Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Amisulbrom (ISO); 3-(3-bromo-6-fluoro-2-methylindol-1-ylsulfonyl)-*N,N*-dimethyl-*1H*-1,2,4-triazole-1-sulfonamide

EC Number: -
CAS Number: 348635-87-0

CLH-O-0000001412-86-104/F

Adopted

10 March 2016

10 March 2016

CLH-O-0000001412-86-104/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **Amisulbrom (ISO); 3-(3-bromo-6-fluoro-2-methylindol-1-ylsulfonyl)-*N,N*-dimethyl-1*H*-1,2,4-triazole-1-sulfonamide**

EC Number: **-**

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

The proposal was submitted by the **United Kingdom** and received by RAC on **3 June 2015.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

The United Kingdom has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **16 June 2015**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **31 July 2015**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Pietro Paris**

Co-Rapporteur, appointed by RAC: **Radu Branisteanu**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **10 March 2016** by **consensus.**

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	**No current Annex VI entry**										
Dossier submitters proposal	616-RST-00-Y	Amisulbrom (ISO); 3-(3-bromo-6-fluoro-2-methylindol-1-ylsulfonyl)-N,N-dimethyl-1H-1,2,4-triazole-1-sulfonamide	-	348635-87-0	Eye Irrit. 2 Carc. 2 Aquatic Acute 1 Aquatic Chronic 1	H319 H351 H400 H410	GHS07 GHS08 GHS09 Warning	H319 H351 H410		M=10 M=10	-
RAC opinion	616-RST-00-Y	Amisulbrom (ISO); 3-(3-bromo-6-fluoro-2-methylindol-1-ylsulfonyl)-N,N-dimethyl-1H-1,2,4-triazole-1-sulfonamide	-	348635-87-0	Eye Irrit. 2 Carc. 2 Aquatic Acute 1 Aquatic Chronic 1	H319 H351 H400 H410	GHS07 GHS08 GHS09 Warning	H319 H351 H410		M=10 M=10	-
Resulting Annex VI entry if agreed by COM	616-RST-00-Y	Amisulbrom (ISO); 3-(3-bromo-6-fluoro-2-methylindol-1-ylsulfonyl)-N,N-dimethyl-1H-1,2,4-triazole-1-sulfonamide	-	348635-87-0	Eye Irrit. 2 Carc. 2 Aquatic Acute 1 Aquatic Chronic 1	H319 H351 H400 H410	GHS07 GHS08 GHS09 Warning	H319 H351 H410		M=10 M=10	-

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Amisulbrom is a pesticidal active substance within the scope of Directive 91/414/EEC (and now Regulation 1107/2009). There are no existing entries in Annex VI of CLP Regulation for amisulbrom and the harmonised classification and labelling has not previously been considered at the EU level. At the time of submission the substance was not registered under REACH.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

In a standard study (EEC A10, Comb, 2003(e)), amisulbrom melted and burned but did not sustain combustion on removal of the ignition source. In a standard study (EEC A14, Comb, 2003(g)), amisulbrom was not found to be sensitive to the effects of flame, shock or friction. In a standard study (EEC A17, Comb, 2003(h)), amisulbrom did not sustain combustion on removal of the ignition source

In summary, in standard tests amisulbrom did not exhibit pyrophoric, explosive or oxidising properties. The DS proposed no classification.

Comments received during public consultation

No comments were received addressing the physical hazards.

Assessment and comparison with the classification criteria

Following standard testing amisulbrom did not meet any of the criteria for classification for physical hazards. Consequently, RAC agrees with the proposal for **no classification**.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

A total of three guideline studies, one for each acute toxicity endpoint, were summarised in the CLH report:

Oral acute toxicity (2003a)

Amisulbrom (purity 99.1%) was administered by oral gavage to fasted male and female Sprague-Dawley rats. During the study period of 14 days, no deaths occurred and no signs of toxicity were present; at gross necropsy, no treatment-related signs were found. The LD_{50} was established at > 5000 mg/kg bw. The study was GLP and OECD TG 423 compliant.

Inhalation acute toxicity (2003)

Five male and five female Sprague-Dawley rats were exposed nose-only to amisulbrom (purity 99.1%) at a concentration of 2.85 mg/L for a period of 4 hours. This concentration was the maximum achievable in this study. No deaths occurred during the study period and signs of toxicity included exaggerated breathing during exposure and temporary wet fur and brown facial staining on days 1 and 2 only. At gross necropsy, no treatment-related findings were found. Since no higher concentration of exposure could be achieved, the 4h-LC_{50} was considered to be > 2.85 mg/L. The study was GLP and OECD TG 403 (1981) compliant, using particles with an mass median aerodynamic diameter (MMAD) of 4.3 ± 1.2 µm.

Acute dermal toxicity (2003a)

Amisulbrom of 99.1% purity formulated as 80% concentration in 1% methylcellulose aq. was applied to the shorn dorsal skin of male and female rats (5/sex) for 24h. The application site was then washed with water. No deaths occurred and no signs of systemic toxicity appeared during the study period of 14 days. Grade 1-2 erythema was observed in all animals on days 2-3 and reduced weight gain in all females was shown. At gross necropsy, no treatment-related findings were found. The study is GLP and OECD TG 402 compliant and the LD_{50} was established at > 5000 mg/kg bw.

The DS proposed no classification.

Comments received during public consultation

One MSCA supported the ”no classification“ proposal of the DS and no other comments were received.

Assessment and comparison with the classification criteria

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

Classification as Acute Tox. 4 (inhalation of dusts and mists) is applicable where 1 < 4h-LC_{50} ≤ 5 mg/L. Amisulbrom was tested at the maximum achievable concentration of 2.85 mg/L at which no deaths occurred. Moreover, the rats experienced exaggerated breathing during exposure only and the minor toxicity signs lasted only for 2 days after exposure. These effects were not seen as significant and no classification is considered warranted.

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

In conclusion, RAC agrees with the argumentation presented by the DS **for no classification for acute toxicity**.

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

Summary of the Dossier Submitter’s proposal

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 only during the 4 h exposure period. On days

1 and 2 following exposure, the 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.

The DS proposed no classification.

Comments received during public consultation

One MS supported the ”no classification“ proposal of the DS and no other comments were received.

Assessment and comparison with the classification criteria

No significant specific target effects on a target organ or tissue were observed after single oral, dermal or inhalation exposure with amisulbrom in rats. Therefore, RAC agrees with the proposal of the DS to **not classify amisulbrom for STOT SE**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter’s proposal

The skin irritation potential of amisulbrom has been investigated in a single standard study in rabbits. The study protocol was OECD TG 404 (1992) compliant and involved the usage of 0.5 g of amisulbrom of 99.1% purity. No signs of irritation were observed in any animal at any time point. Consequently, no signs of corrosion could be seen.
The DS proposed no classification.

Comments received during public consultation

One MSCA supported the ”no classification“ proposal of the DS and no other comments were received.

Assessment and comparison with the classification criteria

Since the results of the **skin corrosion/irritation** test were negative, RAC agrees with the DS’ proposal for **no classification**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter’s proposal

The DS proposed classification as an Eye irritant, Category 2, based on the evaluation of the study as described below.

A single (OECD TG 405 compliant) study is presented for assessment. A quantity of 100 mg amisulbrom of 99.1% purity 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.

No effects on cornea and iris were registered. Also no conjunctival oedema was present. The scores registered for conjunctival erythema are shown in the table below:

Conjunctival erythema in the guideline study

Observation	**1h**	**24h**	**48h**	**72h**	**Mean 24-72h**	**7d**	**14d**	**21d**
Unwashed eyes	2,1,1	1,0,1	0,1,1	0,1,0	0.56	1,1,0	1,1,0	1,1,0
Washed eyes	1,2,2	1,1,1	1,0,0	0,1,0	0.56	0,0,0	0,1,0	0,1,0

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 one 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 DS stated that the results of the washed rabbits were not included in the classification for eye irritancy because they did not follow the study guidelines. With this in mind, it is noted that a similar effect was observed 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.

