Committee for Risk Assessment

RAC

Annex 2

Response to comments document (RCOM)

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

Flumioxazin (ISO); N-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboximide

EC number: -

CAS number: 103361-09-7

CLH-O-0000004153-83-03/F

Adopted

06 June 2014

ANNEX 2 - COMMENTS AND RESPONSE TO COMMENTS ON CLH PROPOSAL ON FLUMIOXAZIN (ISO); N-(7-FLUORO-3,4-DIHYDRO-3-OXO-4-PROP-2-YNYL-2H-1,4-BENZOXAZIN-6-YL)CYCLOHEX-1-ENE-1,2-DICARBOXAMIDE

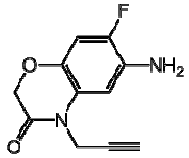
COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

Comments provided during public consultation are made available in this table as submitted by the webform. Please note that some attachments received may have been copied in the table below. The attachments received have been provided in full to the dossier submitter and RAC.

ECHA accepts no responsibility or liability for the content of this table.

Substance name: Flumioxazin (ISO); N-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboxamide
CAS number: 103361-09-7
EC number: -
Dossier submitter: Czech Republic (RMS)

GENERAL COMMENTS

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Sweden		MemberState	1
Comment received				
A general comment is that too little detail is presented in the dossier to allow an independent analysis. A figure of the metabolites would be helpful. The abbreviation APF (one of the metabolites) should be explained.				
Dossier Submitter's Response				
Response see comment No 10 Abbreviation APF (one of the metabolites) is explained as follows. APF: 6-amino-7-fluoro-4-(2-propynyl)-2H-1,4-benzoxazin-3(4H)-one  Flumioxazin is hydrolyzed to APF via opening of the cyclic imide moiety with the subsequent cleavage of the amide linkage. APF is a major degraded or metabolized product in water, soil, animals and plants.				
RAC's response				
RAC notes the extensive discussion of the data presented under point 10.				

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	France		MemberState	2
Comment received				
FR agrees with the classification proposal: Removal of Repr. 1B H360D (May damage the unborn child) and addition of M factor chronic = 1000.				
Dossier Submitter's Response				
Agreed				
RAC's response				
RAC considers that a classification as Repr. 1B should be removed and replaced by Repr. 2				

on the basis that although when compared to the rat, a significant difference in sensitivity of human development toxicity is envisaged, the difference is quantitative and the same mechanism is likely to exist in humans.

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Germany		MemberState	3
Comment received				
The German CA supports the proposed environmental classification and labeling as H410 and H400. We support the M-factors and concentration limits too. We do not support the proposal to delete the classification as Repr. 1B, H360D of flumioxazin without further clarifications.				
Dossier Submitter's Response				
Noted. Response see comment No.11				
RAC's response				
RAC considers that the justification for removing the classification is not adequate.While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.				

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	United States		Individual	4
Comment received				
Classification as Repr 1B (p5, Table 2, existing entry) is not appropriate. *(ECHA note: The following attachment was provided:* *"Flumioxazin:Classification for Developmental Toxicity" [Attachment 1])*				
Dossier Submitter's Response				
Agreed				
RAC's response				
RAC considers that the criteria for removing the classification as proposed by the DS **are not met**. While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.				

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	United States		Individual	5
Comment received				
Classifications for flumioxazin regarding its potential reproductive hazard should be removed. Experimental data demonstrate that the rat is particularly sensitive to the toxic				

effects of flumioxazin whereas this is unlikely to be the case for humans.

In developing rat embryos, flumioxazin targets protoporphyrinogen oxidase (PPO), an enzyme located in yolk sac-derived erythroblasts (precursors of red blood cells). Inhibition of PPO interferes with heme synthesis and causes subsequent loss of the affected erythroblasts from the fetal blood stream. This is potentially catastrophic to rat embryos because their yolk sac-derived erythroblast develop synchronously and entire segments of erythroblasts would be lost leading to anemia. Due to the resulting fetal anemia and hypoxia, the developing heart subsequently becomes enlarged and somewhat distorted. This interferes with proper closure of the interventricular septum. Flumioxazin treatment during in utero development also reduces rat fetal serum protein concentrations, which affects bone development and causes reduced ossification, wavy ribs, and bent bones.

