPLC and TLC. Non-extractable residues remaining in the fish were determined by combustion and subsequent radioassay of any released $^{14}CO_2$ by LSC. The lipid content was also determined in selected fish.

Temperature was measured daily in test solutions and continuously in the solvent control. Dissolved oxygen concentration and total organic carbon were determined at regular intervals. The pH was determined at the beginning and end of the uptake and depuration phase and on Day 24. Total hardness of the solvent control and 0.5 µg [triazole-^{14}C] amisulbrom/l and conductivity of dilution water were measured. The test area was illuminated at a light intensity of 250 to 470 lux.

Results

One fish in the 0.5 µg [indole-^{14}C]amisulbrom/l treatment group died during the depuration phase. No sub-lethal effects were observed during the study.

Analysis of concentrations in water:

Daily measurements of TRR generally remained within ±20% of the mean measured concentration for all test groups. Mean measured concentrations over the uptake phase were 108.3, 113.1 and 100.8% of nominal for 0.05 and 0.5 µg [triazole-^{14}C]amisulbrom/l, and 0.5 µg [indole-^{14}C]amisulbrom/l treatment groups, respectively. After the addition of the fish there was a temporary drop in the measured concentration due to immediate binding of amisulbrom to them. This was compensated by using stock solutions at 120% of nominal for 2 days.

Mean measured concentrations of amisulbrom in test media over the uptake phase were 0.0370, 0.370 and 0.379, for 0.5 and 0.05 µg [triazole-^{14}C]amisulbrom/l test media, and 0.5 µg/l in the [indole-^{14}C]amisulbrom test media, respectively.

On Day 1 of the depuration phase concentrations of 0.1 and 0.04 µg/l were detected in water of the 0.5 µg [triazole-^{14}C] and [indole-^{14}C]amisulbrom/l treatment groups, respectively. No radioactivity was detected in any of the test aquaria on Day 2. Following extraction, 14% of radioactivity was organosoluble and 86% of radioactivity remained in the aqueous fraction.

Analysis of concentrations in fish:

Uptake Phase

For the [triazole-^{14}C]amisulbrom treatment groups, mean concentrations of TRRs varied between 17.7-24.7 µg/kg in the 0.05 µg/l group, and 167-239 µg/kg in the 0.5 µg/l group. Concentrations in

fish treated at 0.5 µg/l were approximately 10 times greater than those exposed to 0.05 µg/l. The mean concentration in the [indole-^{14}C]amisulbrom treatment group was 143 µg/kg on Day 14.

For the [triazole-^{14}C]amisulbrom treatment groups, mean concentrations of amisulbrom varied between 2.36-6.52 µg a.s./kg in the 0.05 µg/l group, and 31.9-53.4 µg a.s./kg in the 0.5 µg/l group. The concentration in the [indole-^{14}C]amisulbrom treatment group was 43.8 µg a.s./kg on Day 14.

The mean lipid content in fish was 4.5%. It is not clear whether lipid normalization or growth correction was performed, however as the lipid content was close to 5% this is unlikely to significantly affect results or its relevance to classification. The short duration of the test also means growth is unlikely to be relevant as a depuration mechanism. Based on this, the maximum mean concentrations of amisulbrom in the 0.05 and 0.5 µg [triazole-^{14}C]amisulbrom/l treatment groups were 145 (Day 3) and 1187 µg a.s./kg (Day 12), respectively. The highest mean concentration in the [indole-^{14}C]amisulbrom treatment group was 973 µg a.s./kg on Day 14.

Steady State

Since the test substance was rapidly metabolised and excreted by the fish, no plateau value was obtained. As there was no increase in TRR or concentration of amisulbrom in fish between Day 12 and 14, it was concluded that a maximum concentration had been reached. Therefore, the uptake phase was terminated after 14 days.

Depuration Phase

For both low and high concentrations, rapid excretion of the radioactivity during the first 5 days of the depuration phase was followed by a slower excretion between Days 5 and 28. For the 0.05 µg [triazole-^{14}C]amisulbrom/l treatment group, the mean concentrations of TRRs on Day 1 and 28 (depuration phase) were 53.5 and 5.4% of the TRR found on Day 14 (uptake phase), respectively. For the 0.5 µg [triazole-^{14}C]amisulbrom/l treatment group, the mean concentrations of TRR on Day 1 and 28 were 73.4 and 9.3% of the TRR found on Day 14, respectively. The mean concentration in the [indole-^{14}C]amisulbrom treatment group on Day 28 was 5.6% of the TRR found on Day 14.

Analysis of edible and non-edible tissues on Day 28 showed no radioactivity in the extractable fraction of the edible tissues; radioactivity was mainly found in the non-extractable fraction of the non-edible tissues. As for the TRR, there was a rapid decline of the amisulbrom concentration in fish at the start of the depuration phase. On Day 1 the mean concentrations in the 0.05 and 0.5 µg [triazole-^{14}C]amisulbrom/l treatment groups were 7.3 and 16.1% of the concentration found on Day 14 (uptake phase), respectively. By Day 2 and 5 these had decreased to <2.7 and <0.2%, respectively, both <LOD of 0.1 µg amisulbrom/kg. The mean concentration in the [indole-^{14}C]amisulbrom treatment group on Day 28 was also <LOD.

Metabolism

The major metabolites (>10% TRR) found in fish extracts were IT-4 and a polar fraction with a retention time of approximately 3 min in both [triazole-^{14}C] and [indole-^{14}C]amisulbrom treatment groups (FT1 and FI1). The [triazole-^{14}C] polar fraction did not contain the metabolites T-3 or T-4, but consisted of three different fractions, one of which exceeded 10%. The [indole-^{14}C] polar fraction consisted of at least six different fractions, none of which exceeded 10%. All other degradates were detected at <10% TRR.

Bioconcentration factors (BCF):

BCFs based on measured TRR and amisulbrom found in fish are summarised in Table 27. The BCF of amisulbrom determined on Day 14 ranged from 100 to 141.

Table 27: BCF values in bluegill sunfish based on measured TRRs and amisulbrom

Treatment	Bioconcentration factor				
	Day 3	Day 8	Day 10	Day 12	Day 14
TRR					
0.05 µg [triazole-^{14}C] amisulbrom/l	431	457	394	327	373
0.5 µg [triazole-^{14}C] amisulbrom/l	423	416	400	350	296
0.5 µg [indole-^{14}C] amisulbrom/l	ND	ND	ND	ND	284
Amisulbrom					
0.05 µg [triazole-^{14}C] amisulbrom/l	176	144	119	63.8	100
0.5 µg [triazole-^{14}C] amisulbrom/l	86.2	120	108	144	132
0.5 µg [indole-^{14}C] amisulbrom/l	ND	ND	ND	ND	141

ND: The BCF was not determined until Day14 for the indole-labelled material

Conclusion

The worse case whole fish bioconcentration factor of amisulbrom in bluegill sunfish was 176 (or 457 based on TRR). Amisulbrom was readily metabolised by the fish. During the depuration phase its concentration in fish declined rapidly so that within 3 days it was below 1% of the concentration at the end of the uptake phase.

Major metabolites (>10% TRR) were IT-4 and an unidentified single polar fraction. All other metabolites were detected at <10% TRR. No radioactivity was detected in the extractable fraction of the edible tissues. It was mainly associated with the non-extractable fraction of the non-edible tissues.

5.3.3 Summary and discussion of aquatic bioaccumulation

The Log K_{ow} of amisulbrom is 4.4, this is greater than the trigger in the CLP Regulation of 4 which could influence the chronic aquatic classification for amisulbrom, particularly if adequate chronic data were not available. However, a reliable experimental fish bioconcentration factor (BCF) is available from the study by Van der Kolk (2005). The maximum whole fish BCF for amisulbrom in bluegill sunfish was 176 and there was rapid metabolism and depuration of the parent substance. Some radioactivity associated with metabolites of amisulbrom was found mainly, at low levels, in the non-extractable fraction of the non-edible tissues.