Comments received during public consultation

One MSCA agreed with the proposed classification as Eye Irritant category 2. Two MS disagreed with the proposed classification and suggested Eye Damage 1; both proposals were motivated by the effects remaining on day 21.

Two additional studies have been submitted by the applicant during the public consultation. Two study reports with formulations of 20% and 50% amisulbrom were provided. It was stated that each test material included other irritant substances, which have not been further described. The mild conjunctival erythema was first seen in all animals but completely disappeared after 72 h or 6 days. None of the studies were carried out for 21 days.

Assessment and comparison with the classification criteria

In the comment, the applicant concluded that neither the technical grade amisulbrom nor its formulations have an irritative effect on the eye. The intermittent weak erythema observed in the OECD compliant study was not regarded as related to treatment and therefore amisulbrom does not meet the criteria for eye irritation classification.
All the studies showed a similar pattern of effects over a 72 hour period. None of the studies revealed effects upon the iris. The OECD compliant study and one of the amisulbrom formulation studies showed no effects on the cornea. The remaining formulation study showed a mild (grade 1) opacity that completely disappeared after 5 days. All the studies revealed conjunctival erythema but no oedema. In the 99.1% purity amisulbrom study, the conjunctival redness recurred in 2 out of 3 animals and persisted until day 21 when the test was terminated.

The *severity* of effects is low. Grade 2 erythema had been registered after 1 h from the application and this resolved to grade 1 for the rest of the observation period in 2 out of the 3 given test studies. The average score for the 72 h observation period was below 1. Only in the study with 20% amisulbrom formulation transient corneal opacity was observed. Since it was stated that this formulation contained other irritant components (not further described) and the pattern of effects is slightly different, this study will not be further considered for analysis.

The *reversibility* of the effects over 21 days could only be assessed for the guidance compliant study conducted with 99.1% amisulbrom. The aetiology of conjunctival erythema may be attributed to amisulbrom exposure since the untreated eye did not exhibit any effect at all. Moreover, the recurrence and persistence after day 7 could be observed in 1 animal out of 3 in the washed eyes group. Therefore RAC does not agree that this could have been an artefact arising from local irritation due to hairs liberated through grooming, as stated in the CLH report.

In general, no irritation potential of amisulbrom could be demonstrated. No skin irritant properties could be detected in an OECD TG 404 (1992) compliant study. Also, no respiratory tract irritation was clearly shown in the acute inhalation test.

When severity is taken into account, RAC acknowledges the lack of effects on cornea and iris as well as the absence of conjunctival oedema. Also, RAC recognises the low severity of conjunctival erythema. In addition, the irritation potential of amisulbrom could not be detected in skin and acute inhalation tests. Therefore, based on severity, RAC considers that the irritation potential of amisulbrom is at least very low.

When taking into account reversibility, the persistence of the effect until the day 21 shown in one study suggests that amisulbrom should be regarded as an eye damaging compound. However, taking into account that the persistence is inconsistent throughout the study, the severity of effects is very low and amisulbrom did not show skin and respiratory irritant properties, RAC agrees with the DS proposal for classification of amisulbrom as **Eye Irrit. 2; H319 – Causes serious eye irritation**.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

There are no data available with respect to respiratory sensitisation; consequently the DS proposed no classification.

Comments received during public consultation

One MSCA noted the lack of relevant data to conclude on this hazard.

Assessment and comparison with the classification criteria

RAC agrees that **no classification** is warranted based on lack of data.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

In a single guideline-compliant 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, Freunds Complete Adjuvant (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 sodium dodecyl sulfate (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.
The DS proposed no classification.

Comments received during public consultation

One MSCA supported the ”no classification“ proposal of the DS.

Assessment and comparison with the classification criteria

Since no evidence of skin sensitisation was observed RAC agrees with the proposal for **no classification**.

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

Summary of the Dossier Submitter’s proposal

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-year study). A 21-day dermal study in rat is also available.

Rat

28-day study (amisulbrom of 99% purity, method EU B7, not GLP)

Groups of 5 male and 5 female Sprague-Dawley (SD) rats were fed dietary concentrations of 0, 2500, 5000, 10000 or 20000 ppm amisulbrom for 4 weeks. The overall mean achieved daily intakes were 206, 424, 833 and 1699 mg/kg bw/d in males and 224, 459, 875 and 1816 mg/kg bw/d in females, respectively.

Transient decreases in food consumption were observed during the first three days of administration, at 20000 ppm (1699 mg/kg bw/d) in males and at 10000 (875 mg/kg bw/d) and 20000 ppm (1816 mg/kg bw/d) in females. Clinical chemistry showed modifications of some parameters in the range of historical controls and were not considered to be of toxicological significance.
Haematologic changes were detected from a dose of 10000 ppm (833-875 mg/kg bw/d).
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.

The target organs identified in this study were the liver and blood; the established NOAELs were of 2500 ppm (206 mg/kg bw/d) for males and 5000 ppm (459 mg/kg bw/d) for females.

90-day study (amisulbrom of 98% purity, method EU B26, GLP)

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, 171, 525 and 1715 mg/kg bw/d in males and 0, 187, 587 and 1880 mg/kg bw/d in females, respectively.
Dosages of 6300 ppm (525 mg/kg bw/d) and 20000 ppm (1715-1880 mg/kg bw/d) 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. The study authors considered that food scatter during the first two weeks of treatment 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 (1715-1880 mg/kg bw/d) compared with none in controls. It was noted that there were no significant histopathological changes in the eye.
Clinical chemistry changes were indicative of effects on liver function with slight differences between sexes at 20000 ppm (1715-1880 mg/kg bw/d).
Haematological changes (slightly increased platelet counts) with statistical significance only in males were detected at 20000 ppm (1715-1880 mg/kg bw/d).
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 (1715 mg/kg bw/d). Females were unaffected. An equivocal increased incidence of sinus erythrocytosis/erythrophagocytosis was also seen in the mandibular and mesenteric lymph nodes of males at the same dose.

The identified target organs were the liver, blood and mesenteric lymph nodes; the established NOAELs were of 2000 ppm (170 mg/kg bw/d) for males and 6300 ppm (587 mg/kg bw/d) for females.

2-year chronic toxicity/carcinogenicity study (amisulbrom 99.1% purity, method EC B33GLP, GLP). Chronic component of the study.

Groups of 20 male and 20 female Han Wistar rats were treated with amisulbrom in the diet with 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 2 years.
There was a dose-related reduction in bodyweight gain during the 52 week treatment at doses $\geq$ 2000 ppm (112 m/147 f mg/kg bw/d). It was marked in 20000 ppm males (77% of controls) and in females at both 10000 (735 mg/kg bw/d) and 20000 ppm (1503 mg/kg bw/d) (70% and 68% of controls respectively). At doses $\geq$ 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 $\geq$ 10000 ppm during week 1 and suggested initial unpalatability of the test diets. Overall food conversion efficiency was slightly lower during the first 16 weeks of treatment, particularly in males given doses $\geq$ 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.

Clinical chemistry showed significantly high gamma-glutamyl transpeptidase activities in week 26 in animals treated with ≥ 10000 ppm (735 mg/kg bw/d) and in males in week 52 at doses ≥ 2000 ppm. Several individual values in these groups exceeded the historical control range.
Histopathological examination of the liver showed 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).

The target organs identified in this study were the liver (also the biliary system) and kidneys; the NOAEL for toxicity was established as 100 ppm (11/14 mg/kg bw/d in M/F)

Mouse

28-day study (amisulbrom of 99.7% purity, method EU B7, not GLP)

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, 470, 904 and 1860 mg/kg bw/d in males and 0, 296, 543, 1077 and 2192 mg/kg bw/d in females, respectively.
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 (amisulbrom of 98.7% purity, range finding for carcinogenicity study, GLP)

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 119, 400 and 1280 mg/kg bw/d in males and 163, 505 and 1638 mg/kg bw/d in females at 800, 2500 and 8000 ppm, respectively. No histopathological examinations were conducted in this study.
The overall bodyweight gain of females receiving 2500 (506 mg/kg bw/d) or 8000 ppm (1638 mg/kg bw/d) was lower than that of the controls. In males, lower gains were observed from 800 ppm (119 mg/kg bw/d), 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 same doses induced slight haematology changes and findings indicative of liver effects including increased liver weight and slightly decreased plasma cholesterol.

