Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

clofentezine (ISO); 3,6-bis(*o*-chlorophenyl)-1,2,4,5-tetrazine

EC Number: 277-728-2
CAS Number: 74115-24-5

CLH-O-0000006816-65-01/F

Adopted

11 June 2020

11 June 2020

CLH-O-0000006816-65-01/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: **clofentezine (ISO); 3,6-bis(*o*-chlorophenyl)-1,2,4,5-tetrazine**

EC Number: **277-728-2**

CAS Number: **74115-24-5**

The proposal was submitted by **Spain** and received by RAC on **21 June 2019.**

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

PROCESS FOR ADOPTION OF THE OPINION

Spain 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 **24 July 2019**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **24 September 2019**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Peter Hammer Sørensen**

Co-Rapporteur, appointed by RAC: **Irina Karadjova**

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 **11 June 2020** by **consensus**.

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

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	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	TBD	clofentezine (ISO); 3,6-bis(*o*-chlorophenyl)-1,2,4,5-tetrazine	277-728-2	74115-24-5	Carc. 2 Aquatic Chronic 1	H351 H410	GHS08 GHS09 Wng	H351 H410		M=1	
RAC opinion	TBD	clofentezine (ISO); 3,6-bis(*o*-chlorophenyl)-1,2,4,5-tetrazine	277-728-2	74115-24-5	Aquatic Chronic 1	H410	GHS09 Wng	H410		M=1	
Resulting Annex VI entry if agreed by COM	TBD	clofentezine (ISO); 3,6-bis(*o*-chlorophenyl)-1,2,4,5-tetrazine	277-728-2	74115-24-5	Aquatic Chronic 1	H410	GHS09 Wng	H410		M=1	

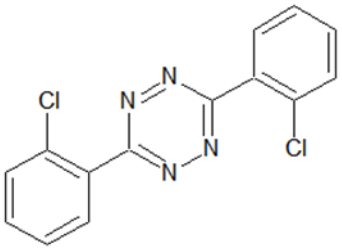
GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Clofentezine is an acaricide used as an active substance in plant protection products (PPP). It was included in Annex I to Directive 91/414/EEC EFSA previously finalised a conclusion on this active substance on 4 June 2009 (EFSA Scientific Report (2009) 269, 1-113) and proposed classification of clofentezine as R53 according to Directive 67/548.

Clofentezine is not currently listed in Annex VI of the CLP regulation.

A Renewal Assessment Report (RAR) in accordance with Commission Regulation (EC) No. 844/2012 has been developed by the Spanish CA. The content of this CLH Report is therefore based on data included in the RAR.



RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Explosive

The DS originally proposed no classification based on an ECC A.14 negative study. However, after the consultation they changed the conclusion to no classification due to lack of data.

Flammable solids

Clofentezine did not meet the pass criteria of an EEC A.10 test and consequently, the CLP criterion. Therefore, the DS concluded that clofentezine is not a flammable solid.

Self-reactive substances

The DS originally proposed no classification based on perceived available data. However, after the consultation they changed the conclusion to no classification due to lack of data.

Pyrophoric solids

Although no test data are provided, there is no information to indicate that clofentezine ignites after 5 minutes in contact with air. Consequently, the DS concluded that no classification was warranted.

Self-heating substances

The DS originally proposed no classification based on a negative ECC A.16 study and perceived available data in line with ECHA guidance on the application of the CLP criteria. However, after the consultation they changed the conclusion to no classification due to lack of data.

Substances which in contact with water emit flammable gases

Although no test data are provided, there is no information to indicate that clofentezine emits flammable gases in contact with water. Consequently, the DS concluded that no classification was warranted.

Oxidising solids

Clofentezine was not oxidising according to the results of an EEC A.17 test and therefore did not meet the CLP criterion for this hazard class. Consequently, the DS concluded that clofentezine did not warrant classification as an oxidising solid.

Corrosive to metals

Although there are no test data available, clofentezine is a solid that decomposes before boiling (exothermic effect is observed at 233.6ºC). Consequently, the DS concluded that clofentezine did not warrant classification as corrosive to metals.

Comments received during public consultation

One MSCA commented on the lack of data for precise classification of clofentezine for physical hazards: explosive, self-reactive and self-heating properties. The MSCA highlighted that a negative result from the EEC A.14 did not automatically mean that substance should not be classified as explosive under CLP. The MSCA requested as a minimum a DSC measurement for justifying that the classification procedures for explosives or self-reactive substances does not need to be performed if the exothermic decomposition energy is less than 300 J/g. The MSCA recommended new testing to determine the exothermic decomposition energy and if this is higher than 300 J/g (but less than 500 J/g) also the SADT. They also recommend the following changes to the CLH Report:

Explosives: Reason for no classification - data lacking

Self-reactive substances: Reason for no classification - data lacking

Self-heating substances: Reason for no classification - data lacking

The MSCA pointed that the EU test method A.16 as described in Regulation (EC) No 440/2008 is generally inappropriate for substances with a low melting point and that the findings do not lead to a classification.

The DS agreed that the data gap should be reflected as a "data lacking" statement.

Assessment and comparison with the classification criteria

RAC agrees in general with MSCA comments for appropriate data lacking but noted some important points regarding explosive, self-reactive and self-heating properties.

Explosives

- Clofentezine does not contain aliphatic azo groups (-R-N=N-R-) shown in Table 6.1 indicating explosive properties (Table A6.1 in Appendix 6 of the UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria)
- Clofentezine is not strictly speaking a nitrogen rich compound – nitrogen content 18%.
- In the paper, Löbbecke, 1999, not one of diaryl-substituted tetrazines showed exothermic decomposition energy above 300 J/g. In addition, according to the mechanism of decomposition of diaryl-substituted tetrazines explosive decomposition should not be expected. Almost the same should be valid for clofentezine.
- In the Bretherick's Handbooks no examples for explosive diaryl-substituted tetrazines are given.
- A negative EC A.14 study is available and might be accepted as supportive evidence.
- The substance is on the market for more than 15 years without incidents.

Consequently, RAC is of the opinion that **clofentezine does not warrant classification for explosive properties**.

Flammable solids

The screening part of the studies for UN test N.1 and the EEC A.10 method are equivalent, therefore a result as 'not highly flammable' from the EEC A.10 method is considered sufficient to conclude on the classification (see ECHA Chapter R.7a: Endpoint specific guidance, R.7.1.10.3). Consequently, RAC is of the opinion that **clofentezine does not warrant classification as a flammable solid**.

Self-reactive substances

Thermally unstable substances or mixtures that are not classified as explosives should be considered for classification as self-reactive substances and mixtures.

RAC notes:

- Clofentezine does not contain aliphatic azo groups shown in Table A6.1 of UN RTGD.
- Clofentezine does not contain chemical groups shown in Table A6.3 UN RTGD (equivalent to A6.2 on a previous version) indicating self-reactive properties.
- However, an accurate determination of the exothermic decomposition energy and SADT of the substance is lacking.

In conclusion, **no classification due to lack of data** is the only appropriate recommendation RAC can make.

Pyrophoric solids

If a substance does not ignite upon contact with a very hot flame (as in an EEC A.10 test) or upon heating, it will not ignite spontaneously at room temperature. Therefore, RAC considers **clofentezine does not warrant classification for this hazard class**.

Self-heating substances

RAC agrees that EU test method A.16 is in generally inappropriate for substances with low melting points (Guidance on the application of CLP criteria, 2017).

The conclusion from the test method A.16 should be: No self-ignition temperature up to the melting range (180-195 °C).

In conclusion, **no classification due to lack of data** is the only appropriate recommendation RAC can make.

Substances which in contact with water emit flammable gases

The substance does not contain metals or metalloid groups, hence it fulfils the criteria for no classification in the CLP regulation 2.12.4.1. Therefore, RAC agrees that **clofentezine does not warrant classification for this hazard class**.

Oxidising solids

Clofentezine does contain fluorine atoms which are chemically bound only to carbon hence it fulfils the no classification criteria in 2.14.4.1(a). Therefore, RAC considers **clofentezine does not warrant classification for this hazard class**.

Corrosive to metals

Clofentezine has a melting point of 180°C, which is above the 55°C indicated in the CLP guidance, and has no acid or basic groups. Overall, RAC agrees that **clofentezine does not warrant classification for this hazard class.**

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute oral toxicity

The LD_{50} values obtained in the six studies performed in four species (rats, mice, hamster and dogs) are clearly above the threshold value of 2000 mg/kg bw for triggering an acute oral toxicity classification according to CLP Regulation. The Dossier Submitter (DS) proposed no classification for acute oral toxicity.

Acute dermal toxicity

The LD_{50} observed in two studies both in rats showed LD_{50} greater than 1332 mg/kg bw (limit dose) and 2100 mg/kg bw, which is above the threshold value of 2000 mg/kg bw for triggering an acute dermal toxicity classification. The DS proposed no classification for acute dermal toxicity.

Acute inhalation toxicity

Two acute inhalation studies in rats resulted in 4-hour inhalation LC_{50} values of > 5.20 mg/L, which is above the value for classification in the CLP Regulation (i.e. 5 mg/L dust/mist). The DS proposed no classification for acute inhalation toxicity.

Comments received during public consultation

No comments were received

Assessment and comparison with the classification criteria

Acute oral toxicity

Summary of animal studies on acute oral toxicity:

Method	Specie	Value LD_{50}	Reference
Method comparable to OECD TG 401 GLP: No Study acceptable as supporting information	Purity: 99% Rat strain: Sprague-Dawley Oral (gavage) 3 rats/sex/dose Doses: 0, 800, 1131, 1600, 2261 and 3200 mg/kg bw 14-day observation period	Mortality: not occurred. Clinical signs: slight urinary incontinence in 1♂ and 1♀ at 3200 mg/kg bw and slight salivation in 1♂ at 1131 mg/kg bw. Pink coloration of faeces (attributed to the test chemical) between 20 and 22 hours was seen after dosing in females at all dose levels and in males at ≥ 2261 mg/kg bw. LD_{50}: > 3200 mg/kg bw for both sexes	Anonymous 26 (1980)
Acute oral toxicity study in rats Method: OECD TG 401 GLP: No Study acceptable	Purity: 99.3% Rat strain: Sprague-Dawley Oral (gavage) 5 rats/sex/dose Doses: 5200 mg/kg bw (limit test) and controls 14-day observation period	Mortality: not occurred. Clinical signs: not observed. Bodyweight: no effects. Necropsy: no treatment related effects. LD_{50}: > 5200 mg/kg bw for both sexes	Anonymous 27 (1986a)
Acute oral toxicity study in mice Method comparable to OECD TG 401 GLP: No Study acceptable	Purity: 99.1% Mice strain: CD-1 Oral (gavage) 6 mice/sex/dose Doses: 3200 mg/kg bw (limit test) and controls 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: (↓) significant in ♀ (days 1-8). Necropsy: spleen with pale and pitted appearance or apparently small in 4/6♂ and 1/6♀ *vs.* 0/6♂ and 1/6♀ in controls. LD_{50}: > 3200 mg/kg bw for both sexes	Anonymous 28 (1986a)
Acute oral toxicity study in mice Method: OECD TG 401 GLP: No (predates GLP) Study acceptable	Purity: 99.3% Mice strain: Swiss CR1:CD1 (ICR) BR Oral (gavage) 5 mice/sex/dose Doses: 5200 mg/kg bw (limit test) and controls 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: no effects. Necropsy: no treatment related effects. LD_{50}: > 5200 mg/kg bw for both sexes	Anonymous 29 (1986b)
Acute oral toxicity study in hamster Method comparable to OECD TG 401 GLP: No (predates GLP) Study acceptable	Purity: 99.1% Hamster strain: Syrian Oral (gavage) 6 hamsters/sex/dose Doses: 3200 mg/kg bw (limit test) and controls 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: no effects. Necropsy: no treatment related effects. LD_{50}: > 3200 mg/kg bw for both sexes	Anonymous 30 (1980)
Acute oral toxicity study in dogs Method comparable to OECD TG 401 GLP: No (predates GLP) Study acceptable	Purity: 98.8-99.6% Dog strain: Beagle Oral (gavage) 2 dogs/sex in controls and at 2000 mg/kg bw and 1♂ at 1000 mg/kg bw 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: no effects. Necropsy: slight focal hyperplasia of the renal papillary epithelium was observed amongst treated male and female dogs. Although such changes were not evident in controls, this is a common, spontaneous lesion in laboratory dogs. LD_{50}: > 2000 mg/kg bw for both sexes	Anonymous 31 (1981)

The LD_{50} values obtained from the six studies performed in four species (rats, mice, hamster and dogs) are clearly above the threshold value of 2000 mg/kg bw for triggering acute oral toxicity classification according to the CLP Regulation. RAC concludes, in line with the DS, that **no classification for acute oral toxicity is warranted**.

Acute dermal toxicity

Summary of animal studies on acute dermal toxicity:

Method	Species	Value LD_{50}	Reference
Acute dermal toxicity study in rats Method comparable to OECD TG 402 GLP: No Supporting information	Purity: 99.1% Rat strain: Sprague Dawley 6 rats /sex/dose Doses: 1332 mg/kg bw (limit test) and controls 21 h of exposition (occlusive dressing) 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: no effects. Necropsy: no treatment related effects. LD_{50}: > 1332 mg/kg bw for both sexes	Anonymous 32 (1980b)
Acute dermal toxicity study in rats Method comparable to OECD TG 402 GLP: No (prior to GLP enforcement) Study acceptable	Purity: 99.3% Rat strain: Sprague Dawley 5 rats /sex/dose Doses: 2100 mg/kg bw (limit test) and controls 24 h of exposition (occlusive dressing) 14-day observation period	Mortality: none. Clinical signs: not observed. Bodyweight: no effects. Necropsy: no treatment related effects. LD_{50}: > 2100 mg/kg bw for both sexes	Anonymous 33 (1987)

The acute dermal toxicity was tested in two studies, both in rats, and the observed LD_{50} were greater than 1332 mg/kg bw (limit dose) and 2100 mg/kg bw. The value is above the threshold value of 2000 mg/kg bw for triggering acute dermal toxicity classification. RAC agrees with the DS and concludes **no classification for acute dermal toxicity is warranted**.

Acute inhalation toxicity

Summary of animal studies on acute inhalation toxicity.

