Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

Cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol

EC Number: -
CAS Number: 94361-06-5

CLH-O-0000001412-86-73/F

Adopted

11 September 2015

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11 September 2015

CLH-O-0000001412-86-73/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 harmonized classification and labelling (CLH) of:

Chemical name: **Cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1 H-1,2,4-triazol-1-yl)butan-2-ol**

EC Number: **-**

CAS Number: **94361-06-5**

The proposal was submitted by **Ireland** and received by RAC on **18 November 2014.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation; the notation of 67/548/EEC, the Dangerous Substances Directive (DSD) is no longer provided.

PROCESS FOR ADOPTION OF THE OPINION

Ireland 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 **13 January 2015**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **27 February 2015**.

ADOPTION OF THE OPINION OF THE RAC

Rapporteur, appointed by RAC: **Stephanie Copin (Vivier)**

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 harmonized classification and labelling was adopted on **11 September 2015** by **consensus**.

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

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard state-ment Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	650-032-00-X	cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H—1,2,4-triazol-1-yl)butan-2-ol	-	94361-06-5	Repr. 2 Acute Tox 4 * Aquatic Acute 1 Aquatic Chronic 1	H361d *** H302 H400 H410	GHS08 GHS07 GHS09 Wng	H361d *** H302 H410			
Dossier submitters proposal	650-032-00-X	cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol	-	94361-06-5	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Add** Carc. 2 STOT RE 2 **Modify** Repr. 1B Acute Tox 4	**Retain** H400 H410 **Add** H351 H373 (liver)(oral) **Modify** H360D H302	**Retain** GHS08 GHS07 GHS09 **Modify** Dgr	**Retain** H410 **Add** H351 H373 (liver)(oral) **Modify** H360D H302		**Add** M (Acute) = 10 M (Chronic) = 10	
RAC opinion	650-032-00-X	cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol	-	94361-06-5	**Retain** Aquatic Acute 1 Aquatic Chronic 1 **Add** STOT RE 2 **Modify** Repr. 1B Acute Tox. 3	**Retain** H400 H410 **Add** H373 (liver) **Modify** H360D H301	**Retain** GHS08 GHS07 GHS09 **Modify** Dgr	**Retain** H410 **Add** H373 (liver) **Modify** H360D H301		**Add** M (Acute) = 10 M (Chronic) = 1	
Resulting Annex VI entry if agreed by COM	650-032-00-X	cyproconazole (ISO); (2RS,3RS;2RS,3SR)-2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol	-	94361-06-5	Repr. 1B Acute Tox. 3 STOT RE 2 Aquatic Acute 1 Aquatic Chronic 1	H360D H301 H373 (liver) H400 H410	GHS08 GHS07 GHS09 Dgr	H360D H301 H373 (liver) H410		M=10 M=1	

GROUNDS FOR ADOPTION OF THE OPINION

HUMAN HEALTH HAZARD ASSESSMENT

RAC evaluation of physical hazards

Summary of the Dossier submitter's proposal

Cyproconazole is not considered explosive, oxidising, flammable or self-ignitable and does not fulfil the classification criteria for physico-chemical properties, based on negative standard tests. Therefore, no classification was proposed by the dossier submitter (DS).

Comments received during public consultation

Physical hazards were not specifically commented on.

Assessment and comparison with the classification criteria

Cyproconazole does not meet the classification criteria for physical hazards according to CLP. RAC agreed with the DS that **no classification for physical hazards** was warranted.

RAC evaluation of acute toxicity

Summary of the Dossier submitter's proposal

Cyproconazole is currently classified as Acute Tox. 4 *; H302 (Harmful if swallowed).

The acute oral toxicity of cyproconazole has been tested in the rat, mouse and rabbit, with the following results:

Acute oral studies	Results (LD_{50})
Han Wistar rat (male and female) OECD 401 (1987)	Male: 1115 mg/kg bw Female: 1342 mg/kg bw Combined: 1290 mg/kg bw
Sprague Dawley rat (female) OECD 425 (2001)[1]	Female: 350 mg/kg bw
NMRI mouse (male and female) OECD 401 (1987)	Male: 200 mg/kg bw Female: 218 mg/kg bw
CD-1 mouse (male) OECD 401 (1987)	Male: 270 mg/kg bw
NZW rabbit (female) OECD 401 (1987)	Female: 460 mg/kg bw

[1] Study conducted in 2001, after the previous decision on classification and labelling by TC C&L

The DS proposed to classify cyproconazole in category 4 for acute oral toxicity according to CLP, based on the most recently published (new) LD_{50} value in rats of 350 mg/kg which is within the CLP limits in the CLP Regulation defining that category (CLP cut-off value: 300<ATE or $LD_{50} \leqslant$ 2000 mg/kg). The DS emphasised that although the mouse was the most sensitive species, the rat is generally the preferred species and category 4 was hence considered more reflective of the data, based on weight of evidence considerations.

The acute 4-hour inhalation LC_{50} in rats was >5.65 mg/L in a study from 1985 and >2.03 mg/L in a more recent study (conducted in 2005, i.e. after the previous decision on classification and

labelling); no mortalities occurred at the highest doses tested. No classification is therefore required for acute inhalation toxicity.

Based on a dermal LD_{50} > 2000 mg/kg bw in two studies in rats and one study in rabbits, cyproconazole does not meet the classification criteria for acute dermal toxicity.

Comments received during public consultation

Four Member State Competent Authorities (MSCAs) commented and suggested to classify cyproconazole in category 3 instead of category 4 for the oral route, based on the results in mice, which appear to be the most sensitive species. The DS acknowledged that the classification could be based on these results.

Assessment and comparison with the classification criteria

For acute oral toxicity, the available LD_{50} values in mice were in the range of 200 – 270 mg/kg in two studies which would lead to category 3, as also indicated during the public consultation. According to the CLP Regulation, although the rat is the preferred species, when experimental data for acute toxicity are available from several animal species, scientific judgement shall be used in selecting the most appropriate LD_{50} value from available valid, well-performed tests. According to CLP guidance, in general, classification is based on the lowest acute toxicity estimate (ATE) available in the most sensitive appropriate species tested (i.e. the mouse in the case of cyproconazole). It further states that expert judgment may allow another value to be used, provided this can be supported by a robust justification. For cyproconazole, there is no evidence that the oral rat study would be more relevant to humans than the mouse study. Therefore, RAC agreed to classify cyproconazole as Acute Tox. 3; H301 (Toxic if swallowed), based on the oral LD_{50} in mice.

RAC supported the proposal **not to classify for acute dermal or inhalation toxicity**, given that the results from standard studies were above the threshold values for classification.

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

Summary of the Dossier submitter's proposal

There is no indication from the data presented that specific target organ toxicity will result from a single exposure. As regards respiratory tract irritation, classification for skin or eye irritation is not justified, and respiratory tract irritation is not anticipated. There was no evidence of respiratory tract irritation in the data from the acute and subacute studies.

Comments received during public consultation

One MSCA supported the proposal for no classification.

Assessment and comparison with the classification criteria

As no evidence of specific target organ toxicity after single exposure was observed in the available acute standard toxicity studies or in the acute neurotoxicity study (including neurotoxicity and respiratory tract irritation), RAC agreed with the DS that **no classification for STOT SE** is warranted.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier submitter's proposal

The skin irritation potential of cyproconazole was tested in two studies on rabbits (New Zealand White; NZW) in accordance with or similar to OECD TG 404. In the first study from 1985 (3 males, 4 h exposure to cyproconazole in DMSO), no signs of either erythema or oedema was reported at any point throughout the course of the study. In the second study from 2005 (2 males and 1 female, 4 h exposure to cyproconazole in distilled water), very slight erythema was noted for all three treated sites one hour after patch removal. The erythema was reversible within 24 h. It was concluded by the DS that no classification for skin irritation or corrosion was required according to CLP.

Comments received during public consultation

One MSCA supported the proposal for no classification.

Assessment and comparison with the classification criteria

No skin oedema or erythema were seen in any of 3 rabbits following exposure to cyproconazole in the first OECD TG 404 study (1985), and only slight and transient erythema was reported in the second OECD TG 404 study (2005). Therefore, cyproconazole is not considered irritating or corrosive to skin. RAC therefore agreed with the conclusion of the DS that cyproconazole **should not be classified for skin corrosion/irritation**.

RAC evaluation of eye corrosion/irritation

Summary of the Dossier submitter's proposal

Cyproconazole was tested in two studies in rabbits (NZW) which were in accordance with or similar to OECD TG 405. In the first study, from 1985 (3 animals, single instillation in the conjunctival sac), a slight redness of the conjunctivae (score 1) was noted 0.5 h after treatment. No other effects were reported thereafter. In the second study, from 2005 (3 animals, single instillation in conjunctival sac), no corneal opacity was seen. Iritis (score 1) and conjunctivitis (score 2) were noted for all treated eyes after one hour. Iritis was reversible within 1 h (mean score over 24-72 h: 0-0-0), conjunctivitis regressed to score 1 within 24 h and to score 0 within 72 h (mean score over 24-72 h: 0.7-0.7-0.3). No classification for eye irritation or corrosion was proposed.

Comments received during public consultation

One MSCA supported the proposal for no classification.

Assessment and comparison with the classification criteria

Only slight erythema of the conjunctiva was reported after 0.5 h in the first study, reversible within 24 h. In the second study, mild to moderate transient irritation to the eye was noted (score 1 for iritis and score 2 for redness of the conjunctiva after 1 h in all animals) which regressed progressively and had reversed by 72 h. The mean scores over the period of 24-72 h for iritis (0-0-0) and conjunctival redness (0.7-0.7-0.3) in all three animals were below the threshold values for classification as Eye Irrit. 2; H319 according to CLP (≥1 for iritis and ≥2 for redness of conjunctiva). RAC therefore supported the conclusion of the DS that cyproconazole is considered not to be irritating to rabbit eyes and **does not meet the classification criteria for serious eye damage/eye irritation**.

RAC evaluation of respiratory sensitisation

Summary of the Dossier submitter's proposal

No information was available.

Comments received during public consultation

One MSCA supported the proposal for no classification based on the lack of relevant data.

Assessment and comparison with the classification criteria

Based on the lack of respiratory sensitisation data, classification of the substance is not warranted.

RAC evaluation of skin sensitisation

Summary of the Dossier submitter's proposal

Based on a Magnusson & Kligman Guinea Pig Maximisation Test (GPMT; OECD TG 406) and a Buehler test (OECD TG 406), the DS concluded that there were no responses consistent with skin sensitisation and therefore proposed not to classify for this hazard class.
In the GPMT, no positive reactions were evident at the application site in any of the animals at 24 and 48 h after challenge (cyproconazole 25%) application. In the Buehler test, following challenge (cyproconazole 75%), very mild erythema (0.5) was noted for 5 of 20 test sites after 24 h. Irritation cleared from all affected sites by 48 h. The induction phase was conducted with cyproconazole 75%.

Comments received during public consultation

One MSCA supported the proposal for no classification.

Assessment and comparison with the classification criteria

According to the CLP Regulation, a substance should be classified as a skin sensitiser in category 1B; H317, when in a GPMT ≥30% of the animals respond at >1% intradermal induction dose or when in a Buehler test, ≥15% of the animals respond at >20% topical dose. An animal is considered as positive if a score ≥1 for erythema is obtained after the challenge phase. No animal responded (score <1) after the challenge phase for cyproconazole and therefore RAC agreed with the DS that **no classification for skin sensitisation** was warranted.

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

Summary of the Dossier submitter's proposal

The DS proposed to classify cyproconazole as STOT RE 2; H373 based on liver effects observed in the short-term studies in rat, mouse and dog.