The main target organs identified in this study were the liver and blood; the NOAEL was established as 800 ppm, corresponding to 119 and 163 mg/kg bw/d in males and females respectively.

78-weeks study (amisulbrom of 99.1% purity, method EU B33, GLP)

50 male and 50 female CD-1 mice were fed dietary concentrations of 0, 100, 800, 4000 or 8000 ppm of amisulbrom for 78 weeks. The oberall achieved test material intakes were 0, 12, 98, 494 and 1035 mg/kg bw/d in males and 0, 14 121, 594 and 1255 mg/kg bw/d in females, respectively.

In males, bodyweight gains were 77% and 63% of controls at 4000 and 8000 ppm, respectively, and 87% of controls in females at 8000 ppm. There was also a non-statistically significant decrease in body weight gain (86% of controls) in males given 800 ppm. At termination, there was a dose-related increase in relative liver weight in males fed >800 ppm and in females at >4000 ppm. Increased incidence of focal hepatocyte necrosis were found in males at 8000 ppm. Slight to mild, not dose-related centrilobular hypertrophy of hepatocytes was observed in a few male and female mice dosed at levels >2500 ppm.
Also slight increase in the incidence of cortical hypertrophy of the adrenal in males given >800 ppm was found. 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 >800 ppm. In the kidney, the incidence of cortical tubular basophilia was increased in females at dose levels of >4000 ppm. Incidences of perivascular lymphoid aggregations were also slightly increased in females fed >800 ppm, and in males at 8000 ppm
Overall, kidney (lymphoid aggregation), caecum (pigmentation of submucosal venules) and adrenal glands (cortical hypertrophy) were identified as target organs of toxicity from a dose of 800 ppm (98 (M)/121 (F) mg/kg bw/d).

Dog

90-day study (amisulbrom of 98.7% purity, method EU B27, GLP)
In a guideline compliant 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 gelatin capsules for 13 consecutive weeks. The administration of amisulbrom was well-tolerated for all doses, 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.

The target organ identified in this study was the liver and a NOAEL of 300 mg/kg bw/d was established.

1-year study (amisulbrom of 99.1% purity, method EU B30, GLP)

In a guideline compliant 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 gelatin capsules for 52 consecutive weeks. The administration of amisulbrom to dogs up to the highest dose of 1000 mg/kg bw resulted in initial effects on bodyweight gain, food consumption and on the liver (reduced total protein concentration) at the top dose.
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.
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.

The main target organ identified in this study was the liver. In addition, a stress response, with effects on the adrenal glands, spleen and thymus was seen. The established NOAEL was of 100 mg/kg bw/d.

Repeated dose toxicity: dermal

A guideline compliant short-term (21-day) dermal toxicity study is available in the rat. In this study, groups of 10 male and 10 female Sprague-Dawley (SD) 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.
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. A NOAEL of 300 mg/kg bw/d was identified from the study.

Following the assessment, the DS proposed no classification.

Comments received during public consultation

One comment supporting the no classification proposal of the DS was received.

Assessment and comparison with the classification criteria

Amisulbrom induced significant and specific target organ toxicity mainly in the rat and mouse but at relatively high dose levels. These doses are 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, RAC supports the proposal of the DS **not to classify amisulbrom for STOT-RE**.

Following the STOT RE studies, there are two aspects with possible influence in the classification of other endpoints:

1. The liver was identified as the main target organ in all the oral administration studies. The clinical chemistry showed functional disturbances and the histopathological examinations revealed centrilobular hepatocyte hypertrophy.
2. In the rat, the low body weight gain cannot be attributed solely to bad palatability; the food conversion efficiency finding indicates that reduced bodyweight gain was not entirely due to low food consumption and was evidence of an underlying toxic effect. This conclusion was drawn in the 28 and 90 days studies and in the 2-year chronic toxicity/carcinogenicity study. Also, in the dermal toxicity study, the same effect, but of a lower magnitude, was observed.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The mutagenicity potential of amisulbrom was tested in three *in vitro* guideline studies and three *in vivo* tests. In addition, to further investigate the genotoxic potential and mechanism of adenoma formation, four studies (one liver micronucleus assay in rats and three liver comet assays in rats and mice) have been carried out.

In vitro data

The tests are summarized in the following table:

Summary of mutagenicity *in vitro* tests for amisulbrom

Method	Organism/strain	Concentrations tested	Result
Ames – bacterial reverse mutation test (n = 3) OECD TG 471 GLP Purity 99.1% May (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 (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 (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

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. Overall, amisulbrom was negative for genotoxicity in the presence or absence of S9.

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 an oral OECD TG 474 compliant micronucleus test male CD-1 mice received amisulbrom with doses of 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 using intraperitoneal administration. 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 (GLP compliant) test in rats was performed in accordance with OECD TG 486; the results were negative with no evidence of unscheduled DNA synthesis in liver, no increase in the net number of nuclear grains and no increase in the proportion of cells in repair.

Additional tests

Rat liver micronucleus assay

Amisulbrom of 99.1% purity was administered by gavage at dose levels of 500 and 2000 mg/kg to female F344 rats. The test material did not induce the formation of micronuclei in the rat liver up to the highest dose that caused cytotoxicity. The positive control group (diethylnitrosamine 50 mg/kg bw) showed statistically significantly increased proportions of micronucleated hepatocytes.

Rat liver comet assay

Amisulbrom of 99.1% purity was administered by gavage at dose levels of 500 and 2000 mg/kg bw to female Han Wistar rats and was not found to cause DNA damage within the tested dose range.

Mouse liver comet assay

Amisulbrom of 99.1% purity was administered by gavage at dose levels of 500 and 2000 mg/kg to male CD-1 mice and did not caused DNA damage at the tested doses.

In both comet assays the positive control (diethylnitrosamine or methyl methanesulphonate) showed significantly greater tail moment in hepatocyte nuclei, thus demonstrating the validity of the test. The comet assay on rat forestomach tissue was not included in the analysis since it is not considered to be relevant to humans.

The DS proposed no classification.

Comments received during public consultation

The two comments received expressed support for no classification of amisulbrom for mutagenicity.

Assessment and comparison with the classification criteria

The DS proposed not to classify amisulbrom for mutagenic effects but expressed a slight reservation in terms of limitations of the studies due to poor solubility and oral absorption of the test material. However, the available studies showed no evidence of mutagenicity and RAC agrees with the proposal for **no classification of amisulbrom as a germ cell mutagen**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The carcinogenic potential of amisulbrom was assessed in two guideline compliant carcinogenicity studies. To further investigate the mode of action (MoA) a number of mechanistic studies including one carcinogenesis assay and several enzyme induction and replicative DNA synthesis have been added. In addition, the applicant provided one additional MoA study and one analysis of the existing data performed by an expert panel. The DS proposed the classification of amisulbrom as Carcinogen 2.

Carcinogenicity studies

Rat

A guideline EC B33 chronic toxicity/carcinogenicity was performed on Han Wistar rats; the carcinogenicity group consisted of 50/sex/group and amisulbrom was administered as 99.1% purity in diet. The dosages for the carcinogenicity phase were: 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). The duration of the study was of 104 weeks (24 months).
Treatment-related neoplastic findings were found in the liver and stomach; for the liver, the findings are summarized in the following table:

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)								
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 historical control data (HCD): 0 – 6% (M, F)

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. 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% and 2%) were within the historical control range (males: mean = 1.6%; range = 0 - 6%; n = 365; females: mean = 1.9%; range = 0- 6%; n = 365). The study authors consider that the increased incidences of hepatocellular tumours at doses $\geq$ 10000 ppm were a consequence of persistent hypertrophic change.

In females, the increase in liver adenoma incidence was observed at dose levels causing high 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).

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 NOAEL established for carcinogenicity was 2000 ppm (96/129 mg/kg bw/day).