<table>
<tr><th>Method</th><th>Species</th><th>Test substance, Dose levels, form and particle size (MMAD)</th><th>Value LC_{50}</th><th>Reference</th></tr>
<tr>
<td>Acute inhalation toxicity study in rats
Method comparable to OECD TG 403
GLP: No
Deviations: 6 h of exposure instead of 4 h
Supporting information</td>
<td>Rat strain: Sprague Dawley
Whole body exposure system for 6 hour and thereafter 14-day observation
5 animals/sex/dose</td>
<td>The study was performed with a preparation: wettable powder (WP) containing 77.6-82.4% w/w of clofentezine.
<table>
<tr><th rowspan="2">Parameter</th><th colspan="2">Value</th></tr>
<tr><th>Active ingredient</th><th>Preparation</th></tr>
<tr><td>Nominal concentration (mg/L)</td><td>9.08</td><td>11.35</td></tr>
<tr><td>Mean achieved atmosphere concentration (mg/L)</td><td>1.51</td><td>1.89</td></tr>
<tr><td>Particle size (MMAD ± GSD)</td><td colspan="2">2.7 ± 0.2</td></tr>
<tr><td>% inspirable (< 4 μm)</td><td colspan="2">> 79.6%</td></tr>
</table>
</td>
<td>Mortality: none.
Clinical signs: coolness to touch and pale eyes on removal of animals from the exposure chamber.
LC_{50} > 1.51 mg/L</td>
<td>Anonymous 34 (1982)</td>
</tr>
</table>

<table>
<tr><th>Method</th><th>Species</th><th>Test substance, Dose levels, form and particle size (MMAD)</th><th>Value LC_{50}</th><th>Reference</th></tr>
<tr>
<td>Acute inhalation toxicity study in rats
Method comparable to OECD TG 403
GLP: Yes
Study acceptable</td>
<td>Rat strain: HsdHanTM: WIST rats
Nose-only for 4 hour exposure and thereafter 14-day observation
5 animals/sex/dose</td>
<td>Purity: 98.2%.
<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Nominal concentration (mg/L)</td><td>6.17</td></tr>
<tr><td>Mean achieved atmosphere concentration (mg/L)</td><td>5.20</td></tr>
<tr><td>Chamber flow rate (L/min)</td><td>50</td></tr>
<tr><td>Particle size (MMAD ± GSD)</td><td>3.24 ± 2.45</td></tr>
<tr><td>% inspirable (< 4 μm)</td><td>59.3%</td></tr>
</table>
</td>
<td>Mortality: not seen.
Necropsy: abnormally dark lungs in 2/5 ♀ .
LC_{50} > 5.20 mg/L</td>
<td>Anonymous 35 (2010)</td>
</tr>
</table>

In Anonymous 34 (1982), an acute inhalation toxicity study in rats, performed with a preparation consisting of a wettable powder of clofentezine and deemed acceptable only as supporting information, the LC_{50} was found to be greater than 1.51 mg/L after 6-hour exposition.

In Anonymous 35 (2010), an acute inhalation toxicity study in rats carried out with clofentezine, the LC_{50} was found to be greater than 5.20 mg/L after 4-hour exposition.

Based on the 4-hour inhalation LC_{50} of > 5.20 mg/L for rats is above the value for classification in the CLP Regulation (i.e. 5 mg/L dust/mist) RAC agrees with the DS that **no classification for acute inhalation toxicity is warranted**.

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

Summary of the Dossier Submitter's proposal

The only effects observed in the range for STOT SE classification after oral administration were slight salivation in rats and slight focal hyperplasia of the renal papillary epithelium in dogs. The effects in dogs are a common, spontaneous lesion, while the findings in rats were not considered by the DS to be sufficiently adverse to justify classification. No relevant effects were observed after dermal application or inhalation of clofentezine.

No clinical signs were observed which are relevant for classification for STOT SE 3 according to CLP Regulation (respiratory tract irritation and narcotic effects).

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the DS. The slight clinical effects seen in one rat (slight salivation) and the observed slight focal hyperplasia of the renal papillary epithelium in dogs were not relevant for classification. The renal hyperplasia is commonly observed and occurs spontaneously in laboratory dogs. Therefore, **no classification for specific target organ toxicity – single exposure is warranted**.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

In a guinea pig skin irritation study, slight oedema (not graded) was observed in 2/12 application sites of 6 animals (two sites/animal). It is not known if these two positive responses at two application sites occurred in one or two animals. The results indicated negligible primary irritation of clofentezine. The study is considered acceptable as additional supporting information only due to a large number of deficiencies.

An *in vitro* human skin irritation assay based on an OECD TG 439 compliant method resulted in a mean relative tissue viability > 50%, indicating that clofentezine did not have skin -irritating potential. The DS noted that according to the ECHA Guidance on the application of the CLP criteria (July 2017) this method can reliably distinguish non-classified from classified substances and it is considered valid for the evaluation of skin irritation potential of substances.

The DS suggested, based on data available, that clofentezine does not require classification as skin irritant.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

A summary table of the submitted studies relating to skin corrosion/irritation is presented below:

<table>
<tr><th>Method</th><th>Species</th><th>Results</th><th>Reference</th></tr>
<tr><td>Skin irritation study in guinea pigs
Method comparable to OECD TG 404
GLP: No (predates GLP)
Study acceptable as supporting information</td><td>Purity: 99.1%
Guinea pig strain: Dunkin-Hartley
6 animals (female)
Vehicle: 0.5% gum tragacanth (aq)
Test preparation: 333 mg/mL of the test chemical in the vehicle.
4 sites of application/animal: 0.2 mL of the test preparation in two sites (A and D), 1 with 0.2 mL of vehicle (B) and one for blank (C).
24 h of exposition and 7 days of observation.</td><td>Only slight oedema (not graded) was observed in 2/12 test application areas:
– 1 until 2 ½ days after washing application area.
– 1 from 2 ½ hours after washing application area until 2 ½ days.

It is not known according to data available if both areas corresponded to 1 or 2 animals.

Conclusion: Negligible primary skin irritation</td><td>Anonymous 36 (1980c)</td></tr>
<tr><td>In vitro skin irritation: human skin model test
Method OECD TG 439: EPISKIN-SMTM
GLP: Yes
Study acceptable</td><td>Purity: 98.7%
Skin model: (non-cancerous), adult human-derived epidermal keratinocytes (NHEK) cultured to form a multi-layered, highly differentiated model of the human epidermis
Control negative (10 µL): Phosphate Buffered Saline
Control positive (10 µL) : 5% sodium dodecyl sulphate
Clofentezine: (10 mg + 10 µL distilled water)</td><td><table><tr><th></th><th>Negative Control</th><th>Positive Control</th><th>Test Chemical</th></tr><tr><td>Mean relative tissue viability (%) ± SD</td><td>100 ± 4.8</td><td>18.5 ± 6.1</td><td>101.4 ± 4.5</td></tr></table>Evaluation criteria according to the method:
Irritant: ≤ 50% mean tissue viability (% negative control).
Non-Irritant: > 50% mean tissue viability (% negative control)

Conclusion: Non-irritant</td><td>Gehrke (2015)
(AS)
B.6.2.4-02</td></tr>
</table>

In the guinea pig skin irritation study slight oedema (not graded) was observed in 2/12 application sites of 6 animals. The result indicated negligible primary irritation of clofentezine. The study is considered acceptable only as additional information due to a large number of deficiencies.

In the *in vitro* human skin irritation assay based on an OECD TG 439 method the result was a mean relative tissue viability > 50%, indicating the non-irritative potential of clofentezine.

Based on the negative results in the available studies, RAC agrees with the DS that **clofentezine does not warrant classification as a skin irritant.**

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

The DS proposed no classification for eye damage/irritation. According to the ECHA Guidance on the application of the CLP criteria (July 2017), when 6 rabbits are used in the eye irritation study the test material is considered irritant to the eye when conjunctival erythema is ≥ 2 in at least 4/6 animals. The erythema of 0.33 in 2/6 animals obtained in the study does not meet the criteria for classification as irritating to the eyes according to CLP.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

A summary table of the submitted study on eye damage/irritation is presented below:

<table>
<tr><th>Method</th><th>Species</th><th>Results</th><th>Reference</th></tr>
<tr>
<td>Eye irritation study in rabbits

Guideline: US EPA 81-4 comparable to OECD TG 405

GLP: No (predates GLP enforcement)
Study acceptable</td>
<td>Purity: 99.3%
Rabbit strain: New Zealand albino
6 animals (female)
70 mg of undiluted test material equivalent to a volume of 0.1 mL instilled into one eye. The other one served as control.
Eyes remained unwashed after instillation.</td>
<td>Results of animals with unwashed eyes after instillation:

<table>
<tr><th></th><th colspan="6">Cornea</th><th colspan="6">Iris</th><th colspan="12">Conjunctiva</th></tr>
<tr><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th></th><th colspan="6">Redness</th><th colspan="6">Chemosis</th></tr>
<tr><td>After 24 hours</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>1</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>After 48 hours</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>After 72 hours</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
</table>

2/6 animals showed slight erythema of 0.33 (mean score after 24, 48 and 72 h).

Conclusion: Not eye irritant.</td>
<td>Anonymous 37 (1986)

(AS) B.6.2.5-01</td>
</tr>
</table>

RAC agrees with the DS that based on the erythema score of 0.33 in 2/6 animals, the criteria for classification as irritating to the eyes according to CLP are not met. Therefore, RAC considers that **clofentezine does not warrant classification for Serious Eye Damage / Irritation**.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The DS proposed no classification for skin sensitisation based on a guinea pig maximisation test (GPMT) performed with 20 animals for the tested and control groups. After challenge with 0.5 g of the test material moistened with 0.5 mL of ethanol a positive response was observed in 2/20 animals (10%). The response in these two animals was observed 24 hours after challenge but not after 48 hours. No response was observed in the 20 animals of the control group (0%). According to the results of the study, clofentezine did not show skin sensitization potential.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

A summary of the submitted maximisation test on guinea pigs is presented below:

<table>
<tr><th>Method</th><th>Test substance</th><th>Dose levels
duration of exposure and results</th><th>Reference</th></tr>
<tr>
<td>Guinea pig maximisation test

Guideline: OECD TG 406.

Deviations: An additional topical application of the test chemical was applied immediately post-intradermal injection on day 1.

GLP: No (predates GLP enforcement)
Study acceptable</td>
<td>Purity: not stated

Female Dunkin Hartley guinea pigs (females)

20 animals for main tested group and 20 for control

Vehicle: ethanol</td>
<td><u>Preliminary test</u>:
Intradermal injection (induction): no test performed. A saturated solution (80 g/L) of the test compound in ethanol equivalent to a concentration of 8% p/v was used.

Topical application (induction and challenge): 4 guinea pigs with 4 application sites/animal were treated (occlusive patch 24 h) with the following concentrations in ethanol:
– 0.5 g of the neat test chemical moistened with 0.5 mL of ethanol.
– 50% and 25% suspensions.
– A saturated solution (0.8 g/L) in ethanol.
Results: individual irritation scores after 24/48 h were 0 (no reaction) in the 4 guinea pigs.

<u>Main test:</u>
<table>
<tr><th>Induction intradermal injection Day 1</th><th>Test</th></tr>
<tr><td>1</td><td>FCA (Freund's Complete Adjuvant)</td></tr>
<tr><td>2</td><td>Saturated solution (8 % p/v)</td></tr>
<tr><td>3</td><td>Saturated solution (8 % p/v) mixed with FCA in proportion 1:1</td></tr>
<tr><td>Induction topical application day 1 (occlusive patch 48 h)</td><td>0.5 g of the neat test chemical moistened with 0.5 mL of ethanol</td></tr>
<tr><td>Induction topical application day 8 (occlusive patch 48 h)</td><td>0.5 g of the neat test chemical moistened with 0.5 mL of ethanol</td></tr>
<tr><td>Challenge topical application day 22 (occlusive patch 24 h)</td><td>- 0.5 g of the neat test chemical moistened with 0.5 mL of ethanol
- 50% suspension of the test material in ethanol*</td></tr>
</table>
*Used to ensure that a non-irritant concentration was used and</td>
<td>Anonymous 38 (1982)

(AS) B.6.2.6-01</td>
</tr>
</table>

<table>
<tr><th>Method</th><th>Test substance</th><th>Dose levels
duration of exposure and results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>applied on the other flank of the animal

Control group had the same treatment on day 1 and 8 using ethanol instead of active substance.

<u>Results</u>

2/20 (10%) animals showed a weak response (grade 1) 24 h after challenge with the neat test chemical but not at 48 hours. No response in the controls was observed (0/20 → 0%)

<table>
<tr><th colspan="2">Challenge phase</th><th colspan="2">Incidence of significant responses</th></tr>
<tr><th colspan="2">Group</th><th>24 hours</th><th>48 hours</th></tr>
<tr><td rowspan="2">0.5 g of neat test chemical moistened with 0.5 mL ethanol</td><td>Control</td><td>0/20</td><td>0/20</td></tr>
<tr><td>Test</td><td>2/20 [a]</td><td>0/20</td></tr>
<tr><td rowspan="2">50% suspension of the test chemical in ethanol</td><td>Control</td><td>0/20</td><td>0/20</td></tr>
<tr><td>Test</td><td>0/20</td><td>0/20</td></tr>
</table>
[a] : The two positive responses are of grade 1

Conclusion: Not sensitising.</td><td></td></tr>
</table>

RAC notes that no positive control group was included in this study and the maximum concentration used was 50%.

Based on the results from the GPMT showing a positive response of 2/10 animals 24 h after challenge but not after 48 hours, RAC agrees with the DS that clofentezine did not show skin sensitization potential, **therefore no classification for skin sensitisation is warranted.**

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

Summary of the Dossier Submitter's proposal

The DS proposed no classification for STOT RE based several studies conducted by oral administration including:

One 17-d dose range finding, two 90-d, one 13-week neurotoxicity studies in rats, one 17-d dose range finding, one 28-d dose range finding, one 13-week neurotoxicity and one year studies in dogs and one 90-d in mice. Palatability studies were performed with rats (21-d), in mice (42-d) and in dogs (28-d). A multigenerational study in rats and two carcinogenicity studies, one in rats and one in mice, were also considered for STOT RE effects.

The main target organ was the liver according to the results of the available studies listed above. However, the only effects deemed relevant for STOT RE classification were found in the oral 90-day dietary study in rat with centrilobular hepatocyte enlargement in males and increases in the absolute and relative weights of liver and in the level of cholesterol at 26.2/29.3 mg/kg bw/day in both sexes. Effects were below the guidance value for STOT RE 2 (100 mg/kg bw/day) classification. The DS pointed out that no more doses were tested from 26.2/29.3 mg/kg bw/day to the limit dose of 100 mg/kg bw/day since the next tested dose level in the study was 265/292 mg/kg bw/day. It could be sufficient that the severity of the effects in liver can increase from 26.2/29.3 mg/kg bw/day onwards. Taking into account the observed effects in liver at this dose

level and the lack of data at higher doses below the cut-off value for STOT RE 2, the DS considered that there is some uncertainty on the potential of clofentezine for causing adverse effects in liver. However, the weight of the evidence based on the available information from all studies in several species indicate that clofentezine does not cause liver toxicity at dose levels below guidance values for STOT RE classification. Consequently, the DS did not propose STOT RE classification.

Comments received during public consultation

One comment from the company / manufacturer related to the centrilobular hepatocyte enlargement observed amongst male rats at doses ≥ 400 ppm but not in females which was reversible after cessation of treatment and dosing with control diet for six weeks.

Although the effects observed at this dose level fall within the concentration range for a STOT RE 2 classification, these findings were an adaptive response of the liver and reversible after cessation of treatment and thus considered non-adverse. According to this commenter's opinion, no STOT RE classification is warranted.