Species/study	NOAEL/LOAEL	Main findings	CLP GV (extrapolated)	Reference
Rat 28-day feeding study	LOAEL: 300 ppm (25.32/31.54 mg/kg bw/d)	-reduced bw gain -significant hepatotoxicity (liver weight, some histopathology,	≤300	Skinner *et al.,* 1984

Species/study	NOAEL/LOAEL	Main findings	CLP GV (extrapolated)	Reference
OECD TG 407 (1995)	NOAEL: 100 ppm = 8.1 mg/kg	LDH levels) -other findings at higher doses: haematological changes		
Rat 1st 90-day feeding study OECD TG 408 (1998)	LOAEL: 320 ppm (23.8/31.1 mg/kg bw/day) NOAEL: 80 ppm = 6.4 mg/kg	-slight reduced bw gain -hepatotoxicity (liver weight, histopathology) reversible after recovery period -other effects: haematological changes, disturbed calcium and creatinine levels	≤100	Skinner *et al*., 1985
Rat 2nd 90-day feeding study OECD TG 408 (1998)	LOAEL: 350 ppm (24.7/29.6 mg/kg bw/d) NOAEL: 20 ppm = 1.4 mg/kg Note: large spacing of dose	-reduced bw gain -hepatotoxicity (fatty changes, hypertrophy) -disturbed lipid metabolism -other findings on thyroid, pituitary and adrenal glands, and, at higher doses: haematological changes	≤100	Gerspach, 1999.
Mouse 13-week dose-range finding feeding study Similar to OECD TG 408 (1998) No haematology or clinical chemistry investigated	LOAEL: 300 ppm (43.8/70.2 mg/kg bw/d) NOAEL: 15 ppm = 2.2 mg/kg Note: large spacing of doses	-reduced bw gain -significant hepatotoxicity (liver enlargement, single cell necrosis, hepatocyte eosinophilia)	≤100	Warren *et al*; 1987
Dog: 13-week feeding study in Beagle dogs Similar to OECD TG 452 (1981)	LOAEL: 500 ppm (18.18/19.17 mg/kg bw/d) NOAEL: 100 ppm= 3.3 mg/kg	-significant hepatotoxicity (liver weight, hepatomegaly, hepatocyte degeneration) -disturbed lipid metabolism	≤100	Warren *et al*., 1986
Dog: 1-year dietary study OECD TG 452 (1981)	LOAEL: 350 ppm (12.05/12.58 mg/kg bw/d) NOAEL: 100 ppm = 3.2 mg/kg	-hepatotoxicity -disturbed lipid metabolism	≤12.5	Warren *et al.,* 1988 (Amended report 1992)
Rat: Subacute (16-day) inhalation OECD TG 412	LOAEL: 0.099 mg/L NOAEL: 0.017 mg/L ai = 4.9 mg/kg	-hepatocellular hypertrophy (and fatty changes)	≤0.6	Bernstein *et al.,* 1987
Rat: 28-day dermal OECD TG 410	LOAEL: 100 mg/kg NOAEL: 10 mg/kg bw/day	-clinical chemistry -histopathology (incl. centrilobular hepatocellular hypertrophy) -haematological changes	≤600	Sommer, 2000

LDH=Lactate dehydrogenase
CLP GV=CLP guidance value

According to the DS the overall data from the sub-chronic testing of cyproconazole in rats, mice and dogs clearly identify the liver as the main target organ, with evidence of adaptive changes, but also with evidence of hepatocyte toxicity. Increases in relative liver weight were seen with histopathological changes such as hepatocellular hypertrophy, vacuolation, fatty change, and single cell necrosis. These effects were generally accompanied by evidence of functional impairment such as alterations in clinical chemistry and marker enzymes.

Other main effects reported consistently in the studies were in the blood system. Anaemia was reported in dogs but at the highest dose only and was of minimal severity. Disturbance of the haematopoietic system was observed in several studies in rats but only at or above the Maximum Tolerable Dose (MTD) and without anaemia. Hence, the DS did not propose to classify for haematological effects.

As the doses at which significant adverse effects were seen in the liver were generally low, with evidence of significant hepatotoxicity seen at doses below the cut-off levels in the rat, the DS considered that the effects in the three species are consistent with the criteria for STOT RE 2; H373 (May cause damage to organs (liver) through prolonged or repeated exposure via the oral route). The findings in the inhalation and dermal route studies in the rat also were considered as supportive.

Comments received during public consultation

Five comments were received for this hazard class. Four MSCA supported the proposal for classification as STOT RE 2, based on the severe liver findings. Two MSCAs also emphasized that the classification should not be limited to the oral route only since hepatotoxicity was also reported after exposure via the dermal and inhalation routes. The DS agreed to this in their response to comments (RCOM).

One comment from Industry did not support the proposal. Although it was acknowledged that the target organ in all species is the liver, they commented that the effects reflect adaptive responses due to xenobiotic metabolism and are not of toxicological concern; therefore, the findings in the liver do not justify STOT RE classification. Further information (including tables) was provided to justify this position. The DS responded in the RCOM, that although it is not clear whether the effects observed (such as fatty change/hepatocellular vacuolation/single cell necrosis/single hepatocyte degeneration, accompanied in most cases by altered clinical chemistry and/or functional changes) were adaptive or toxic in nature, overall the data justify classification.

Assessment and comparison with the classification criteria

Repeated dose toxicity of cyproconazole was investigated in several species (rats, dogs and mice). The liver was identified as the target organ with a consistent pattern of increased liver weight associated with macroscopic and microscopic changes and modified clinical chemistry.

According to the CLP criteria, substances should be classified for repeated dose toxicity if significant adverse effects, which indicate functional impairment, occur at dose levels ≤100 mg/kg bw/d in a 90-day oral rodent study. Such effects may include significant consistent and adverse changes in clinical biochemistry, haematology, or urinalysis parameters; significant organ damage noted at necropsy and/or subsequently seen or confirmed at microscopic examination; or morphological changes that are potentially reversible but provide clear evidence of marked organ dysfunction. In contrast, adaptive responses that are not considered toxicologically relevant do not warrant classification.

In the *28-day oral study in rats* (Skinner *et al.*, 1984), rats received cyproconazole in the diet at 0, 0.8, 2.3, 8.1, 25 and 96 mg/kg bw/d for males and 0, 0.9, 2.9, 9.8, 32 and 128 mg/kg bw/d for females. Exposure to 25/32 and 96/128 mg/kg bw/d resulted in decreases in body weight gain in both sexes, being marked only at the high dose (during the first week: 89% in males and 155% in females at the high dose, and 37% in males and 1% in females at the lower dose level of 25/32 mg/kg bw/d), and did not correlate with reductions in food consumption, or with increases in liver weights.

The change in liver weight at 25 mg/kg bw/d for males and 32 mg/kg bw/d for females was associated with hepatocellular vacuoles. In the highest dose group (i.e. 96 mg/kg bw/d for males and 128 mg/kg bw/d for females), it was associated with increased architectural visibility macroscopically, and with hepatocellular vacuoles in microscopic investigations in the majority of males, as well as hepatocytomegaly (predominantly centrilobular) in 10/16 males and 2/16 females. Clinical chemistry revealed dose-related increases in LDH levels, statistically significant at doses of 25/32 and 96/128 mg/kg bw, and at the highest dose, increases were seen in ALAT, ASAT (males), blood urea nitrogen, total protein (females) as well as increased cholesterol and decreased bilirubin (the latter statistically significant in females).

Based on the decreased body weight gain and liver effects (increased liver weight and histopathological findings, which correlated with the increased ALAT/ASAT and probably LDH), the LOAEL was considered to be 25 mg/kg bw/d (the extrapolated guidance value for STOT RE 2 is ≤ 300 mg/kg bw/d for a 28-day study).

In the *first 90-day oral study in rats* (Skinner, 1985), animals received cyproconazole in the diet for 90 days, at 0, 1.5, 6.4 and 24 mg/kg bw/d for males and 0, 1.9, 7.0 and 31 mg/kg bw/d for females. Oral exposure at the top dose of 24 mg/kg bw resulted in a slightly lower body weight gain in males. Relative liver weights were increased in both sexes (together with absolute liver weights in females), and correlated with the microscopic finding of vacuolated hepatocytes in 6/15 males (single large or several small vacuoles, predominantly centrilobular). At macroscopic examination, a distinct lobular pattern (5 males, 4 females) was observed but this was reversible within the recovery period. Hence, the effects were observed at doses below the guidance value of ≤ 100mg/kg bw/d, but were reversible. Clinical chemistry parameters were inconsistent and fluctuated without a clear dose-response relationship, and therefore did not provide information on liver impairment.

In the *second 90-day oral study in rats* (Gerspach, 1999), animals received cyproconazole in the diet for 90 days, at 0, 1.4, 25, 53 and 107 mg/kg bw/d for males and 0, 1.6, 30, 57 and 118 mg/kg bw/d for females. A decrease in body weight gain during the first four weeks of treatment was observed at all dose levels but was slight (2.5% decrease at 25/30 mg/kg bw/d and up to 28% in males at the highest dose of 107 mg/Kg bw/d); it was associated with a slight decrease in food consumption (statistically significant only at the two highest doses, maximum of 16% at the highest dose).

Exposure to 25/30 mg/kg bw/d cyproconazole and above resulted in increased liver weights in both sexes. The increases in liver weight correlated with histopathological findings, namely hepatocellular hypertrophy in both sexes and increased incidence and severity grading of fatty changes in males, in most of the animals (6/10 at 25mg/kg bw/d and 9/10 at 53 and 107 mg/kg bw/d, mean severity grading from 1.8 to 2.6). Liver toxicity was also indicated by clinical chemistry parameters, such as the increased ALAT/ASAT. Clinical chemistry following exposure to 25/30 mg/kg bw/d cyproconazole and above, revealed increased urea, protein, globulin, Na/K/Ca as well as ALP, GGT, cholesterol, and at the two highest doses, decreased triglycerides and bilirubin. Some of these findings could be related to liver effects. However, at these dose levels the following were reported, which may also have contributed to the findings: adverse effects on the adrenal gland (increased adrenal gland weight, fatty changes, deposition of ceroid and single cell necrosis) and pituitary gland (increased pituitary gland weight, hypertrophy of the distal lobe).

The LOAEL for the range of effects observed was set at 25/30 mg/kg bw/d but it is also emphasized that the starting dose (1.4/1.6 mg/kg bw/d) was too low when compared to the next dose (25/30 mg/kg bw/d) which showed the large range of adverse effects, including the liver effects.

In the *90-day oral study in mice* (Warren *et al.*, 1980c), mice received cyproconazole in the diet for 90 days, at 0, 0.7, 2.2, 44 and 89 mg/kg bw/d for males and 0, 1, 3.2, 70 and 128 mg/kg bw/d for females. Haematology and clinical chemistry were not investigated in that study which is therefore considered as supportive information only. Oral exposure at all dose levels resulted in a lower body weight gain, significant at the two highest doses, and more pronounced in males (43% and 68% in males at 44 and 89 mg/kg bw/d, respectively, and 22% and 35% in females at 70.2 and 128 mg/kg, respectively) which was partly associated with a decreased food consumption (decreased in week 1). It resulted in a terminal bodyweight lower than controls (-9.5% in males only at the dose of 44 mg/kg, both sexes at the high doses: -11% in males, -7.4% in females), however the magnitude of the decrease (ca.10%) was not considered to reflect excessive general toxicity. Absolute and relative liver weights were increased in both sexes from 2.2/3.2 mg/kg bw/d, which correlated from 44/70 mg/kg bw/d with the microscopic finding of vacuolation, which was more marked in females (0/10 and 4/10 males at 44 and 89 mg/kg bw/d, respectively, and 1/10 and 10/10 females at 70 and 128 mg/kg bw/d), eosinophilia of hepatocytes whiach was more frequent in males (7/10 and 8/10 males at 44 and 89 mg/kg bw/d, respectively, and 3/10

and 1/10 females at 70 and 128 mg/kg bw/d) and single cell necrosis (2/10 and 5/10 males at 44 and 89 mg/kg bw/d, respectively, and 3/10 and 7/10 females at 70 and 128 mg/kg bw/d). In the highest dose group, liver showed an accentuated lobular pattern at macroscopic examination in both sexes (5/10 males vs 2 in controls, 9/10 females vs 1 in controls). It is noted that there is a large span between the NOAEL (2.2 and 3.2 mg/kg bw/d for males and females, respectively) and the LOAEL (44 and 70 mg/kg bw/d for males and females, respectively).