The available evidence, including the Sponsor's mechanistic data combined with literature studies, is adequate to support the proposed mode of action in the rat. Importantly, the available information is sufficient to establish that the developmental effects seen in the rat are not relevant to humans not only because possible exposure levels to human embryos will be far lower than the effective levels in pregnant rats, but also because human yolk sac-derived erythroblasts do not develop synchronously and therefore any loss of erythroblasts would be small and would not lead to anemia or hypoxia.

Dossier Submitter's Response

Agreed

RAC's response

The Sponsor's proposed mode of action in the rat is supported by the mechanistic data (both old and new) presented. The particular sensitivity of the rat embryo due to the synchronous development of erythroblasts is recognised. However, inhibition of PPO also occurs in humans (IC50 = 7.15 and 17.3 nM in rats and humans respectively) and will also inhibit haem synthesis in humans and the potential exists to cause adverse effects in the human foetus. Even though the concentration in the human embryo may be less and a smaller population of developing erythroblasts targeted, a potential hazard still exists. The proposal that classification in Cat 1B could be downgraded because of the significant evidence for the shared mechanism and the existence of some doubt about the relevance of the effect to humans was considered. However, RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	Japan	Sumitomo Chemical	Company-Manufacturer	6

Comment received

Sumitomo has conducted additional studies to better address human fetal hazard and risk after dossier submission. Here we submit two study reports using human CD36+ cells1) and rat erythroleukemia cells2).

Sumitomo established test conditions and conducted a study with CD36+ cells which are derived from human cord blood1). CD36+ cells are precursor of erythroblasts, which can be differentiated into heme-synthesizing cells under appropriate culture conditions, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells, hence better addressing effect on heme biosynthesis in human fetal erythroid cells. The results of the study demonstrated that there were no effects on heme

content and cell number of human heme-synthesizing cells treated with flumioxazin at up to 5 µM.

Sumitomo has also conducted a study with rat erythroleukemia cell line which can be differentiated into heme-synthesizing cells by treatment with inducers and it can be corresponded to human K562 cells2). The result revealed that flumioxazin at 0.1µM and above reduced heme production in the rat heme-synthesizing cells.

Considering results from these 2 studies as well as negative result of study with human K562 cells, rat fetus are much susceptible than human. PBPK model, which is recommended to clarify the difference of pharmacokinetics between animals and humans in the guideline of OECD, suggests 5 µM is much higher than expected concentration in human embryos whose mothers are exposed to 1000 mg/kg flumioxazin. We used PBPK model developed by EPA3) since there are no harmonized PBPK models in EU level at present.

Sumitomo believes these results provide further evidence that human fetuses would not be affected by exposure to maternal doses as high as 1000 mg/kg.

1) Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0126.
2) Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0125.
3) Godin SJ, DeVito MJ, Hughes MF, Ross DG, Scollon EJ, Starr JM, Setzer RW, Conolly RB, Tornero-Velez R., 2010 Physiologically based pharmacokinetic modeling of deltamethrin: development of a rat and human diffusion-limited model. Toxicol Sci. 115, 330-343.

(ECHA note: The following confidential attachments were provided:
"Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells", Kawamura S., 2013.
"Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells", Kawamura S., 2013.
[Attachments 2 and 3]

Dossier Submitter's Response

Agreed. New studies will be cited and results evaluated.

RAC's response

The analysis of the new studies is noted. The results of the new *in vitro* studies support the findings of the K562 cells and the possibility that erythroblast cells in the human foetus may be less sensitive to PPO inhibition-associated anaemia. However, there is considerable uncertainty associated with extrapolation of such *in vitro* observations to the situation *in vivo* in the human foetus. The use of the PBPK model in this context provides some insight into possible risk considerations for the human foetus, but is not sufficient evidence to disregard a potential hazard. The evidence is considered by the RAC to support the discussion on downgrading of the Cat 1B classification to Cat 2 on the basis of evidence of a plausible mechanism in conjunction with some doubt concerning the human relevance on the basis of quantitative differences in enzyme sensitivity and the relationship between PPO inhibition and altered haem production in humans. However, RAC concludes that the doubts with regard to human relevance are not sufficiently removed by the additional *in vitro* data to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained

Date	Country	Organisation	Type of Organisation	Comment number

17.10.2013	United States		Individual	7

Comment received

I fully agree with the evaluation in the CLH Report justifying that flumioxazin does not require classification regarding reproductive and developmental toxicology. The CLH Report presents a well delineated mode of action utilizing the IPCS framework regarding mode of action analysis in animals in the evaluation of its human relevance.