Assessment and comparison with the classification criteria

A summary of studies witch shows relevant findings for classification for STOT RE are listed below:

Dose levels and duration of exposure	**Effect relevant for STOT RE** Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr)/ncdr (not clearly dose-related)	**Reference**
Oral 17-day range-finding study in rats Doses: 0, 5, 20, 80, 320 and 1280 mg/kg bw/day daily for 17 days	**STOT RE 2 (≤ 529.4 mg/kg bw/day (Calculated))** 320 mg/kg bw/day ▪ Liver: (↑) absolute weight in ♀ (16%) and (↑) relative weight in ♂/♀ (7% n.s./10% ncdr). 80 mg/kg bw/day ▪ Liver: (↑n.s.) absolute weight in ♀ (14%) and (↑) relative weight in ♂/♀ (9% ncdr/12% ncdr). **STOT RE 1 (≤ 52.9 mg/kg bw/day):** 20 mg/kg bw/day ▪ Liver: (↑) absolute (14%) and relative (9% ncdr) weight in ♀.	Anonymous 68 (1980)
Oral 90-day dietary study in rat Doses of 0, 40, 400 and 4000 equivalent to 0, 2.65/2.91, 26.2/29.3 and 265/292 mg/kg bw/day for ♂/♀	**STOT RE 2 (≤ 100 mg/kg bw/day):** 400 ppm (26.2♂/29.3♀ mg/kg bw/day) ▪ (↑) cholesterol in ♂/♀ [week 12 (18%/50%)] and in ♀ [week 4 (34%) and 8 (23%)]. ▪ Liver: (↑) absolute weight in ♂/♀ [week 13 (11%/13%)] and (↑) relative weight in ♂/♀ [week 13 (13%/9%)]. ▪ Centrilobular hepatocyte enlargement: 13/20 ♂ (reversible)	Anonymous 74 (1981) Anonymous 75, 1983 (Additional examination of the liver histology)
Oral 28-day range-finding study in dog Doses of 200, 2000 and 20000 ppm equivalent to 10, 100 and 1000 mg/kg bw/day (no control group)	**STOT RE 2 (≤ 300 mg/kg bw/day):** 2000 ppm (100 mg/kg bw/day) ▪ Increase in ♂/♀ in the liver absolute (38%/7%) and relative weight (34%/21%) with respect to the lowest dose but only clearly dose-related in ♀.	Anonymous 73 (1983)

Dose levels and duration of exposure	Effect relevant for STOT RE Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr)/ncdr (not clearly dose-related)	Reference
Oral 13-week dietary study in dog Doses of 0, 3200, 8000 and 20000 ppm equivalent to 0, 80, 200 and 500 mg/kg bw/day	**STOT RE 2 (≤ 100 mg/kg bw/day and > 10 mg/kg bw/day):** 3200 ppm (80 mg/kg bw/day) ▪ (↑) AP in ♀ [day 30 (94% ndr) and day 86 (44% ndr)]. ▪ Liver: in ♂ (↑) abs wt (31% ndr) and (↑n.s) rel wt (20% ndr) and in ♀ (↑n.s) abs wt and rel wt (13% and 12%).	Anonymous 78 (1981)
Multigenerational study in rats F1 treatment for 33 weeks Doses: $F_{1\ males}$→ F2A and F2B: 36.1 mg/kg bw/day	**STOT RE 2 (≤ 39 mg/kg bw/day and > 3.9 mg/kg bw/day):** ▪ ↑ Relative liver weight (16%) in ♂. ▪ Increased incidence of minimal centrilobular hepatocyte enlargement in ♂ (4/10 *vs* 0/10 in controls).	Anonymous 61 (1984)
Long-term oral toxicity and carcinogenicity study in rats (27 months) Doses of 0, 10, 40 and 400 ppm, equivalent to 0.43, 1.72 and 17.3 mg/kg bw/day.	**STOT RE 2 (≤ 12.5 mg/kg bw/day):** 400 ppm (17.3 mg/kg bw/day) Thyroid: follicular cell tumours	Anonymous 42 (1985a) Anonymous 43 (1985-88) Anonymous 44 (1988)
Long-term oral toxicity and carcinogenicity study in mice (105 weeks) Doses of 0, 50, 500 and 5000 ppm, equivalent to 0, 5.3, 56.9, 557.1 mg/kg bw/day.	**STOT RE 2 (≤ 12.5 mg/kg bw/day):** 5000 ppm (557.1 mg/kg bw/day) Liver: Hepatocellular tumours	Anonymous 42 (1985a)

RAC agrees with the DS that the main target is the liver. In the 17-d rat study, absolute and relative liver weights were increased, however relative weight were 7 and 10%, in male and females respectively, and were not accompanied by histopathological findings or any clinical chemistry parameters.

In the 90-d rat study, centrilobular hepatocyte enlargement was observed in males at 400 ppm (26.2/29.3 mg/kg bw/day) along with increases in the absolute and relative weights of liver (both sexes) and significant and dose-dependent increases in the plasma cholesterol level in both sexes. These effects show a pattern of liver damage even if they were reversible after the recovery period on week 19. It has to be noted that liver hypertrophy starts in the centrilobular hepatocytes, spreading to the intermediate zone as it progresses, and is eventually observed as diffuse hypertrophy all around the lobule of the liver. The liver effects observed in the 90-day rat repeated dose toxicity study could be considered as indicative of adverse effects. No doses in this study were tested from 26/29 mg/kg bw/day to 265/292 mg/kg bw/day, where centrilobular hepatocyte enlargement was observed in 20/20 in both males and females together with significantly increased relative liver weight (> 50%).

In the 28-d dose range finding study in dogs, the increases in liver weight were not accompanied by changes in clinical chemistry or histopathological findings.

In the 13 week study in dogs, some changes in clinical chemistry were observed, however these were not dose related. Increased liver weight was also noted but without dose dependency in females and all liver effects were not accompanied by histopathological findings.

In the multigenerational study in rats, increases in relative liver weights (16%) were observed in females, while increased incidences of minimal centrilobular hepatocyte enlargement was observed in males.

In the 2-year long-term toxicity and carcinogenicity study in rats the target organs were liver and thyroid at 400 ppm, equivalent to 17.3 and 22.1 mg/kg bw/day for males and females, respectively. The dose of 17.3 mg/kg bw/day at which effects in male liver and thyroid were observed is above the extrapolated boundary guidance value for a 2-year study for STOT RE 2 (12.5 mg/kg bw/day).

In the 2-year carcinogenicity study in mice, the liver was the target organ from 500 ppm, equivalent to 50.7 and 56.9 mg/kg bw/day for males and females, respectively. Effects in liver at 56.9 mg/kg bw/day in females are of doubtful toxicological relevance. Besides, they are above the extrapolated boundary guidance value for STOT RE 2 classification for a 2-year carcinogenicity study (12.5 mg/kg bw/day). Consequently, the effects were not regarded relevant for STOT RE classification.

The effects observed in the 90-d rat study could indicate adverse effects in the liver. However, even if this effect is regarded adverse for liver, the weight of the evidence based on the whole available information on all studies in several species indicate that clofentezine does not cause a pattern of liver toxicity at dose levels below guidance values sufficient for STOT RE classification. Therefore RAC agrees with the DS that **clofentezine does not warrant classification for STOT RE**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

Clofentezine was not mutagenic in a valid *in vivo* somatic cell mutagenicity test and therefore the DS proposed no classification. The overall body of toxicological data from a number of *in vitro* and *in vivo* assays indicates that clofentezine is of no genotoxic concern. Therefore, no classification for mutagenicity under the CLP regulation was proposed.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

A summary of submitted mutagenicity / genotoxicity test *in vitro* is presented below:

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Reference
Bacterial gene mutation (Ames Test) Pre-OECD TG 471 (1983) Study not acceptable, due to the inadequacy of the positive controls.	*Salmonella typhimurium:* TA1535, TA100, TA1538, TA98, TA1537. S9 from livers of rats induced with Aroclor 1254.	Clofentezine Purity: Not stated 10, 33, 100, 330, 1000, 3300 μg/plate (± S9) Solvent: DMSO	Negative	McConville (1980) B.6.4.1.1-01 (AS)
Bacterial gene mutation (Ames Test) OECD TG 471 (1997)	*Salmonella typhimurium:* TA1535, TA100, TA1537, TA98, TA102 S9 from livers of rats	Clofentezine, Purity: 98.4% 50, 150, 500, 1500, 5000 μg/plate (± S9)	Negative	Bowles (2005) B.6.4.1.1-02 (AS)

Deviations: None GLP: Yes Study acceptable	induced with phenobarbitone and β-naphthoflavone	Solvent: DMF		
Mammalian cell gene mutation test. Pre-OECD TG 476 (1984) Study not acceptable.	Mouse lymphoma L5178Y $TK^{+/-}$ cells. S9 from livers of rats induced with Aroclor 1254	Clofentezine Purity: 98.4% 4 h (-S9): 15, 30, 70, 100, 128 μg/mL 4 h (+S9): 2, 10, 30, 80, 128 μg/mL Solvent: Acetone	Negative	Bootman and Rees (1982) B.6.4.1.2-01 (AS)
Mammalian cell gene mutation test. OECD TG 476 (1997) Deviations: None GLP: Yes Study acceptable	Chinese hamster V79 cells (*Hprt* locus) S9 from livers of rats induced with phenobarbital and β-naphthoflavone	Clofentezine, Purity: 98.7% 4 h (±S9): 0.30, 0.76, 1.52, 2.27, 3.03, 7.58, 15.15, 22.73 μg/mL 20 h (-S9): 0.30, 0.61, 0.91, 1.21, 3.03, 6.06, 15.15, 18.18 μg/mL 4h (+S9): 15.15, 16.67, 18.18, 19.70, 21.21, 22.73, 24.24, 27.27 μg/mL Solvent: DMSO	Negative	Wallner (2015a) B.6.4.1.2-02 (AS)
Mammalian cell chromosome aberrations test OECD TG 473 (1983) Deviations: None GLP: No (predates GLP enforcement) Supporting information.	Chinese hamster ovary cells (CHO - K1- BH_4) S9 from livers of rats of Sprague-Dawley origin induced with Aroclor 1254	Clofentezine Purity: 99.6% 20 h (-S9): 0.4, 2, 4 μg/mL 2 h (+S9): 0.4, 2, 4 μg/mL Solvent: DMSO	Negative	Allen *et al.* (1987) B.6.4.1.3 (AS)
Gene conversion and mitotic recombination test in yeast Pre-OECD TG 481 (1986) Deviations: None GLP: No (predates GLP enforcement) Study acceptable only as supplementary information, since this study is not required and OECD TG 481 (1986) was deleted on 2 April 2014.	*Saccharomyces cerevisiae,* D7 strain S9 from livers of rats induced with Aroclor 1254	Clofentezine Purity: 98.4% 12.5, 25, 50, 100, 200 μg/mL Solvent: DMF:ethanol (1:9)	Negative	Riach and McGregor (1983) B.6.4.1.4-01 (AS)
Rec-assay No test guideline available GLP: No Study acceptable only as supplementary information, since it is not required.	*Bacillus subtilis* H17 (Rec^+) and M45 (Rec^-) S9 from livers of rats induced with phenobarbital and β-naphthoflavone	Clofentezine Purity: Not stated 156, 313, 625, 1250, 2500 μg/disk (-S9) 78.1, 156, 313, 625, 1250 μg/disk (+S9) Solvent: DMSO	Negative	Inoue and Nakajima (1986) B.6.4.1.4-02 (AS)

A summary of submitted mutagenicity / genotoxicity test *in vivo* is presented below:

Method, guideline, deviations if any	Test system	Test substance and dosage	Results	Reference
Micronucleus test (somatic cells) Pre-OECD TG 474 (1983) Deviations: A single sex (male). A single sampling time. GLP: No (predates GLP enforcement) Study not acceptable, because the bone marrow sampling used (6 h post the 2nd dose) is insufficient.	Mice, CD-1 strain	Clofentezine Purity: 99.6% Doses: 800, 1600, 3200 mg/kg bw/day (two oral administrations separated by 24 h). Vehicle: 0.5% aqueous gum tragacanth	Negative	Anonymous 39 (1982) B.6.4.2.1-01 (AS)
Micronucleus test (somatic cells) OECD TG 474 (1983) Deviations: None GLP: No (predates to GLP enforcement) Study acceptable	Mice, CD-1 strain	Clofentezine Purity: 99.6% Dose: 8000 mg/kg bw (single oral administration) Vehicle: 0.5% sodium carboxymethyl cellulose	Negative	Anonymous 40 (1987) B.6.4.2.1-02 (AS)
Rodent dominant lethal test (germ cells). Pre-OECD TG 478 (1984) Deviations: No inclusion of a positive control. Exposure for 10 weeks exceeded the recommended one. GLP: No (predates to GLP enforcement) Study acceptable only as supporting information since this study is not required and by some deficiencies in methodology.	Rats, Sprague Dawley strain	Clofentezine Purity: ≥ 98.1% Diet containing 0.28, 2.81 and 27.8 mg/kg bw/day for 10 weeks following by pairing each treated male with two untreated females for up to14 days.	Negative	Anonymous 41 (1983) B.6.4.3-01 (AS)

The genotoxic potential of clofentezine has been investigated in a series of *in vitro* and *in vivo* studies.

The *in vitro* bacterial gene mutation study (Ames test) (Bowles, 2005) showed no evidence of mutagenicity following testing in five Salmonella strains when tested up to 5000 µg/plate in the absence and presence of metabolic activation using the plate incorporation method.

In the *in vitro* mammalian cell gene mutation study (Wallner, 2015a) clofentezine did not induce forward mutation at the Hprt locus of V79 Chinese hamster cells. These conditions included treatment in both the absence (4 and 20 hours) and presence (4 hours) of a rat liver metabolic activation system, which was limited by toxicity (a reduction of relative total growth below 70%).

Although both the Ames study (McConville, 1980) and the mammalian cell gene mutation assay with mouse lymphoma L5178Y cells (Bootman and Rees, 1982) were not considered acceptable for the assessment due to deficiencies noted, both gave negative results.

Regarding DNA damage studies, as supplementary information, clofentezine was negative in a gene conversion and mitotic recombination test in yeast (Riach and McGregor, 1983) and in a Rec-assay with H17 (Rec+) and M45 (Rec-) strains of *Bacillus subtilis* (Inoue and Nakajima, 1986).

Three studies have been considered to assess the chromosomal aberration potential: the *in vitro* clastogenicity test in Chinese hamster ovary (CHO) cells (Allen *et al.*, 1987), the *in vivo* mouse bone marrow micronucleus test (Anonymous 40, 1987) and the *in vivo* rat lethal dominant mutation assay (Anonymous 41, 1983). Clofentezine did not induce either micronuclei or bone marrow cell toxicity in the mouse (single oral dose at 8000 mg/kg bw). Clofentezine was also negative in both an *in vitro* clastogenicity test and an *in vivo* lethal dominant mutation assay.