The maximum fish BCF value for amisulbrom (inc. TRR) is less than the trigger of 500 in the CLP regulation requiring consideration of the impact of bioconcentration on its chronic classification. However, since amisulbrom is ‘not rapidly degradable’ and adequate chronic data are available, this would not in any case affect the decision on chronic classification and M-factor.

5.4 Aquatic toxicity

Toxicity studies are available for fish, aquatic invertebrates, sediment-dwelling organisms and algae on amisulbrom and its main degradants IT-4 and IT-15. Although I-1 was a major hydrolysis degradant it was not considered major or relavant in natural water/sediment systems and so no ecotoxicological studies were conducted on it. These studies are evaluated in detail at Section B.9.2, Vol. 3 of the pesticide DAR for amisulbrom. They are summarized in the following table and additional detail on the key studies considered for classification purposes is given further below.

Table 28: Summary of relevant information on aquatic toxicity

Organism group/ species	Time-scale, guideline and GLP status	Test substance	End point	Toxicity level* (mg/l)	Reference[#]
Fish					
Cyprinus carpio	**Acute** 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	**0.0229**	2003c (DAR B.9.2.1.1.1(v))
Pimephales promelas	Acute 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	0.0363	2003b (DAR B.9.2.1.1.1(iv))
Lepomis macrochirus	Acute 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	0.0407	2004b (DAR B.9.2.1.1.1(iii))
Oncorhynchus mykiss	Acute 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	0.0515	2003a (DAR B.9.2.1.1.1(i))
Danio rerio	Acute 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	0.12	2006 (DAR B.9.2.1.1.1(vi))
Gasterosteus aculeatus	Acute 96 hr flow-through, OECD 203, GLP	amisulbrom	Mortality, LC_{50}	0.17	2004a (DAR B.9.2.1.1.1(ii))
Pimephales promelas	**Chronic** FELS, 28 d flow-through, OECD 210, GLP	amisulbrom	Growth, NOEC	**0.037**	2005a (DAR B.9.2.1.4.1(i))
Cyprinus carpio	Acute 96 hr semi-static, OECD 203, GLP	IT-4	Mortality, LC_{50}	0.232	2005a (DAR B.9.2.1.2.1(i))
Pimephales promelas	Chronic FELS, 28 d flow-through, OECD 210, GLP	IT-4	Growth, NOEC	0.16	2008 (DAR B.9.2.1.5.1(i))
Cyprinus carpio	Acute 96 hr semi-static, OECD 203, GLP	IT-15	Mortality, LC_{50}	11.0	2005b (DAR B.9.2.1.2.1(ii))

Organism group/ species	Time-scale, guideline and GLP status	Test substance	End point	Toxicity level* (mg/l)	Reference#
Aquatic invertebrate					
Daphnia magna	**Acute** 48 hr static, OECD 202, GLP	amisulbrom	Mortality, EC_{50}	**0.0368**	Jenkins, 2003d (DAR B.9.2.1.1.2(i))
Daphnia magna	**Chronic** 21 d semi-static, OECD 211, GLP	amisulbrom	Reproduction, mortality, NOEC	**0.0197**	Jenkins, 2004c (DAR B.9.2.1.4.1(i))
Daphnia magna	Acute 48 hr static, OECD 202, GLP	IT-4	Mortality, EC_{50}	4.39	Jenkins, 2005c (DAR B.9.2.1.2.2(i))
Daphnia magna	Acute 48 hr static, OECD 202, GLP	IT-15	Mortality, EC_{50}	22	Jenkins, 2005d (DAR B.9.2.1.2.2(ii))
Sediment-dwelling organisms					
Chironomus riparius	28 d static, OECD 219, spiked-water study	amisulbrom	Development and emergence, NOEC	**0.1114**	Cockroft, 2005b (DAR B.9.2.1.6(i))
Algae					
Pseudokirchneriella subcapitata	96 hr static, OECD 201, GLP	amisulbrom	Growth rate: 72 hr E_rC_{50}: 96 hr E_rC_{50}: NOE_rC:	 **0.0521** **0.057** **0.0139**	Jenkins, 2003e (DAR B.9.2.1.1.3(ii))
Pseudokirchneriella subcapitata	72 hr static, OECD 201, GLP	IT-4	Growth rate: E_rC_{50}: NOE_rC:	 3.28 0.467	Jenkins, 2005e (DAR B.9.2.1.2.3(i))
Pseudokirchneriella subcapitata	72 hr static, OECD 201, GLP	IT-15	Growth rate: E_rC_{50}: NOE_rC:	 24.9 2.16	Jenkins, 2005f (DAR B.9.2.1.2.3(ii))

* Concentrations are in terms of measured concentrations

Study references, e.g. Jenkins, 2003b or c, follow the numbering system used in the amisulbron DAR for consistency and ease of reference

Figures in **bold** are key endpoints for each group considered for acute and chronic aquatic hazard classification

5.4.1 Fish

5.4.1.1 Short-term toxicity to fish

The lowest acute fish toxicity endpoint is derived from a study on common carp. The next most acutely sensitive species is fathead minnow. As fathead minnow was also used for chronic testing of amisulbrom, both the carp and minnow acute studies are summarised below in greater detail:

i) A study is available on the acute toxicity of amisulbrom (98.6% pure) to common carp (*Cyprinus carpio*) (2003c). The study was carried out according to guidelines EU 92/69/EEC, C.1 (1992), OECD 203 (1992), JMAFF 12 Nohsan no. 8147, 2-7-1 (2000) and in compliance with GLP.

Method

Groups of seven juvenile common carp were exposed to amisulbrom for 96 hours under flow-through conditions at nominal concentrations of 5.98, 13.1, 28.9, 63.6 and 140 µg/l. Negative and solvent (DMF at 0.1 ml/l) control groups were also included.

Test aquaria received 18.4 to 20.4 volume replacements every 24 hours. Fish were not fed for 26 hours prior to exposure. Observations were made on mortality and the incidence and type of any sub-lethal effects at approximately 2, 4, 24, 48, 72 and 96 hours after the start of the study. Daily records of pH, temperature and dissolved oxygen were kept for each control and test vessel together with measurements of total hardness for selected vessels at 0 hours. Temperature in the water control vessel was continuously monitored during the study

Test concentrations were verified by HPLC using spectrophotometric detection. Samples were taken from each control and test vessel immediately prior to fish introduction and at 48 and 96 hours.

Results

Overall geometric mean measured levels of amisulbrom were 5.04, 12.1, 24.5, 62.8 and 122 µg/l. These ranged from 84 to 99% of the nominal concentrations, but although nominal concentrations were acceptable, mean measured concentrations were used. Environmental parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable limits throughout the duration of the study. At all test concentrations, the test media were clear and colourless

Based on measured levels, no sub-lethal effects occurred at concentrations up to and including 12.1 µg/l. At 24.5 µg/l and above, symptoms of toxicity comprised lethargy, effects on respiration and swimming and loss of co-ordination.

No mortality occurred in any control groups and at concentrations up to and including a measured 12.1 µg/l of amisulbrom. At 24.5 µg/l, mortalities were first recorded at 96 hours (57%). At 62.8 µg/l, mortalities were first recorded at 48 hours (29%) and by 72 hours, all fish were dead. In fish exposed to 122 µg/l, mortalities were first recorded at 48 hours (100%). The mortality results are summarized in table 29.