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 of 99.1% purity for 78 weeks. The overall mean achieved test material intakes were 0, 12, 98, 494 and 1035 mg/kg bw/d in males and 14, 121, 594 and 1255 mg/kg bw/d in females. The findings are summarized in the following table:

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)										
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

A statistically significant increase in hepatocellular adenomas appeared in males at doses ≥ 800 ppm. 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%) fitted 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 in which the mean control incidence was 26% (n=50). Incidences of hepatocellular carcinomas were unaffected. The trend analysis for male data was significant when all groups were included and if the data for the 8000 ppm group 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.

The increase in liver adenoma at 8000 ppm (36%) occurred at a dose level causing high toxicity (37% reduction in body weight gain).

Mechanistic studies

Medium-term liver carcinogenesis (Ito) rat bioassay

A medium-term liver carcinogenesis using the Ito model bioassay was conducted on F344 male rats. This study aimed 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. Hepatocarcinogenesis was initiated by a single intraperitoneal injection of 200 mg/kg bw N-nitrosodiethylamine (DEN). Two weeks later, they received the diet containing amisulbrom at 0 (control) ppm, 200 ppm (12 mg/kg bw), 2000 ppm

(120 mg/kg bw) and 20000 ppm (1448 mg/kg bw) for 6 weeks. The positive control group was administered a diet containing 500 ppm of phenobarbital (PB) sodium salt as a promoter. Groups without DEN initiation also received 0 (control) or 20000 ppm of the test material.

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 tumorigenic activity in the liver. No such activity was demonstrated at 200 ppm.

Enzyme induction assays

A. 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. The corresponding intakes are 21 and 1946 mg/kg bw/d in males and 214 and 2083mg/kg bw/d in females. The target enzymes were EROD (Ethoxyresorufin-O-deethylation), PROD (Pentoxyresorufin-O-deethylation), MFCOD (7-Methoxy-4-trifluoromethylcoumarin-O-deethylation) and T-OH (Testosterone-6B-hydroxylation). Control groups were untreated and positive control groups received phenobarbital by gavage at a dose of 50 mg/kg bw.

The 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; however, with the exception of EROD, the values of enzymatic activities were lower when compared with PB and administration of amisulbrom at a dose of 200 ppm did not significantly affect hepatic drug metabolizing enzyme activities.

B. Mouse liver

Amisulbrom was administered in diet at doses of 100 or 8000 ppm for 7 days to male and female mice. The corresponding intakes are 14 and 1079 mg/kg bw/d in males and 17 and 1310 mg/kg bw/d in females.

The 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 similar to that of phenobarbital administration; however, the extent of induction is different between the two substances. Also, administration of amisulbrom at a dose of 100 ppm did not affect hepatic drug metabolizing enzyme activities.

Replicative DNA synthesis (RDS) assays

A. Single dose - in male rats

Amisulbrom was administered by gavage at 0 and 1000 or 2000 mg/kg bw. The dose of phenobarbital was 50 mg/kg bw. Two hours prior to termination the animals were given a single intraperitoneal injection of 10 mg/100 g bw of 5-bromo 2'-deoxyuridine (BrdU) in physiological saline. 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 errors of the mean (+3 x SEM) of the control group.

At 2000 mg/kg bw 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. 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.

B. Single dose – in female rats

The dosages and the experimental protocol were the same as 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. In the phenobarbital-treated group, the incidence was increased at all-time points. In summary, a single oral dose by gavage of 1000 or 2000 mg/kg bw/d amilsulbrom induced RDS activity in female rats.

C. Repeated dose- in male rats

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. Another group of 8 males was given 3 or 7 daily oral doses, by gavage, of 50 mg/kg bw of phenobarbital in water. The experimental protocol was the same as in single dose studies.
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, it is concluded that the 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.

D. Repeated dose – in female rats

The testing protocol was the same as in the male rats and the equivalent dosages are 17, 150 and 656 mg/kg bw/d. 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.
Consequently, it is concluded that the 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.

E. Repeated dose – in male mice

The testing protocol was the same as in repeated dose studies in rats; the dosages were of 0, 100 or 8000 ppm amisulbrom (equivalent to 15 and 1021 mg/kg bw/d) for 7 days.
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 significant. 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.

F. Repeated dose – in female mice

The same working protocol was applied and the equivalent dosages were of 17 and 1234 mg/kg bw/d.
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.

Summary of mechanistic studies

Amisulbrom has promotional tumorigenic activity in the liver of male Fisher 344 rats. Also, amisulbrom at high doses (threshold indicative) 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); however, the extent of induction is different between the two substances, with a generally lower potency of amisulbrom. 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.

Comments received during public consultation

Two MSCAs supported the proposal for classification as Carcinogen 2.
The applicant commented that amisulbrom should not be considered as a human carcinogen.

Assessment and comparison with the classification criteria

Tumour profile

Amisulbrom induced both malignant and benign tumours in two species: rats and mice. In rats, one female out of 50 developed forestomach squamous cell carcinoma at the highest dose. Other squamous cell tumours were papillomas seen in 1 female at 10000 ppm and 2 females at 20000 ppm. Although the incidences were very low, a trend test for combined incidence of benign squamous cell papilloma and malignant squamous cell carcinoma proved statistical significance in the Fisher's Exact test ($p < 0.0183$). In general, the forestomach tumours in rodents are not considered relevant to humans because they occur in a tissue with no human equivalent. However, the criteria in the CLP Regulation, Annex I, paragraph 3.6.2.3.2 state (under "*Additional considerations on classification"*) that "*tumours occurring in such tissues indicate that the substance has the potential to induce carcinogenic effects in the species tested*". Moreover, a specific example is given: "*forestomach tumours in rodents following the administration by gavage of irritating or corrosive, non-mutagenic substance*". Amisulbrom is not mutagenic and it also was not shown to have irritant properties. Therefore, RAC took into account the nature and low incidence of these tumours but noted that they appeared concurrent with liver carcinomas and adenomas following the exposure to a non-irritant compound. This observation is considered as an element of uncertainty in the assessment for classification.

Liver carcinomas were induced in rats in both sexes at the two highest doses. Since the incidence was low and not related to dose, the DS considered that it was not treatment related; however, RAC notes that for this tumour type there is no HCD. In mice, hepatocellular carcinomas were present in males only. Although higher than in rats, the incidence is comparable to the unexposed group; no dose response relationship can be observed but no HCD is given either. Overall, the lack of HCD for liver carcinomas is considered as an element of uncertainty in the assessment.

Liver adenomas were statistically significant in rats in both sexes at the highest two doses. The number of tumours was more than double in females. The greater number of tumours in females was concurrent with greater general toxicity. In mice, hepatocellular adenomas exceeding the HCD were seen only in males. Also, a high background of liver adenomas was noted in the HCD.

Liver tumours in both species occurred concurrently with the increased liver weight and increased incidence of centrilobular hepatocyte hypertrophy. By contrast, histopathological examination performed in the Study in chimeric mice with humanized liver (PXB) showed no changes in the PXB mice while in the control mice the same hypertrophy of centrilobular hepatocytes was observed. This finding is further used as an argument in favour of the proposed mode of action.

There was no evidence of tumour progression into malignancy; according to the CLP Criteria, the induction of only benign tumours will usually support Category 2 (CLP Annex I, 3.6.2.2.3).

Mode of action

Amisulbrom is not genotoxic, as was shown in the mutagenesis assays and its tumorigenic potency is threshold mediated.

Amisulbrom showed only promotional tumorigenic activity in the liver as assessed by a medium-term liver carcinogenesis bioassay in rats.