Based on all the data, it can be concluded that clofentezine showed no evidence for chromosomal aberration induction.

In the other *in vivo* mouse bone marrow micronucleus test (Anonymous 39, 1982), the results were negative, but it was not considered acceptable because the bone marrow sampling used was insufficient in order to evaluate chromosomal aberrations.

In conclusion, RAC agrees with the DS that **clofentezine does not warrant classification for germ cell mutagenicity**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

Two long-term toxicity/oncogenicity studies were conducted with clofentezine, one in rats and one in mice.

In a 27-month long-term toxicity and carcinogenicity study in rats the doses tested were 0, 10, 40 and 400 ppm equivalent to 0, 0.43, 1.72 and 17.3 mg/kg bw/day, respectively, for males and 0, 0.55, 2.18 and 22.1 mg/kg bw/day, respectively, for females.

No mortality or clinical signs were associated with the treatment.

Blood chemistry revealed a statistically significant and dose-dependent increase in free thyroxine (T4) in males at 400 ppm in month 27. Other parameters of thyroid function were not affected. Other statistically significant differences of the biochemical parameters compared with controls at 400 ppm were only marginal and not dose-dependent.

The absolute liver weight was increased in month 27 from 10 ppm in males and at 400 ppm in females, though clearly dose-related only in males. Relative liver weights were increased in both sexes in months 12 and 27 at 400 ppm though the variation was greater than 10% only in males and dose-dependency was not clear.

Histopathology revealed observations in male liver at the high dose level of 400 ppm with significant (pairwise and dose-trend) centrilobular hepatocyte vacuolation (observed at interim sacrifice and in the total number of animals including the incidence at interim sacrifice, interim deaths and terminal sacrifice) and centrilobular hepatocyte enlargement (interim sacrifice, terminal sacrifice and total number of animals). Also observed was focal cyst degeneration of hepatocytes and fat deposits in a non-specific distribution, non dose-trend significant for the total number of animals and for focal hepatocyte necrosis, dose-trend significant for interim sacrifices and the total number of animals. Telagiectasia was present in both sexes but this was only dose-trend significant in females for the total number of animals.

Thyroid effects in males were manifested at 400 ppm by dose-trend significant agglomeration of colloid at interim sacrifice, terminal sacrifice and for the total number of animals which was also pairwise significant for terminal sacrifices. Besides, follicular cell hyperplasia was observed from 40 ppm in males but this was not dose-related and not statistically significant.

Other pairwise and dose-trend significant histopathological findings at 400 ppm were glomerular nephropathy in females in the interim deaths and interstitial mononuclear infiltration of the Harderian gland in males at the interim sacrifice and total number of animals.

At 400 ppm in males, there was a slight increase in the number of follicular cell tumours (combined adenomas and carcinomas) in the thyroid at terminal sacrifice (8/50 vs. 2/50 in controls). This may have been associated with the pairwise and dose-trend significant increase

of agglomeration of colloid at terminal sacrifice (18/21 vs 12/24 in control). These tumours are not pairwise significant but exhibited dose-trend significance. Besides, it should be emphasized that the spontaneous rate of thyroid tumour development in rats increases rapidly after the animal exceeded 2 years of age. The DS concluded that the mechanism of action (MoA) thoroughly developed in the CLP report shows that follicular cell tumours are not relevant for humans.

Additionally, the DS noted the occurrence of several rare tumour incidence which were not regarded relevant:

Malignant mixed glioma: single occurrences were restricted to the mid and high dose group interim decedent males. Concurrent laboratory historical control data were not available, therefore contemporary historical control data were obtained from where the stock animals originated with an upper background level of 1.92%. In this present study the incidence was marginally above (2.0%, 1/50) this level. These effects were considered not to be treatment related but rather incidental in their occurrence because the incidences were not statistically different for the concurrent controls, and these effects were not replicated in females.

Astrocytoma: an incidence of 2 (1 in interim deaths, 1 for terminal deaths), 1 (interim death) and 1 (interim death) were observed in the low, mid and high dose group males, respectively. In females incidences of 2 and 1 were observed in the low and high dose groups, respectively, at termination. Concurrent laboratory historical control data were not available, but contemporary historical control data obtained from where the stock animals originated showed upper background levels of 4.92% and 2.31% for males and females, respectively. In this study, the tumour incidence was within the stated background range (1.90% for males 2.14% for females). These effects were considered not to be treatment related.

Leydig cell tumours, testes: the single incidence of this malignant tumour type in the high dose males falls within the historical control incidence value of 5% (equivalent to 2/50) and is not considered to be treatment-related.

In a 2-year carcinogenicity study in mice the tested dose levels were 0, 50, 500 and 5000 ppm equivalent, respectively, to 0, 5.0, 50.7 and 543.4 mg/kg bw/day for males and 0, 5.3, 56.9 and 557.1 mg/kg bw/day for females.

At 5000 ppm, a higher proportion of deaths was observed in females (42 vs. 27 in controls) during the latter part of the study, which was attributed to amyloidosis. Bodyweight and food consumption were not affected by treatment. Only bodyweight gain at 5000 ppm was slightly reduced in males mainly during the first half of the study. No effects were observed from week 52.

The only significant effects on haematology were observed at 5000 ppm in males on week 52 with a decrease in red blood cells (12%) not seen at terminal sacrifice.

Analysis of organ weights of mice killed after 105 weeks of treatment revealed slightly increased absolute liver weights in females (18%) at 5000 ppm that was not clearly dose-related. This increase may be correlated with a dose-related increased incidence of foci/areas of altered hepatocytes (eosinophilic) noted from 500 ppm in females at above historical control incidences. A slightly increased incidence of foci/areas of altered hepatocytes above historical controls was seen in males for decedent animals from 500 ppm. The increased incidence of this lesion in terminal males was not dose-related.

Non neoplastic findings liver		Males				HCD	Females				HCD
		Dose level (ppm)					Dose level (ppm)				
		0	50	500	5000		0	50	500	5000	
Number of animals examined	D	**39**	**35**	**36**	**41**	**1775**	**27**	**24**	**25**	**42**	**1773**
	T	**13**	**17**	**16**	**11**		**25**	**28**	**27**	**10**	
Eosinophilic hepatocytes	D	2 (5.1%)	3 (8.6%)	4 (11.1%)	8 (19.5%)	46 0.0-9.8%	1 (3.7%)	1 (4.1%)	2 (8%)	4 (9.5%)	35 0.0-9.1%
	T	1 (7.7%)	4 (23.5%)	4 (25%)	2 (18.2%)		2 (8%)	2 (7.1%)	5 (18.5%)	5 (50%)	

D: animals dying or killed during study, T: animals killed at termination

At 5000 ppm an increased incidence of amyloidosis in females was observed (19/42 vs 6/27 in controls). There was no evidence of this effect in males.

A higher number of benign liver cell tumours was observed in females at 5000 ppm. The incidence (7/52; 13.5%) was slightly higher than the concurrent control incidence in females in this study (4/52; 7.7%) and outside the provided historical control range (0-7.7%) obtained from 26 studies with duration ≥ 92 weeks conducted at Huntingdon Research Centre (1980-83). The incidence was not significant after pairwise comparison ($p > 0.05$) but showed a positive trend after trend analysis ($p < 0.01$). The DS noted that the incidence in controls is equal to the upper HCD value of 7.7%. There was an increase in the malignant hepatic tumours (1/52; 1.9%) in females with respect to controls (0/52), however, it was within the range of historical controls and was not statistically significant. The combined analysis of benign and/or malignant hepatic tumours in females (8/52 vs. 4/52 in controls) was significant after pairwise comparison ($p < 0.05$) and showed a positive trend after trend analysis ($p < 0.01$).

The toxicological relevance of these liver tumours in females seems doubtful since they correspond to non-significant (pairwise) benign tumours occurring in one sex and one species and at high dose levels of treatment (557.1 mg/kg bw/day for females). However, the increase in liver tumours cannot be dismissed as non-relevant to humans as the mechanism of action for formation of liver tumours in female CD-1 mice developed in the CLH report remains unclear.

On this basis, the DS proposed classification for carcinogenicity in Cat. 2.

Comments received during public consultation

Comments were received from two MSCA and the company/manufacture.

One MSCA supported the proposal for classification for carcinogenicity based on the observed thyroid and hepatocellular neoplasia in rats and mice. The MSCA agreed with the DS that despite extensive and elaborate MoA analysis, the mechanistic data is not complete and thus findings cannot be dismissed. In view of the unlikely relevance of the MoA postulated for the thyroid tumours, likely lack of genotoxicity and the substantial data on the MoA for liver tumours, Cat. 1B however is clearly not justified. Therefore, the MSCA support the proposal for Carc. 2.

Another MSCA agreed with that the MoA via induction of UDP-glucuronyltransferase is sufficiently well demonstrated and therefore agreed that the slightly increased incidence of thyroid tumours as observed in the rat carcinogenicity study is not relevant for humans. The MSCA supported the DS in their assessment of the liver tumours, which cannot be fully discarded and classification for carcinogenicity in Cat. 2 is supported.

The company/manufacturer agreed with the DS that the thyroid follicular cell tumours in male rats were considered relevant to humans. However, the company/manufacturer disagreed with the DS in their assessment of the MoA for the liver findings. The slight increase in benign liver

tumours seen in females only in the mouse carcinogenicity study are likely to be via a phenobarbital-like MoA, and therefore can be considered non-relevant for humans. They highlighted the absence of a clear dose response relationship (dose levels were spaced 10-fold apart), the malignant hepatocellular tumours falling within the HCD, the increase in benign hepatocellular tumours, which were slightly above the HCD, and these tumours were observed in one species (mice) and one sex (females). Furthermore, control animals were at the higher end of the HCD range and the study duration was longer (104 weeks) compared to contemporary studies (78 weeks), which reduces the concerns regarding these tumours. Based on the available data it was the company/manufacturer's position that a carcinogenicity classification for clofentezine is not warranted.

The company/manufacturer pointed out that further work to strengthen this conclusion for mice will be conducted shortly, including an *in vitro* comparative hepatocyte proliferation study (mouse, human) to investigate enzyme induction, cell proliferation and species differences, and a short-term repeat dose mouse toxicity study to investigate enzyme activity and hepatocyte proliferation, with results from this work expected in the first half of 2020.

Assessment and comparison with the classification criteria

RAC identifies the following endpoints to be critical for assessment of carcinogenicity in the two year carcinogenicity studies conducted in rats and mice

- Thyroid follicular cell tumours in male rats and,
- Hepatocellular tumours in female mice

Thyroid follicular cell tumours in male rats

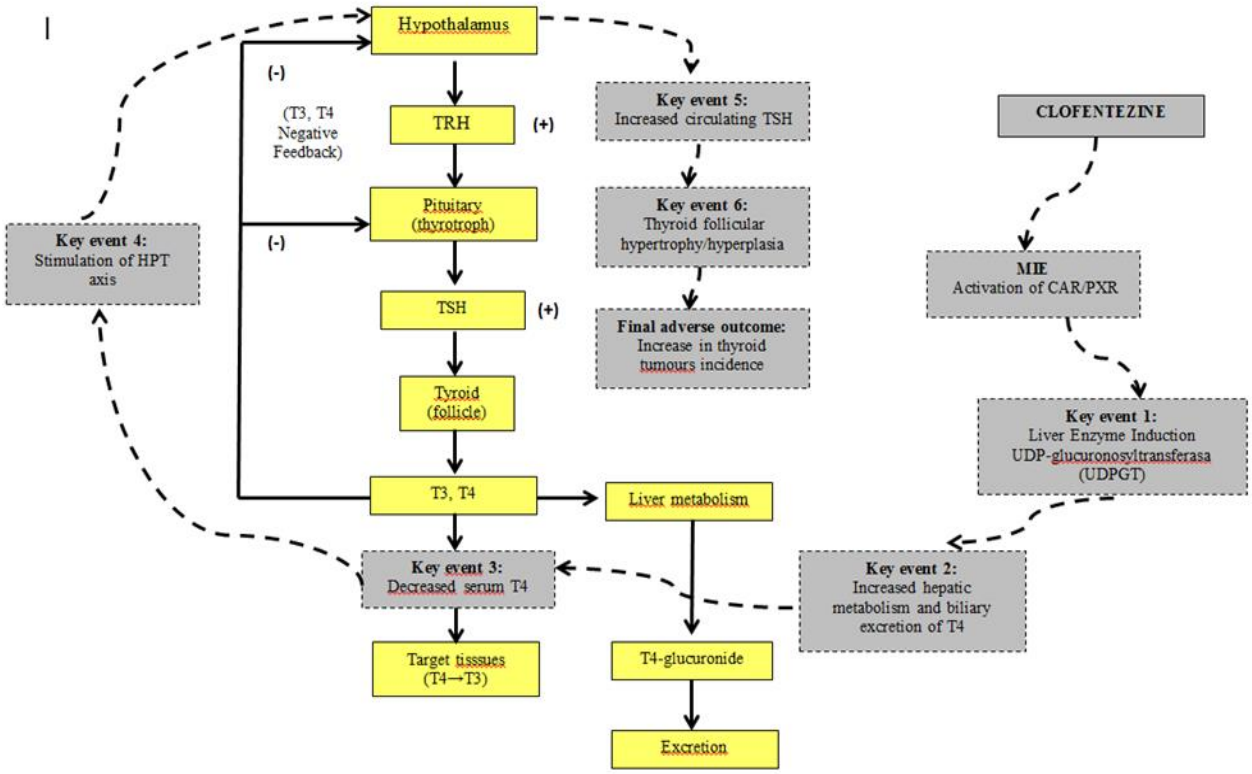
Thyroid follicular cell tumours in males	**Doses**											
	0 ppm			**10 ppm**			**40 ppm**			**400 ppm**		
	0 mg/kg bw/day			**0.43 mg/kg bw/day**			**1.72 mg/kg bw/day**			**17.3 mg/kg bw/day**		
	Time of death											
	I	**D**	**T**	**I**	**D**	**T**	**I**	**D**	**T**	**I**	**D**	**T**
Rat carcinogenicity study for 27 months (March 1982-June 1984); *(Anonymous 42.; 1985a)*												
No animals examined	20	26	24	20	26	24	20	23	27	20	29	21
Benign	0	1	0	0	0	1	0	0	0	0	0	3
Probably malignant	0	0	0	0	0	0	0	0	0	0	0	2
Malignant	0	1	0	0	0	1	0	1	1	0	0	3
Total tumours (D+T)	2/50†			2/50			2/50			**8/50**		
TOX 82074 (1982-4) Study conducted at the same laboratory (December, 1982-March 1985)												
No animals examined	25	32	17									
Benign	0	2	2									
Probably malignant	0	0	0									
Malignant	0	1	1									
Total tumours (D+T)	**6/49**											

I: Interim sacrifice, D: Interim deaths during treatment, T: Terminal sacrifice
† Positive after trend analysis

At 400 ppm in males, there was a slight increase in the number of follicular cell tumours (combined adenomas and carcinomas) in the thyroid at terminal sacrifice (8/50 vs. 2/50 in control). This may have been associated with the pairwise and dose-trend significant increase in agglomeration of colloid at terminal sacrifice (18/21 vs 12/24 in control). These tumours are not pairwise significant but exhibited dose-trend significance. No HCD are available. The only background data included in the CLH report were obtained from a single concurrently run study (TOX 82074) performed at the same laboratory with the same procedure, strain, housing condition, diet and pathologist. The incidence of thyroid follicular cell tumours in high dose males treated with clofentezine was only marginally higher than the incidence in the control group from the other concurrently run study (8/50 vs. 6/49). Besides that, the DS concluded that the thyroid

follicular cell tumours are not relevant for humans due to the UDP-glucuronosyltransferase (UDPGT) MoA.