Table 29: Cumulative mortality of *Cyprinus carpio* exposed to amisulbrom over 96 hours

Amisulbrom concentration (µg a.s./l)		**Cumulative mortality (initial population = 7 fish)**			
Nominal	Measured	24 hour	48 hour	72 hour	96 hour
0	nd	0	0	0	0
0S	nd	0	0	0	0
5.98	5.04	0	0	0	0
13.1	12.1	0	0	0	0
28.9	24.5	0	0	0	4
63.3	62.8	0	2	7	7
140	122	0	7	7	7

nd = none detected
0S = solvent control

Conclusion

Based on measured concentrations, the acute 96-hour LC_{50} of amisulbrom to common carp (*Cyprinus carpio*) was 22.9 µg/l (95% confidence limits: 12.1 and 62.8 µg/l). This corresponds to 0.0229 mg amisulbrom/l. The acute NOEC was 12.1 µg/l (0.0121 mg/l).

ii) A study is available on the acute toxicity of amisulbrom (98.6% pure) to fathead minnow (*Pimephales promelas*) (2003b). The study was carried out according to guidelines EU 92/69/EEC, C.1 (1992), OECD 203 (1992), JMAFF 12 Nohsan no. 8147, 2-7-1 (2000) and in compliance with GLP.

Method

Groups of 20 juvenile fathead minnow were exposed to amisulbrom for 96 hours under flow-through conditions at nominal concentrations of 2.72, 5.98, 13.1, 28.9, 63.6 and 140 µg/l. Negative and solvent (DMF at 0.1 ml/l) control groups were also included.

Based on range finding tests, duplicate groups of 10 Fathead minnow were exposed to amisulbrom for 96 hours under flow-through conditions at nominal concentrations of 2.72, 5.98, 13.1, 28.9, 63.6 and 140 µg/l. Test aquaria received 11.4 to 13.7 volume replacements every 24 hours. Fish were not fed 48 hours prior to exposure. Observations were made on mortality and the incidence and type of any sub-lethal effects at approximately 15 minutes, 3, 6, 24, 48, 72 and 96 hours after the start of the study. Daily records of pH, temperature and dissolved oxygen were kept for each control and test vessel together with measurements of total hardness for selected vessels at 0 hours. Temperature of the water in one of the control vessels was continuously monitored during the study.

Test concentrations were verified by HPLC using spectrophotometric detection. Samples were taken from each control and test vessel immediately prior to fish introduction and at 48 and 96 hours.

Results

Overall geometric mean measured levels of amisulbrom were 2.11, 4.81, 9.96, 24.7, 59.9 and 116 µg/l. These ranged from 76 to 94% of nominal concentrations, so mean measured concentrations were used. Environmental parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable limits throughout the duration of the study. At all test concentrations, the test media were clear and colourless.

Based on measured levels, no significant sub-lethal effects occurred at concentrations up to and including 4.81 µg/l. At 9.96 µg/l and above, symptoms of toxicity comprised lethargy, effects on respiration, haemorrhaging, nervous/erratic swimming and loss of co-ordination.

No mortality occurred at concentrations up to and including a measured 9.96 µg/l of amisulbrom. At 24.7 µg/l, mortality was first recorded at 96 hours (5%). At 59.9 µg/l, mortalities were first recorded at 48 hours (35%). By 96 hours, 100% mortality had occurred. When fish were exposed to 116 µg/l, mortalities were first recorded at 24 hours (55%) and by 48 hours, all fish were dead. The mortality results are summarized in table 30.

Table 30: Cumulative mortality of *Pimephales promelas* exposed to amisulbrom over 96 hours

Amisulbrom concentration (µg a.s./l)		Cumulative mortality (initial population = 7 fish)			
Nominal	Measured	24 hour	48 hour	72 hour	96 hour
0	nd	0	0	0	0
0S	nd	0	0	0	0
2.72	2.11	0	0	0	0
5.98	4.81	0	0	0	0
13.1	9.96	0	0	0	0
28.9	24.7	0	0	0	1
63.6	59.9	0	7	19	20
140	116	11	20	20	20

nd = none detected
0S = solvent control

Conclusion

Based on measured concentrations, the acute 96-hour LC_{50} of amisulbrom to fathead minnow was 36.3 µg/l (95% confidence limits: 24.7 and 59.9 µg/l). This corresponds to 0.0363 mg amisulbrom/l. The acute NOEC was 4.81 µg/l (0.00481 mg/l).

Acute studies on additional fish species:

Four other reliable acute fish toxicity studies on amisulbrom (98.6% pure) have been undertaken on bluegill sunfish (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*), zebra fish (*Danio rerio*) and three-spined stickleback (*Gasterosteus aculeatus*). These studies reported acute 96 hr LC_{50} values of 0.0407 to 0.17 mg amisulbrom/l (see table 28). They were all undertaken to OECD 203 and GLP and were performed and reported by the same study author in an almost identical manner to the above studies on the most sensitive fish species. As these are considered supporting data, they have not, therefore, been reported here in detail but they are evaluated in full at B.9.2.1.1.1 in the amisulbrom DAR.

5.4.1.2 Long-term toxicity to fish

A fish early life stage study has been submitted to determine the chronic toxicity of amisulbrom (98.6% pure) to fathead minnow (*Pimephales promelas*). The study was carried out according to US EPA OPPTS 850.1400 (1996), OECD 210 (1992) and to GLP.

Method

Groups of approximately 60 newly fertilised fathead minnow eggs were exposed to amisulbrom under flow-through conditions at nominal concentrations of 1.1, 2.6, 6.3, 15 and 36 µg/l. Developing embryos were exposed for five days pre-hatch and 28 days post-hatch. Control groups, each also comprising approximately 60 newly fertilised eggs, were placed into diluent water alone or diluent water containing DMF solvent (0.05 ml/l). Test aquaria held approximately 8.8 litres of exposure medium and had renewal rates of 110 ml/min. Fish were fed twice daily during exposure.

The number of healthy and dead larvae together with the number of dead eggs was recorded daily. Post-hatch, the incidence and type of sub-lethal effects, as well as mortality was recorded on a daily basis. At study termination all surviving larvae were sacrificed. The length and dry weight of each

individual fish was subsequently recorded. Daily records were taken of pH, temperature, dissolved oxygen, total hardness, as well as total and free chlorine.

Results

Measured concentrations of amisulbrom were 0.96, 2.2, 5.7, 15 and 37 µg/l, equivalent to 87, 85, 90, 100 and 103% of nominal respectively, but although nominal concentrations were acceptable, mean measured concentrations were used. Environmental parameters (pH, temperature, dissolved oxygen and total hardness) remained within acceptable limits throughout the duration of the study.

Hatching success:

Hatching commenced on the third day after initiation of the study with 3.3% of eggs in the 15 µg/l level having hatched successfully. By Day 0 (post-hatch), hatching was complete for all egg chambers. Hatching success ranged from 90-97% across all test levels. Embryo development therefore appeared to be unaffected by the presence of amisulbrom. Table 31 shows the hatching success. Hatching success was 96.7% and post-hatch survival rate was 91.4% in the solvent control group. Therefore, the study validity criteria were met.

Post-hatch survival:

No significant mortality was observed in any test concentration or solvent control. Based on measured concentrations, the 28-day LC_{50} and NOEC values for amisulbrom were calculated to be >37 and 37 µg/l, respectively. Table 31 also shows the percentage survival from day 0 to day 28 post hatch.

Table 31: Percentage survival of fry exposed to amisulbrom from day 0 to day 28 post hatch

Nominal conc. (µg/L)	Measured conc. (µg/L)	Starting number of eggs	Number of hatched larvae	Number of surviving fry at d28	Post hatch survival to d28 (%)	Overall survival to d28 (%)
0	NS	60	57	55	96.5	91.7
0S	ND	60	58	53	91.4	88.3
1.1	0.96	60	56	51	91.1	85.0
2.6	2.2	60	54	50	92.6	83.3
6.3	5.7	60	54	50	92.6	83.3
15	15	60	55	53	96.4	88.3
36	37	60	55	53	96.4	88.3

ND = None detected
NS = Not sampled
0S = solvent control

Sub-lethal observations:

Observed sub-lethal effects were considered to be negligible. Very few individual larvae were noted as being lethargic or exhibited reduced size. Sub-lethal observations declined in frequency towards the end of the exposure period, with only one fish at 15 µg/l exhibiting reduced size from Day 10 post-hatch to termination. All sub-lethal observations were within acceptable limits for background occurrence and so were not considered to have resulted from exposure to amisulbrom.