In following this type of analysis, both of the endpoints (defects on heart and defects on ribs) are presented separately, as suggested by the framework. The report also presents the details regarding the mode of action involved with these. The evidence strongly indicates that the chemical inhibits the enzyme protoporphyrinogen oxidase (PPO) which leads to subsequent changes, including an inhibition of heme synthesis, leading to anemia, leading to secondary enlargement of the heart and its embryotoxicity. Similarly, the inhibition of this enzyme leads to a decrease in serum proteins in the fetus (a secondary effect via hypoxia) which leads to edema and incomplete and delayed ossification giving rise to the effects on the ribs. The evidence regarding the modes of action is well described. The lack of relevance to humans is also well described, indicating a lack of relevance based on effects on quantitative aspects for the human enzyme.

I am particularly pleased to see this type of analysis for a non-cancer endpoint regarding mode of action and human relevance. I have been involved with the development of this framework, which started in the 1990's by the International Program on Chemical Safety (IPCS), initially focused on analysis of mode of action of effects in animals. This led to the publication by Sonich-Mullin et al. in 2001 (Reg. Toxicol. Pharmacol.). The application of this mode of action analysis was then further developed into a framework regarding extrapolation to humans and evaluation of human relevance, a framework that was initially developed by the International Life Sciences Institute (ILSI), Risk Science Institute (RSI) through a group sponsored by the US EPA and Health Canada and involving individuals from of those agencies, in addition to scientists from academia and industry. This led to the initial human relevance framework proposed by Meek et al. (Crit. Rev. Toxicol., 2003), which largely focused on non-genotoxic cancer endpoints. This framework was further developed for application to genotoxic carcinogens and for non-cancer toxicologic endpoints, published by Seed et al. (Crit. Rev. Toxicol., 2005). This framework was then further refined by the IPCS, first for use for non-genotoxic carcinogens (Boobis et al., Crit. Rev. Toxicol., 2006) and ultimately for all toxicologic endpoints (Boobis et al. Crit. Rev. Toxicol., 2008), the reference that is being applied in the CLH report. It is particularly gratifying to see application of this framework to a non-cancer toxicologic endpoint, especially one for which a considerable amount of research has been performed. The data clearly show the unique nature of the effect in rat which was not seen in rabbits and can clearly be demonstrated to not occur in humans. The use of a susceptible and resistance species for comparison of the effect is highly useful in trying to establish the likely response in humans. Also, the availability of a known genetic abnormality in humans greatly enhances our understanding of the likely effects in humans, but in this case, clear evidence for a lack of a toxicologic effect.

Dossier Submitter's Response

Agreed

RAC's response

Noted. RAC agrees that the evidence for the mode of action for developmental toxicity in the rat is well supported. The proposed lack of relevance to humans for this mechanism is based on quantitative aspects for the human enzyme. The criteria state that … *where there is mechanistic information that* ***raises doubt about the relevance of the effect for humans****, classification in Category 2 may be more appropriate.* While all arguments related to relative

sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
16.10.2013	United States		Individual	8
Comment received				
I agree that flumioxazin should not be classified for reproduction. Flumioxazin produces developmental toxicity in rats manifested as fetal death, impaired fetal growth, and ventricular septal defect. There is clear and compelling evidence that this toxicity is due to inhibition of protoporphyrinogen oxidase with consequent impairment of heme synthesis and profound embryofetal anemia. Fetal swelling and cardiac dilatation occur as a consequence of the severe anemia, and the cardiac dilatation distorts the normal relationships of the elements that contribute to the periventricular septum. This mechanism of developmental effects is not relevant to humans, because there is no suppression of heme synthesis by flumioxazin at up to 5 µM in cultures of human erythroid cells. This concentration is higher than the flumioxazin concentration that could be achieved in humans after a flumioxazin dose of 1000 mg/kg bw/day. The margin of safety provided by this model is substantial and reassuring.				
Dossier Submitter's Response				
Agreed				
RAC's response				
Classification according to the CLP Regulation is based on hazard assessment and aspects of risk assessment such as 'the margin of safety' cannot generally be considered. Although the *in vitro* data presented support a possible difference in sensitivity of the PPO enzyme between rats and humans, no explanation is offered as to why this might occur. There is considerable uncertainty in extrapolated results from studies *in vitro* on human erythroleukemia derived cells (K562) to the situation in human embryos *in vivo*. The new studies conducted on the (human) CD36+ cells and rat erythroleukemia cells also point towards differing sensitivities in PPO inhibion between humans and rats. The new data could be considered to support a lower classification as a 'doubt' is raised as to the relevance to humans. RAC considers that the criteria to reduce the classification to zero as proposed by the DS **are not met**. While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.				