In the *90-day oral study in dogs* (Warren *et al.*, 1986), groups of 4 animals/sex/dose received cyproconazole in the diet for 90 days, at doses equal to 0, 0.8, 4 and 18.2 mg/kg bw/d for males and 0, 0.7, 3.3 and 19.2 mg/kg bw/d for females. A reduction in body weight gain associated with a decrease in food consumption was found in both sexes of the highest dose group (33% males, 58% in females). It resulted in a terminal bodyweight lower than controls (-11% in males, -9% in females, but was already 6% lower at the beginning of treatment in males) but the magnitude of decrease (ca. 10%) is not considered as excessive toxicity. At this dose level, absolute and relative liver weights were increased in both sexes, which corresponded with mild/moderate hepatocytomegaly in all animals. Single cell degeneration (1 male, 1 female) was also observed. At the same high dose of 18.2 mg/kg bw/d in males and 19.2 mg/kg bw/d in females, liver toxicity and impairment were also reflected by clinical chemistry findings: marked increases in GLDH (+1000% in males, +250% in females), as well as decreased protein and albumin; decreased bilirubin and cholesterol levels noted at week 4 and 8 (had begun to recover by week 13), increase in GGT and alkaline phosphatase (ALP) throughout the treatment period with, for ALP, values tending to increase with time and dose. In the last week of treatment, additional blood sampling revealed higher plasma cortisol levels. However, this was observed in all treated groups, was without any dose-response relationship and no histological change in the adrenal gland was reported, therefore it is not considered that the findings would be related to a disturbance of the adrenocortical system. The LOAEL was set at 18.2/19.2 mg/kg bw/d (guidance value of ≤ 100 mg/kg bw/d for a 90-day study).

In the *1-year study with Beagle dogs* (Warren at al., amended report, 1992), groups of 4 animals/sex/dose received cyproconazole in the diet at doses equal to 0, 1.0, 3.2 and 12.1 mg/kg bw/d for males and 0, 1.0, 3.2 and 12.6 mg/kg bw/d for females.

During the first 9 weeks of the study there was a slight reduction in the bodyweight gain of both males and females at the high dose (-25% in males, -20% in females); the body weight of males returned to control levels at termination whereas female bodyweights remained depressed (-22%). This did not correlate with the changes in food consumption (increase in males, no change in females when compared to controls).

Absolute and relative liver weights in males showed a dose-related increase, statistically significant only at the high dose of 12.1 mg/kg bw/d. A minor, non-significant effect was seen in the liver weight of females. This slight liver weight change was associated with laminar eosinophilic intrahepatocytic bodies (all males and 2/4 females at 12.1/12.6 mg/kg bw/d, also 1 male at 3.15 mg/kg bw/d). Although it could be argued that this finding only reflected increased activity of the endoplasmic reticulum due to adaptive metabolism at the same dose levels of 12.1/12.6 mg/kg bw/d, an increase in intrahepatocytic pigment (3/4 males, also reported in 1 male at 3.2 mg/kg bw/d, in the same animal presenting the intrahepatocytic bodies) was observed, indicating toxicity instead. Total protein and albumin were also reduced. Moreover, canalicular bile plugs were found in 2/4 males. These findings were associated with clinical chemistry consistent with disturbed lipid metabolism: markedly increased ALP, decreased cholesterol and decreased triglycerides (females), although the bilirubin levels were found to be reduced (trend towards a decrease). Therefore, the observed effects in this 1-year-long study seem to correlate with the progression from adaptive to toxic liver response. No changes were reported in the adrenal gland. The LOAEL is 12.1/12.6 mg/kg bw/d for liver effects (extrapolated guidance value of $\leqslant$ 24 mg/kg bw/d for a 1-year study).

Liver toxicity was also reported in the *2-year combined chronic toxicity/carcinogenicity study in rats* (Warren *et al.*, 1988) and in *the 18-month oncogenicity study in mice* (Warren *et al.*, 1989). In rats, liver effects occurred at the high dose of 15.6 mg/kg bw/d in males and 22 mg/kg bw/d in females with increased relative liver weight associated with histological changes, which

included hepatocellular hypertrophy in females at the interim kill on week 78 only and fatty changes in males from week 52 (increasing in incidence and severity) and in clinical chemistry findings: decreased bilirubin, increased plasma γ-GT (both sexes), increased ALAT/ASAT (males) and cholesterol levels (females). No tumours occurred in that study. In mice, liver effects occurred from 13.2/17.7 mg/kg bw/d with increased relative liver weight associated with accentuated lobular pattern and histological changes: focal hepatocytic inflammation (males), single cell necrosis (both sexes) and diffuse hypertrophy, centriacinar and periacinar vacuolation (females). Hypertrophy, vacuolation (in both sexes) and focal inflammation (males) were also reported in the satellite group exposed for 90 days. However, liver adenomas and carcinomas in male mice also occurred in that study, at similar dose levels. In both carcinogenicity studies, the findings were observed at above the guidance value of 12.5 mg/kg bw/d (extrapolation from the guidance value of 100 mg/kg bw/d for 90-day studies to a 2-year study), and in mice effects co-occurred with tumours. Therefore these results alone would not justify classification as STOT RE 2, but they support the liver being the most sensitive target organ after exposure to cyproconazole.

Similar liver effects were reported in investigative studies. In a mechanistic study to investigate liver cell proliferation (Warren, 1995), rats and mice received cyproconazole during 28 days and showed similar results with increases in liver weight associated with hepatocyte enlargement and vacuolation and slight increases in liver enzymes (from 25 mg/kg bw/d in rats and 14 mg/kg bw/d in mice). In a study investigating biochemistry of the liver (Trendelenberg, 2001), mice exposed during 14 days *via* the diet showed a marked increase in liver weight (ca. 50%) associated with a dose-dependent increase in hypertrophy, vacuolation and necrosis, which included single necrotic hepatocytes as well as small groups of necrotic hepatocytes, frequently accompanied by inflammatory cells (granulocytes) (9.0/ 12.7 mg/kg bw/d). In another mechanistic study to compare effects of cyproconazole in three strains of mice (Milburn, 2006), wild type mice were exposed during 7 or 14 days at 38 mg/kg bw/d: increased liver weight was associated with hypertrophy, fat vacuolation, single cell necrosis and changes in clinical chemistry markers of disturbed lipid metabolism (ALP, GGT, cholesterol). These studies were not guideline studies and since they used a low number of animals per study, they could not be considered sufficient for classification. However, they provide further support for liver damage occurring after repeated exposure at low doses of cyproconazole.

In the *16-day inhalation toxicity study in rats* (Bernstein, 1987), groups of 5 rats/sex/dose were exposed nose-only for 2 weeks at doses of 0.01, 0.1, 1.00 mg/L. The highest aerosol concentration of 1 mg/L caused substantial mortality in male and female rats. The respiratory tract, the spleen and the liver (increased mild/moderate hepatocellular hypertrophy - 4/5 males and 2/5 females - and hepatocytes with fatty change - 5/5 males and 4/5 females vs 1 in controls) were affected at this dose level. At the lower concentration of 0.1 mg/L, the liver was the main target organ, with increases in liver weight accompanied by slight changes in liver enzymes (increased ASAT/ALAT/LDH in females, not statistically significant), decreased bilirubin in males and histological changes in the form of hypertrophy (2/3 males and 1/3 females); all these effects were reversible. The NOAEL was set at 0.01 mg/L and the LOAEL at 0.1 mg/L for the range of effects observed, including liver effects. Hence, although the effects were observed at doses below the guidance value of $\leqslant$ 0.6 mg/L, the low severity of the effects in the liver (no clear sign of liver impairment was indicated by clinical chemistry, and the changes were reversible) would not warrant classification. However, the effects identify the liver as a target organ.

In the *28-day dermal toxicity study in rats* (Sommer, 2000), groups of 6 CD rats/sex/dose received the test substance in distilled water at dose levels 0, 10, 100 and 1000 mg/kg bw/d, 6 h/day and 5 days/week for 3 weeks and then daily for the last week of treatment. Dermal exposure of rats to cyproconazole at 1000 mg/kg bw/d resulted in an increase in liver weight (ca. 25%) and hypertrophy was observed in both sexes (10/10 males and 5/10 females). These findings were associated with altered clinical chemistry: increased ALAT/LDH ratio (1.6 fold; males), slightly increased protein and decreased globulin (and ratio of both), increased cholesterol in females and decreased bilirubin in males. At 100 mg/kg bw/d, centrilobular hypertrophy in males was also reported (7/10). Body weight gain was not affected. These data do not justify classification as STOT RE 2, since liver effects were seen at exposure level higher than

the extrapolated guidance value of ca 600 mg/kg bw/d. However, these data identify the liver as a target organ.

In addition to the liver effects, some alterations of the haematological parameters were reported in all studies (rats and dogs; mice not investigated in the 90-day study) but they were minimal to slight and/or transient and/or not consistent among studies. In the 28-day study in the rat (Skinner, 1985) at the high dose of 96/128 mg/kg bw/d, a significant increase of white blood cell counts (WBC) was reported at the high dose at week 4 (+35% in males, +26% in females), reversible upon recovery. In the first 90-day study in the rat (Skinner, 1985), at the two highest doses in males, reduced haematocrit (Ht, max 8%) and increased mean corpuscular haemoglobin concentration (MCHC, max 6%) were noted at week 4 and 8, but none were affected at week 13 and haemoglobin (Hb) and red blood cell count (RBC) were not affected. In the second 90-day study in rats (Gerspach, 1999), a number of dose-related changes in blood parameters were noted in both males and females, which were significant at the two highest doses (53/57 and 107/118 mg/kg bw/d). There was a decrease in haemoglobin (Hb), which was dose-related but reaching a maximum of 5% decrease at the high dose, a 9% decrease in both the mean corpuscular volume (MCV, 9%) and mean corpuscular haemoglobin (MCH), and increases in white blood cell counts (76%), Red Cell Distribution Width (RDW, 24%), haemoglobin concentration distribution width (HDW, 54%), as well as increased prothrombin time, significant from 52.8 mg/kg bw in males and from 29.6 mg/kg bw/d in females (up to 84% at the top dose). In the 90-day study in dogs (Warren, 1986), haematological analysis at the high dose of 107 mg/kg bw/d revealed very slight anaemia in males (3% reduction in haemoglobin) in the first week of treatment together with decreased haematocrit and red blood cell count, and this reduction progressed but reached a maximum of a 8% reduction by week 13 in males at the high dose of 107 mg/kg bw/d (lesser extent in females, not statistically significant). A significant increase in platelets was also reported in the two dog studies. In the 90-day study via the dermal route in rats, at the top dose of 1000 mg/kg, haematological changes observed were increased prothrombin time (ca. 30% in both sexes), increased monocytes (50% in males, 30% in females) and elevated HDW (35%). In some of these studies in rats, also slightly increased grading of splenic haemosiderosis was observed (from 25/30 mg/kg bw/d), as well as reduction in incidence/grading of extramedullary haematopoiesis in one study, possibly related to these small changes observed in some haematological parameters.