Weight and length:

Weight and length of fish were unaffected by amisulbrom. The NOEC for both weight and length was calculated to be 37 µg/l. The mean weight and length parameters for surviving fry at day 28 are shown in table 32.

Table 32: Mean weight and length for surviving fry exposed to amisulbrom for 28 days

Nominal conc. (µg/L)	Measured conc. (µg/L)	Length (mm)		Dry weight (mg)	
		mean	sd	mean	sd
0	NS	16.3	1.8	5.39	1.81
0S	ND	16.9	2.4	7.19	3.24
1.1	0.96	18.0	2.0	8.99	2.93
2.6	2.2	18.5	2.2	9.08	3.70
6.3	5.7	18.8	2.0	9.14	3.36
15	15	18.4	2.2	9.31	3.61
36	37	17.2	2.3	6.47	2.57

ND = None detected
NS = Not sampled
0S = solvent control
sd = standard deviation

Conclusion

Based on measured concentrations, the 28-day NOEC for post-hatch survival, length and dry weight for newly fertilised fathead minnow fry exposed to amisulbrom was 37 µg/l, this corresponds to 0.037 mg amisulbrom/l.

5.4.1.3 Toxicity of degradants to fish

Two studies have been submitted on the acute toxicity of the amisulbrom degradants IT-4 and IT-15 to common carp (*Cyprinus carpio*) (2005a/b). One fish early life stage (FELS) test has also been submitted on the chronic toxicity of IT-4 to fathead minnow (*Pimephales promelas*). These studies were undertaken according to relevant OECD guidelines and to GLP and are considered reliable. They reported acute 96 hr LC_{50} values of 0.232 and 11.0 mg/l for IT-4 and IT-15 respectively and a chronic NOEC for IT-4 of 0.16 mg/l (see table 28). It is appropriate that these were conducted on the same most acutely and chronically sensitive fish species tested with the parent compound. The data indicate that IT-4 and IT-15 are less toxic to fish than the parent amisulbrom. As they are considered supporting data, they have not been reported here in detail but they are evaluated in full in section B.9.2 of the amisulbrom DAR.

5.4.2 Aquatic invertebrates

5.4.2.1 Short-term toxicity to aquatic invertebrates

A study has been submitted on the acute toxicity of amisulbrom (98.6% pure) to *Daphnia magna* (Jenkins, C. A., 2003d). This was carried out according to EU 92/69/EEC, C.2 (1992), OECD 202 (1984), US EPA OPPTS 850.1010 (1996), JMAFF 12 Nohsan no. 8147, 2-7-2 (2000) and to GLP.

Method

Groups of 20 first instar *Daphnia magna* were exposed to amisulbrom for 48 hours under static conditions at nominal concentrations of 10, 20, 40, 80 and 160 µg/l.

Four replicate groups of 5 first instar *Daphnia* were exposed to amisulbrom for 48 hours under static conditions at nominal concentrations of 10, 20, 40, 80 and 160 µg/l. A negative control and a solvent control (DMF at 0.1 ml/l) group were also included. Test vessels contained 125 ml of medium and the daphnids were not fed during exposure.

The numbers of mobile, immobile and floating *Daphnia* were counted approximately 24 and 48 hours after the start of the study. The pH, temperature and dissolved oxygen of control and test media were recorded at the start and at the end of the test. Total hardness and alkalinity of the dilution medium was checked before use.

Test concentrations were verified by HPLC using spectrophotometric detection. Samples were taken from freshly prepared control and test media at the start of the test and again from each test vessel at 48 hours.

Results

In freshly prepared media measured concentrations ranged from 81-107% of nominal. In expired media, (48 hours post preparation), concentrations were 91-106% of nominal at 10, 20 and 40 µg/l respectively, and between 47 and 74% of nominal at 80 and 160 µg/l respectively. The decrease in the measured concentrations at the two highest test levels suggested that the limit of aqueous solubility of amisulbrom had been exceeded. Overall geometric mean measured levels of amisulbrom were 10.3, 19.6, 38.3, 65.3 and 104 µg/l and these mean measured concentrations were used to describe the endpoints.

Environmental parameters (pH, temperature, dissolved oxygen, total hardness and alkalinity) remained within acceptable limits throughout the duration of the study. At all test concentrations, the test media were clear and colourless.

There was no effect on immobilisation at concentrations up to and including 19.6 µg/l. At 38.3 and 65.3 µg/l, immobilisation was 0% after 24 hours, and 70 and 80% after 48 hours. At 104 µg/l, immobilisation was 25% after 24 hours, and 95% after 48 hours. No immobilisation occurred in dilution media or solvent control groups. The immobilisation results are summarised in table 33.

Table 33: Cumulative immobilisation of *Daphnia magna* exposed to amisulbrom over 48 hours

Amisulbrom concentration (µg a.s./L)		**Cumulative mortality (initial population = 20 daphnids)**	
Nominal	Measured	24 hour	48 hour
0	nd	0	0
0S	*	0	0
10	10.3	0	0
20	19.6	0	0
40	38.3	0	14
80	65.3	0	16
160	104	5	19

nd = none detected
OS = solvent control
* Measured concentration was estimated to be ca. 4 µg/L

The 48-hour EC50 value was 36.8 µg/l (95% confidence limits: 31.0 and 43.6 µg/l). The NOEC was 19.6 µg/l.

Conclusion

Based on mean measured concentrations, the 48-hour EC_{50} of amisulbrom to *Daphnia magna* was 36.8 µg/l. This corresponds to 0.0368 mg amisulbrom/l. The NOEC was 19.6 µg/l (0.0196 mg/l).

5.4.2.2 Long-term toxicity to aquatic invertebrates

A study has been submitted on the chronic toxicity of amisulbrom (98.6% pure) to *Daphnia magna* (Jenkins, C A, 2004c). This was carried out according to OECD 211 (1998), US EPA OPPTS 850.1300 (1996), JMAFF 12 Nohsan no. 8147, 2-7-2-2 (2001) and to GLP.

Method

Groups of 10, individually-housed first instar *Daphnia magna* were exposed to amisulbrom for 21 days under semi-static conditions at nominal concentrations of 3.13, 6.25, 12.5, 25, 50 and 100 µg/l. Control groups, also comprising 10 *Daphnia*, were placed into dilution media alone or dilution media containing DMF (0.1 ml/l). The medium within test vessels was renewed on days 3, 6, 8, 10, 13, 15, 17 and 19. *Daphnia* were fed suspensions of *Chlorella vulgaris* algae on a daily basis. Amisulbrom concentrations in fresh and expired control and test media were verified by HPLC using spectrophotometric detection.

The numbers of mobile, immobile, dead and gravid parental *Daphnia* were recorded daily, together with observations on size and appearance. The number of moults produced by the parental generation was also recorded throughout the test. From Day 8, the numbers of live and dead neonates and the presence of unhatched eggs were recorded daily. The pH, temperature, total hardness and dissolved oxygen of freshly prepared control and test media were determined at the start of the test and at each media renewal.

Results

Measured concentrations in freshly prepared media were 81-118% of nominal, except at the lowest concentration (3.13 µg/l), which ranged between 107 and 135% of nominal. In expired media, measured levels ranged from 48-103% of initial values. Losses were primarily attributed to adsorption onto neonates and algal cells. Overall time-weighted mean measured levels of amisulbrom were 2.98, 5.59, 10.5, 19.7, 41.3 and 79.6 µg/l, these mean measured levels were used to describe the endpoints.