Date	Country	Organisation	Type of Organisation	Comment number
16.10.2013	United States		Individual	9
Comment received				
Developmental effects seen in rats, but not rabbits, treated with flumioxazin are unlikely to occur in humans and the rat is an inappropriate model for humans, supporting no				

classification for reproductive toxicity in humans. The MOA in rats is very unlikely in humans.

Effects in the rat specifically included reduced prenatal survival and growth retardation of survivors including reduced skeletal ossification, interventricular septal defects (VSDs) and wavy ribs in the absence of maternal toxicity, with the most sensitive period for exposure on gestation day 12. Thinning of cardiac ventricular walls occurred prior to interventricular (IV) septal closure. Iron deposits in mitochondria and erythroblastic lesions (sideroblastic ethrythrocytes) leading to anemia, could result in altered cardiodynamics, leading to enlargement of the heart, in turn leading to decreased IV septal closure because of the altered juxtaposition of structures involved in formation of the IV septum. Increased fetal death occurred prior IV septal closure. Increased wavy ribs were associated with reduced serum protein concentrations leading to reduced or delayed ossification.

Pharmacokinetic and metabolism studies showed minor differences between rats and rabbits, but inhibition of PPO was significantly greater in rats, and corresponded to the critical period for induction of VSDs and other developmental effects in rats. PPO inhibition leads to anemia in rats which can result in the other changes seen in the sequence of developmental effects. PPO inhibition in liver cells from rats, rabbits and humans showed that cells from rats and rabbits appeared to be good surrogates for PPO inhibition in the embryo, with greatest sensitivity to PPO inhibition in rats, followed by human and then rabbit cells. PPIX accumulation was much greater in rats than in humans, and no accumulation occurred in rabbits or monkeys. Because PPO activity in humans is much higher than other rate-limiting enzymes in the heme biosynthetic pathway, it is unlikely that anemia would result. PBPK modeling in the rat and human suggested that humans would not be susceptible to anemia or the developmental effects of flumioxazin.

The synchronicity of erythroid cells in the rat yolk sac, and resultant loss of a large population of blood cells after flumioxazin exposure would lead to anemia not easily compensated. Due to several populations of erythroid cells found simultaneously in humans, loss of one particular population would have much less impact than in rats.

The sequence of biological events leading to fetal death, growth retardation, VSDs and wavy ribs in the rat following flumioxazin exposure has been clearly demonstrated. The critical period for the induction of developmental effects, including the relative timing of anemia, ventricular hypertrophy, IV septal closure, and death of the fetus, correlates well with the timing of PPO inhibition and subsequent accumulation of PPIX. Comparative PPO inhibition in rat, rabbit, and human liver cells and extrapolation to the human embryo/fetus demonstrated that the rat is an inappropriate model for human developmental effects of flumioxazin. PBPK modeling predicted that human erythroblasts would not be affected by exposure to doses as high as 1000 mg/kg, an unlikely exposure scenario.

Dossier Submitter's Response

Agreed

RAC's response

RAC considers that the criteria for removing the classification as proposed by the DS **are not met**. While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1 should be retained.