Therefore, some disturbance of the haemapoietic system and slight anaemia (3-8% reduction in Hb) was reported in several studies. However, the changes were considered as minor effects. In addition, some of the findings, such as the increase in prothrombin time, could be related to liver dysfunction.

According to CLP criteria (Annex I, Section 3.9.2.8.1), it is recognised that effects may be seen in humans and/or animals that do not justify classification. Such effects include small changes in haematological parameters and/or transient effects, when such changes or effects are of doubtful or minimal toxicological importance. RAC agreed with the DS that no significant, adverse effects meeting the classification criteria were seen and concluded that the small changes in haematology do not provide sufficient evidence for classification of cyproconazole as STOT RE 2 for effects on blood.

Some effects on reproductive organs were sporadically observed and are discussed in the section on fertility.

In summary, RAC notes that the findings reflect the adaptive capacity of the liver being overwhelmed by cyproconazole, leading to liver damage, at doses well below the guidance value for classification and is therefore of the opinion that these liver effects of cyproconazole warrant classification. RAC takes into account in particular the consistency of the effects, across the species and the studies, including the effects observed in the inhalation and dermal studies, which also support the evidence for the liver as a target organ after repeated exposure to cyproconazole. In conclusion, RAC agrees with the proposal of DS **to classify cyproconazole as STOT RE 2; H373** (May cause damage to organs (liver) through prolonged or repeated exposure).

RAC evaluation of germ cell mutagenicity

Summary of the Dossier submitter's proposal

The DS did not propose to classify for mutagenicity as cyproconazole was negative in all systems tested *in vitro* and *in vivo*.

In tests conducted *in vitro*, cyproconazole did not induce gene mutations in bacterial or in mammalian cells (Chinese hamster fibroblasts V79), did not show evidence for an aneugenic activity in yeast, did not induce unscheduled DNA synthesis in rat hepatocytes and did not cause transformation of Syrian hamster embryo (SHE) cells. As regards clastogenicity, the first *in vitro* chromosome aberration test in Chinese hamster ovary (CHO) cells revealed a weak positive result. However, this finding was not confirmed when re-evaluated by a second, independent investigator and two additional *in vitro* chromosome aberration tests revealed clear negative results. The absence of genotoxic potential of cyproconazole was further supported by three *in vivo* studies in mice. Both the bone marrow micronucleus test and a chromosome aberration assay were negative. In addition, cyproconazole was negative in a dominant lethal assay in male germinal cells. It was concluded by the DS that no classification for mutagenicity was required according to CLP.

Comments received during public consultation

Two MSCAs supported the proposal for no classification.

Assessment and comparison with the classification criteria

Cyproconazole was negative for mutagenic properties in several *in vitro* assays and *in vivo* assays. RAC supported the conclusion of the dossier submitter that classification of cyproconazole for **germ cell mutagenicity is not warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier submitter's proposal

Cyproconazole caused a significant increase in the incidence of hepatocytic adenomas and carcinomas, from 13.2 mg/kg bw/d in male mice and at doses of 36.3 mg/kg/d in females. These tumours occurred at doses that were also toxic to the liver. Cyproconazole was not considered to be carcinogenic in rats since there were no treatment-related neoplasms observed in the two year combined chronic toxicity and carcinogenicity study in the rat at doses up to 15.6 mg/kg/day in males and 21.8 mg/kg bw/d in females.

In addition, cyproconazole was not genotoxic and, based on supplementary investigative studies conducted to explore the mode of action (MoA) using the CAR null C3H mouse, it was concluded that CAR activation occurred with cyproconazole and it is generally agreed that the CAR-mediated MoA is not associated with an increased incidence of liver tumours in humans. However, it was further concluded from the investigative studies that an alternative cytotoxic MoA occurred with a well-defined sequence of events, starting with perturbation of hepatic homeostasis (cytotoxicity) and resulting in degenerative lesions with subsequent liver cell proliferation leading to pre-neoplastic lesions and finally hepatocellular tumours. Still, although several of these events were also observed in rats treated with cyproconazole, the incidence of liver tumours did not increase. It was therefore concluded that no MoA for these tumours could be established with certainty (a CAR-mediated MoA, of questionable relevance to humans, being involved only in part).

Therefore, the DS considered that the data provided limited evidence of carcinogenicity (effects in a single species with no clear MoA demonstrated) and proposed to classify in category 2 for carcinogenicity.

Comments received during public consultation

Four comments were received for this hazard class. Three MSCAs supported the proposal for category 2:

- One MSCA supported the conclusion based on an increased incidence of liver adenoma and carcinoma in mice and the uncertainty as to the CAR-mediated MoA (an alternative MoA such as cytotoxicity could not be excluded).
- One MSCA agreed with Category 2 based on (i) the significant increase in the incidence of hepatic adenomas and carcinomas in female and male mice, while no tumours were observed in rats; (ii) the limited mechanistic information with regard to the human relevance (cytotoxicity and/or CAR events); and (iii) lack of genotoxicity.
- One MSCA provided a detailed analysis of the data, arriving at a similar conclusion to that of the DS for category 2: (i) clear increase incidence in both benign and malignant tumours in both sexes in mice and questions on the adequacy of the rat study because of the low dose levels used; and (ii) results on the MoA were insufficient to exclude relevance to humans. Although the available mechanistic studies in mice indicated a crucial role for the CAR receptor (the CAR-null genotype negates some of the effects of cyproconazole, implying that initial activation of the CAR receptor is required), the relative contribution of CAR to the total cyproconazole effect is not known (some other responses imply that not all the effects of cyproconazole are completely mediated by CAR activation).

One comment from Industry disagreed with the proposal for classification due to supporting data demonstrating a human non-relevant MoA via CAR-activation. This comment underlined that since the adoption of the current classification, no new data demonstrating an increased risk of tumours from administration of cyproconazole have been generated and therefore argued that the previous decision of 'no classification' was still justified, all the more since the investigative studies subsequently generated have strengthened the MoA case for cyproconazole and hence the non-relevance to humans. The company emphasized that they specifically disagreed with the proposal that the tumour MoA could involve cytotoxicity (relevant to humans) and provided additional argumentation quoting the publication of Tamura *et al.* (2015). In that study, no evidence of increased altered foci or adenoma formation was observed in the knock-out animals treated with cyproconazole, confirming, in the commenter's view, the crucial role of CAR in liver tumour development following cyproconazole exposure.

The DS clarified in the response to comment document that they agreed that the involvement of CAR was an important event but questioned whether it was the primary or sole cause. The DS further responded that this literature study did not follow a standard protocol, the animals having been pre-exposed to a genotoxic compound. In addition, the data from the quoted publication did not change their position: subtle cytotoxicity working in concert with a CAR-mediated MoA to promote liver tumours was still considered as plausible since, even in the Tamura *et al.* (2015) study, not all the effects of cyproconazole were negated in CAR knock-out mice. The DS emphasized that the previous decision on classification was considered in the overall weight of evidence.

Assessment and comparison with the classification criteria

Carcinogenic data

In the 18-month oncogenicity study in CD-1 mice (Warren *et al.*, 1989), increased incidences in liver adenomas and carcinomas were observed from 100 ppm (13.2/17.7 mg/kg bw/d) in males and at the high dose of 200 ppm (36.3 mg/kg bw/d) in females. The liver tumours were seen together with non-neoplastic liver lesions, as discussed in the STOT RE section, with increased relative liver weight associated with accentuated lobular pattern and histological changes: focal hepatocytic inflammation (males), single cell necrosis (both sexes) and (in females) diffuse hypertrophy and centriacinar and periacinar vacuolation.

Liver tumour incidences are provided in the table below (no other treatment-related tumours were reported and there were no tumours at the interim kill on week 13):

18-month oncongenicity study in mice		Dose (ppm)					
50 tissues examined		0	0	5	15	100	200
MALES	Adenomas	3	3	4	5	**12***	**12***
	Carcinomas	0	0	0	3	3	1
	Combined age-related	3	3	4	8	**15***	**13***
FEMALES	Adenomas	0	0	0	0	2	**6***
	Carcinomas	0	0	0	0	0	**7***
	Combined age-related	0	0	0	0	2	**13***

* statistically significant

At doses where the liver tumours occurred (from 100 ppm in males), body weight gain was affected (>10%) by cyproconazole in both sexes. This was not related with food consumption, and the effects were more pronounced in males with a clear retardation in bodyweight gain from week 13 in males (see table below).

Doses	Bodyweight gain reduction as compared to controls (%)				Tumours
	Week 13	Week 26	Week 52	End of study	
Females					
100 ppm	0	14	20	30	No
200 ppm	20	14	20	30	Yes
Males					
100 ppm	37	33	29	37	Yes
200 ppm	25	33	21	37	Yes

The Guidance on the application of the CLP criteria (CLP guidance) states that in lifetime bioassays "...*the highest dose needs to induce minimal toxicity, such as characterised by an approximately 10% reduction in body weight gain (maximal tolerated dose, MTD dose). The MTD is the highest dose of the test agent during the bioassay that can be predicted not to alter the animal's normal longevity from effects other than carcinogenicity."* In the case of cyproconazole, the bodyweight gain reduction in females was similar at both 100 ppm and 200 ppm and tumours only occurred at 200 ppm. Body weight gain reduction in males did not increase with time and the mean terminal body weight was no more than 10% below controls. In addition, two control groups were used in that study and the second control group showed a decreased body weight gain during the end of the treatment period, therefore when compared to that second control, decrease body weight gain would be 28% (males) and 18% (females) instead of 37% and 30%, respectively. Furthermore, treatment did not increase mortality rate. Therefore, RAC did not consider that these reductions in bodyweight gain were indicators of excessive toxicity and a confounding factor for tumours.

Cyproconazole was not carcinogenic in rats in an OECD/GLP-compliant study. Therefore, it is considered that the animal studies provided limited evidence of carcinogenicity (the definition for which in the CLP Regulation includes that "*the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment")*.

Mechanistic studies investigating the mode of action for the liver tumours in mice
Supplementary studies were carried out to investigate the MoA for the induction of liver tumours in mice and their relevance to humans. The MoA investigated was the activation of the CAR nuclear receptor (mitogenic MoA). This MoA is similar to the MoA established for phenobarbital.

Two ***in vitro* studies** from Elcomb (2011) investigated the ability of cyproconazole (0.2, 1, 5, 25, 125 and 500 µM) to induce **Cyp2b transcript levels** and Cyp3a transcript levels and **cell**

proliferation in mouse and human hepatocytes. Phenobarbital was one of the positive controls (10, 100 and 1000 µM).
In both human and mouse hepatocytes, cyproconazole induced Cyp2b:

- In mouse hepatocytes: increase of 2.7 fold for cyproconazole (dose-related induction for phenobarbital: 2.0 to 2.8-fold)
- In human hepatocytes: marked dose dependent increases up to 4.0-fold for cyproconazole (up to 5.2-fold for phenobarbital).

Cyproconazole also induced Cyp3a, similar to phenobarbital:

- In mouse hepatocytes (Cyp3a11): cyproconazole induced a 'stronger' increase at low doses up to 25 µM (1 - 1.8-fold versus control) rather than at higher doses (a small increase was observed with phenobarbital at the high dose of 1000 ppm only (1.3-fold versus control).
- In human hepatocytes (CYP3A4): cyproconazole induced (up to 5.6-fold) at up to 25 µM and then a decrease at higher concentrations, correlating with cytotoxicity (up to 10.1-fold induction for phenobarbital)

Cell proliferation was induced with both cyproconazole and phenobarbital in mouse hepatocytes but not in human hepatocytes:

- In mouse hepatocytes: cyproconazole induced cell proliferation by up to 3.9-fold versus control, and phenobarbital by 2-fold versus control.
- In human hepatocytes: no statistically significant changes in replicative DNA synthesis were observed following treatment with either cyproconazole or phenobarbital at any concentration tested.