The postulated MoA for effects on the thyroid and induction of thyroid follicular tumours in rats by clofentezine can be summarised as follows. Briefly, activation of the CAR/PXR nuclear receptors by clofentezine leads to induction of hepatic UDPGT resulting in increased conjugation and excretion of thyroxine (T4) and a decrease in serum T4 levels. A compensatory increase in thyroid stimulating hormone (TSH) levels secreted via the hypothalamus-pituitary-thyroid (HPT) axis results in the chronic proliferative stimulus of thyroid follicular cells by TSH prompting hypertrophy and hyperplasia, and eventually progress to form follicular cell adenomas and/or carcinomas. The figure below shows the normal steps in functioning of the HPT axis.



Listing of key events identified in experimental animals

The key events and associative events in this process have been observed and measured in male rats in several short-term and MoA studies and in the carcinogenicity study in rats (Anonymous 42, 1985a). The essential Key Events (KE) and Associative Events (AE) for the MoA of clofentezine:

Key Events	Associative events
MIE (Molecular Initiating Event) : Activation of CAR/PXR activation	
Key event 1: Induction of hepatic UDP-glucuronosyltransferase (UDPGT)	
Key event 2: Increased hepatic metabolism, increased glucuronidation and biliary excretion of T4	Increased liver weight Liver histopathology
Key event 3: Decreased serum T4 half-live and concentration	

Key Events	Associative events
Key event 4: Stimulation of HPT axis	Increased pituitary weight Pituitary histopathology
Key event 5: Increasing circulating TSH concentration	Increased Thyroid weight Thyroids enlargement /Hypertrophy
Key event 6: Increased thyroid follicular cell proliferation (hyperplasia)	Increased colloid depletion Increased mitotic activity follicular cells (lining)
Final adverse outcome (AO): Increase in thyroid tumours incidence	

A summary for each steps in the key events and the associative processes are listed below:

- The Molecular Initiation event: Activation of CAR/PXE activity: No data
- KE1: Three MoA studies in male rats showed a 5 fold increase at 30000 ppm and 2 fold at 400 ppm.
- KE2: two MoA studies in rats showed increased bile flow rate, increased excretion into bile and increased clearance together with increased excretion of thyroxine glucuronide.
- AE1: Several MoA, in subchronic studies and in the carcinogenicity study, consistent findings of increased absolute and relative liver weights associated with centrilobular hepatocyte enlargement starting at doses around 400 ppm.
- KE3: five MoA studies and the carcinogenicity study again showed slight decreases in serum T4 (6.8% in week 4) and T3. Levels returned to control.
- KE4: No data.
- AE2: Two MoA studies showed pituitary histopathology and this corresponded with increased TSH. Indication for stimulation of the HPT axis.
- KE5: Three MoA studies showed increased circulation of TSH.
- AE3: Two MoA studies showed consistent increased absolute and relative thyroid weights. Another MoA study did not show any effects on thyroid weight.
- AE4: five MoA studies and one subchronic study showed consistent hypertrophy and enlargement of the thyroid and increasing in follicular cell size.
- KE6: Three MoA studies and one subchronic study showed follicular cell hyperplasia and agglomeration of colloid.
- AE5. Four MoA studies and one subchronic study showed marked to moderate depletion of colloid.
- AE6: Two MoA studies were positive in findings for increased mitotic activity of the follicular cell.
- Final adverse outcome: In the carcinogenicity study, slightly increased thyroid tumour incidences in male rats at 400 ppm.

RAC concludes that the overall picture of the assessed MoA across all the studies are moderate to strong and the proposed MoA indicating that the liver is the primary target organ and that its response to clofentezine by hepatic enzyme induction (UDPGT) and increased metabolic activity generation catabolism of T4 and increase the excretion of thyroxine metabolites which involves an turnover of thyroid hormones is partly confirmed. Stimulation of the HPT axis generates an increase in TSH which leads to increases in thyroid weight and hyperplasia and/or hypertrophy which progress to tumours. The 2-fold induction of UDPGT at 400 ppm is significant ($p < 0.01$ at days 5 and 15 and $p < 0.05$ at days 8 and 29) and 5-fold at 30000 ppm. The increased mitotic activity follicular cells (lining) showed by the cellular division was maximal at 7 days and some activity was still evident at 14 days (4 days: 5/10 rats vs 1/10 control; 7 days 7/10 vs 0/10 control; 14 days 5/10 vs 0/10 control). Based on the observed increase in induction of UDPGT, decrease in serum T4 and thyroid follicular hyperplasia, RAC concludes that the thyroid follicular

cell tumours occurring at 27 months in male rats (i.e. very late) are of little relevance to humans and therefore not considered for classification based on the incidences of the thyroid tumours.

RAC overall conclusion of the 27-month long-term toxicity and carcinogenicity study in rats

RAC concludes that the dose levels in the rat carcinogenicity study were too low. The highest dose tested was only up to 400 ppm, ~ 17.3 / 22.1 mg/kg bw/d, with no mortality or clinical signs associated with the treatment; only slight liver toxicity and slightly decreased body weight gain were observed. The highest dose in the 90-day rat studies of 265/292 mg/kg bw/d indicates that MTD in the carcinogenicity study has not been reached and the carcinogenicity study in rats was not adequate for assessment of carcinogenicity potential. The conclusion is no classification based on inconclusive data.

Hepatocellular tumours in female mice

A table presenting the results from the 105 week long term toxicity/ carcinogenicity test in mice:

Neoplastic findings liver		**Females**				**HCD**
		Dose level (ppm)				
		0	**50**	**500**	**5000**	
Number of animals examined	D	**27**	**24**	**25**	**42**	
	T	**25**	**28**	**27**	**10**	
Benign tumour	D	0	1	0	3	
	T	4	2	1	2	
Benign tumour (two)	D	0	0	0	1	
	T	0	0	0	0	
Benign tumour (multiple)	D	0	0	1	0	
	T	0	0	1	1	
Benign tumour sub-total	D	0	1	1	4 (9.5%)	**0-7.7%**
	T	4 (16%)	2 (7%)	2 (7.4%)	3 (30%)	
Benign tumour overall total		4/52 (7.7%)	3/52 (5.8%)	3/52 (5.8%)	7/52 (13.5%)	
Malignant tumour (two)	D	0	0	0	1 (2.4%)	
	T	0	0	0	0	**0-3.8%**
Total malignant tumour		0/52	0/52	0/52	1/52 (1.9%)	

HCD (Huntingdon Research Centre between March 1980 and July 1983). Study duration ≥ 92 weeks.
D: Animals dying or killed during study, T: Animals killed at termination

RAC agrees with the DS that the toxicological relevance of these liver tumours in female mice seems doubtful since they correspond to non-significant (pairwise) benign tumours occurring in one sex and one species, at high dose levels (557 mg/kg bw/day). The DS concluded that the slight increase in liver tumours could not be dismissed as non-relevant to humans as the MoA for the liver tumours in female mice remains unclear.

The applicant / manufacturer provided data for the postulated MoA. The proposed MoA for clofentezine liver tumours consists of the activation of the CAR/PXR in the liver. CAR/PXR activation induces increased expression of pro-proliferative and anti-apoptotic genes in the liver and an early, transient, increase in hepatocellular proliferation. Over time, the increased hepatocellular foci because of clonal expansion of spontaneously mutated cells in the mouse results in slight increases in liver tumour incidence compared to concurrent controls.

Listing of Key Events (KE) and Associative Events (AE) identified in experimental animals:

Key events	Associative events
Key event 1: CAR nuclear receptor activation	
Key event 2: Altered gene expression specific to CAR activation	Enzyme induction (CYP2B) Hepatocellular hypertrophy Liver weight increase Inhibition of apoptosis Epigenetic changes
Key event 3: Increased cell proliferation	
Key event 4: Clonal expansion leading to foci/areas of altered hepatocytes (eosinophilic)	
Key event 5: Liver adenomas/carcinomas	

The submitted data to provide justification for KE and AE of a CAR-mediated induction of the findings in liver in mice are listed below.

Reference	**Dose** **(mg/kg bw/day)** ♂/♀	**Key event 1** Enzyme induction	**Associative event[1]** Hepatocellular hypertrophy	**Associative event[1]** Increased liver weight	**Key event 4** Clonal expansion leading to altered foci	**Key event 5** Liver adenomas/ carcinomas
	Ordered from low to high dosage	**Key and associative events are shown in order from earliest event to later (left to right). Results show the time that the event was observed. Quantitative changes in severity are not shown**.				
MICE						
Anonymous 45, 1985	**5/5.3**	ND	- 105 weeks	- 105 weeks	+ 105 weeks (♂)	- 105 weeks
Anonymous 45, 1985	**50.7/56.9**	ND	- 105 weeks	- 105 weeks	+ 105 weeks (♂/♀)	- 105 weeks
Anonymous 77, 1982	**151.4/176.5**	ND	- 13 weeks	+ 13 weeks (Rel in ♂/♀)	NA	NA
Anonymous 45, 1985)	**543.4/557.1**	ND	- 105 weeks	+ 105 weeks (abs in ♀)	+ 105 weeks (♂/♀)	+ 105 weeks (♀)
Anonymous 77, 1982	**757.1/884.9**	ND	+ 13 weeks [a] (♂)	+ 13 weeks (Abs in ♂ Rel in ♂/♀)	NA	NA
Anonymous 70, 1982	**766/912**	ND	+ 6 weeks [b] (♂)	+ 6 weeks (abs and rel in ♂)	NA	NA
Anonymous 70, 1982	**5149/5395**	ND	+ 6 weeks [b] (♂)	+ 6 weeks (abs and rel in ♂)	NA	NA

-: negative response, +: positive response, ND: Not determined, NA: Not applicable, [a]: Observed as centrilobular hepatocyte enlargement, [b]: observed as centrilobular hepatocytomegaly.
[1]Associative events are referred to key event 2 (altered gene expression specific to CAR activation)

Clofentezine treatment first led to early events such as enzymatic activation, hypertrophy and liver weight increase. The final adverse outcome effect (Key Event 5) of formation of hepatocellular tumours and the key event 4 of formation of eosinophilic foci only occurs in mice. At same doses these two effects are always late events, only observed at 104 weeks (no evidence at 52 weeks). Although eosinophilic foci and hepatocellular tumours do not have sufficient time points to distinguish temporally between both, the incidence of foci occurred at lower doses at

which tumours have not been developed yet. A sex difference for the final adverse outcome was evident (female mice having a higher tumour incidence than males).

The weight of evidence linking the key and associative events with the toxicological response is quite consistent with the hepatic effects observed in mice. The succession of key and associative events, including liver enzyme induction (only tested in rats), proliferation of smooth endoplasmic reticulum, increased liver weight with associated histopathological hepatocellular changes (hypertrophy, centrilobular enlargement), foci/ areas of altered hepatocytes (eosinophilic) and liver benign tumours in mice is consistent with a phenobarbital (PB)-like mechanism.

Reversibility is also consistent with the proposed CAR MoA where the non-neoplastic cellular changes may be reset by the normal feedback-control systems and reversed. Liver effects observed might be considered as an adaptive effect if they are caused by induction of enzyme activities; if it is not associated with any other liver toxicity and if it is a transient phenomenon, which is fully reversible. In this sense, there are studies with clofentezine that showed reversible effects. The 90-day study in rats (Anonymous 63, 1982b) showed reversibility in the increases in liver weight, liver macroscopic pathology and histopathological changes (centrilobular hepatocyte enlargement). Besides, in the other 90-day study in rats (Anonymous 74, 1981) there was also reversibility in the increase in liver weight and the centrilobular hepatocellular enlargement.

Hepatic tumours in mice are preceded by foci/ areas of altered hepatocytes (eosinophilic) that occurred at lower doses and at a higher incidence than seen for tumours. In the chronic toxicity/ oncogenicity study at 5000 ppm in female mice (557 mg/kg bw/day), the incidence of hepatocellular adenomas was 13.5% (7/52 animals) while the incidence in foci of animal dying or killed during study and at termination was 17% (9/52 animals). These observations also fit with the MoA, where, at similar doses, the incidence/severity of later key events would not be expected to be greater than that of earlier key events.

The key and associative events occurred in a logical temporal sequence and in a dose-dependent manner and were reversible when exposure was discontinued. At similar doses, the incidence of later key events is not greater than that of earlier key events. All these factors provide support for the proposed MoA. However, there is not evidence for all key events of this MoA and the strength, consistency and specificity of association of the hepatic tumour response with key events suggested that the MoA is only partially convincing.

Overall, the key and associative events observed in mice and rats receiving clofentezine occurred in a logical temporal sequence and in a dose-dependent manner. However, there is not evidence for all key events of this MoA.

<u>Other possible modes of action</u>

Mutagenicity: DNA reactivity can be excluded since the genotoxicity testing *in vivo* and *in vitro* gave no evidence of a genotoxic potential.

Cytotoxicity and regenerative hyperplasia: In a 42-day study in mice (Anonymous 70, 1982) localized areas of hepatic necrosis were observed in male at 5149 mg/kg bw/day. In addition, slight incidences of degenerative lesions (vacuolization, focal cyst degeneration of hepatocytes and focal hepatocyte necrosis) were reported in males of the 27-month chronic toxicity rat study (Anonymous 42, 1985a) at 17 mg/kg bw/day. Tumours observed in the chronic/ carcinogenicity

study in mice were observed in female at dose levels of 557 mg/kg bw/day. However, there were not increases in other necrosis indicators (e.g., alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) and, in contrast with classic cytotoxic carcinogens, there was reversibility in the early key events (hepatocellular hypertrophy and increased liver weight).

Oestrogen activity: There was no structural similarity with oestrogens, no changes in clinical chemistry parameters and no hepatic necrosis or other histological changes suggestive of other receptor (oestrogen, statins) and non-receptor-mediated (apoptosis, infections or metal accumulation) involvement. Data evaluation indicate that these alternative possible MoAs are not likely to be relevant.

PPARα or AhR MoA: these MoAs have not been experimentally ruled out since there are no measures in lauric acid 12α-hydroxylase activity and Cyp4a protein levels to determine PPARα activation, neither were there measurements of EROD activity and Cyp1a protein levels to determine AhR activation in mouse liver microsomes.