TOXICITY TO REPRODUCTION

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Sweden		MemberState	10

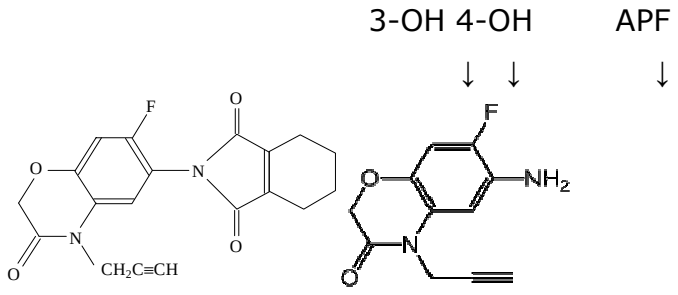
Comment received

The Swedish CA does not agree with the suggested declassification for reproductive toxicity. Based on the available data, we do not think that the information presented in the CLH report have shown convincingly enough that the effects seen are not relevant to humans. The described mode of action (MoA) for the developmental toxicity in rat is plausible, and IF this is the only MoA, the information indicates that this MoA also operates in humans even though there might be quantitative difference in sensitivity between species. However, information (met abolic/kinetic/dynamics) showing that humans are not sensitive is lacking. Considering that flumioxazin is rather potent in rats, classification in category 1B is relevant even if there would be a difference in sensitivity between species.
It would be interesting to get more information on why PPO from different species seemingly displays different sensitivity (structural differences of PPO?) to flumioxazin. The sensitivity of human fetal primary erythroblasts to flumioxazin is also unknown. We also miss a discussion about other potential MoA, such as inhibition of the other closely related enzymes involved in the heme biosynthesis. What developmental effects are seen in the cardiovascular system from other substances when the flow is increased in the developing fetus and how do they correspond with the picture given by flumioxazin? Can other effects be anticipated in humans by PPIX accumulation? We also note that humans might be differently sensitive to this effect, making species comparisons based on some in vitro experiments quite uncertain. For instance, humans (adults/fetuses) having porphyria is likely a specific risk group for this substance.
Table 32 (p. 43) It says in one of the table column headings "Time after administration (h)" – shouldn't this be "day of exposure"?
Table 34 (p. 50): Is the word "selected" in the title to this table correct? In that case how are they selected, maybe it should be "measured"?

Dossier Submitter's Response

Headings of tables 32 and 34 will be corrected: Table 32 - Day of exposure, Table 34 – Selected (flumioxazin administered on GD12) . .

Flumioxazin (S-53482) and its major metabolites: 3-OH S-53482, 4-OH S-53482, APF- (6-amino-7-fluoro-4-(2-propynyl)-1,4,-benzoxazin-3(2H)-one)



S-53482

Mode of action

Flumioxazin is a specific inhibitor of protoporphyrinogen oxidase. Inhibition of mitochondrial PPO in mammals results in cumulation of protoporphyrinogen IX, dislocation

and non-enzymatic oxidation of protoporphyrinogen IX to protoporphyrin IX.. This mechanism explains all observed external effects and no other primary mechanism has been proposed to explain the pathogenesis of flumioxazin toxicity. Direct interaction with any other enzyme of the porphyrine metabolism chain (i.e. ALA synthase – transaminase, porphobilinogen synthase – deaminase, uroporhyrinogen III synthase , uroporhyrinogen III decarboxylase and coproporphyrinogen oxidase) is practically ruled out and no cumulation or excretion of the respective porphyrine intermediates has been reported in flumioxazin administered animals.

Species specific higher rat sensitivity to haematological toxicity of flumioxazin is supported by absence of anaemia in adult mice, rabbits and dogs after repeated exposure to higher doses than doses inducing anaemia in adult rat, and absence of anaemia in patients with various forms of porphyria, including variagate porphyria patients.
Direct comparison with human subjects in *in vivo* experiments is not feasible. *In vitro* studies confirm substantial differences (see table). Two new studies of this type have been recently conducted. CD36+ cells, derived from human cord blood, are precursor of erythroblasts which can be stimulated to differentiate into haem-synthesizing cells under appropriate culture conditions, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells. The results of the study demonstrated that there were no effects on haem content and cell number of human haem-synthesizing cells at up to 5 µM flumioxazin although PPIX concentration in them increased more than ten times. Similarly, rat erythroleukemia cells can also differentiate into haem-synthesizing cells and may be more convincingly compared to human K562 cells: flumioxazin at 0.1µM and above reduced haem production in them, whereas no such effect was observed at 5.0 µM in human K562 cells..