It was however noted that at the dose of 125 µM, while the highest rate of cell proliferation was observed, it corresponded to the lowest expression in Cyp2b (and Cyp3a, no more details available).
In addition, in these two studies, the high dose of 500 µM cyproconazole was considered to be cytotoxic to mouse and human hepatocytes (intracellular ATP levels reduced to 2% of control in mouse hepatocytes and 1% of controls in human hepatocytes) while no cytotoxicity was reported with phenobarbital. In human hepatocytes, 125 µM cyproconazole was also considered to be cytotoxic to human hepatocytes (intracellular ATP levels reduced to 67% of control).

In the study used to determine the extent of **liver enzyme induction in rats and mice** after treatment with cyproconazole (Dorobek *et al.*, 1995), it was clearly shown that cyproconazole did induce enzyme activities in both species, mainly phase I enzymes (total CYP450, EROD, PROD), and to a larger extent in rats than in mice, specifically for PROD (which reflects CYP2B activity). Liver metabolic induction is presented in the table below (% of controls).

	Rats		Mice	
Parameter	**4 days**	**21 days**	**4 days**	**21 days**
Total Cyt P-450	453**	444**	296**	283**
EROD	305**	247**	176	155
PROD	9754**	10653**	424*	323**
NCPR	140	126*	270**	306**
UDPGT	97	180*	87	76
GST	139	260**	133*	140**
GSH	152*	94	111	108

* $p < 0.05$; ** $p < 0.01$

Liver **enzyme induction and activity in male and female mice** after diet treatment with **cyproconazole** and **phenobarbital** were compared in the study of Trendelenberg (2001). Groups of CD-1 mice (5/sex) were exposed for 14 consecutive days to cyproconazole at doses of 50, 100 and 200 ppm. An additional group was exposed to phenobarbital (850 ppm).
In this study, increases in liver weight, hepatocyte hypertrophy (dose-related increase in incidence in females and in severity in both sexes), fat vacuolation, necrosis (single necrotic or small groups of necrotic hepatocytes) and inflammatory cells were reported.

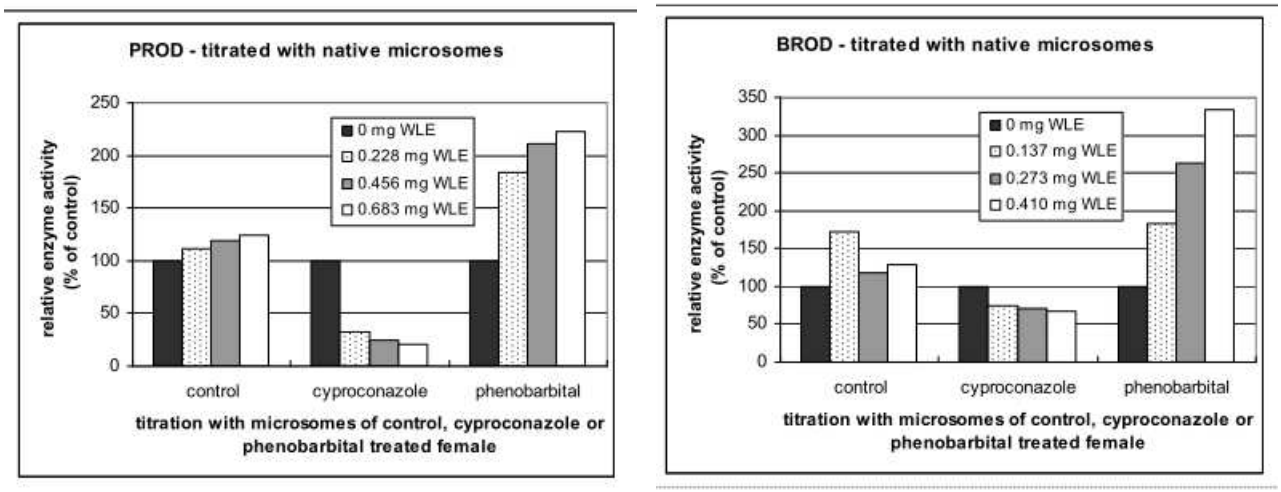
As regards enzyme induction, immunoblot analyses (amount of enzymes) revealed a strong increase in CYP2B (PROD, BROD) for cyproconazole and phenobarbital, as shown below (no data provided for controls).

Treatment	Cyproconazole			Phenobarbital
Dose (ppm)	50	100	200	850
Enzymes amount (% controls)				
CYP2B males	1063	1140	1661	2421
CYP 2B females	2492	3679	3983	4253

Activity was only minimally increased for cyproconazole (unlike for phenobarbital where the increase was higher):

Treatment	Cyproconazole			Phenobarbital
Dose (ppm)	50	100	200	850
Enzymes activity (% of controls) – males only				
PROD	257	343	404	2448
BROD	520	694	825	4431

However, further analyses were conducted to investigate the limited activity of PROD/BROD and the hypothesis was the presence of an inhibitor in microsomal fractions from mice treated with cyproconazole. Activity of PROD and BROD were measured in microsomes from a phenobarbital-treated female further 'enriched' with microsomal protein (dosed in WLE ie Wet Liver Equivalent) from control, 200 ppm cyproconazole, or 850 ppm phenobarbital treated animals. The analysis of PROD and BROD from 'mixed' microsomes showed that the adjunction of cyproconazole clearly reduced the level of PROD/BROD activity when compared to phenobarbital alone or phenobarbital with a control (indicating the presence of an inhibitory factor), as shown in the figure below.



Therefore, it may be concluded from this study that cyproconazole is a strong inducer of PROD/BROD synthesis (as shown by the increase amount of enzymes by immunoblot) and that the low activity was due to lack of accuracy of measurements in microsomal fractions (in relation to the presence of inhibitor), although this inhibition of PROD/BROD could be more complex.
In addition to these CYP2B proteins, cyproconazole also induced CYP3A and CYP2A, but did not induce CYP1A or CYP4A. This induction profile was similar to phenobarbital (which also induced CYP3A).

In the study used to determine time-dependent effects of cyproconazole on **liver cell proliferation** during continuous application through diet for 28-days in both rats and CD-1 mice (Warren *et al.*, 1995), no evidence of increased cell proliferation was seen in rats (at 20, 350 or 1400 ppm) while a transient, early increase (day 3) was observed in mice (12-fold) at all dose levels: at 15 ppm (eq. to 2.2 mg/kg/day), 100 and 200 ppm. This cell proliferation was lower than with phenobarbital (95-fold). Cell proliferation was measured by bromodeoxyuridine (BrdU)-pulse labeling technique (immunostaining after animals were injected BrdU by intraperitoneal route).

In this study, in both rats and mice, increased liver weight, lobular pattern, hepatocyte enlargement and vacuolation were reported.

In the study conducted so as to compare the liver histopathological effects of cyproconazole and phenobarbital, a known CAR-activator (Weber, 1999; re-evaluation of Warren, 1995), a similar pattern of histopathological changes was observed with cyproconazole (up to 200 ppm) and phenobarbital (850 ppm) (the comparison focused on liver weights/hypertrophy degree/ mitotic activity/ necrosis/ vacuolation).

It was noteworthy that **hepatocyte hypertrophy** was reported, but some small differences were emphasised regardless: the hypertrophy was centrilobular or mid zonal with phenobarbital when the distribution was less defined with cyproconazole. In addition, no vacuolation was reported after phenobarbital exposure (1 animal amongst all groups) when marked or moderate centrilobular vacuolation was observed in all animals with cyproconazole (day 4 and onwards) as well as panlobular vacuolation in single animals given the top dose of 200 ppm (each time point) which was mainly micro vesicular. Minimal necrosis was reported after exposure with cyproconazole. No mitotic activity was reported with cyproconazole in contrast to phenobarbital but this was explained by lack of sensitivity of the method (haematoxylin & eosin staining) when compared to BrdU used in the Warren study, also considering that the proliferation of cyproconazole was rather low. In addition, the slides examined were those from the positive cell proliferation study (Warren, 1995).

In a study (Milburn, 2006b) to determine whether the tumours observed with cyproconazole were a consequence of CAR receptor activation (and its subsequent downstream effects on other gene targets) as seen for phenobarbital, cyproconazole was administered in the diet for 7 days to both **wild type and CAR-knockout animals.** The study was carried out on the C3H strain, chosen as a suitable surrogate to CD-1 mice (strain used for subchronic and oncogenicity studies with cyproconazole) since knockout mice for the CAR receptor were not available in the CD-1 strain. The choice for this surrogate was based on the results of the study of Milburn (2006a) which compared the liver effects (organ weight/ clinical chemistry/ histology) between three strains. In the wild-type a marked increase in expression of Cyp2b transcription levels and CYP2B enzyme were reported, as well as an increase in cell proliferation (KI67 labelling index). The CAR-null genotype negated a variety of effects of cyproconazole (increases in liver weight, ALT, hypertrophy) and this was noteworthy along with the expression of CYP2b transcription levels (vs marked increase in wild-type). Some of the parameters were reduced only, including the KI67 labelling index (cell proliferation) while in the study of same author (Milburn, 2006c) the CAR-null genotype negated the effects of phenobarbital, including the cell proliferation measured by Ki67. However, cell proliferation (KI67) was still clearly reduced in knockout-mice treated with cyproconazole.

Observation	***Wild type (mg/kg bw)***			***CAR-null (mg/kg bw)***		
	0	***38.6***	***71.5***	***0***	***34.0***	***54.7***
		(200ppm)	***(450ppm)***		***(200ppm)***	***(450ppm)***
plasma cholesterol	100%	41%	29%	100%	103%	73%
plasma triglycerides	100%	79%	41%	100%	101%	39%
plasma ALP	100%	130%	154%	100%	112%	147%
plasma ALT	100%	196%	1004%	100%	94.9%	52.6%
plasma AST	100%	192%	461%	100%	147%	173%
liver weight	100%	129%	135%	100%	107%	100%
Ki67 labeling index	100%	209%	495%	100%	142%	189%
Mdm2 mRNA levels	1	0.93	1	1	0.93	1.23
Gadd45β mRNA levels	1	2.46	6.06	1	1	4.29
Cyp2b10 mRNA levels	1	147	294	1	2.5	4
Cyp 2b relative protein levels	0	2.3	1.6	0	0	0
(upper band)	1	2.6	1.6	1	1.2	1
(lower band)						
Coumarin 7-hydroxylase (CYP 2a enzyme activity)	100%	849%	1135%	100%	472%	458%

Conclusion

Several possible MoAs for the hepatocellular carcinogenesis observed can be dismissed: cyproconazole is not genotoxic and a polycyclic aromatic hydrocarbon-or peroxisome-type inducer is considered unlikely since CYP1A (MROD, EROD) and CYP4A (lauric acid 12-hydroxylase) were not at all or were only slightly induced (Trendelenberg, 2001).
RAC agrees with DS that the pivotal role of CAR-activation MoA was well investigated and demonstrated. According to Elcombe *et al.* (2014) cited by the DS in the RCOM, the CAR-mediated pathway is associated with key events and associated events as follows: activation of the CAR nuclear receptor (key event #1), followed by:
- liver enzyme induction,
- increased liver weight, hepatocyte hypertrophy, and
- hepatocyte proliferation (key event #2), leading to
- pre-neoplastic, altered hepatic foci and
- formation of adenomas and carcinomas (key event #3).