A PB-like MoA was assumed and investigated. The argumentation mirrored with the literature model is shown in the following table:

Constitutive Androstane Receptor (CAR) activation mode of action versus the argumentation for amisulbrom

Events according to *Elcombe 2014*	**Amisulbrom assessment**
Key event 1 - CAR activation	Shown via CYP2B induction in rats and mice in both males and females
Key event 2 - altered gene expression specific to CAR activation	CYP2B and CYP3A mRNA levels were statistically significantly higher in both PXB and SCID mice. In addition, the Gadd45b mRNA level tended to be higher in the SCID mice group.
Key event 3 – increased cell proliferation	Shown only in rats (both males and females), male mice and SCID mice. In female rats RDS was inconclusive and absent in chimeric mice with humanized liver.
Key event 4 – clonal expansion leading to altered foci	- No data
Key event 5- liver adenomas/carcinomas	Liver adenomas in male and female rats (concurrent with high toxicity) and male mice.
Associative event -Enzyme induction	• EROD, PROD, MFCOD, and T-OH activities in both male and female rats • increased EROD and PROD but not T-OH activities in both male and female mice • PROD activities were significantly higher in both the PXB and SCID mice. In addition, the Gadd45b mRNA level tended to be higher in the SCID mice group. Overall, a lower potency of enzyme induction in amisulbrom than in PB
Associative event -Liver hypertrophy	Only in rats

The hepatic enzyme induction potential was shown in both rats and mice. The finding was reconfirmed in the Study Report for both PXR and SCID mice. In addition, in this study the gene expression analysis showed that CYP2B and CYP3A mRNA levels were higher in both PXR and SCID mice. The potential was dose dependent and lower for amisulbrom when compared to PB.

The cell proliferation was assessed by RDS assays in both single and repeated dose in males and female rats. All the tests showed increased hepatic replicative DNA synthesis. By contrast, tests on mice showed increased RDS in males but were inconclusive in females. Also, the RDS was dose dependent. The cell proliferative potential was also different in PXR versus SCID mice: while in SCID mice the findings were confirmed, in the chimeric mice with humanized liver no potential could be detected.

Overall, the potential of CAR activation and specific gene expression alteration (key events #1 and 2, Elcombe, 2014) was emphasized in rats and mice in both sexes, while the increased cell proliferation (key event #3, Elcombe, 2014) was shown only in rats (both sexes), male mice and SCID mice. In female mice the potential was inconclusive and in chimeric mice with humanized liver no RDS could be detected.

Conclusions

The applicant considered that the tumour profile "*can be linked principally to adaptive hepatic changes*". In addition, the MoA is similar to PB and therefore irrelevant to humans. Consequently, amisulbrom should not be considered as a human carcinogen.

The DS considered that the PB-like MoA still has its intrinsic uncertainties with respect to human relevance. In addition, the DS considered that other MoAs might be responsible for the observed liver tumours. In the 2-yr rat study there were signs of concurrent liver toxicity (increased γGTP, bile duct hyperplasia, hepatocyte vacuolation, cystic degeneration and decreased basophilic foci) which may have contributed to the induction of tumours. Consequently, the data in the mechanistic studies considered are not sufficient to exclude the relevance for humans of the liver adenomas in rodents. It has to be noted that the DS did not assess the two confidential studies provided by the applicant during PC.

When taking into account the tumour profile, RAC acknowledges that, according to the CLP Annex I, 3.6.2.2.3, the tumour profile has some elements that might lower the level of concern: tumour type and site (liver adenomas), the lack of evidence of progression into malignancy of the adenomas, a strong response only in males. However, the uncertainties previously presented, cannot be dismissed. Also, due to the histopathological changes concurrent with toxicity, enzymatic induction, liver gene expression and cell proliferation, the process cannot be seen as simple adaptive.
Concerning the MoA, RAC acknowledges that alternative MoAs such as DNA reactivity, estrogenic activity and infections are not appropriate for amisulbrom. The MoA claimed by the applicant is CAR-activation with subsequent cell proliferation; the mechanistic data presented seem to support the assumption. However, as presented the table above, not all key events were presented and within the associative events there were some inconsistencies: female mice showed enzyme induction and increased liver weight comparable to male mice but they had no increase in adenomas and liver hypertrophy was shown in rats but not in mice. While the first two key events in the PB-like MoA (as defined in Elcombe, 2014) resemble the behaviour of PB, the intrinsic potential of enzymatic induction is lower for amisulbrom. With respect to the increased cell proliferation (key event #3 in Elcombe, 2015), there is a notable differentiation between species and strains: RDS was inconclusive in the female rats and, most importantly, absent in chimeric rats with humanized liver. The absence of both increased cell proliferative potential and hypertrophy of the middle lobe cells are good arguments in favour of non-relevance to humans and subsequently could suggest no classification. However, the overall assessment has elements of uncertainty that cannot be overruled.

In conclusion, RAC considered that the mechanistic data provided were ultimately insufficiently robust to dismiss the elements of uncertainty in the tumour profile and contribution of other MoA than CAR. Consequently RAC agrees with the proposal of the DS for the classification of amisulbrom as **Carc. 2; H351 - Suspected of causing cancer**.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Fertility

The potential effects of amisulbrom on fertility and reproductive performance have been investigated in a guideline multigenerational study in the rat. Additionally, several mechanistic studies have been performed in order to clarify the aetiology of the effects detected in this two generation study.

In an OECD TG 416 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 (corresponding to 0, 9.8, 48.5, 240 and 1200 mg/kg bw/d in males and 0, 10.5, 53, 261, 1291 mg/kg bw/d in females).

The top dose level of 15000 ppm (1200-1291 mg/kg bw/d) had a clear and marked effect on reproduction in F1 females. 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 was registered. After weaning, 2 F0 dams were humanely killed due to poor physical condition. Also, reduced ovarian weight and function and associated histopathology (cystic, marked follicle count) was shown. Oestrus cycle analysis prior to mating revealed a significantly increased proportion of F1 females with extended oestrus; four to five weeks following mating, extended oestrus was apparent in nearly all females in this group. As a result of the poor mating performance, all of the 15000 ppm F1 males were re-mated with untreated females: all of the males subsequently mated successfully to produce litters.

The middle dose of 3000 ppm (240/261 mg/kg bw/d in M/F) caused significantly delayed sexual maturation in females. Pre-mating bodyweights were significantly lower due to lower initial weights and reduced weight gain in both sexes. Mean bodyweights of the females were significantly lower throughout gestation and lactation; weight gain was slightly (but significantly) lower during gestation. The ovarian and uterine effects occurred in a small number of females from litters with relatively poor body weight performance before or after weaning. For instance, atrophic ovaries (4 F vs. 0 in controls) and uterus metaplasia (2 F vs. 0 in controls) were noticed. These responses did not result in impaired mating performance and fertility and hence were not considered of relevance to the establishment of the reproductive toxicity NOAEL. No treatment-related findings were apparent in males.

The dose of 600 ppm (49/53 mg/kg bw/d in M/F) showed no parental toxicity and no treatment-related effects in both generations. The only effect in the offspring was a slight but significant decrease in pups body weights (8% M) during postnatal days 1-14 in F1 males.
The dose of 120 ppm (9.8/10.5 mg/kg bw/d) showed no treatment-related effects.

The following fertility indices were established: 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).

The effects upon fertility seen in the two high doses are associated with the marked decrease of the body weight. To further investigate the specific influence of amisulbrom, some mechanistic studies have been performed analysing the effects on the ovary in foetuses and young rats, the sex hormone in adult rats and the uterotrophic effect and aromatase activity in young female rats. In addition, to further distinguish between the amisulbrom induced effects and those attributable to restricted food intake on rat ovarian and uterine development, a comparison study was performed. Moreover, to assess the effects of oral gavage administration (as opposed to the dietary studies) on rat ovarian and uterine development during gestation, lactation and weaning up to 40 days, a separate study was performed.

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 the 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) resulted in 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. In contrast

to dietary administration, the 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.

Based on these findings, the DS 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. The differences in outcome between the dietary study and the gavage study at similar dose levels are likely to be due to the low 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 the observed effects.

The assumption that the findings are a secondary effect of food impairment (fasting) is further sustained by citations from two feed-restriction studies in rats from the open literature. Reduced bodyweight by 29-30% is associated with disturbances of the oestrous cycle, decreased ovary weight and reduced number of corpora lutea. Decreased mating performance and fertility is also mentioned.

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).

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. Maternal effects were a 6% decrease of bodyweight in the top dose only. No foetal effects were noted in any dose.

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. 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 except for the following.

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 (7 and 5, respectively, 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 in 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. However, it should be noted that the foetal and litter incidences of cleft palate in this study barely exceeded the laboratory historical control range (5 pups/litter).