Table: Flumioxazin effect in rat and man compared *in vitro*

Dose of flumioxazin	Tissue concentr.	PPIX liver – rat	PPIX liver – man	PPIX ELR - rat	Haem ELR rat	PPIX K562 - man	Haem K562+ man	PPIX CD36+ man	Haem CD36+ man
mg/kg bw *	µM	pg /mg protein	pg /mg protein	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6 cells	ng/ 10^6cells
0	0	293	180	0.56	127	0.19	208	1.18	1777
0.018 +	0.00007 +								
4	0.01			0.58	116	0.19	202	1.77	1706
15	0.03	370	190						
30 repeated	0.06 mean								
50	0.1			0.80	91	0.18	186	1.59	2198
30 repeated	0.2 peak								
200	0.3	1200	400	0.95	85				
	1.0	3000	800	2.67	60	0.44	224	2.82	1882
1000	2.4/1.9**								
>1000	5.0			8.32	47	3.0	213	14.0	1535

+ AOEL; absorption rate for oral dose levels ≤ 0.01 mg/kg bw is presumed to be almost 100%
*Flumioxazin oral dose X) corresponding to average tissue concentration. Systemic dose (Y) = X x (oral absorption rate); tissue concentration = Y / MW. Measured oral absorption rates were 89, 50,

35 and 12.4% for oral doses of 1, 30, 100 and 1000 mg/kg bw, respectively. Decrease in absorption rate with dose is represented by regression equation: Y = 130 x (1 – EXP(-0.003xX)) ; dose in the last line is estimated for absorption rate ≤ 10% (≤0.1)
**peak concentration in maternal blood /foetal tissue predicted by PBPK model (Takaku, 2012c)

Species specific sensitivity to flumioxazin effects on haematopoiesis appeared in subchronic and chronic studies in adult rats. No clinical findings are reported in flumioxazin exposed rats up to the highest concentrations in diet (3000 ppm) corresponding to daily doses of about 200 mg/kg bw. Significant (but slight) changes in the haematopoiesis included moderate decrease in haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and hematocrit values for males in the 1000 (≈60 mg/kg bw) and 3000 ppm groups, and for females at 300 ppm (≈20 mg/kg bw) and greater; extramedullary haematopoiesis was observed in the spleen of males at 1000 ppm and greater and in females at levels of 300 ppm and greater.

Higher sensitivity of rat PPO to flumioxazin is only a secondary factor involved in the species specificity of observed developmental toxicity.
In the two-generation study, P_1female rats receiving 300 ppm diet both body weight and body weight gain were reduced and there was an increase in litter resorptions.
Flumioxazin oral dose of 30 mg/kg bw per day caused evident developmental toxicity in rats. No such effects have been observed in rabbits at doses per kg bw by two orders of magnitude higher. In contrast, the differences between these two species in flumioxazin absorption, distribution and elimination were small: after repeated oral administration of the same dose per kg bw the concentration in blood, maternal tissues and fetus were lower in rabbits than in rats by about one third 2 hours after the first dose and by more than 50% 24 hours after the first dose and after repeated dosing. The concentration of flumioxazin in foetuses of rabbits was half that in rat foetuses. In contrast, accumulation of PPIX under the effect of flumioxazin has been observed in rat foetuses but not in rabbit foetuses.
The developmental effects in foetuses of rats administered flumioxazin on GD 12 (at 400 mg/kg) included: severe anaemia (decreased red blood cell count to approx. 1/3 and 1/2 of values in controls on gestation days 14 and 16, respectively), enlarged heart (by 60% on days 14 and 15), oedema, delayed closure of the interventricular foramen (0% and 21% compared to 72% in controls on day 16, and 86% on day 17, see table), reduced serum protein and incomplete/delayed ossification.