The first key event in this MoA is activation of the CAR nuclear receptor, and it was demonstrated by the observed increase in Cyp2b transcription levels and with the associative event of enzyme expression and activation upon cyproconazole treatment. Indeed, cyproconazole did induce the expression of Cyp2b transcription levels *in vitro* in mouse and human hepatocytes and *in vivo* in the wild-type mice of the study of Milburn (2006). Cyproconazole was a strong inducer of CYP2B enzymes in two *in vivo* studies in mice (Dorobek, 1995; Trendelenberg, 2001). Supportive, associative events to the first key event included increased liver weight and microscopic hepatocellular hypertrophy that were identified following cyproconazole treatment: increased liver weight and hepatocyte hypertrophy were consistently reported after repeated exposure with cyproconazole (see also STOT RE section).

The second key event is an increase in hepatocellular proliferation and was identified following cyproconazole treatment. In the study of Warren (1995), early increase of cell proliferation (day 3) was observed in mice (12-fold) and not in rats. This transient increase in cell proliferation in mice followed a similar pattern to mitogen compounds such as phenobarbital, a known CAR-activator (despite phenobarbital being more potent). In addition, RAC noted that the absence of cell proliferation in rats in this study could explain the absence of tumours observed in that species. Cell proliferation was also reported in the study of Milburn (2006) investigating the CAR-pathway by exposure to both wild type and CAR-knockout animals. Cell proliferation was also induced with both cyproconazole and phenobarbital *in vitro* in mouse hepatocytes but not in human hepatocytes as it could be expected for CAR-activation.

Moreover, a study investigating the effects in both wild-type and CAR- knockout mice (Milburn, 2006) is part of the data set available and most of the effects reported after exposure to cyproconazole in the wild-type mice were not reported in the CAR- knockout mice, including the induction on Cyp2b transcription levels. Based on these results in CAR- knockout mice, RAC acknowledges that some of the findings were not fully negated which cannot be fully explained however, from this study, RAC put more emphasize on the fact that most of the effects were negated (including the key induction of Cyp2b transcription levels) and all were at least reduced, clearly indicating the involvement of CAR.

Cyproconazole also induced the Cyp3a transcription levels and CYP3A proteins which may be related to PXR receptor. Indeed, while induction of CYP2B is an indicator of CAR activation, PXR activation can also produce an induction of CYP2B enzymes, along with a greater induction of CYP3A enzymes. However, according to the recent review of Elcombe (2014), PXR activation also leads to increased expression of specific genes including xenobiotic metabolizing enzymes, many of which are also CAR-responsive. Many of the molecules that can activate CAR may also activate PXR, producing a combined response pattern of gene expression and functional change. However, while there can be considerable crosstalk between CAR and PXR receptors, the key and associative events for phenobarbital-induced liver tumour formation are considered as predominantly CAR-dependent as such effects are absent in mice lacking CAR. The induction of CYP3A observed with cyproconazole was reported similarly to phenobarbital, both *in vitro* and *in vivo* studies and similarly to phenobarbital, following exposure to cyproconazole, most effects

were negated in CAR- knockout mice. Overall, RAC is of the opinion that CAR activation is the most plausible mechanism behind the liver tumour formation in the mice, given the evidence presented for the key events and some of the associative events.

The DS suggested another MoA in addition to the CAR-activation: cytotoxicity, resulting in degenerative lesions and subsequent liver cell proliferation, with such conditions creating an environment where spontaneously mutated liver cells have a proliferative advantage, leading to the development of pre-neoplastic foci after long-term treatment to form tumours. This hypothesis is based on different observations. In two *in vitro* studies, the high dose of 500 µM cyproconazole was considered to be cytotoxic to mouse and human hepatocytes (intracellular ATP levels reduced to 2% of control in mouse hepatocytes and 1% of controls in human hepatocytes) while no cytotoxicity was reported with phenobarbital. In human hepatocytes, 125 µM cyproconazole was also considered to be cytotoxic to human hepatocytes (intracellular ATP levels, reduced to 67% of control). Furthermore, degenerative lesions (inflammation and vacuolation, single cell necrosis) were reported in repeated toxicity studies with cyproconazole at the same dose levels as tumours. Industry in their response commented that the cyproconazole data are not consistent with a cytotoxic MoA since persistent regenerative growth and sustained proliferation were not observed. According to their argumentation, substantial hepatocellular death observed after exposure with a cytotoxicant is characterised:

- Biochemically, by clearly elevated hepatic clinical chemistry parameters (ALT/AST). In the example provided, CCl4, the increase was 200-fold while cyproconazole induced these only by a factor of 2.6. The DS in its response to comments argued that the comparison with CCl4 is an extreme one and that the effects of cyproconazole are much more subtle.
- at an organ level by gross distortion of lobular shape and increased liver weight while cyproconazole did not alter lobular architecture.

RAC noted, in accordance with industry comments that the global pattern may not be consistent with a cytotoxicant: widespread multifocal hepatocyte death was not observed with cyproconazole (single cell necrosis) and a difference in hepatocellular proliferative response was observed: sustained (cytotoxicant) vs transient early burst (mitogen like CAR-activator). In addition, despite the similar toxic effects that were observed in rats, no tumours occurred. Therefore, RAC did not consider that cytotoxicity was an additional MoA involved in the tumour formation.

In summary, RAC considered that the CAR activation is the most plausible mechanism behind the liver tumour formation in the mice, given the evidence presented for the key events and some of the associative events.

RAC further took into consideration that similarly to phenobarbital, for cyproconazole the prerequisite for tumour formation, i.e. DNA replication, does not seem to occur in human hepatocytes following induction of human CAR, in contrast to mice. Due to this qualitative difference, the liver tumours as a result of CAR-activation by cyproconazole are considered to be of little relevance to humans. This is in line with a recent review of the human relevance of CAR-mediated liver toxicity, for which phenobarbital is the example substance (Elcombe *et al.* 2014).

In conclusion, RAC is of the opinion that **no classification for carcinogenicity** is warranted for cyproconazole.

RAC evaluation of reproductive toxicity

Summary of the Dossier submitter's proposal

The DS proposed not to classify for fertility since cyproconazole did not affect fertility in rats in a guideline compliant two-generation study.

The DS however, proposed to classify cyproconazole in category 1B for development, based on serious malformations and embryo/foetal toxicity observed in two species (rat, rabbit), at doses causing limited maternal toxicity. Cyproconazole is currently classified in category 2 but the second rabbit study was not part of the set of data on which the classification was previously agreed.

Comments received during public consultation

Four MSCA supported the classification in category 1B for development. One Industry comment disagreed with the proposal and suggested to maintain classification in category 2 since the new rabbit study is not considered to add significant new information and the combined data are insufficient to trigger a category 1B classification. Industry considered that category 2 for "some evidence" is considered more appropriate based on the inconsistency in the database among species (main argument: only one malformation per species; palatoschisis in rat and malrotated hind limb in rabbit), the incidence of hydrocephalus that did not follow a dose-response relationship and with incidences similar to historical controls, and the adverse findings observed only at maternally toxic doses.

Of the four MSCA commenting, one agreed with the proposal of no classification for fertility but questioned the dose levels used in the two-generation study, as they were 10 fold lower than those used in repeated dose studies and wondered whether higher dose levels would have affected the conclusion on fertility. One MSCA suggested to classify in category 2 for fertility mainly based on the MoA of cyproconazole (similar to other triazole compounds), and with supportive data from repeated studies and disregarding the 2-generation study (due to the low dose levels used). Although cyproconazole is a member of the triazole group of substances (thereby inhibiting aromatase activity), the DS pointed out the difference in potency and effects among these substances and suggested to classify based on the data for each substance rather than use a group approach. The DS clarified that the dose levels in the 2-generation study induced minimal parental toxicity and slight alteration of fertility parameters (treatment related) and that the effects observed in repeated dose studies, on a weight of evidence approach, do not warrant classification.

Assessment and comparison with the classification criteria

Fertility

In the two-generation study in rats (Eschbach, 1987), rats received cyproconazole in the diet, at concentrations of 4, 20, 120 ppm (equivalent to doses of 0.3, 1.4 and 8.3 mg/kg bw/d, respectively, for F0 males, 0.3, 1.7 and 9.9 mg/kg bw/d for F0 females, and 0.4, 1.8, 10.9 mg/kg bw/d, respectively, for F1 males and 0. 5, 2.2 and 13.3 mg/kg bw/d for F1 females). No deaths and no clinical signs were reported in parental animals; parental effects at the high dose of 8.3/9.9 mg/kg bw/d were limited to a slight increase in liver weight (5.5% in males, 4.4% in females, statistically significant only in males), associated with increased incidence in liver fatty changes (in males only according to the DAR report), which is not considered as severe parental toxicity. Mating and fertility indices were not affected by treatment.

The mean gestation period was increased in some F0 females of the mid and high dose group (mean gestation period in F0: 22.4, 22.6, 22.5 at 0.3, 1.7 and 9.9 mg/kg bw, respectively, vs 22.3 in controls); delivery occurred on day 22 for 66% of the controls and 50% of the dams in the mid and high dose groups. One F0 female of the high dose group failed to give birth (sacrificed day 25 *post coitum*). However, there was no such increase in the F1 generation. A slight reduction (7% vs control, not statistically significant) in the number of implantation sites was reported in F0 high

dose animals, which was within the historical range (9.8 – 13.00) according to the DS in response to information provided during public consultation. A decreased in litter size at birth (12% less than controls) was also recorded. Such effects were not observed in the F1 generation.

A dose-related increased in the pre-/perinatal mortality in F1 pups was reported at the mid and high dose group [13.6% and 16.3%, respectively vs. 10.7% in controls (high mortality in controls)]. Pre-/perinatal mortality is not reported as such in the study but on a general basis may include post-implantation losses, stillbirths and neonatal deaths. At the mid dose, a single female lost all its pups (12/13 on pre-/perinatal losses, 1/13 shortly after birth), which may explain the increase at this dose level according to the DS. However, one total litter loss also occurred in one F0 female in the high dose group (day 5 *post-partum*) as well as in one F1 female of the high dose group (day 4 *post-partum*). Additionally, at the high dose, this finding was also observed in F2 pups: the pre/perinatal mortality was increased (12.6% vs 11.3% in controls). An increase in post-natal mortality was also reported in both generations (dose-related in F0: 1.6, 6.6% and 8.1%, respectively vs 0.3% in controls; high dose only in F2: 5.9%, 2.9% and 7.6%, respectively, vs 2.2% in controls). This finding is considered as treatment-related but will be covered under the discussion of the classification for development (below).

Parameter	Generation	0 ppm	4 ppm	20 ppm	120 ppm
Mean live pups/dam at days					
0	F1	11.2	11.5	10.8	9.8↓
4[a)]		7.8	7.7	7.5	7.2↓
21		7.8	7.7	7.4	7.1↓
0	F2	11.0	11.7	12.0	10.9↓
4[a)]		7.7	7.7	7.8	7.2↓
21		7.7	7.6	7.7	7.1↓
Pre- and perinatal loss (%)	F1	10.7	7.8	13.6↑	16.3↑
	F2	11.3	7.3	5.6	12.6↑
Mean postnatal loss (%) at days					
0 - 4	F1	0.3	1.6	5.6	7.6↑
0 - 21		0.3	1.6	6.6	8.1↑
0 - 4	F2	2.2	4.2	1.4	5.8↑
0 - 21		2.2	5.9	2.9	7.6↑

a) After culling

In the 18-month oncogenicity study in mice (Warren *et al.*, 1989), testicular germinal epithelial deficit and aspermia of the epididymides were found at and above 13.2 mg/kg bw/day (NOAEL = 1.8 mg/kg bw/day). However, no effect was observed in the 90-day study in mice at doses up to 89 mg/kg bw/d. The testis was not affected either in the dog studies (except for degeneration of the testicular germinal epithelium in a single male of the mid-dose group only in the 1-year study). In the 28-day study in rat, the relative testes weight was minimally increased in the two higher dose groups (+3% and +8%, respectively) but this effect was a consequence of the reduced body weight (absolute testes weight not changed), it did not correlate with histopathological changes and this finding was not reported in the 2-year combined chronic toxicity/carcinogenicity study.