In another guideline compliant 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. Animals were terminated on Day 20 and the uterine contents investigated. Maternal toxicity is reflected by the mean terminal bodyweight of treated females; the value was slightly lower than in controls due to a slightly (but significantly) reduced body weight gain (by 7%) during the dosing period. Food consumption in treated females was also significantly lower (by 13%) than controls. No foetal effects were noted.

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. 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 $\geq$ 300 mg/kg bw/d. Maternal Toxicity: 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. Foetal toxicity: 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.

Based on an assessment of the data, the DS proposed no classification.

Comments received during public consultation

Two MSCAs challenged the proposal for no classification recommended by the DS. With respect to fertility, it was argued that the limited incidence of ovarian atrophy seen in the 261 mg/kg bw/d group, the dose at which the reduction in bodyweight was much lower, is not consistent with the overall range of findings conclusion. Also, a study from the public literature that could not associate malnutrition with reduced fertility was noted. Moreover, the second comment considered that the observed effects on body weight and food consumption during the 2 generation study were not severe enough to exclude a specific effect of amisulbrom on female fertility.

Assessment and comparison with the classification criteria

Fertility

The association between malnutrition and decreased fertility in female rats is shown in a 2 generation guideline study, the supportive mechanistic studies and two literature citations. 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 during lactation, weaning and sexual maturation. While the influence of amisulbrom could explain these differences, the poor palatability and thus severe food impairment was raised by the DS as an explanation. However, the STOT RE studies showed that the lower body weight gain cannot be attributed solely to poor palatability; the food conversion efficiency indicates that reduced bodyweight gain was not entirely attributable to low food consumption and provides evidence for an underlying toxic effect. This conclusion was drawn in the 28 and 90 days studies and in the 2-year chronic toxicity/carcinogenicity study. Also, in the dermal toxicity study, the same effect but of a lower magnitude was observed.
The low body weight and fertility impairment are dose-dependent, as shown by the findings at the top and middle doses. Also, it is noted that the relative liver weight in the offspring is higher and also appears to be dose-dependent. Two of the findings, the atrophic ovaries and uterus metaplasia, could be regarded as amisulbrom-related. The atrophic ovaries seen at the top and middle doses in the 2 generation study decrease with the dose; however, in the three dietary mechanistic studies which focused on ovary development (foetal, young and juvenile rats) this association could not be supported. Moreover, in the gavage mechanistic study, which focused on the effects on ovary development in juvenile rats, no effect on follicular development in the ovary could be found. With respect to uterus metaplasia, the uterotrophic assay based on gavage administration showed no effect on the level of uterine mucosal proliferative activity.

Overall, an impaired fertility effect associated with severe bodyweight decrease was shown at the highest and middle doses. The findings are explained by the DS to be attributable to malnutrition caused by poor palatability. RAC agrees with the argumentation provided and the proposal for **no classification for the fertility** endpoint for amisulbrom.

Developmental

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. The incidence of cleft palate slightly exceeded the laboratory historical control range (1-5 foetuses/litter) and is attributed by the DS to a spontaneous (genetic) aetiology typical of this strain/colony. It is to be noted that the incidence of cleft palate is low, considering that the study used 22 animals per group for each dose. Also, in the pups that died before termination in the rat multi-generation study (Han Wistar from the Harlan, UK colony), 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; the observed incidence was within the laboratory historical control range. Furthermore, the incidence of chondrodystrophy for this colony is analysed in a comprehensive table summarising 42 embryofoetal studies performed between 1996 and 2010. The syndrome was detected in various studies but the historical control range was exceeded in 2 studies: the study with 7 affected pups in one litter and 5 affected pups in another litter (technically still in the control range) and a study in which 7 affected pups in one litter out of 22 litters was registered. In the rest of the studies the chondrodystrophy with cleft palate did not follow a dose-dependent pattern.

On the other hand, 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. The same applies to the range-finding study in SD rats with doses up to 1000 mg/kg bw/d.
The rabbit study performed at doses where no maternal toxicity was did not reveal treatment-induced effects.

In general, in teratogenicity studies, the cleft palate in rats is a dose-dependent deformity with consistent reproducibility. In the case of amisulbrom, the presence of cleft palate has an incidence at the limit of the historical control range of the testing laboratory; it was shown that the Han Wistar, Harlan, UK breeding colony has an occurrence of the chondrodystrophy syndrome. Moreover, in a test performed on a different breeding colony in a different laboratory cleft palate was not observed. Testing on rabbits could also not confirm the presence of cleft palate. Therefore, the observed cleft palate in rats in the first study cannot be strongly associated with the chemical treatment. In summary, the observed developmental effects are not attributable to amisulbrom but rather to a genetic cause. Therefore, RAC agrees with the DS proposal **not to classify amisulbrom as a developmental toxicant**.

Overall conclusion

Based on the presented argumentation, RAC agrees that **no classification for amisulbrom is warranted for reproductive toxicity**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Amisulbrom is not currently listed on Annex VI of CLP (Regulation (EC) 1272/2008). The DS proposed to classify the substance as Aquatic Acute 1; H400 with an M-factor =10 and Aquatic Chronic 1; H410 with an M-factor =10.

Degradation

A hydrolysis study carried out according to EU guideline 92/69/EEC, C.7 (1992) and in compliance with GLP (Wicks, 2004a) indicated that amisulbrom is hydrolytically stable at 25 °C and pH 7 (DT_{50} = 76.5 days) and 4 (DT_{50} = 78.5 days). The rate of hydrolysis was shown to be pH dependent and more rapid at pH 9 (DT_{50} = 5.0 days at 25 °C). At pH 4 and 7, the sole degradant occurring at levels > 10% AR was IT-4[1], with maxima of 17.7% AR and 15.9% AR respectively. At pH 9, degradant I-1[2] occurred at a maximum of 70.1% AR, IT-4 occurred at a maximum of 17.8% AR and T-1[3] at 39.8% AR. These degradants are stable to hydrolysis since they were at their maxima at study end. The hydrolytic degradation rates of amisulbrom when converted to 20 ºC 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). It was therefore concluded by the DS that degradant formation due to hydrolysis, including that at pH 9, will not be significant under field conditions, and that the degradants formed in this hydrolysis study do not need further consideration. Furthermore, as the hydrolysis half-life was not < 16 days for all the relevant pH, the DS concluded that amisulbrom is not rapidly degradable.

The photodegradation of amisulbrom in water was studied according to Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) guideline 12 Nohsan no. 8147 and in compliance with GLP (Takehara, 2004). In the study conducted at 25 °C under artificial sunlight conditions

[1] IT-4: 3-bromo-6-fluoro-2-methyl-1- (1H-1,2,4-triazol-3-ylsulfonyl)indole

[2] I-1: 3-bromo-6-fluoro-2-methylindole

[3] T-1: 1-(N,Ndimethylaminosulfonyl)-1,2,4-triazole-3-sulfonic acid

equivalent to south-central EU in spring-summer, amisulbrom was shown to be photolytically degraded with DT_{50} values in sterilised river water at pH 6.7 of 4.2 (indole labelled amisulbrom) and 4.4 hours (triazole labelled amisulbrom). Degradants T-3[1] (50.6% AR), T-1 (22.8% AR), T-4[2] (15.2% AR) and IT-12[3] (6.7% AR) were produced, along with others at lower levels. 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.

In a second aquatic photodegradation study (Wicks, 2004b) performed according to a SETAC Europe guideline and in compliance with GLP, amisulbrom was degraded with DT_{50} value of 4.9 hours at 25 °C in a sterile buffered solution at pH 4 under artificial sunlight equivalent to south-central EU in spring-summer. Major photolytic degradates which accounted for > 10% AR were I-2[4], I-8[5], I-9[6], T-1 and T-7[7]. Of these, T-1, I-2 and I-8 were all at their maxima at study end.

A reliable ready biodegradation study (Barnes, 2004) following OECD TG 301B (modified Sturm test) was conducted at 22 °C and pH 7.5-7.8 for 29 days. 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. Validation criteria for the reference and toxicity controls were met. There was no evidence of biodegradation of amisulbrom (0%) at 10 mg C/L by the end of the test on day 29 and amisulbrom was considered to be not readily biodegradable under the conditions of this test.