Missing data: data providing evidence for microsomal enzyme induction, CAR activation (KE1) and *in vitro* studies in different hepatocyte cultures (mouse wild type, mouse CAR/PXR KO and human) would have strengthened the evidence for CAR/PXR MoA.

RAC overall conclusion for liver effects

When proposing a CAR MoA for liver tumours induced by a test compound, there are critical parameters to be included in the final mechanistic data package, which should (at a minimum) include demonstration of the molecular initiating event (CAR activation, KE1) and the obligatory key event of increased cell proliferation (KE3). However, in the analysis of postulated MoA for hepatocellular tumour caused by clofentezine, there is not experimental evidence for these crucial key events as CAR activation (KE1) and increased cell proliferation (KE3).

Therefore, there is no clear evidence that CAR receptor activation is involved in the tumourigenic action of clofentezine in the liver of CD-1 mice. The absolute certainty on CAR involvement could have been confirmed with a CAR-knock-out mouse study and the evidence for cell proliferation could have been strengthened with an *in vitro* comparative cell proliferation study (mouse, rat, human). Further enzyme induction studies might also have been done.

However, the available data suggest that the slight increase in benign liver tumours seen in females only in the mouse carcinogenicity study are likely to be via a CAR mode of action. Increased PROD, BROD and BQ activity and increased CYP2b and CYB3a enzymes, indicate CAR activation, was found in rats and a similar MoA could be plausible between mice and rats.

A clear dose response relationship was absent (dose levels were spaced 10-fold apart), the single case of malignant hepatocellular tumours fell within the HCD, only a slight increase in benign hepatocellular tumours was seen, which were slightly above the HCD and these tumours were only observed in one species (mice) and one sex (females) at the highest dose tested.

The historical control data for hepatocellular tumours in mice were from six studies started in 1980-1981, but new data have been submitted to RAC from 26 studies started in 1980-1983. The incidence of 7/52 benign hepatocellular tumours (13.5%) is above of the historical control range for females, where the range was 0-7.7% (see table below):

Tumour data (liver tumour) for females and HCD for both sexes:

Tumour data	Females (ppm)							
	0		50		500		5000	
	D	Ter	D	Ter	D	Ter	D	Ter
Benign tumour	0/27	4/25	1/24	2/28	0/25	1/27	3/42	2/10
Benign tumour (two)	0/27	0/25	0/24	0/28	0/25	0/27	1/42	0/10
Benign tumour (multiple)	0/27	0/25	0/24	0/28	1/25	1/27	0/42	1/10
Benign tumour sub-total	0/27	4/25	1/24	2/28	1/25	2/27	4/42	3/10
Benign tumour overall total	4/52 (7.7%)		3/52 (5.8%)		3/52 (5.8%)		7/52 (13.5%)	
Malignant tumour (two)	0/27	0/25	0/24	0/28	0/25	0/27	1/42	0/10
Total malignant tumour	0/52 (0%)		0/52 (0%)		0/52 (0%)		1/52 (1.9%)	
Historical control data for hepatocellular tumours in mouse studies started in 1980-1983[a]								
Tumour type	**Males**				**Females**			
	Low (%)		**High (%)**		**Low (%)**		**High (%)**	
Benign liver cell tumour	3.8		36.5		0		7.7	
Malignant liver cell tumour	8.7		38.5		0		3.8	
Infarcted liver cell tumour	0		1.9		-		-	

D: decedents, Ter: terminal; [a] : Study duration is ≥ 92 weeks.

The tumour profile has some factors that reduce considerably the level of concern regarding the carcinogenicity. These include the lack of progression to malignance of the adenomas, as well as the long time of latency, as the adenomas were only observed at the end of the study. The incidence of the liver adenomas was not statistically significant after pairwise comparison ($p > 0.05$) and there was no clear dose-response and only slightly above the historical control.

The occurrence of the benign hepatocellular tumours were limited to one sex of one species, only at the highest dose tested and slightly above the HCD without statistical significance after pairwise comparison. In addition, the lack of progression to malignancy further decreases the concern. On a weight of evidence basis, RAC concludes that **classification for carcinogenicity is not warranted**.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Sexual function and fertility

The potential effects of clofentezine on fertility and reproductive performance have been investigated in a reliable standard 2-generation study in rat. Information from this study in rats (summarised in the table below) showed that clofentezine has no effects on fertility and reproductive performance. Consequently, the DS was of the opinion that classification is not warranted.

Summary of the submitted study on adverse effects on sexual function and fertility (Anonymous 61, 1984; Seamons and Crofts, 1984 (dietary concentrations), Anonymous 62, 1986).

Method	Test substance, dose levels duration of exposure	Results
Multigeneration study in the rat Guideline: OECD TG 416 GLP: No (predates GLP enforcement) Rat strain: Charles River Sprague Dawley Crl CD BR No. animals (groups): F0: 30 rats/sex/dose (0, 4, 40 ppm) and 40 rats/sex/dose (400 ppm) F1: 25 rats/sex/dose F2: 20 rats/sex/dose **Study acceptable**	Purity: 97.9 – 99.3% Oral (diet) Study scheme F0 →F1A and F1B ↓ F1 →F2A and F2B ↓ F2 Doses: 0, 4, 40 or 400 ppm equivalent to: F0 → F1A and F1B ♂: 0, 0.28, 2.79, or 27.8 mg/kg bw/day ♀: 0, 0.33, 3.22, 31.7 mg/kg bw/day. F1 → F2A and F2B ♂: 0, 0.35, 3.57, or 36.1 mg/kg bw/day ♀: 0, 0.39, 3.85 or 38.5 mg/kg bw/day F2 ♂: 0, 0.36, 3.55, or 36.1 mg/kg bw/day ♀: 0, 0.38, 3.85 or 39.3 mg/kg bw/day. Exposure: Pre-mating treatment: F0 (74 days) →F1A F1 (88 days) →F2A Treatment continued in F1 and F0 throughout gestation and lactation. 14 days after weaning, animals of F0 and F1 were remated again to give F1B and F2B. Treatment did not stop until weaning of the 2nd generation. ↓ Total treatment for both sexes: F0 (32 weeks) and F1 (33 weeks) F2 (maturation of 82-84 days). Parameters observed: *F0, F1 and F2 parental*: Mortality, clinical signs, bodyweights, food consumption, organ weights, gross pathology, histopathology and reproductive parameters (mating, fertility and pregnancy index and gestation period).	**PARENTAL ANIMALS** ***Parental toxicity*** *Mortality* ***F0:*** 2 ♀ died during the breeding phase: 1 ♀ (0 ppm) during gestation of the F_{1A} litter and 1 ♀ (400 ppm) during parturition of the F_{1B} litter. 1 ♂ (40 ppm) was killed *in extremis* following weight loss. This animal was found to have to a fractured upper jaw. ***F1:*** 2 ♀ (40 ppm) died during their first breeding phase: during parturition and 1 ♀ killed in extremis on day 3 *post-partum* after total litter loss. 1 ♂ (400 ppm) died during the maturation period a 1 ♂ (4 ppm) during lactation of F2A offspring. **400 ppm** ***F0:*** ▪ ↓ bw gain in ♀ between days 4-7 *post coital* to give F1B (33%). ***F1:*** ▪ ↓ bw in ♂ at week 1 and 5 of pre-mating period (11 and 7% respectively) and in ♀ following the birth of F2A (10% and 7% on days 10 and 14 of *post-partum*) and F2B litters (6-7% days 4-14 *post coital* and 7-9% days 4-21 *post-partum*). ▪ ↓ bw gain in ♀ at week 12 of pre-mating period (*ndr* 43%) and on days 4-10 *post-partum* (37%) following the birth of F2A. ▪ ↓ Terminal bw in ♀ (10%). ▪ ↑ Relative liver weight (16%) in ♂ and ↓ absolute liver weight (*ndr* 11%) in ♀ ▪ ↑ Relative ovary weight (*ndr* 15%) in ♀. ▪ Increased incidence of minimal centrilobular hepatocyte enlargement in ♂ (4/10 *vs* 0/10 in controls). ***F2 (maturation):*** ▪ ↓ bw in ♂ at week 1 (12%), week 2 (11%), week 3 (10%) and week 6 (7%) and in ♀ at week 2 and 3 (10% and 7% respectively). ▪ ↑ Relative liver weight (14%) in ♂. In the absence of histopathological change, this marginal effect is of no toxicological relevance. **40 ppm** ***F0:*** There were no treatment-related effects. ***F1:*** ▪ ↓ bw gain in ♀ at week 12 (*ndr* 43%) and on days 4-10 *post-partum* (33%) following the birth of F2A (33%). ▪ ↓ Absolute liver weight (*ndr* 11%) in ♀. ▪ ↑ Relative ovarys weight (*ndr* 18%) in ♀. ***F2:*** ▪ ↑ Relative liver weight (9%) in ♂. 4 ppm ***F0:*** There were no treatment-related effects. ***F1:*** ▪ ↓ bw gain in ♀ following the birth of F2A (*ndr* 41%) on days 4-10 *post-partum*. ▪ ↓ Absolute liver weight (*ndr* 12%) in ♀. ▪ ↑ Relative ovary weight (*ndr* 15%) in ♀. ***F2:*** There were no treatment-related effects. NOAEL $_{\text{parental toxicity}}$: **40 ppm** (equivalent to approx. **4 mg/kg bw/day**) **REPRODUCTIVE PARAMETERS** ***F0 and F1***

	F0→F1A/F1B and F1→F2A/F2B litter: Mortality, clinical signs, body weights, litter size, pup developmental (pinna detachment, tooth eruption and eye opening), organ weights, gross pathology and histopathology	All reproductive parameters were similar to controls NOAEL reproductive toxicity **> 400 ppm (>27.8 mg/kg bw/day)** **LITTER DATA** **400 ppm** ***F0→F1A and F1B:*** There were no treatment-related effects. ***F1→F2A and F2B*** ***F1→F2A*** ▪ ↓ Pup weights (17%) at day 21 *post-partum.* ***F1→F2B*** ▪ ↓Mean litter size: born pups (12%), live pups (16% on day 1 *post-partum* and 18% on day 21 *post-partum*). ▪ ↓ Litter weights between days 4 and 21 *post-partum* (16-18%). **40 ppm** ***F0→F1A and F1B:*** There were no treatment-related effects. ***F1→F2A and F2B*** ***F1→F2A*** ▪ ↓ Pup weights (11%) at day 21 *post-partum.* This slight reduction reflected the slightly higher mean litter size at this dose and is of no toxicological concern ***F1→F2B:*** There were no treatment-related effects. **NOAEL neonatal toxicity: 40 ppm** (equivalent to approx. **4 mg/kg bw/day**)

n.s: not significant, ndr: not dose-related, ncdr: not clearly dose-related

Comments received during public consultation

No comments were received for any reproductive endpoints.

Assessment and comparison with the classification criteria

A standard 2-generation study in rats (Anonymous 61, 1984) was performed with doses up to 400 ppm (approx. equivalent to 27.8 / 39.3 mg/kg bw/day for males/females).

Parental toxicity was evident in F1 and F2 parents at 400 ppm (in F0 there were no treatment-related effects):

In F1 parents, body weights were lower than control values in both sexes: throughout the maturation phase in males (11% and 7% at week 1 and 5 respectively) and gestation and lactation period in females [F1→F2A (10% and 7% on post-partum days 0 and 14) and F1→F2B (6-7% during period 4-14 days *post-coitum* and 7-9% during period 4-21 days *post-partum*]. In addition, in females it was observed a decrease in body weight gain F1→F2A (37%) during on days 4-10 days *post-partum* and in terminal body weight (10%). In males, an increase in relative liver weight (16%) was seen which was associated with histopathology (increased incidence of minimal centrilobular hepatocyte enlargement).

In F2 parents, there were a decrease in bodyweight from week 1 to week 6 in males (7-12%) and at week 2 and 3 (10% and 7% respectively) in females. In males, an increase in relative liver weight (14%) was seen, but this increase was not associated with histopathological changes, so this marginal effect was of no toxicological relevance. NOAEL for maternal toxicity was set at 40 ppm (equivalent to approximately 4 mg/kg bw/day).

Neonatal toxicity was evident in F2 pups at 400 ppm (in F1 pups there were no treatment-related effects). In F2A pups, a decrease in pup's weights (17%) on day 21 post-partum was observed. In F2B pups, a decrease in mean litter size [born pups (12%) and live pups (16% and 18% on days 1 and 21 post-partum respectively)] and litter weights (16-18% between days 4 and 21 post-partum) were seen. The NOAEL for development was set at 40 ppm (equivalent to approx. 4 mg/kg bw/day).

RAC concludes that the dose levels in the rat multi-generation study have been too low. The highest dose tested was only up to 400 ppm ~ 36.1 / 39.3 mg/kg bw/day where slight clinical signs associated with treatment, only slight liver toxicity and slightly decreased body weight gain, were observed. The highest dose in the teratology study in rats was up to 3200 mg/kg bw/day and a maternal NOAEL from this study was 1280 mg/kg bw/day, indicating that the MTD in the multi-generation study had not been reached and the multi-generation study in rats is not considered to be adequate for assessment of reproductive toxicological potential.

RAC concludes on **no classification for sexual function / fertility based on inconclusive data**.

Developmental toxicity

The DS proposed no classification for developmental toxicity as no evidence of teratogenicity was observed in two prenatal developmental toxicity studies, one performed in rats (Anonymous 65, 1982) and the other in rabbits (Anonymous 66, 1983). Both studies predate the current OECD TG 414 (2001) and do not include the recommended extended dosing period (i.e. from implantation to one day prior to the day of scheduled kill). However, both studies are considered adequate and relevant for evaluation of the potential of clofentezine to induce developmental effects.