Some occasional effects were observed on the ovaries in the dog studies, which were not consistent. In the 90-day study, while absolute and relative weight of the ovaries were considerably increased in low and mid dose group, with macroscopic enlargement for one female only, they were reduced in the high dose group, with no associated macroscopic findings. In the ovaries there was decreased follicular activity and an inactive uterine endometrium was also noted in some dogs. However, no such effects were observed in the 1-year study. In that study, one female in the high dose group did not come into heat and histology revealed immature ovaries. These findings may represent treatment-related findings on the oestrous cycle but all other females had normal heat during the cycle.

In summary, the findings from the two-generation study (increase in gestation period, decreased implantation sites, decreased litter size) reported at the high dose (8-13 mg/kg bw/d) were slight and were not reported in the second generation, therefore, RAC agrees with DS that they do not

provide sufficient basis for classification for fertility. Despite the effect observed in the long-term study in mice, the evidence from other repeated toxicity studies does not indicate the testis as a target organ for cyproconazole and therefore, RAC agrees with DS that the available testis data do not support classification for fertility. Some occasional effects were observed on the ovaries in the dog studies, which were not consistent and RAC agrees with the DS that the available ovary data do not provide sufficient evidence to support classification for fertility. Overall, RAC agreed with the DS that available data does not justify a classification for fertility for cyproconazole.

Development

Two main adverse effects of cyproconazole on development are considered critical for the classification for developmental toxicity: malformations (mainly cleft palates in rats, but also malrotated forelimb in rabbits as well as hydrocephalus in both species) and post implantation losses/resorptions.

A preliminary range-finding study (Becker, 1985a) was carried out in the Han Wistar rat by gavage at doses of 7.5, 30, 75 and 120 mg/kg bw/d over gestation days (GD) 6-15.
Maternal toxicity was reported at the high dose of 120 mg/kg bw/d with a significant reduction in body weight gain (82%) during the early part of the treatment period (GD 6-11), which was associated with reduced food consumption (16.5% vs controls). Maternal body weight gain was also reduced by 41% at 75 mg/kg bw/d, which was associated with a slight reduction in food consumption of 10%. However, the reduction in body weight gain was not statistically significant (29%, 29%, 41% and 82% of controls at 7.5, 30, 75 and 120 mg/kg bw/d, respectively).

Developmental effects were reported from 30 mg/kg bw. Malformations, including palatoschisis (cleft palate), occurred from 30 mg/kg bw with incidences of 1/47, 1/16 and 10/11 foetuses at 30, 75 and 120 mg/kg bw, respectively. Severe embryotoxicity was manifested as a dose related increase in post-implantation losses from 30 mg/kg: 2.1%, 24.2%, 64%, 82.3% at 7.5, 30, 75 and 120 mg/kg bw respectively, versus 7.8% in controls. They were mainly related to early resorptions. Total litter resorptions occurred in 2/5 females at 75 mg/kg bw and in 3/5 dams at 120 mg/kg bw.

In the main rat developmental toxicity study (Becker, 1985b), Han Wistar rats received doses of 6, 12, 24, and 48 mg/kg bw over GD 6-15.

Maternal effects were observed from 24 mg/kg bw/d, however details on severity were not reported. Body weight loss was reported in dams at the two highest doses at the beginning of the treatment (GD 6-7 at 24 mg/kg bw/d; GD 7-8 at 48 mg/kg bw/d, but there were no data on the extent, and body weight gain recovered in the following days with an overall reduction in body weight gain for the period GD 6-11 (-29% and -35% at 24 and 48 mg/kg when compared to controls). Mean food consumption was also reduced in this period (no details) and recovered thereafter.

Developmental effects were observed from 24 mg/kg bw/d. Treatment at 24 and 48 mg/kg bw/d resulted in an increased number of early resorptions (20.1% and 25.5% at 24 and 48 mg/kg bw/d, respectively, versus 4.6% in controls) and post-implantation losses (22.2% and 30.6 % at 24 and 48 mg/kg bw/d, respectively vs 5% in controls) and a decreased percentage of live foetuses (19% and 26% less than controls at 24 and 48 mg/kg bw/d, respectively). Significant malformations occurred at these dose levels with the following incidences of hydrocephalus: 1 foetus in 1 litter at 24 mg/kg bw and 2 foetuses in 2 litters at the high dose of 48 mg/kg bw (one being a runt with both hydrocephalus and palatoschisis). At the high dose, 2 foetuses in 2 litters had palatoschisis (cleft palate), of which one was a runt (both palatoschisis and hydrocephalus). At these dose levels, foetal bodyweight was reduced (-8.3% bw as compared to controls at both 24 and 48 mg/kg bw/d) and incomplete/absent ossification of phalangeal nuclei and in calcanea were also observed.

In the second developmental study in Wistar rats (Machera, 1995), animals were exposed at 20, 50 and 75 mg/kg bw over GD 6-15. This study is considered as supplementary only since it was not GLP-compliant and did not follow any guideline although it was considered as similar to OECD 414 with limitations (no analytical data of the test substance was conducted, maternal

parameters not reported). A preliminary test was carried out at 100 mg/kg bw/d, which was severely toxic to dams (no details); at this dose, total resorption occurred in 7/10 pregnant dams and from the 12 foetuses obtained, 8 were found dead.

In the main study, maternal effects were seen from 20 mg/kg bw/d, which were more marked at the high dose of 75 mg/kg bw/d: reduced body weight gain was observed from the beginning of treatment and until day 16 (days 6-11: -37%, -37%, -63% as compared to controls; days 11-16: -51%, -53% and -63% at 20, 50 and 75 mg/kg bw/d, respectively) but, as individual animal data were not provided to the DS, this assessment could not be verified.

Developmental effects were observed from 20 mg/kg bw/d. Treatment at all dose levels resulted in a dose-dependent increase in litter resorptions: 1.4, 3.4 and 4.3 resorptions per litter at 20, 50 and 75 mg/kg bw/d respectively, vs 0.4 in controls (statistically significant from 50 mg/kg bw/d) and a decreased percentage of live foetuses: 7.6, 5.0 and 4.7 live foetuses per litter at 20, 50 and 75 mg/kg bw, respectively, vs 9.5 in controls (statistically significant from 50 mg/kg bw/d). Treatment at all dose levels resulted in a dose-dependent increase in malformations: palatoschisis (cleft palate: 2 foetuses in 2 litters at 20 mg/kg bw, 11 foetuses in 5 litters at 50 mg/kg bw, 9 foetuses in 4 litters at 75 mg/kg bw/d), hydrocephalus and hydronephrosis of the ureter. Reduced foetal weights (10.8%, 11.4% and 12.1% less than controls at 20, 50 and 75 mg/kg bw/d, respectively) and foetal size were observed at all dose levels as well as absence of the 13^{th} rib and delayed ossification (absence of one or more ossification centres of sternum, reduced metatarsal ossification centres). External observation of the foetuses also showed anophtlamia in 1 foetus at dose of 50 mg/kg bw/d and microphtalmia at the high dose of 75 mg/kg bw/d, again in 1 foetus. This effect was also reported for other azole compounds but considering the incidence and the absence of a dose response relationship, it may not be treatment-related.

In the first rabbit developmental study (Becker, 1986), Chinchilla rabbits received cyproconazole at doses of 2, 10 and 50 mg/kg bw/d over GD 6-18. In that study, the actual exposure levels were uncertain: there was a large deviation in homogeneity (*ca*. 30% around the mean value) and dose levels differed when measured after preparation or 90 minutes after, therefore the results should be interpreted with caution.
Maternal effects were observed at the high dose of 50 mg/kg bw/d with a slight loss in bodyweight during the first two days of treatment (weight gain resumed by day 8, therefore body weight gain over GD 6-29 was not altered). Food consumption was decreased over GD 6-11.
Developmental effects were observed from 10 mg/kg bw/d. A significantly increase in post-implantation losses was recorded at both 10 and 50 mg/kg bw/d: 7.0% and 16.4% of implantations, respectively, vs 2.4% in controls, which was statistically significant at 50 mg/kg bw/d. They were related to both early and late resorptions (at 50 mg/kg bw/d: 7.8% early resorptions and 8.6% late resorptions).

Some malformations were reported but they were of low incidence (similar to the incidence in controls) and were evenly distributed: 1 shortened tail in the high dose group, 1 omphalocele in the 10 mg/kg bw group and 1 thoraco-gastrochisis in controls; 1 agenesia of the left kidney ureter in the high dose group, 1 agenesia of the diaphragm in the 10 mg/kg bw group, 1 agenesia of the diaphragm in controls. Among these malformations, the occurrence of hydrocephalus in all treated groups should be noted (1 foetus in each group), which is a rare malformation in rabbits according to historical controls (7/3202 controls, 3/7733 treated animals).

In a second rabbit study (Muller, 1991), NZW rabbits were exposed to 2, 10 and 50 mg/kg bw/d over GD 6-18. Concentration, homogeneity and stability of the substance were considered acceptable.

Maternal effects were seen at the high dose of 50 mg/kg bw/d. Administration of cyproconazole early during the treatment period resulted in a 13% body weight loss (GD 6-9) associated with significantly reduced food consumption.

Developmental effects were observed from 10 mg/kg bw/d. Foetal malformations and variations were observed in both treated and control animals, but they were of different type and incidences. Among the findings, an increase in the incidence of a severe malformation, malrotated hindlimb,

was observed at both 10 mg/kg bw/d (1 foetus) and 50 mg/kg bw/d (4 foetuses in 4 litters). Although this rare malformation may occur spontaneously and the incidence at the dose of 10 mg/kg was low, given the occurrence at the high dose group, this malformation was considered treatment-related at both 10 mg/ kg bw/d and 50 mg/kg bw/d. In addition, the increase in the total number of foetuses with malformations was statistically significant at the high dose (50 mg/kg bw/d), with 15 foetuses from 7 litters malformed, when compared to controls (3 foetuses in 3 litters). At 10 mg/kg bw, 5 foetuses out of 3 litters were affected.

In summary, treatment with cyproconazole led to a severe and rare malformation in rat (palatoschisis; cleft palate), in all three studies. Additionally, hydrocephalus was reported in both rats and rabbits. Although the incidence observed in rabbit for this malformation was low, it is considered treatment-related as it is rare in this species. Furthermore, the second rabbit study, which was not available when the first assessment of classification was conducted, is considered as providing additional information on malformations, since malrotated hindlimb was reported at a high incidence at the high dose.

A marked dose-related increase in resorptions (as post-implantation losses) observed in several studies in rats (including the 2-generation study which reported pre-natal mortality) are also considered to be a critical effect. The first rabbit study, despite the poor reliability in the dose levels, also showed increased resorptions and is considered as supportive to this finding.

Developmental effects were observed in the presence of maternal toxicity. However, RAC considered that these effects are not secondary to maternal toxicity. Indeed, in rats, decreased maternal body weight gain also occurred in absence of developmental toxicity: for instance, in the preliminary study, a 30% reduction in maternal body weight gain was reported at both 7.5 and 30 mg/kg bw/d while developmental effects were only reported from 30 mg/kg bw/d. In rabbits, in the first study (Becker, 1986), some developmental effects were reported from 10 mg/kg bw/d while maternal body weight loss occurred only at the dose of 50 mg/kg bw/d. Moreover, even with a markedly decreased body weight gain, given the severity of the effects (malformations, post-implantation losses), it is considered unlikely that it would be caused by maternal toxicity. Indeed, the OECD guidance document number 43 (from 2008) indicates that in the latest available feed restrictions studies (Fleeman *et al.* and Cappon *et al.*, published in 2005), no malformations occurred in either rat or rabbit even in presence of severe reduction in body weight gain with weight loss, and in rats, no effect on embryo viability was observed (but abortions were seen in rabbits).