An aerobic water/sediment simulation study, carried out according to OECD TG 308 and in compliance with GLP, was run for 120 days in the dark at 20 °C (pH range 5.4-8.0) using two natural systems (Unsworth, 2004c). Amisulbrom dissipated in both systems from the water phase to the sediment layer (dissipation DT_{50}s 6.4-7.1 days). In the sediments, some degradation of amisulbrom plus dissipation back to the water and limited mineralisation was noted (volatile radioactivity/mineralization only accounted for 1.2-1.3% AR by day 120). Whole system DT_{50} values were calculated to be 64.2 days for the clay loam system and 156.1 days for the clay system, indicating that amisulbrom is not ultimately degraded (half-life of > 16 days). In the water/sediment systems, the only degradants occurring at levels ≥ 5% AR were IT-4 and IT-15[8]. 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 a decline phase for IT-15 in either system, DT_{50}s could not be determined for this degradant.

Overall, the DS concluded that amisulbrom is not rapidly degradable for the purposes of classification under the CLP Regulation.

Bioaccumulation

The experimental logKow value is 4.4 at pH 6.4 and 25°C (Ogi, 2003d), carried out according to EU guideline 92/69/EEC, A.8. (1992). This value is greater than the CLP trigger value of 4 intended to identify substances with a potential to bioaccumulate. However, a reliable experimental BCF value is available from the study by Van der Kolk (2005), carried out according

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[1] T-3: 1H-1,2,4-triazole-3-sulfonic acid

[2] T-4: 1H-1,2,4-triazole

[3] IT-12: 3-(1H-1,2,4-triazol-3-ylsulfonyl)-6-fluoro-2-methylindole

[4] I-2: 2-acetylamino-4-fluorobenzoic acid

[5] I-8 (hydroxylated I-2): 2-acetylamino-4-fluorohydroxybenzoic acid

[6] I-9 (I-5 dimer): 2,2'-oxybis(6-fluoro-2-methylindolin-3-one)

[7] T-7 (T-2 isomer): 5-(N,Ndimethylaminosulfonyl)-1H-1,2,4-triazole

[8] IT-15: 6-fluoro-2-methyl-1-(1H-1,2,4-triazol-3-ylsulfonyl)indole

to OECD TG 305 and US EPA OPPTS 850.1730 (draft 1996) and in compliance with GLP. The bioaccumulation of [triazole-^{14}C]amisulbrom and [indole-^{14}C]amisulbrom in bluegill sunfish (*Lepomis macrochirus*) was determined under flow-through conditions for 14 days, followed by a depuration period of 28 days. The nominal exposure concentrations were 0.05 and 0.5 µg [triazole-^{14}C]amisulbrom/L (for 48 and 88 fish respectively) and 0.5 µg [indole-^{14}C]amisulbrom/L (for 56 fish). The BCF values were based on measured TRR (Total Radioactive Residue) and concentration of amisulbrom. The test substance was rapidly metabolised and excreted by the fish, so no plateau value was obtained: as after day 12 the values of TRR and the concentration of amisulbrom in fish did not increase it was concluded that a maximum concentration had been reached. Therefore the uptake phase was terminated after 14 days. The worst case whole fish BCF was 176 L/kg (or 457 L/kg based on TRR) and as such less than the trigger of 500 according to the CLP Regulation. Major metabolites (>10% TRR) were IT-4 and an unidentified single polar fraction, associated with the non-extractable fraction of the non-edible tissues. All other metabolites were detected at < 10% TRR. No radioactivity was found in the extractable fraction of the edible tissues of fish.
It was not clear to the DS whether lipid normalisation or growth correction was performed. However, as the lipid content was close to 5% (4.5%) the DS concluded that it 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.

Aquatic toxicity

Toxicity studies on amisulbrom and its main degradants IT-4 and IT-15 are available for fish, aquatic invertebrates, algae and sediment-dwelling organisms. Although I-1 was a major hydrolysis degradant, it was not considered major or relevant in natural water/sediment systems and so no ecotoxicological studies were conducted on it.

All tests followed standard guidelines and were in compliance with GLP. A summary of relevant information on aquatic toxicity studies is reported in the Table below.

Summary of relevant information on aquatic toxicity

Method, test substance	**Test organism**	**Conditions**	**Endpoint**	**Toxicity values in [mg a.s./L]**	**Reference**
Short-term toxicity to aquatic fish					
OECD TG 203 amisulbrom	*Cyprinus carpio*	Flow-through	96-h LC_{50}	**0.0229**	Jenkins, 2003c (DAR B.9.2.1.1.1(v))
OECD TG 203 amisulbrom	*Pimephales promelas*	Flow-through	96-h LC_{50}	0.0363	Jenkins, 2003b (DAR B.9.2.1.1.1(iv))
OECD TG 203 amisulbrom	*Lepomis macrochirus*	Flow-through	96-h LC_{50}	0.0407	Jenkins, 2004b (DAR B.9.2.1.1.1(iii))
OECD TG 203 amisulbrom	*Oncorhynchus mykiss*	Flow-through	96-h LC_{50}	0.0515	Jenkins, 2003a (DAR B.9.2.1.1.1(i))
OECD TG 203 amisulbrom	*Danio rerio*	Flow-through	96-h LC_{50}	0.12	Jenkins, 2006 (DAR B.9.2.1.1.1(vi))

Method, test substance	Test organism	Conditions	Endpoint	Toxicity values in [mg a.s./L]	Reference
OECD TG 203 amisulbrom	*Gasterosteus aculeatus*	Flow-through	96-h LC_{50}	0.17	Jenkins, 2004a (DAR B.9.2.1.1.1(ii))
OECD TG 203 IT-4	*Cyprinus carpio*	Semi-Static	96-h LC_{50}	0.232	Jenkins, 2005a (DAR B.9.2.1.2.1(i))
OECD TG 203 IT-5	*Cyprinus carpio*	Semi-Static	96-h LC_{50}	11.0	Jenkins, 2005b (DAR B.9.2.1.2.1(ii))
Long-term toxicity to aquatic fish					
OECD TG 210 Amisulbrom	*Pimephales promelas*	Flow-through	28-d NOEC (based on growth)	**0.037**	Cockroft, 2005a (DAR B.9.2.1.4.1(i))
OECD TG 210 IT-4	*Pimephales promelas*	Flow-through	28-d NOEC (based on growth)	0.16	Cafarella, 2008 (DAR B.9.2.1.5.1(i))
Short-term toxicity to aquatic invertebrate					
OECD TG 202 amisulbrom	*Daphnia magna*	Static	48 hr EC_{50}	**0.0368**	Jenkins, 2003d (DAR B.9.2.1.1.2(i))
OECD TG 202 IT-4	*Daphnia magna*	Static	48 hr EC_{50}	4.39	Jenkins, 2005c (DAR B.9.2.1.2.2(i))
OECD TG 202 IT-15	*Daphnia magna*	Static	48 hr EC_{50}	22	Jenkins, 2005d (DAR B.9.2.1.2.2(ii))
Long-term toxicity to aquatic invertebrate					
OECD TG 211 amisulbrom	*Daphnia magna*	Semi-static	21 d NOEC (Reproduction, mortality)	**0.0197**	Jenkins, 2004c (DAR B.9.2.1.4.1(i))
Sediment-dwelling organisms					
OECD TG 219 amisulbrom	*Chironomus riparius*	Static, spiked-water	28 d NOEC (development, emergence)	**0.1114**	Cockroff, 2005b (DAR B.9.2.1.6(i))
Algae					
OECD TG 201 amisulbrom	*Pseudokirchneriella subcapitata*	Static	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))
OECD TG 201 IT-4	*Pseudokirchneriella subcapitata*	Static	72 hr E_rC_{50} NOE_rC	3.28 0.467	Jenkins, 2005e (DAR B.9.2.1.2.3(i))
OECD TG 201	*Pseudokirchneriella subcapitata*	Static	72 hr E_rC_{50}	24.9	Jenkins, 2005f (DAR

Method, test substance	Test organism	Conditions	Endpoint	Toxicity values in [mg a.s./L]	Reference
IT-15			NOE_rC	2.16	B.9.2.1.2.3(ii))

The key endpoints for each group are given in bold text

Fish

Six acute and one chronic aquatic toxicity studies carried out with amisulbrom on freshwater fish are available. Three other studies are also available with the degradants IT-4 and IT-5.