Environmental parameters (pH, temperature, dissolved oxygen, total hardness and alkalinity) remained within acceptable limits throughout the duration of the study. At all test concentrations, the test media were clear and colourless on the days of preparation.

Mortality:

The numbers of dead parental *Daphnia* observed during the test are summarised in table 34.

Table 34: Summary of survival of parental *Daphnia magna* following exposure amisulbrom

Observation time (day)	% cumulative mortality / measured amisulbrom concentrations (µg a.s./l)							
	0	0S	2.98	5.59	10.5	19.7	41.3	79.6
7	0	0	0	0	0	0	0	20
14	0	0	10	20	30	10	10	50
21	10	0	10	20	40	20	50	80

0S: Solvent control

Since only 20% mortality occurred at 5.59 and 19.7 µg/l at 21 days (which is the limit of acceptable mortality in the controls), the higher level of mortality (40%) noted at 10.5 µg/l was considered unlikely to have occurred solely due to exposure to amisulbrom. Therefore, statistical analysis excluded this group. The NOEC for adult mortality was therefore determined to be 19.7 µg/l.

Sub-lethal observations:

There was no significant difference in adult body length between adults exposed to amisulbrom at any concentration and those of the solvent control. There was no evidence of any treatment-related effect on moulting or other growth effects. Therefore the NOEC for body length and moulting was determined to be 79.6 µg/l, the highest concentration tested.

Reproduction:

All adults were gravid between Days 5 and 6, and produced first broods between Days 8 and 10. There was no biologically significant effect on the time to production of the first brood. There was a significant reduction in live neonate production at 41.3 and 79.6 µg/l compared to the solvent control. There was no significant difference at concentrations up to an including 19.7 µg/l.

There were no statistically significant effects on live neonates produced per adult at up to 19.7 µg/l. At 41.3 and 79.6 µg/l, significant numbers of dead neonates were recorded. Dead neonates were first recorded between Days 14 and 17. Neonate production is shown in table 35.

Table 35: Summary of cumulative total numbers of live neonates produced by parental *Daphnia magna* following exposure amisulbrom

Measured amisulbrom concentration (µg/l)	Total number live neonates / adults	Standard deviation
Control	109	± 9.6
0S	102	± 6.9
2.98	107	± 10.1
5.59	101	± 8.46
10.5	108	± 10.7
19.7	101	± 15.9
41.3	86.4*	± 11.7
79.6	80.5*	± 4.95

0S: Solvent control
*: $p<0.05$

Conclusion

The results for mortality are not clear cut. There is 20% mortality at 5.59 and 19.7 µg/l, which is within the level of mortality considered acceptable for this method. There was also 40% mortality seen at the 10.5 µg/l treatment level. This does not follow a dose response, since mortality was only 20% at the next highest treatment level. When the other measured parameters are considered there are no indications of other effects at 19.7 µg/l and below. Therefore, it was considered that the mortality at 10.5 µg/l is a result of chance and is not treatment-related.

Overall, based on time-weighted mean measured concentrations, the 21-day NOEC of amisulbrom to *Daphna magna* for both adult mortality and reproduction was determined to be 19.7 µg/l. This corresponds to 0.0197 mg amisulbrom/l.

Toxicity of degradants to aquatic invertebrates

Two studies have been submitted on the acute toxicity of the amisulbrom degradants IT-4 and IT-15 to *Daphnia magna* (Jenkins, 2005c/d). These studies were undertaken according to OECD 202 and to GLP and are considered reliable. The reported acute 48 hr EC_{50} values were 4.39 and 22.0 mg/l for IT-4 and IT-15 respectively (see table 28) indicating that IT-4 and IT-15 are less toxic to *Daphnia* than the parent amisulbrom. They are, therefore, considered supporting data and have not been reported here in detail but they are evaluated in full in section B.9.2 of the amisulbrom DAR.

5.4.3 Algae and aquatic plants

An algal growth inhibition test has been conducted on *Pseudokirchneriella subcapitata* using amisulbrom (98.6% pure) (Jenkins, C. A., 2003e). It was performed according to EU 92/69/EEC, C.3 (1992), OECD 201 (1984), US EPA OPPTS 850.5400 (1996), JMAFF 12 Nohsan no. 8147, 2-7-3 (2000) and to GLP.

Method

Three replicate cultures of *Pseudokirchneriella subcapitata* (initial cell density 1 x 10^4/ml) were exposed to amisulbrom for 96 hours without medium renewal, at nominal concentrations of 5, 10, 20, 40, 80 and 160 µg/l. A negative control group and a solvent control (0.1 ml/l DMF) group were also included. The extent of adsorption/absorption of the test substance by algal cells was determined. A supplementary replicate of each test concentration was prepared for chemical analysis at 48 hours. Cell densities were estimated at 0, 24, 48, 72 and 96 hours. The presence of any abnormal cells was noted during counting. In addition, any algistatic/algicidal activity was assessed in fresh medium after 3 and 5 days using cultures exposed to the highest test concentration (160 µg/l).

Temperature and pH of control and test media was recorded at the start and end of the test. The temperature in the incubator was continuously monitored during the study. The light intensity within the incubator was measured daily. Test concentrations were verified by HPLC using spectrophotometric detection. Samples were taken from control and test media at the start of the test and at 48 and 96 hours. At 0 and 96 hours, samples containing no algal cells were assessed.

Results

Measured concentrations of amisulbrom ranged from 68 to 82% of nominal at the start of the test and 54 to 71% of nominal after 96 hours. At nominal concentrations of 5 to 80 µg/l, losses of amisulbrom were attributed to adsorption/absorption by algal cells but may also relate to the photolysis noted in Section 5.1.1. At 160 µg/l, where the mean measured concentration decreased from 60% to 32% of nominal after 96 hours, the limit of aqueous solubility of amisulbrom had been exceeded and there was some precipitation of test substance. Overall geometric mean measured levels of amisulbrom were 3.39, 6.97, 13.9, 27.6, 49.6 and 69.8 µg/l and these were used to describe the effect levels.

Growth rate was significantly reduced at nominal concentrations of 40 µg/l and above (see table 36). The area under the growth curve and the average specific growth rate were calculated using the geometric mean measured concentrations.

Table 36: Mean cell densities, percentage inhibition of cell growth and percentage reduction in growth rate of *P. subcapitata* when exposed to amisulbrom for 96 hours

Nominal conc. (µg/l)	Measured conc. (µg/l)	Mean cell density (10^4 cells/ml)				Inhibition of growth			
		24 hrs	48 hrs	72 hrs	96 hrs	Area[a]	% inhibition	Rate[b]	% inhibition
0	-	3.26	12.4	51.4	154	3378	-	5.248	-
0S	-	3.30	12.2	47.6	144	3160	-	5.165	-
5	3.39	3.15	12.4	51.9	147	3295	0	5.195	0
10	6.97	2.55	10.5	47.3	138	3013	5	5.125	1
20	13.9	3.02	9.70	42.5	111	2569	19	4.902	5
40	27.6	2.53	5.59	19.2	56.3	1246*	61*	4.143*	20*
80	49.6	1.32	2.71	9.90	23.1	526.9*	83*	3.146*	39*
160	69.8	1.40	1.42	3.25	6.34	137.6*	96*	1.858*	64*

[a]: Mean area under the curve at 96 hours
[b]: Mean growth rate 0-96 hours
*: $p<0.05$
0S: Solvent control
Note: Calculations were made assuming an initial cell density of 1 x 10^4 cells/ml
-: Not applicable

Environmental parameters (pH and temperature) remained within acceptable limits throughout the duration of the study.

No microscopic abnormalities were detected at concentrations up to and including 40 µg/l. At 80 and 160 µg/l, algal cells were swollen compared to those in the controls. At 160 µg/l (69.8 µg/l measured), amisulbrom was algistatic as algae exposed to this highest test concentration re-established growth after five days of incubation in fresh media.