Table: Time course of haematological effects of flumioxazin (400 mg/kg bw orally, GD12) in rat foetus

Flumioxazin	Gest. Day			13	14	15	16	17		20
0	Ery count	10^6/µL		0.35	0.6	0.6	0.8	1.4		2.3
+	Ery count	10^6/µL		0.15	0.2	0.3	0.35	1.0		2.2
+	Heart size	% contr.		100	160	161	125	110		100
0	IVF closure	% foetuses		0	0	0	72	86		95
+	IVF closure	% foetuses		0	0	0	0	21		55

At this developmental stage, yolk sac erythropoiesis is the primary source of new circulating foetal erythrocytes. As shown in the study by Ihara (2011), in rats are erythroblasts produced in a single wave: GD 11: more than 95% of blood cells were basophilic erythyroblasts; GD12: predominant cell type were polychromatophilic erythroblasts; GD13: polychromatophilic erythroblasts constituted about 95% of embryonic blood cells; GD 14: orthochromatophilic erythroblast population (postmitotic cells) became the predominant cell type. The primary erythroid cell population affected by flumioxazin is

the population of polychromatophilic erythroblasts. A characteristic of hemopoiesis in yolk sac of rat embryos is that erythroid cells undergo synchronous maturation as a relatively homogeneous population. Synchronized differentiation of erythroblasts in rat embryos does not alow for an effective compensation of haem synthesis inhibition in citical gestational days 12 to 14. Fresh blood cells would not be supplied until
haemopoiesis shifts from the yolk sac to the liver (GD 17) : lower output of mature erythrocytes in flumioxazin rats has been completely compensated and no anemia has been observed on GD20.
Such a single wave of erythroblast formation in a short period coinciding with rapid organogenesis does not occur in humans. In humans, erythroblast formation in yolk sac is characteristic for embryonal days 20 – 50 and is extended over a period of several weeks; haematopoiesis then shifts to liver and finally to bone marrow. Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at an exposure equivalent to a maternal dose exceeding 1000 mg/kg/day. Similar association between foetal anaemia, retarded ossification and morphological anomalies of heart incl. delay of timely IVF occlusion in rats has been confirmed, e.g in preclinical studies of antimalaric drugs (e.g. Schmuck et al.) in doses used in pregnant women without adverse developmental effects. Higher doses caused even more severe foetal anaemia and late resorptions of rat foetuses. (Schmuck G, Klaus AM, Krötlinger F, Langewische FW Developmental and reproductive toxicity studies on artemisone. Birth Defects Res B Dev Reprod Toxicol. 2009 Apr;86(2):131-43.)

<u>Clasification of flumioxazin for developmental toxicity</u>.
The teratogenic effect in rats at doses not toxic to mother animals is clearcut, but their relevance for humans is not.
Applicant assessed them as not relevant for humans. RMS agrees that they probably are not relevant..
According to Regulation (EC) No 1272/2008:

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

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely?
Regulation (EC) No 1272/2008, subpoint 3.7.2.3.2: admits not to classify such substances:

> *„ . . If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans <u>or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans</u> then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."*

The developmental effects of flumioxazin in rat foetuses were observed at maternal dose of 30 mg/kg bw per day (systemic dose of 15 mg/kg bw). The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect. RMS agrees that difference in sensitivity of protoporphyrinogen oxidase in human and rat hepatocytes to inhibition by flumioxazin doesn't appear to be very large, namely 2.4. For a systemic dose of 15 mg/kg bw, systemic dose predicted to cause similar PPO inhibition in

human liver would be 15x2.4 = 36 mg/kg bw and respective oral dose 108 mg/kg bw per day (taking into account decrease in absorption rate with oral dose): that means, for instance, pregnant woman receiving orally more than 6000 mg of flumioxazin daily (> 6000 ADI) for two first months of pregnancy. Moreover, this calculation does not consider two more influential differences between prenatal development in man and in rat:
1) Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM, corresponding to doses > 1000 mg/kg bw.
2) Much longer time between the end of yolk sac haematopoiesis and final foetal stages in humans (from 2 to 9 months) compared to rats (from GD 16 to GD 20) allows for a complete compensation of delays - if any - in the morphological development of human foetus (such as development of the interventricular septum).
All these one-direction points of dissimilarity raise doubt about the relevance of this hazard for humans.