The lowest acute fish toxicity value is derived from a study on common carp (LC_{50} = 0.0229 mg/L). The next most acutely sensitive species is fathead minnow (LC_{50} = 0.0363 mg/L), that was the only species used for chronic testing of amisulbrom (NOEC = 0.037 mg/L). The results were based on measured concentrations (95% confidence).

In the chronic study, exposure to amisulbrom lasted 5 days pre-hatch and 28 days post-hatch. No significant mortality was observed in any test concentration or solvent control, as well as weight and length of fry were unaffected by amisulbrom. Based on measured concentrations, the 28-day NOEC for post-hatch survival, length and dry weight for newly fertilised fathead minnow fry was calculated to be 0.037 mg/L.

The amisulbrom degradants IT-4 and IT-15 were tested on both the most sensitive species. The data indicate that IT-4 and IT-15 are less toxic to fish than the parent amisulbrom.

Aquatic invertebrates

Based on mean measured concentrations, the 48-hour EC_{50} value to *Daphnia magna* was 0.0368 mg/L (95% confidence).

In the chronic toxicity study, a 21-day NOEC value to *Daphnia magna* for both adult mortality and reproduction was determined to be 0.0197 mg/L, based on time-weighted mean measured concentrations.

Two reliable studies have been submitted on the acute toxicity of the amisulbrom degradants IT-4 and IT-15 to *Daphnia magna*. The reported acute values indicate that IT-4 and IT-15 are less toxic to *Daphnia* than the parent amisulbrom.

Algae and aquatic plants

Regarding toxicity to algae and aquatic plants, the DS provided three toxicity studies on *Pseudokirchneriella subcapitata*, one with amisulbrom and the other two with its degradants IT-4 and IT-15.

The amisulbrom study was based on mean measured concentrations; both the area under the growth curve and the average specific growth rate were calculated using the geometric mean of measured concentrations. The test revealed losses of test substance, attributed to adsorption/absorption by algal cells but possibly also related to the photolysis.

When the limit of aqueous solubility of amisulbrom had been exceeded, there was some precipitation of test substance, but the values used to describe the effect levels were under that limit. Growth rate was significantly reduced at nominal concentrations of 0.04 mg/L and above, but no abnormalities were detected up to that concentration. At 0.08 and 0.160 mg/L, algal cells were swollen compared to those in the controls.

The 72h E_rC_{50} was 0.0521 mg/L, the 96h E_rC_{50} was 0.057 mg/L and the NOEC (96h) for both biomass and growth rate was 0.0139 mg/L.

The toxicity of amisulbrom degradants IT-4 and IT-15 were tested with two reliable studies. For IT-4 and IT-15 respectively, the reported 72h 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. These data indicate that both degradants are less toxic to algae than the parent amisulbrom.

Sediment-dwelling organisms

A study to the sediment-dwelling phase of the midge *Chironomus riparius* was provided. This was the spiked-water variant of the test and it was conducted using [triazole-^{14}C]amisulbrom with radiochemical purity > 98%.

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/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 0.1114 mg/L.

The chronic toxicity of amisulbrom degradants IT-4 and IT-15 were tested with two reliable studies. The data indicate a low level of toxicity but as they employed the sediment-spiking method (OECD TG 208) they are not considered relevant to the classification of amisulbrom.

Comments received during the public consultation

Three MSCA commented during the public consultation on the proposed environmental classification.

One MSCA supported the environmental classification and the proposed M-factor with a general comment and, in another specific comment, supported the surrogate approach to derive the long-term hazard classification with the lowest acute data for fish. However the provided conclusion on the chronic study in the comment differs from that provided by the DS. In the MSCA's opinion, relevant effects observed on dry weight of fry exposed even to the lowest test concentration of 0.0011 mg/L (25% increase) should be considered. Therefore the lowest relevant NOEC should be < 0.0011 mg/L. As no other chronic toxicity data for fish are available, the use of the surrogate approach for fish was considered justified. The DS referred in his response to the comment to the DAR where these deviations were not highlighted as being statistically significant but also stressed that the conclusion on the proposed environmental classification would not change.

A second MSCA expressed some doubt over the proposed chronic M-factor of 10 being too conservative. Although the fish species tested for chronic toxicity was not the most acutely sensitive, the acute LC_{50} value does not differ significantly from that on the most sensitive species. The DS emphasised that the doubt over the reliability of the chronic study is also related to the lack of difference between the acute and the chronic fish endpoints.

Moreover, another MSCA expressed a general support for the well justified CLH proposal.

Assessment and comparison with the classification criteria

Degradation

The substance is hydrolytically stable at neutral pH, even if hydrolysis is pH dependant and more rapid at pH 9. As the hydrolysis half-life is not < 16 days for all relevant pH, amisulbrom screens as not rapidly degradable. Regarding photodegradation in water, amisulbrom was shown to be photolytically degraded with DT_{50}s of 4.2 to 4.4 hr at pH 6 and 4.9 hr at pH 4. Thus, while photodegradation could be considered a major mechanism of degradation for amisulbrom in

surface waters under certain conditions, this might, however, not be broadly applicable to turbid natural surface waters in lower insolation regions of the EU.

In a ready biodegradation study no degradation 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. Moreover the degradation information from a water/sediment simulation test 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 indicate that key degradants such as IT-4 and IT-15 exhibit some biological activity/ecotoxicity (although less than the parent). In conclusion, RAC agrees with the DS' proposal that amisulbrom is considered to be not rapidly degradable for the purposes of classification under the CLP Regulation.

Bioaccumulation

The logKow of amisulbrom is 4.4, this is greater than the cut off in the CLP Regulation of 4. However, a reliable experimental fish bioconcentration factor (BCF) is available showing that amisulbrom has a low potential to bioaccumulate, based on the worst case whole fish BCF value in bluegill sunfish of 176 L/kg, which is less than the cut off value of 500 in the CLP Regulation.

Aquatic toxicity

Acute toxicity data are available for all three trophic levels. The most acutely sensitive trophic group is fish with a 96-h LC_{50} value for *Cyprinus carpio* of 0.0229 mg/L. This acute endpoint is in the range of 0.01 < L(E)C_{50} ≤ 0.1 mg/L, *i.e.* the range of application of an M-factor of 10 .

Chronic toxicity data are available for all three trophic levels. 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. RAC agrees with the DS' proposal to use the surrogate approach for chronic classification:

- the lowest chronic endpoint is reported for algae, with a 96h NOE_rC for *Pseudokirchneriella subcapitata* of 0.0139 mg/L. This value is in the range 0.01 < NOEC ≤0.1 mg/L and amisulbrom is not rapidly degradable, therefore it would be classified as Chronic 1 with a chronic M-factor of 1;
- the lowest acute endpoint for fish falls between 0.01 < L(E)C_{50} ≤ 0.1 mg/L and amisulbrom is not rapidly degradable, therefore it would be classified as Chronic 1 with a chronic M-factor of 10.

Conclusion on the classification

Amisulbrom is considered not rapidly degradable and does not fulfil the criteria for bioaccumulation. The lowest acute toxicity value falls in the range 0.01 < L(E)C_{50} ≤ 0.1 mg/L, therefore amisulbrom fulfils the CLP criteria for classification as **Aquatic Acute 1**; **H400** with an **M-factor of 10**. Based on the most stringent outcome of the surrogate approach (lowest acute endpoint for fish falls between 0.01 < L(E)C_{50} ≤ 0.1 mg/L range and amisulbrom is not rapidly degradable), amisulbrom fulfils the CLP criteria for classification as **Aquatic Chronic 1**; **H410** with an **M-factor of 10.**

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and by RAC (excluding confidential information).