Conclusion

Based on mean measured concentrations, the E_rC_{50} (0-72 hr) of amisulbrom to *Pseudokirchneriella subcapitata* was 52.1 µg/l (95% confidence limits: 46.3 and 59.8 µg/l). The E_rC_{50} (0-96 hr) was 57.0 µg/l (95% confidence limits: 50.4 and 67.2 µg/l). The lower 72-hr growth rate endpoint corresponds to 0.0521 mg amisulbrom/l; this was not presented in the DAR where a lower biomass E_bC_{50} was used but this is not standard practice for classification. The mean measured NOEC for both biomass and growth rate was 13.9 µg/l (0.0139 mg/l).

Toxicity of degradants to algae

Two studies have been submitted on the toxicity of the amisulbrom degradants IT-4 and IT-15 to *Pseudokirchneriella subcapitata* (Jenkins, 2005e/f). These studies were undertaken according to OECD 201 and to GLP and are considered reliable. For IT-4 and IT-15 respectively, the reported 72 hr E_rC_{50} values were 3.28 and 24.9 mg/l and NOE_rC values were 0.467 and 2.16 mg/l, respectively (see table 28). These data indicate that IT-4 and IT-15 are less toxic to algae than the parent amisulbrom. They are, therefore, considered supporting data and have not been reported in detail here but they are evaluated in full in section B.9.2 of the amisulbrom DAR.

5.4.4 Other aquatic organisms (including sediment)

5.4.4.1 Toxicity of amisulbrom to sediment-dwelling organisms

The toxicity of amisulbrom to the sediment-dwelling phase of the midge *Chironomus riparius* was assessed according to OECD 219 (draft 2001) and to GLP. This was the spiked-water variant of the test and it was conducted using [triazole-^{14}C]amisulbrom with radiochemical purity >98%.

Method

Groups of 20 first instar *Chironomus riparius* were exposed for 28 days, without medium renewal, to nominal concentrations of ^{14}C-amisulbrom of 6.9, 14, 28, 55 and 110 µg a.s./l applied to the water column in a water/sediment system. An artificial sediment (in accordance with OECD 219) was used, comprising 5% *Sphagnum* peat, 20% kaolin clay and 75% acid-washed fine sand and this was adjusted to pH 6.7. Negative control and solvent control (DMF 0.1 ml/l) groups were also included. Destructive samples were prepared for regular analysis of radioactivity in overlying water, sediment and pore water. The radioactivity in dosing solutions was determined by LSC.

Larvae were fed daily, test vessels were aerated and pH, temperature, dissolved oxygen, water hardness and ammonia content were assessed during or at the beginning and end of the test.

Chironomid growth and development was monitored daily. From Day 13, when the first midges emerged, adults were sexed and removed from the vessels. The 28-day percentage emergence success was calculated for each treatment.

Results

The measured applied concentrations of ^{14}C-amisulbrom were 6.9, 13.9, 28.5, 56.8 and 111.4 µg/l. Concentration of ^{14}C- amisulbrom in overlying water decreased from 88.9-99.6% AR on Day 0 to 16.5-20.4% AR on Day 28. The concentration of ^{14}C- amisulbrom in the sediment increased from 7.8-9.4% AR on Day 0 to 67.3-73.9% AR on Day 28. Radioactivity in pore water accounted for ≤0.3% AR throughout the study. In all fractions, total radioactivity on Day 28 accounted for 85.8 to 96.5% AR.

Temperature, DO, hardness and ammonia remained within acceptable limits throughout the duration of the study. The pH of overlying water remained within the limits of 6 to 9 on Days 0 and 7. From Day 14, a number of vessels gave pH measurements of <pH6 (lowest pH 4.6), this is not considered to have affected the results.

Mean emergence from control and treatment groups were 96.6 to 99.0% and 92.8 to 99.7% respectively. There was no effect on the development rate and sex ratio of emerged midges from any group. The results are summarised in table 37.

Table 37: Emergence and development rate of *Chironomus riparius* exposed for 28-days to amisulbrom added to the water phase of a water/sediment system.

Actual applied concentration (µg a.s./l)	Mean % emergence	Mean development rate (day-1)
Solvent control	96.6	0.0605
Control	99.0	0.0629
6.9	96.8	0.0598
13.9	92.8	0.0607
28.5	99.7	0.0620
56.8	96.3	0.0625
111.4	98.1	0.0623

EC_{50} values for emergence and development rate could not be derived because no test level showed significantly reduced emergence or delayed development (they would be >111.4 µg a.s./l). Based on measured applied concentrations and the lack of treatment-related effects at the highest level, the NOEC for emergence and development rate was 111.4 µg amisulbrom/l. This corresponds to 0.1114 mg amisulbrom/l.

5.4.4.2 Toxicity of degradants to sediment-dwelling organisms

Two reliable studies are available on the chronic toxicity of the amisulbrom degradants IT-4 and IT-15 to *Chironomus riparius* (Cockroft, 2005c/d in DAR Section B.9.2). They indicate a low level of toxicity (28-day NOECs of 27 and 25 mg/kg sediment for IT-4 and IT-15 respectively) but as they employed the sediment-spiking method (OECD 208) they are not considered relevant to the classification of amisulbrom.

5.5 Comparison with criteria for environmental hazards (sections 5.1 – 5.4)

Despite evidence of photolysis under certain aqueous conditions, the overall abiotic and biotic degradation information does not indicate that amisulbrom is ultimately degraded (>70%) within 28 days and so it is considered 'not readily degradable'. The whole system half live in a natural water/sediment study is ≥64.2 days with little mineralisation. Consequently, amisulbrom is considered to be 'not rapidly degradable' for the purposes of classification under the CLP Regulation. The identified main degradants are also less acutely toxic than the parent substance (>10 x according to table 28) and therefore they are not considered further in relation to the classification of amisulbrom.

Amisulbrom has a log K_{ow} value of 4.4 but an experimental fish bioconcentration study reported whole fish BCFs less than 500 for both amisulbrom and total radioactive residue. Amisulbrom was extensively metabolised and then rapidly depurated. It is therefore not considered bioaccumulative according to the CLP criteria.

Reliable acute/short-term aquatic toxicity data on amisulbrom are available for fish, invertebrates and algae. The most acutely sensitive trophic group is fish with a 96 hr LC_{50} value for *Cyprinus carpio* of 0.0229 mg/l. On the basis of this acute fish endpoint being in the range 0.01 mg/l <L(E)C_{50} ≤0.1 mg/l, amisulbrom should be classified for acute environmental (aquatic) effects under CLP as:

Acute category 1 with an acute M-factor of 10

Chronic/long-term toxicity data are also available for fish, invertebrates and algae. The most acutely sensitive species (*Cyprinus carpio*) was not tested in chronic exposures but the next most sensitive fish species (*Pimephales promelas*, 96-hr LC_{50}: 0.0363 mg/l) was used in a FELS test. Given the slight difference in acute LC_{50}, species difference alone is not considered likely to significantly affect the relevance of the chronic fish endpoint, which was a 28-d NOEC for *P. promelas* of 0.037 mg amisulbrom/l (ref. table 28).

Overall, the lowest chronic classification endpoint is for algae, with a 96-hr NOE_rC for *Pseudokirchneriella subcapitata* of 0.0139 mg amisulbrom/l. On the basis of this algal endpoint being in the range 0.01 <NOEC ≤0.1, amisulbrom would be classified as 'Chronic category 1' with a chronic M-factor of 1. However, it is noted that the chronic NOEC for fish (0.037 mg/l) is higher than the acute LC_{50} for fish (0.0229 mg/l) and this calls into question the sensitivity and adequacy of the chronic fish test. Therefore it is proposed that that the surrogate approach to chronic classification using the lowest acute fish endpoint also be undertaken, with the most stringent outcome being used.