Summary of submitted studies on adverse effects on developmental toxicity:

Method	**Test substance, dose levels duration of exposure**	**Results** [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	**Reference**
Teratology study in rats Laboratory: Fisons Limited Pharmaceutical Division Method: *"In house method"* comparable to OECD TG 414 (1981) / B.31 GLP: Yes Oral (gavage) Rat strain: CD Sprague Dawley 34 or 35 mated females/group **Study acceptable** Deviations: The exposure period was from day 7 to 20 of gestation when it should have begun at least from implantation. The highest dose tested, 3000 mg/kg bw/day exceeds the dose	Test substance: Clofentezine (NC 21314, technical material; batch CR 20099/8; purity 100%) Dose levels: 0, 320, 1280, 3200 mg/kg bw/day Vehicle: 0.5 % carboxymethyl cellulose Exposure: dosing on gestation days 7-20 gestation	***Maternal toxicity*** Mortality: During treatment 3♀ were found dead, 2 at 1280 mg/kg bw/day and 1 at 3200 mg/kg bw/day. The cause of these deaths was considered to be misdosing into the respiratory system. **3200 mg/kg bw/day:** ▪ ↓ bw on day 21 for body weight (4%) and corrected bw for uterine contents (5%). ▪ ↓ bw gain during periods days 7-14 (24%) and days 14-21 (9%). ▪ ↑ Relative liver weight (10%) when corrected for the uterine contents, associated with histopathology changes (staining and enlargement of centrilobular hepatocytes). **1280 mg/kg bw/day:** ▪ ↑ Relative liver weight (7%) when corrected for the uterine contents. This increase was < 10% and was not associated with histopathological changes (not toxicologically relevant). **320 mg/kg bw/day:** No effects. **NOAEL** maternal**: 1280 mg/kg/day** ***Developmental toxicity*** **3200 mg/kg bw/day:** ▪ *Skeletal alteration*: ↑Incidence foetuses with: - Incomplete ossification of the hyoid (8.92 *vs* 2.69). - One or less sternebrae incompletely ossified (57.28 *vs* 40.81) - Two or more ossified caudal vertebrae (78.87% *vs* 52.47)	Anonymous 65 (1982) Crofts, (1982a) (Determination concentrations in suspensions) (AS) B.6.6.2.1

<table>
<tr><th>Method</th><th>Test substance, dose levels duration of exposure</th><th>Results
[Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]</th><th>Reference</th></tr>
<tr><td>recommended for a test limit (1000 mg/kg bw/day)</td><td></td><td>
Foetal skeletal alterations
<table>
<tr><th rowspan="2">Parameters</th><th colspan="4">Dose level (mg/kg bw/day)</th></tr>
<tr><th>0</th><th>320</th><th>1280</th><th>3200</th></tr>
<tr><td>Foetuses examined</td><td>223</td><td>207</td><td>193</td><td>213</td></tr>
<tr><td>Litters examined</td><td>35</td><td>33</td><td>30</td><td>33</td></tr>
<tr><td>Observations</td><td colspan="4">Foetal incidence% (Litter incidence %)</td></tr>
<tr><td>Incomplete ossification or absence of hyoid</td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td>2.69 (8.57)</td><td>5.80 (18.18)</td><td>7.25 (26.67)</td><td>8.92** (18.18)</td></tr>
<tr><td>Incomplete ossification of sternebrae - number of bones affected</td><td></td><td></td><td></td><td></td></tr>
<tr><td>≤1</td><td>40.81 (85.71)</td><td>46.38 (81.81)</td><td>37.31 (76.67)</td><td>57.28** (96.97)</td></tr>
<tr><td>2</td><td>53.81 (97.14)</td><td>46.86 (87.88)</td><td>53.37 (96.67)</td><td>41.32* (81.82)</td></tr>
<tr><td>≥3</td><td>5.38 (20.00)</td><td>6.76 (24.24)</td><td>9.33 (40.00)</td><td>1.41* (6.06)</td></tr>
<tr><td>Number of ossified caudal vertebrae</td><td></td><td></td><td></td><td></td></tr>
<tr><td><2</td><td>47.53 (85.71)</td><td>20.77** (57.58)</td><td>17.62** (56.67)</td><td>21.13** (69.70)</td></tr>
<tr><td>≥2</td><td>52.47 (88.57)</td><td>79.23** (96.97)</td><td>82.38** (100.00)</td><td>78.87** (100.00)</td></tr>
</table>
*p≤0.05; **p≤0.01; *** p≤0.001
These increases were not considered to be related to the treatment since:
- No significant difference in the litter incidences of these parameters was detected.
- The number of foetuses with one or less sternebrae incompletely ossified was significantly increased. However, the number of foetuses with 2, 3 or more sternebrae incompletely ossified was reduced at 3200 mg/kg bw/day compared to controls.
- The number of foetuses with two or more ossified caudal vertebrae was significantly increased at all dose levels but these findings were not dose-dependent.

<u>1280 mg/kg bw/day</u>: No effects
NOAEL maternal: 250 mg/kg bw/day
</td><td></td></tr>
<tr><td>Teratology study in rabbit

<u>Laboratory</u>: Schering Agrochemical limited

<u>Method</u>: "In house method" comparable to OECD TG 414 (1981) / B.31
<u>GLP</u>: No (conducted prior to GLP).
Oral (gavage)
<u>Rabbit strain</u>: New Zealand White
14 or 15 mated females/group
Study acceptable</td><td><u>Test substance</u>: Clofentezine (NC 21314), Lot/Batch No.: CR 20099/12, Purity: 98.5%

<u>Dose levels</u>: 0, 250, 1000 and 3000 mg/kg bw/day

<u>Vehicle</u>: 0.5% sodium carboxymethyl cellulose
<u>Exposure</u>: from day 7 to 28 of gestation</td><td>
<u>Maternal toxicity</u>
<u>3000 mg/kg bw/day</u>:
▪ 1/14 dead treatment-related associated with anorexia, reduced faecal output and weight loss.
▪ ↓ bw change relative to the initiation of dosing on day 7 of gestation on day 10 (90%), 14 (51%), 18 (38%), 22 (33%), 26 (29%) and day 29 (19% but not significant).
▪ ↓ Food consumption (~ 20% from day 7 to day 25). There is no statistical calculation for this effect.
▪ Pink discoloration of the GIT.
<u>1000 mg/kg bw/day</u>:
▪ 1/14 dead. The clinical signs observed were indicative of respiratory and gastro-intestinal disorder. 1/14 death was observed in the control group with the same associated symptoms and were considered to be coincidental.
▪ ↓ bw change relative to the initiation of dosing on day 7 of gestation on day 10 (85%) and day 18 (31%).
<u>250 mg/kg bw/day</u>:
▪ ↓ bw change relative to the initiation of dosing on day 7 of gestation on day 18 (27%).

NOAEL maternal: 250 mg/kg bw/day

<u>Developmental toxicity</u>
<u>3000 mg/kg bw/day:</u>
▪ ↓ Mean foetal weight (13%). In consequence, mean litter weights were lower than the control value (12%), although the difference did not attain statistical significance.
</td><td>Anonymous 65 (1983)

Anonymous 67 (1983) (Report addendum)

Crofts, (1982b) (Determination concentrations in suspensions)

(AS) B.6.6.2.2</td></tr>
</table>

Method	Test substance, dose levels duration of exposure	Results [Effects statistically significantly and dose-related unless stated otherwise as not significant (n.s.) or not dose-related (ndr) or not clearly dose-related (ncdr)]	Reference
		1000 mg/kg bw/day: No effects **NOAEL** developmental**: 1000 mg/kg bw/day**	

Assessment and comparison with the classification criteria

In the rat study, the highest dose tested of 3200 mg/kg bw/day induced maternal toxicity. Bodyweights were significantly decreased (also when they were corrected for uterine content) at day 21 (4%) and also body weight gain between days 7-14 (24%) and 14-21 (9%). Dose-related and significantly increased relative liver weights were observed (also when corrected for uterine content). This increase (10%) was associated with histopathology (staining and enlargement of centrilobular hepatocytes).

The foetal skeletal alterations observed were considered to be in the range of variations not related to treatment at this dose level. The number of ossified caudal vertebrae seen at various dose levels was not dose-dependent and the incomplete ossification of sternebrae was higher in the number of foetuses with ≥ 2 bones affected in controls than the highest treated group. Besides, the incomplete ossification or absence of hyoid was seen in foetuses but not in litters (not significant and not dose-related). Consequently, no developmental effects were attributable to this dose level.

At the intermediate dose level of 1280 mg/kg bw/day no maternal toxicity or developmental effects were observed. The NOAEL for maternal toxicity was set at 1280 mg/kg bw/day and NOAEL for development was considered to be higher than 3200 mg/kg bw/day.

In the rabbit study, the highest dose of 3000 mg/kg bw/day was associated with death in one rabbit (with anorexia, reduced faecal output and weight loss), markedly reduced body weight gain and reduced food consumption (~ 20% from day 7 to day 25). Developmental effects included reduced foetal weight (13%) and mean litter weight (12%).

At the intermediate dose, 1000 mg/kg bw/day, a decrease of body weight gain (85% days 7-10 and ~ 20% from day 10 to day 18) was observed.

At the lowest dose, 250 mg/kg bw/day, no maternal toxicity or development effects were observed.

There were no effects triggering classification for clofentezine due to developmental toxicity and therefore RAC agrees with the DS that no evidence for teratogenicity was observed in the submitted studies and **no classification for developmental toxicity is warranted**.

Effects on or via lactation

There were no indication of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk. Overall, based on the limited data, RAC concludes that **no classification for effects on or via lactation is warranted**.

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

Based on the available data for the toxicity of clofentezine included in this dossier as well as the physicochemical properties of the active substance it does not seem to pose an aspiration toxicity hazard to humans. There are no data in humans indicating evidence of this toxicity and clofentezine is a solid organic substance but not a hydrocarbon.

The DS is of the opinion, with the current data available on clofentezine, that classification due to aspiration hazard is not required.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the DS that **classification due to aspiration hazard is not warranted**.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Degradation

Abiotic degradation

Hydrolysis

A study conducted in accordance with OECD TG 111 was provided to assess hydrolysis and was considered valid, although the concentrations used were above the clofentezine water solubility (Previous value: 0.0025 mg/L, current value: 0.034 mg/L, see explanation in consultation section). The hydrolytic behaviour of [^{14}C]-clofentezine was studied at pH 4, 7 and 9 in aqueous solution at different temperatures. A Tier 1 preliminary test was performed at each pH and 50°C: [^{14}C]-clofentezine was stable at pH 4, accounting for 99.4% AR after 168 hours, was degraded rapidly at pH 7, accounting for 19.5% AR after 168 hours, and was degraded rapidly at pH 9, accounting for 3.1% AR after 5 hours. In conclusion, clofentezine is hydrolytically stable at pH 4 but degraded rapidly at pH 7 and 9. The DT_{50} values for the pH 7 samples were 828, 526 and 37.7 hours at 20, 25 and 50°C, respectively. The DT_{50} values for the pH 9 samples were 81.4, 62.8 and 0.574 hours at 20, 25 and 50°C, respectively.

Three further studies to address the hydrolytic degradation of clofentezine were accepted by the DS, with some limitations. However, the main conclusion for hydrolytic stability of clofentezine at low pH values and instability at pH 7 and above are supported.

Photodegradation

Three experimental studies on photodegradation of [^{14}C]-clofentezine in aqueous buffered solution at pH 5 and natural waters were included in the submission of the revised dossier (March 2009). In the first GLP study, performed according to Japanese Ministry of Agriculture, Forestry and Fisheries (JMAFF) test guidelines section 2-6-2, DT_{50} values of 3.54 days (pH 5 buffer) and 5.99 days (natural water), calculated for Europe based on mean maximum summer sunlight at latitude 30ºN, 40ºN and 50ºN were found. Mineralisation was not observed and degradants were identified by HPLC. In the second study, these values from the experimental study were input into the program GCSOLAR from the US EPA Centre of Exposure Modelling to determine the half-lives at three latitudes (30ºN, 40ºN and 50ºN), and at three seasons (spring, summer and autumn). The DT_{50} values were found to be in the range of 2.67 to 5.63 days across the latitudes and seasons. In the third study, the photodegradation and quantum yield of clofentezine was investigated in natural water (river water, collected from Shiny Brook, Saddleworth Moor near Manchester, UK). The DT_{50} values for clofentezine were determined to be 9.56 and 22.32 days in the irradiated and dark samples, respectively.

<u>Biotic degradation</u>

Ready biodegradation

There was one ready biodegradability test available on clofentezine (OECD TG 301B, GLP) showing 12% degradation in 28 days. The test design strictly follows the test guideline requirements. Under the conditions of the test, clofentezine attained 12% degradation after 28 days, and therefore cannot be considered as readily biodegradable.

Aerobic mineralisation

Aerobic mineralisation of [Tetrazine-14C]-clofentezine (4.0 and 41.3 µg/L) was studied in natural German surface water in the dark at 20±2°C under constant bubbling of air through the water (GLP, OECD TG 309). Traps for organic volatiles and carbon dioxide were used. The total mass balance was between 96.3% and 103.8% of the applied radioactivity for low concentrations and between 90.0 and 104.4% for high concentrations. CO_2 and organic volatiles were observed at low concentrations: respectively 10.8% and 4.6% of applied dose for low concentration and 3.6 and 3.1% for high concentration. Clofentezine degraded rapidly in the water from a maximum of 103.8% applied dose initially to 0% after 30 days. AE C593600, 2-CBA and 2-CBZ were identified as major metabolites.

Water/sediment

A GLP study, in line with OECD TG 308 (April 2002) was submitted, which provided information about acidic water bodies of water/sediment systems as a worse case data. The degradation of [Tetrazine-U-14C] clofentezine (0.3 mg/L) under aerobic conditions was investigated in two water sediment systems – Taunton River (sandy loam) and Weweantic River (sand). The major degradation product for the total system was 2-CBA, which had a maximum level at 26.7% of the applied dose at 58 day after treatment. The data generated from these studies was analysed using the CAKE v3.1 and v 3.2 software package according to guidance provided by FOCUS (2006) based on level P-1 and M-1 kinetics (single compartment kinetics) and results calculated were proposed as new endpoints. Resulting total system DT_{50} and DT_{90} values (16.5 – 37 and 76.4 – 123 days, respectively) indicate that clofentezine cannot be considered rapidly biodegradable, according to the DS.

Bioaccumulation

The experimentally determined log K_{OW} (two valid GLP studies according to OECD TG 107 and OECD TG 117) was 4.1 and so a potential for bioaccumulation could not be excluded. The study aimed to determine the accumulation and elimination of [^{14}C]-clofentezine in bluegill sunfish (*Lepomis macrochirus*) was found unreliable and the calculated BCF of 248 was considered not valid. The uncertainties identified include; the concentration of clofentezine not being maintained, it could not be demonstrated that the total radioactivity measured in the water was the parent compound, the concentration of clofentezine is above the water solubility limit, and calculated BCFs were not lipid normalized. In the absence of a reliable experimental bioaccumulation study, the information of the octanol/water partition coefficient should be taken into account to evaluate the substance´s bioaccumulation potential, resulting in the DS's conclusion that clofentezine has a high potential for bioaccumulation.

Acute aquatic toxicity

Acute aquatic toxicity tests for fish and invertebrates with clofentezine (algae presented below)

Method	Species	Substance	Results	Reference
Acute toxicity to fish in line with OECD TG 203	Rainbow trout (*Oncorhynchus mykiss)*	clofentezine (98.6%) [^{14}C]-clofentezine, purity not stated.	96h LC_{50} > 0.0146 mg/L, based on mean measured concentrations.	Anonymous (1986) B.9.2.1/01
Acute toxicity to fish	Bluegill sunfish (*Lepomis macrochirus)*	clofentezine, (99.8%)	96h LC_{50} > 0.25 mg/L, based on mean measured concentrations.	Anonymous (1981) B.9.2.1/02
Aquatic invertebrates Guideline: OECD TG 202 I and US EPA EG1 31: 5007-5009	Water flea (*Daphnia magna)*	clofentezine, (99.8%)	EC_{50} > 0.001123 mg/L, based on mean measured concentrations.	Barrett and Arnold (1988)
Aquatic invertebrates Guideline US EPA 660/3-75-009(1975) and US EPA draft (1978)	Water flea (*Daphnia magna)*	clofentezine, (99.0%)	48h EC_{50} > 0.040 mg/L	Lines (1981)

Acute toxicity studies were also available for different metabolites of clofentezine. The available data indicates no toxicity at the concentrations tested so these substances are not considered further.