RAC therefore considered that the available data provides clear evidence of adverse effects on development according to the CLP criteria and RAC agreed with the DS that classification of cyproconazole as Repr. 1B is warranted. RAC also noted that the findings were similar to those with other substances of the azole class (such as the high incidence of cleft palates in rat foetuses - a malformation which is rarely seen in rats, hydrocephalus, and post-implantation losses).

However, RAC concluded that based on the observed effects across a number of studies for cyproconazole, **classification as Repr. 1B; H360D** is justified.

ENVIRONMENTAL HAZARD ASSESSMENT

RAC evaluation of environmental hazards

Summary of the Dossier submitter's proposal

The DS proposed the environmental hazard classification as Aquatic Acute 1 - H400 with an M-factor of 10 based on acute aquatic toxicity to the alga *Scenedesmus subspicatus* (96-h EbC_{50} = 0.077 mg/L), and as Aquatic Chronic 1 - H410 with an M-factor of 10, based on chronic aquatic

toxicity to the alga *Scenedesmus subspicatus* (96-h NOEC = 0.021 mg/L) and considered cyproconazole to not be rapidly degradable.

Comments received during public consultation

Six comments were received on this hazard class; five from Member States (MS) and one from industry. Four MS supported the proposal of classification but questioned the derivation of M-factors and one MS asked for clarification before the proposed classification and acute and chronic M-factors can be agreed.

Two MS requested the inclusion of additional information in the CLH report (7 day study on aquatic toxicity to *Lemna gibba* (Everett, Wyeth and Powley, 2007 – included in the 2010 pesticides assessment 'Additional Report and EFSA Peer Review conclusion for cyproconazole')) and to consider relevant endpoints from this study for the aquatic classification. This information was provided in the response to comments document (RCOM) but the DS concluded that this will not change the classification. In further comments it was suggested to base the calculation of the algal endpoint on growth rate instead of biomass and to use the ErC_{50} based on measured (rather than nominal) concentrations for classification . In particular, clarification was requested on the algae 96-h ErC_{50} (0.12 mg/L, nominal) by one MS. Three MS suggested to use this ErC_{50} instead of the EbC_{50} (0.077 mg/L), which would result in an acute M-factor of 1 instead of 10. In their response, the DS did not agree, stating that there was no measured ErC_{50} available from this study but clarified that the reported 72-h ErC_{50} is an estimate based on nominal concentrations subsequently submitted by the applicant. The DS further emphasised that, according to the CLP criteria, when the basis of the EC_{50} is not specified or no ErC_{50} is recorded, the lowest EC_{50} shall be used for classification. Furthermore, the DS also underlined that this matter had been discussed with ECHA and EFSA during the peer review process (biocides, PPP) resulting in the acceptance of the EbC_{50} in absence of the ErC_{50}.

The main comment received during public consultation regarded the justification for and the conclusion on M-factors. Several MS noticed that the proposed acute and chronic M-factors of 10 are not justified and considered that both M-factors should be 1.

All MS suggested that the M-factor for chronic toxicity seemed wrong according to the NOEC (0.021 mg/L), suggesting an M-factor of 1 instead of 10. This M-factor of 1 for chronic toxicity was agreed by the DS in the RCOM.

One comment from industry provided corrections and clarifications but stated that these have no impact on the classification proposal (no explicit agreement but no disagreement were expressed on the classification proposal), as follows:

- The evaluation and classification of cyproconazole should be performed independent of metabolites, including 1,2,4-triazole. As a result, the referenced studies and cited data in the classification and labelling proposal regarding 1,2,4-triazole should be disregarded.
- Three studies should be added to take into account the route of soil degradation.
- The non-normalised DT_{50} values (ranging from 26.46-d (DFOP) to 141.3-d (SFO)) that have been evaluated at an EU level and presented in the EFSA conclusions (EFSA Journal 2010;8(11):1987) should be used instead of those currently included in the CLH report as those are deemed to be incorrect. Additionally, four field study summaries should be included.

No response to these comments was provided by the DS in the RCOM document.

Assessment and comparison with the classification criteria

Degradation

Cyproconazole is hydrolytically stable and is expected to be stable to direct photolysis in water.

The photochemical degradation of cyproconazole and its metabolite 1,2,4-triazole was estimated with AOPWin[1] and the photochemical half-life in the atmosphere was determined to be ~ 1 day. Therefore, cyproconazole is not expected to persist in the atmosphere.

[1] Atmospheric Oxidation program (version 1.5, Syracuse Research Corporation, USA)

No valid OECD TG 301 test is available. Nevertheless, an aquatic dissipation study in two dark water/sediment systems at 20°C is available. Degradation in both systems was very slow (DT_{50} >> 1 year). The main dissipation process from the water phase is partitioning to the sediment.

The route of degradation of cyproconazole in soil under dark aerobic conditions at 20 – 22 ºC was investigated in three studies with 14C-triazole-labelled cyproconazole (one soil: pH 7.2, 140 day study), 14C-benzyl-labelled cyproconazole (three soils: pH 4.3 – 7.0, 210 day study), and 14C-phenyl -labelled cyproconazole (one soil: pH 7). In all these studies the degradation of cyproconazole was slow (with half-lives ranging from 72.4 to 192 days), and considerable amounts of radioactivity remained as unmodified cyproconazole at the end of the respective experiments. The degradation of cyproconazole in soil under dark anaerobic conditions showed that cyproconazole is stable under anaerobic conditions.

In conclusion, cyproconazole does not meet the criteria of the CLP Regulation for being rapidly degradable in the environment.

Bioaccumulation

The octanol/water partition coefficient of the cyproconazole metabolite, CGA 71019 (1,2,4 triazole) (log Pow) is –1, indicating that the compound is unlikely to bioaccumulate in fish or other aquatic organisms. Specific studies on the bioaccumulation of CGA 71019 (1,2,4 triazole) are therefore not considered necessary.

In addition, measurements of aquatic and terrestrial bioaccumulation of cyproconazole have been performed. The bioconcentration factor for fish was determined experimentally to be 28 L/kg (Forbis, 1986). With an aquatic bioconcentration factor (BCF) of 28 L/kg, cyproconazole is not considered to be a bioaccumulative substance for classification purposes.

RAC also notes that lipid normalisation is unlikely to change the conclusion about the bioaccumulation behaviour of the parent substance.

Aquatic Toxicity

The lowest reliable ecotoxicity results are included in the table below (the key study is highlighted in bold).

Trophic level/test guideline	**Ecotoxicity result**	**Reference**
Fish: Common carp (*Cyprinus carpio)* (96 h) OECD TG 203 and US EPA Pesticide Assessment Guidelines, FIFRA Subdivision E § 72-1, Hazard Evaluation: Wildlife and Aquatic Organisms, October 1982. / ASTM standard E729-88, 1988	LC_{50}= 18.9 mg a.i/L measured	Hamburger, F. and Klotzsche, C. (1985)
Fish: Rainbow trout (*Oncorhynchus mykiss)* (89 days) US EPA Pesticide Assessment Guidelines, Subdivision E, Section No. 72-4(a) (1982), ASTM Standard E 1241-88 (1988), and U.S. EPA "Standard Evaluation	NOEC = 0.58 mg a.i./L (survival) (measured) 0.16 mg a.i./L LOEC (fry growth) (measured) NOEC < 0.16 mg a.i./L (measured)	Drottar, K.R and Swigert, J.P. (1993a)

Procedure, Fish Early Life-Stage Test" (1986)		
Crustacea: *Daphnia magna* (48 h) US EPA FIFRA Pesticide Assessment Guidelines, Subdivision E, Section No. 72-2 (October 1982)	EC_{50} = >22 mg a.i./L (highest concentration tested) (measured)	Surprenant D.C. (1986)
Eastern oyster (*Crassostrea virginica*) US EPA FIFRA Pesticide Assessment Guidelines, Subdivision E, Section No. 72-2 (October 1982) and ASTM Standard E 729-88 (1988)	EC_{50} = 2.6 mg a.i./L (measured)	Sved D. W. *et al*. (1993)
Daphnia magna (21 days) OECD TG 211	NOEC = 0.023 mg a.s./L (nominal)	Drottar, K.R and Swigert, J.P. (1993c)
Algae: ***Scenedesmus subspicatus* (96 h)** **OECD TG 201**	**NOE_bC =** 0.021 mg a.i./L (measured) **EbC_{50} =** 0.077 mg a.i./L (measured) **Estimated ErC_{50} =** 0.12 mg a.i./L (nominal) **72 h EbC_{50} =** 0.099 mg a.i. /L (measured)	**Ellgehausen, H. (1986a) Acute toxicity of SAN 619F to *Secenedesmus subspicatus* (OECD: Algae Growth Inhibition Test). RCC Itigen Report No: 75521 (unpublished).**
Aquatic plants *Lemna gibba* 7 days	EbC_{50} = 0.059 mg /L (nominal) (frond number)	Growth inhibition to *Lemna gibba* under semi-static conditions Everett, CJ *et al*; 2007

For the short-term aquatic hazard, reliable acute aquatic toxicity data are available for the three trophic levels fish, aquatic invertebrates and algae/aquatic plants. The lowest reliable short-term aquatic toxicity result is a measured 72-h EbC_{50} of 0.099 mg a.i./L for the green algae *Scenedesmus subspicatus*. This result is below the threshold value of 1 mg/L, therefore RAC agrees with the DS proposal to classify cyproconazole as **Aquatic Acute 1; H400**.

According to the OECD TG 201 - Freshwater alga and cyanobacteria growth inhibition test (28 July 2011), the recommended test duration is 72-h although shorter or longer test durations may be used. Furthermore, the CLP Regulation (Annex I, Table 4.1.0, Note 2) indicates that the "*Classification shall be based on the ErC_{50} [= EC_{50} (growth rate)]. In circumstances where the basis of the EC_{50} is not specified or no ErC_{50} is recorded, classification shall be based on the lowest EC_{50} available*". Therefore, RAC agreed with the DS and considered it relevant to take into account the value 72-h EbC_{50}. As this value is between $0.01 < EC_{50} \leq 0.1$ mg/L, **the acute M-factor is 10.** The 7-day EbC_{50} of 0.059 mg/L obtained from the *Lemna gibba* study of 2007 is considered as supportive to the M-factor of 10. RAC noted that if a reliable and appropriate EC_{50} on growth rate were to become available, the acute M-factor might need to be revised.

For the long-term aquatic hazard, reliable long-term aquatic toxicity data are available for fish, aquatic invertebrates and algae/aquatic plants. Cyproconazole is not considered to be rapidly degradable. The lowest long-term aquatic toxicity result is a 96-h NOE_bC of 0.021 mg/L for the green algae *Scenedesmus subspicatus*. As this concentration is below the threshold value of 0.1 mg/L for non-rapidly degradable substances, RAC concludes that a classification as **Aquatic Chronic 1; H410** is justified. As this value is between $0.01 < NOEC \leq 0.1$ mg/L, the chronic M-factor is 1, as proposed by the DS after public consultation.

In summary, RAC agrees with the DS that cyproconazole should be classified as:

Aquatic Acute 1 (H400), M=10
Aquatic Chronic 1 (H410), M=1

Additional references

Tamura *et al.* (2015) Involvement of constitutive androstane receptor in liver hypertrophy and liver tumor development induced by triazole fungicides. Food and Chemical Toxicology, Volume 78, April 2015, Pages 86–95.

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).