If it is assumed that adequate chronic toxicity data are not available and amisulbrom is 'not rapidly degradable' then, on the basis of the acute fish endpoint being in the range 0.01 mg/l <$L(E)C_{50}$ ≤0.1 mg/l, amisulbrom should be classified under CLP as:

Chronic category 1 with a chronic M-factor of 10

In this case, the M-factor of 10 derived for the acute aquatic hazard classification is also applied to the chronic classification and this is the more stringent outcome.

5.6 Conclusions on classification and labelling for environmental hazards (sections 5.1 – 5.4)

Aquatic Acute category 1; H400: Very toxic to aquatic life
Acute M-factor = 10
Aquatic Chronic category 1; H410: Very toxic to aquatic life with long lasting effects
Chronic M-factor = 10

6. OTHER INFORMATION

No additional information of relevance to the CLH proposal is available.

7 REFERENCES

1. Draft Assessment Report – Amisulbrom – Volume 3, Annex B.2; Physical and Chemical Properties – February 2012.

2. Draft Assessment Report – Amisulbrom – Volume 3, Annex B6; Toxicology and Metabolism – February 2012

3. Draft Assessment Report – Amisulbrom - Volume 3, Annex B8: Environmental Fate and Behaviour – February 2012

4. Draft Assessment Report – Amisulbrom - Volume 3, Annex B9; Ecotoxicology – February 2012

5. Conclusion on the peer review of the pesticide risk assessment of the active substance amisulrbom – EFSA Journal 2014; 12(4):3237

Specific references for the physico-chemical, human health and environmental hazard assessments are provided below.

Physico-chemical hazard assessment

CLH report section ref/ DAR Section ref	Author(s)	Year	Title Source (where different from company) Company, Report no. GLP or GEP status (where relevant), Published or not
1.3/ B.2.1.1	Takehara, K.	2003a	NC-224, Melting point Nissan Chemical Industries, Ltd Laboratory no. NCI-2002-054 Nissan Chemical Industries, Ltd Report no. P101 GLP, unpublished
1.3/B.2.1.2	Iijima, K.	2003	Screening test for thermal stability of NC-224 The Institute of Environmental Toxicology, Japan Laboratory no. IET 03-6001-2 Nissan Chemical Industries, Ltd Report no. P102 GLP, unpublished
1.3/B.2.1.4	Comb, A. L.	2003a	NC-224 (pure grade): Relative density Huntingdon Life Sciences Ltd Laboratory no. NAS 446/024103 Nissan Chemical Industries, Ltd Report no. P201 GLP, unpublished
1.3/B.2.1.4	Comb, A. L.	2003b	NC-224 (technical grade): Relative density Huntingdon Life Sciences Ltd Laboratory no. NAS 451/024188 Nissan Chemical Industries, Ltd Report no. P202 GLP, unpublished
1.3/B.2.1.5	Comb, A. L.	2003c	NC-224: Vapour pressure Huntingdon Life Sciences Ltd Laboratory no. NAS 448/024119 Nissan Chemical Industries, Ltd Report no. P301 GLP, unpublished

CLH report section ref/ DAR Section ref	Author(s)	Year	Title Source (where different from company) Company, Report no. GLP or GEP status (where relevant), Published or not
1.3/B2.1.7-2.1.8	Ogi, N.	2003a	NC-224 (pure grade), color, physical state and odor Nissan Chemical Industries, Ltd Laboratory no. NCI-2002-055 Nissan Chemical Industries, Ltd Report no. P401 GLP, unpublished
1.3/B.2.1.11	Takehara, K.	2003b	NC-224, water solubility Nissan Chemical Industries, Ltd Laboratory no. NCI-2002-041 Nissan Chemical Industries, Ltd Report no. P601 GLP, unpublished
1.3/B.2.1.13	Ogi, N.	2003d	NC-224, n-octanol/water partition coefficient Nissan Chemical Industries, Ltd Laboratory no. NCI-2002-073 Nissan Chemical Industries, Ltd Report no. P801 GLP, unpublished
1.3/B.2.1.20	Comb, A. L.	2003e	NC-224: Flammability (solids) Huntingdon Life Sciences Ltd Laboratory no. NAS 450/024105 Nissan Chemical Industries, Ltd Report no. P1101 GLP, unpublished
1.3/B.2.1.20	Comb, A. L.	2003f	NC-224: Relative self-ignition temperature for solids Huntingdon Life Sciences Ltd Laboratory no. NAS 452/024106 Nissan Chemical Industries, Ltd Report no. P1102 GLP, unpublished
1.3/B.2.1.22	Comb, A. L.	2003g	NC-224: Explosive properties Huntingdon Life Sciences Ltd Laboratory no. NAS 453/024107 Nissan Chemical Industries, Ltd Report no. P1301 GLP, unpublished
1.3/B.2.1.23	Comb, A. L.	2003h	NC-224: Oxidising properties (solids) Huntingdon Life Sciences Ltd Laboratory no. NAS 454/024109 Nissan Chemical Industries, Ltd Report no. P1501 GLP, unpublished

Human Health Hazard Assessment

CLH report section ref/ DAR Section ref	Author(s)	Year	Title Source (where different from company) Company, Report no. GLP or GEP status (where relevant), Published or not
4.1/B.6.1	---------	2004	NC-224: Metabolism in rats: single oral administration --------- Nissan Chemical Industries, Ltd Report no. T101 GLP, unpublished
4.1/B.6.1	---------	2005	NC-224: Metabolism in rats: Repeat oral administration --------- Nissan Chemical Industries, Ltd Report no. T102 GLP, unpublished
4.2/B6.2.1	---------	2003a	NC-224: Acute oral toxicity to the rat (Acute toxic class method) --------- Nissan Chemical Industries, Ltd Report no. T201 GLP, unpublished
4.2/B6.2.2	---------	2003b	NC-224: Acute dermal toxicity to the rat --------- Nissan Chemical Industries, Ltd Report no. T202 GLP, unpublished
4.2/B.6.2.3	---------	2003	NC-224: Acute (four-hour) inhalation study in rats --------- Nissan Chemical Industries, Ltd Report no. T203 GLP, unpublished
4.4.1/B.6.2.4	---------	2003a	NC-224: Skin irritation to the rabbit --------- Nissan Chemical Industries, Ltd Report no. T204 GLP, unpublished
4.4.2/B.6.2.5	---------	2003b	NC-224: Eye irritation to the rabbit --------- Nissan Chemical Industries, Ltd Report no. T205 GLP, unpublished
4.6/B.6.2.6	---------	2002	NC-224: Skin sensitization to the guinea-pig (Magnusson & Kligman method) --------- Nissan Chemical Industries, Ltd Report no. T206 GLP, unpublished
4.7/B.6.3.1(a)	---------	2000	Four-week subacute toxicity test of A-992176 in rats --------- Nissan Chemical Industries, Ltd Report no. T301 Not to GLP, unpublished
4.7/B.6.3.2(a)/	---------	2001	Four-week subacute toxicity test of A-992176 in mice --------- Nissan Chemical Industries, Ltd Report no. T302 Not GLP, unpublished