<u>Acute toxicity to fish</u>

Acute toxicity of clofentezine to fish was investigated in two non-guideline studies with rainbow trout (*Oncorhynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus*). In both studies, no effects were reported at the highest concentrations tested - LC_{50} > 0.0146 mg/L mean measured (substance was firstly absorbed to pumice which was then used, via a saturation column, to supply a constant level of dissolved [^{14}C]-labelled clofentezine to the fish) for rainbow trout and LC_{50} > 0.25 mg/L (substance suspension, actual concentration not known) for bluegill sunfish.

The acute toxicity of metabolites 2-chlorobenzoic acid (2-CBA), chlorobenzonitrile, AE C593600, AE F092117 to Rainbow trout (*Oncorhynchus mykiss*) was tested in four separate studies. No toxic effects were observed at highest concentration tested.

Acute toxicity to aquatic invertebrates

Acute toxicity of clofentezine to *Daphnia magna* was studied in two guideline studies under static conditions. In both studies, only one concentration was tested due to the low solubility of clofentezine. Toxic effects were not observed in either investigation: EC_{50} >0.001123 mg/L (measured, representing the maximum solubility attainable at the end of the test) and EC_{50} > 0.040 mg/L (mean measured at the end of the test) were found.

The acute toxicity of metabolites: 2-Chlorobenzoic acid (2-CBA, 2-Chlorobenzonitrile, AE C593600, 2-Chlorobenzamide to Water flea *Daphnia magna* was examined in separate tests. Results did not show toxic effect at highest concentration level tested.

The DS concluded that several toxicity endpoint values derived were above the water solubility limit of clofentezine (previously, 0.0025 mg/L) and therefore the studies could not provide data for classification purposes but were used as supplementary information. In all other cases, regarding the fact that no acute toxicity was recorded at levels up to the substance's solubility in the tests, no acute hazard classification would apply for clofentezine. Algal data are discussed below.

Chronic aquatic toxicity

Chronic aquatic toxicity tests for fish and invertebrates with clofentezine (algae presented below)

Method	Species	Substance	Results	Reference
Fish TG equivalent to OECD TG 210	Rainbow trout (*Oncorhynchus mykiss)*	clofentezine, (99.5%)	97d NOEC > 0.007 mg/L, based on mean measured concentrations.	Anonymous (1993) B.9.2.2/01
Reproductive toxicity to Daphnia Guideline OECD TG 202 II (1984) and US EPA 540/9-86-141	Water flea (*Daphnia magna)*	clofentezine, (99.8%) [^{14}C]-clofentezine, (99.21%)	21d NOEC > 0.025 mg/L, [^{14}C] – clofentezine	Barber and Latimore (1992)
Lifecycle toxicity test Guideline OPPTS 850.1350	Saltwater mysid shrimp (*Americamysis bahia*)	clofentezine, (98.29%)	28d NOEC > 0.0269 mg/L, based on mean measured concentrations. Endpoint recalculated by DS: 28d- NOEC Mean total young per F0-female = 0.0033 mg/L	Aufderhide (2009, 2016)

Chronic toxicity to fish

Chronic toxicity of clofentezine to early life stages of the rainbow trout *Oncorhynchus mykiss* was investigated in a flow through study, which can be considered equivalent to the requirements of OECD TG 210. Due to the low solubility of clofentezine in water, a single maximum attainable concentration was tested. Clofentezine (technical) had no chronic toxicity to early life stages of the rainbow trout at the maximum solubility obtained under study conditions (i.e. 0.007 mg/L) over a 97 day continuous exposure period, so the 97 day-NOEC can be estimated to be > 0.007 mg/L. Several technical protocol deviations were reported for this study; particularly temperature deviated from the specified value, feeding regime, length determination of fish and concentration

measurements during the study. Despite this, the study was considered reliable for use as supportive information.

Chronic toxicity to aquatic invertebrates

The effect of clofentezine to *Daphnia magna* was studied in a GLP study (OECD TG 202 and USEPA). First instar daphnids (less than 24 hours old) were exposed for 21 days to the single concentration of [^{14}C]-clofentezine, 25 µg/L, which represented the maximum sustainable concentration under the test conditions (substance first absorbed to pumice stone that was then used, via a saturation column, to supply dissolved clofentezine to the test chambers). The results indicated that at highest concentration (0.025 mg/L) clofentezine had no effect on survival, growth or reproduction of *Daphnia magna*, therefore the NOEC was concluded to be > 0.025 mg/L. During the peer review, this point was revised and finally it was concluded that this endpoint will not be considered for risk assessment purposes and this endpoint was not considered reliable.

The effects of clofentezine on the survival, growth, and reproduction of saltwater mysid shrimp, *Americamysis bahia* were studied in a GLP guideline study under flow through conditions. Five different clofentezine concentrations were tested, using DMF as solvent to enhance substance solubility. Statistical analysis of results showed that the number of young per female mysid was the only biological parameter that resulted in a statistically significant difference when compared to the negative control data at the concentrations of 0.0067 mg/L and 0.0269 mg/L (mean measured concentrations). Therefore, the NOEC value determined for mean number of total young produced per female was 0.0033 mg/L clofentezine. However, in accordance with the OECD Number 54 Guidance document, the applicant submitted an additional statistical analysis (the Williams' trend test) using the vehicle control data instead of dilution water control. This indicated no statistically significant reduction in the reproductive data for any of the treatment levels tested. Subsequently, following this approach, the NOEC value for mean total number of young per F0-female mysid was > 0.00269 mg/L. In conclusion, as comparing the response data with the water control resulted in a significant dose-response, the DS proposes to maintain the NOEC of 0.0033 mg/L, as a conservative approach.

Acute and chronic toxicity to algae

Available data on algae

Method	Species	Substance	Results	Reference
Effects on growth of green algae Dutch draft standard method NEN 6506	Algae (*Scenedesmus pannonicus*)	NC 21314 Technical clofentezine	120h - EC_{50} > 0.32 mg/L No chronic values derived.	Oldersma *et al.* (1983)
Effects on growth of green algae Guidelines: OECD TG 201 (1984) modified by EG-8 and ES-5	Algae (*Selenastrum capricornutum*)	Apollo 50 SC 50% w/w clofentezine	72h - E_rC_{50} not reported 72h NOEC > 40 mg/L DS recalculation: 92h E_rC50 > 34 mg/L No 96h NOEC provided	Hanstveit (1987)

The toxicity of clofentezine to green algae *Scenedesmus pannonicus* was examined according to the Dutch draft NEN 6506 guideline. Comparison of the growth curves of algal suspension exposed to the test substance with those algal controls (solvent controls with DMSO, no negative controls were used) the NOEC was estimated to be 0.32 mg/L (n), although no statistical treatment of data were reported. When actual concentrations were measured, less than 10% of nominal were found at the end of the test. Therefore, the applicant reported that the results showed that clofentezine in concentrations up to its solubility limit in water did not impair the growth of the alga *Scenedesmus pannonicus* under the conditions of the test. The endpoint was not stated (the effect value was claimed to be greater than the water solubility of clofentezine). No effect values for growth rate are available from this study. This study was already evaluated during Annex I inclusion of clofentezine. However, the EC_{50} endpoint was not stated (claimed to be greater that the water solubility of clofentezine) and it was not used for risk assessment purposes in the DAR (2005). The results are accepted as supplementary information.

The toxicity of metabolites: Metabolite 2-CBA, Metabolite 2-CBA, Metabolite AE C593600, Metabolite AE F092117 to algae (*Pseudokirchneriella subcapitata*) was given in separate studies. Toxic effects were not observed at highest concentration tested.

Conclusions

The DS concluded that based on 28-d NOEC of 0.0033 mg/L for saltwater mysid shrimp, *Americamysis bahia* for reproduction clofentezine meets the CLP Regulation criteria for being classified as Aquatic chronic 1 with M factor of 10

DS proposal: Acute Aquatic Hazard: No classification, Aquatic Chronic 1 (H410), M=10.

Comments received during the consultation

Six MSCAs and one Industry association submitted comments on the DS's proposal during the public consultation.

Two MSCAs commented on the water solubility value and one asked for clarification on the value to be used for assessing the aquatic toxicity. The DS clarified that the value of 0.0025 mg/L was an old value used under the pesticide assessment and that in their view, the newer value of 0.034 mg/L should be used as the water solubility value.

All commenting MSCAs agreed with no classification for aquatic acute toxicity. One MSCA required clarification on the DS opinion on the validity of acute toxicity studies.

Three MSCAs supported aquatic chronic classification as Aquatic Chronic 1, M=10. One commenting MSCA did not support aquatic chronic classification of the substance in category Aquatic Chronic 1 based on NOEC of 0.0033 mg/L derived in chronic toxicity study to mysid shrimp, *Americamysis bahia*. They thought it useful for the DS to compare exposure treatment data with pooled controls (i.e. solvent and procedural controls) in order to clarify if there was a statistically significant effect. One MSCA required clarification for the devaluation of bioaccumulation study given in CLH report in the light of new data for clofentezine water solubility of 0.034 mg/L.

An industry association pointed out that a new study, compliant with OECD TG 305 showed a lipid-normalised growth-corrected kinetic BCF value of 276 L/kg for spiked aqueous solution. The study was considered valid by the RMS (revised dRAR of July 2019). Based on the information presented in this study, the DS concluded that clofentezine does not bioaccumulate in fish.

An industry association disagreed with the DS proposal to classify the substance as Aquatic Chronic 1, M-factor=10 due to inappropriate acceptance of 28d-NOEC of 0.0033 mg/L 28d-NOEC (Mean total young per F0-female), in the chronic toxicity study with mysid shrimp, *Americamysis*

bahia, only taking into account the water control. They commented that a value of 28d-NOEC of 0.0269 mg/L, calculated using the solvent control, would be a more appropriate value for classification. The industry association supported classification Aquatic chronic 1, but proposed M-factor 1 instead of the DS-proposed M-factor of 10.

The DS responded that the proposed aquatic chronic classification in the CLH report is based on a 28d-NOEC (Mean total young per f0-female) of 0.0033 mg clofentezine/L, taking into account only the water control, which is considered as a conservative approach. However, in the course of the EU evaluation for renewal of clofentezine registration, the applicant submitted additional statistical evaluations and data analyses demonstrating that the negative control is not appropriate for statistical comparison and the solvent control is the most suitable to derive a reliable endpoint with a NOEC of 0.0269 mg/L. This endpoint is agreed (revised dRAR of July 2019) and the final classification proposed by DS after PC for clofentezine should be Aquatic Chronic category 1, M=1.

Editorials and minor comments were submitted from 3 MSCAs, these are reflected in the DS response.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS to consider clofentezine as 'not rapidly degradable', as based on the following information:

- 12 % degradation in 28 days in a ready biodegradability test (OECD TG 301B) less than 70% clofentezine is not ultimately degraded to 70% or greater within 28 days (Annex I: 4.1.2.9.5)
- Less than 11% mineralization after 30 days in surface water simulation test.

Based on the hydrolysis and primary degradation data from the surface water simulation study, the substance is rapidly primary degradable. However, as no chronic data are available for all hydrolysis products, it cannot be excluded that the criteria for classification as hazardous to the aquatic environment are not met for the hydrolysis products. The substance therefore cannot be regarded as rapidly degradable for classification via primary degradation.

Bioaccumulation

Based on the log K_{OW} value of 4.09 bioaccumulation could not be excluded. However, the new experimentally determined lipid-normalised growth-corrected kinetic BCF values is 276 L/kg for spiked aqueous solution (as presented by the applicant in a new study), is considered reliable by RAC and is taken as the primary evidence for assessing bioaccumulation in the aquatic environment. Consequently, as the BCF is below 500, RAC agrees with the DS to consider clofentezine as not bioaccumulative.

Acute aquatic toxicity

RAC notes that the reliable water solubility value to be taken into account is 0.034 mg/L and that some endpoint values are in excess of this value. However, in all cases no effects have been recorded up to and including the maximum concentration tested in any acute toxicity test presented.

There are valid acute toxicity data for fish and invertebrates. For algae, no acute toxicity was recorded at levels at any concentrations tested. However, only nominal values are available and the results are far in excess of the water solubility. In conclusion, no reliable ErC_{50} can be determined from this study. In the other available algal study, a formulation was used (Apollo

50 SC) and although the study did not derive an ErC_{50}, the DS recalculated a 96h ErC_{50} of > 34 mg a.s./L (based on mean measured concentration). However, due to difficulty determining the measured concentrations of the test substance, a formulation being used, and the resulting value being far in excess of the water solubility, this value is not considered reliable.

Consequently, due to the available reliable data indicating no toxicity at the concentrations tested, RAC agrees with the DS that **clofentezine does not warrant classification for acute aquatic hazards**.

Chronic aquatic toxicity

RAC concludes that there are reliable chronic toxicity data available for fish and invertebrates. Regarding the invertebrate study with *Americamysis bahia*, RAC agrees with parties commenting during the consultation that the solvent control should be used for deriving the dose-response curve and that the NOEC derived from the study should be 0.0269 mg/L. The DS agreed with commenting parties and changed their proposal after the consultation to use this value for the classification of clofentezine and RAC therefore also agrees to use this value, instead of the previous value of 0.0033 mg/L. Results for algae are not considered reliable for the reasons stated above. Consequently, there are no chronic data for algae and in the absence of reliable acute data, the surrogate approach is not possible. No effects for fish were observed up to the maximum solubility used in the test and therefore the lowest chronic toxicity value is the 28d NOEC of 0.0269 mg/L for mysid shrimp, *A. bahia*.

Comparison with the criteria

Parameter	CLP criteria	Results	Conclusion
Ready biodegradation	< 70% for 28 days	12%	**Not rapidly degradable**
Water simulation study	Ultimate degradation <16 day	< 11% in 30 days	
Bioaccumulation	Log K_{OW} ≥ 4 BCF ≥ 500 L/Kg	Log K_{OW} > 4 BCF < 500 L/Kg	**Not bioaccumulative**
Acute toxicity	$L(E)C_{50}$ ≤ 1 mg/L	No effects observed in fish or invertebrates. No reliable algae data	**No classification**
Chronic toxicity	For a NRD substance NOEC ≤ 0.1 mg/L M-factor - 0.01 < NOEC ≤ 0.1 mg/L	28d-NOEC of 0.0269 mg/L for mysid shrimp, *Americamysis bahia*	**Aquatic Chronic 1 (H410), M=1**

NRD: not readily degradable

In conclusion, RAC agrees with the DS's proposal (modified after the consultation) that **clofentezine warrants classification as Aquatic Chronic 1 (H410), M = 1.**

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 RAC (excluding confidential information).