CLH report section ref/ DAR Section ref	Author(s)	Year	Title Source (where different from company) Company, Report no. GLP or GEP status (where relevant), Published or not
4.7/B.6.3.1(b)	---------	2003a	NC-224: Toxicity study by dietary administration to Han Wistar rats for 13 weeks --------- Nissan Chemical Industries, Ltd Report no. T303 GLP, unpublished
4.7/B.6.3.2(b)	---------	2003b	NC-224: Preliminary toxicity study by dietary administration to CD-1 mice for 13 weeks --------- Nissan Chemical Industries, Ltd Report no. T304 GLP, unpublished
4.7/B.6.3.3(a)	---------	2003	NC-224: Toxicity study by oral capsule administration to Beagle dogs for 13 weeks --------- Nissan Chemical Industries, Ltd Report no. T305 GLP, unpublished
4.7/B.6.3.3(b)	---------	2005	NC-224: Toxicity study by oral capsule administration to Beagle dogs for 52 weeks Amended final report. --------- Nissan Chemical Industries, Ltd Report no. T306 GLP, unpublished
4.7/B.6.3.4	---------	2004	NC-224: Toxicity study by dermal administration to CD rats for 21 days --------- Nissan Chemical Industries, Ltd Report no. T307 GLP, unpublished
4.9/B.6.4.1(a)	May, K.	2002	NC-224: Bacterial reverse mutation test Huntingdon Life Sciences Ltd Laboratory no. NAS 440/023917 Nissan Chemical Industries, Ltd Report no. T401 GLP, unpublished
4.9/B.6.4.1(c)	Kumaravel, T. S.	2004	NC-224: Induction of chromosome aberrations in cultured human peripheral blood lymphocytes Covance Laboratories Ltd Laboratory no. 306/130-D6172 Nissan Chemical Industries, Ltd Report no. T402 GLP, unpublished
4.9/B.6.4.1(b)	Lloyd, M.	2004	NC-224: Mutation at the thymidine kinase (*tk*) locus of mouse lymphoma L5178Y cells (MLA) using the microtitre^{R} fluctuation technique Covance Laboratories Ltd Laboratory no. 306/131-D6173 Nissan Chemical Industries, Ltd Report no. T403 GLP, unpublished
4.9/B.6.4.2(a)	---------	2003	NC-224: Mouse micronucleus test --------- Nissan Chemical Industries, Ltd Report no. T404 GLP, unpublished

CLH report section ref/ DAR Section ref	Author(s)	Year	Title Source (where different from company) Company, Report no. GLP or GEP status (where relevant), Published or not
4.9/B.6.4.2(g)	---------	2007	NC-224: Mouse micronucleus test --------- Nissan Chemical Industries, Ltd Report no. T406 GLP, unpublished
4.9/B.6.4.2(b)	---------	2005	*In vivo*/*in vitro* unscheduled DNA synthesis (UDS) test on NC-224 using rat livers --------- Nissan Chemical Industries, Ltd Report no. T405 GLP, unpublished
4.10/B.6.5.1	---------	2005a	NC-224: Combined carcinogenicity and toxicity study by dietary administration to Han Wistar rats for 104 weeks --------- Nissan Chemical Industries, Ltd Report no. T501 GLP, unpublished
4.10/B.6.5.2(b)	---------	2005b	NC-224: Carcinogenicity study by dietary administration to CD-1 mice for 78 weeks --------- Nissan Chemical Industries, Ltd Report no. T502 GLP, unpublished
4.9/B.6.4.2(c)	---------	2004	Hepatic micronucleus test of NC-224 in young rats --------- Nissan Chemical Industries, Ltd Report no. T512 Not GLP, unpublished
4.9/B.6.4.2(d)	---------	2005a	The comet assay of NC-224 in rats --------- Nissan Chemical Industries, Ltd Report no. T513 Not GLP, unpublished
4.9/B.6.4.2(e)	---------	2005b	The comet assay of NC-224 in mice --------- Nissan Chemical Industries, Ltd Report no. T514 Not GLP, unpublished
4.10/B.6.8.1(a)	---------	2005	Medium-term liver carcinogenesis bioassay of NC-224 --------- Nissan Chemical Industries, Ltd Report no. T503 GLP, unpublished
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4.10/B.6.8.1(f)	---------	2005c	The hepatic replicative DNA synthesis (RDS) study of NC-224 administered in repeat dosing for one week to male rats --------- Nissan Chemical Industries, Ltd Report no. T508 Not GLP, unpublished
4.10/B.6.8.1(g)	---------	2005d	The hepatic replicative DNA synthesis (RDS) study of NC-224 administered in repeat dosing for one week to female rats --------- Nissan Chemical Industries, Ltd Report no. T509 Not GLP, unpublished
4.10/B.6.8.1(h)	---------	2005e	The hepatic replicative DNA synthesis (RDS) study of NC-224 administered in repeat dosing for one week to male mice --------- Nissan Chemical Industries, Ltd Report no. T510 Not GLP, unpublished
4.10/B.6.8.1(i)	---------	2005f	The hepatic replicative DNA synthesis (RDS) study of NC-224 administered in repeat dosing for one week to female mice --------- Nissan Chemical Industries, Ltd Report no. T511 Not GLP, unpublished
4.9/B.6.4.2(f)	---------	2005e	The comet assay of NC-224 in rats: stomach --------- Nissan Chemical Industries, Ltd Report no. T515 Not GLP, unpublished
4.11/B.6.6.1	---------	2005	NC-224; Study of reproductive performance in Han Wistar rats treated continuously through two successive generations by dietary administration --------- Nissan Chemical Industries, Ltd Report no. T603 GLP, unpublished
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4.11/B.6.6.2(c)	---------	2003	Teratogenicity study of NC-224 in Han Wistar rats --------- Nissan Chemical Industries, Ltd Report no. T604 Not GLP, unpublished
4.11/B.6.6.3	---------	2004b	NC-224: Study of effects on embryo-fetal toxicity in the rabbit treated by oral gavage administration --------- Nissan Chemical Industries, Ltd Report no. T602 GLP, unpublished
4.11/B.6.8.2(a)	---------	2005g	The effect of NC-224 on rat fetus ovary (histopathological examination of fetus ovary in NCI03TE-115) --------- Nissan Chemical Industries, Ltd Report no. T605 Not GLP, unpublished
4.11/B.6.8.2(b)	---------	2005h	Confirmative study on the effect of NC-224 on the ovaries of newborn rats Nissan Chemical Industries, Ltd --------- Report no. T606 Not GLP, unpublished
4.11/B.6.8.2(f)	---------	2006a	The effect of NC-224 on ovary development in juvenile rats (dietary administration) --------- Nissan Chemical Industries. Ltd Report no. T610 GLP Unpublished
4.11/B.6.8.2(g)	---------	2006b	The effect of NC-224 on ovary development in juvenile rats (oral gavage administration) --------- Nissan Chemical Industries. Ltd Report no. T612 GLP Unpublished
4.11/B.6.8.2(c)	---------	2005a	Confirmative study of the effect of NC-224 on the genitals of rats (four week administration in diet) --------- Nissan Chemical Industries, Ltd Report no. T607 Not GLP, unpublished
4.11/B.6.8.2(d)	---------	2005b	Uterotrophic assay of NC-224 in female immature rats (antagonism study) Nissan Chemical Industries, Ltd --------- Report no. T608 Not GLP, unpublished

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5.3.2/B.9.2.1.7	----------	2005	NC-224: Bioconcentration study with bluegill sunfish (*Lepomis macrochirus*) under flow-through conditions --------- Nissan Chemical Industries, Ltd Report no. E207 GLP, unpublished
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5.4/B.9.2.1. 2.3(ii)	Jenkins, C. A.	2005f	IT-15: Algal growth inhibition assay Huntingdon Life Sciences Ltd Laboratory no. NAS 680/052413 Nissan Chemical Industries, Ltd Report no. EM402 GLP, unpublished

8 ANNEXES

Annex 1: Proposed degradation of amisulbrom in a natural water/sediment system

Annex 1: Proposed degradation of amisulbrom in a natural water/sediment system (ref. Section 5.1)

The proposed primary route of breakdown of amisulbrom in water/sediment systems is by cleavage of the sulfonamide side chain on the triazole ring to form IT-4, followed by debromination to form IT-15. Additionally, a combination of debromination, oxidation, methylation and indole ring opening reactions result in the production of lower level degradants. Further degradation of these result in residues bound to humic acid and humin fractions in sediment and low levels of carbon dioxide. A proposed degradation pathway is given in Figure 1 below:

Figure 1: Proposed degradation pathway for amisulbrom in water/sediment systems