1)
Anemia, attributable to PPO inhibition, is the primary toxic effect in rats caused both in adults and embryos. Sumitomo has investigated whether or not PPO inhibition in erythroblasts can cause anemia in humans.
To experimentally demonstrate that human erythroids are resistant to the disturbance of heme synthesis and induction of anemia by flumioxazin-induced PPO inhibition, Sumitomo conducted a study with K562 cells, which are derived from human erythroleukemia. They are used as a model for human erythroid maturation since K562 cells can be differentiated into hemoglobin-synthesizing cells by treatment with various inducers. Although accumulation of PPIX resulting from PPO inhibition was observed in K562 cells, no effects were observed on heme content and cell proliferation even when treated with 5 µM of flumioxazin. Sumitomo further conducted a study, the report of which was posted during the period of public comment (see Comment number 6), with CD36+ cells which are isolated from human cord blood. CD36+ Cells are a precursor of erythroblasts, which can be differentiated into heme-synthesizing cells, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells, hence better addressing effects on heme biosynthesis in human fetal erythroid cells. The results of the study demonstrated that there were no effects on heme content and cell number of human heme-synthesizing cells treated with flumioxazin at 5 µM.
A physiologically based pharmacokinetic (PBPK) model for flumioxazin was developed to predict flumioxazin concentration in the maternal blood and fetus of pregnant human. An *in vitro* metabolism study using rat and human liver microsomes was conducted to analyze the species differences in the metabolism of flumioxazin between rat and human. In addition, a biliary excretion study was conducted in bile duct-cannulated female rats to determine the % absorbance of flumioxazin after oral administration at 1000 mg/kg. The developed human pregnant model demonstrated that flumioxazin concentration in the human fetus at dose of 1000 mg/kg po was 0.68 ppm (1.92µM). This concentration is lower than the maximum no effect concentration of 5 µM in K562 and CD36+ cells, supporting the view that humans would not be susceptible to anemia and the developmental effects of flumioxazin.
Human erythroblasts are considered to be non-susceptible to flumioxazin when treated at concentrations as high as 5 µM. These concentrations are expected to far exceed those attained in human embryos following flumioxazin exposure.
2)
As discussed in "An update of a discussion on human relevance of the developmental effects induced by flumioxazin in rats" (SBT-0122), Sumitomo has investigated whether or not PPO

inhibition in erythroblasts can cause anemia in humans. Porphyrias are disorders in which the activities of the enzymes of the heme biosynthetic pathway, including PPO, are deficient. They can be classified as either hepatic or erythropoietic, depending on the principal site of expression of the specific enzymatic defect. The tissue-specific expression of porphyrias is largely due to the tissue-specific control of heme pathway gene expression, especially at the level of aminolevulinate synthase (ALAS), the first and rate-limiting enzyme of heme biosynthesis. In liver, hemoprotein enzymes are rapidly turned over in response to current metabolic needs. The activity of ALAS1, the housekeeping isoenzyme of ALAS, in normal liver is the lowest among all enzymes in the heme biosynthetic pathway. In erythroid cells, the activity of ALAS2 (the erythroid-specific isoenzyme of ALAS) is induced only during the period of active heme synthesis, and is regulated by the amount of free iron present.

Variegate porphyria (VP) is a disease associated with PPO deficiency. VP is categorized as hepatic porphyria and the main symptoms are neuronal manifestation and dermal inflammation. Hepatic porphyrias usually do not include anemia or hematological problems. Anemia was not found in hepatic porphyrias attributable to marked deficiency of delta aminolevulinic acid dehydratase (ALAD), coproporphyrinogen oxidase (CPO), or PPO. This suggests that defective enzymatic activity resulting in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells. Variegate porphyria is associated with reduced PPO content and ALAD activity in erythrocytes. Erythrocytes counts were not affected by VP and hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin in VP were slightly higher than their controls. The low rate of heme production in VP is enough to generate the same, or even greater, quantity of hemoglobin as control women.

In contrast to VP, erythropoietic protoporphyria (EPP) resulting from deficiency of ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway, sometimes includes mild anemia with hypochromia and microcytosis or mild anemia with reticulocytosis. Microcytic anemia occurs in 20% to 60% of patients. Erythropoiesis was impaired in most patients with dominant EPP from the UK and France and all had a downward shift in hemoglobin. FECH deficiency in EPP results in the accumulation of protoporphyrin almost exclusively in erythroid tissue, even though FECH is deficient in all other tissues in these patients. This finding suggests that FECH activity can become rate limiting in erythroid cells, but not in other tissues when the enzyme itself or its substrate, iron, is partially deficient.

Recently families with X-linked, dominant protoporphyria (XLDPP) have been described. Patients with this disorder have normal FECH activities, indicating that protoporphyrin accumulation is not caused by FECH deficiency. Patients showed neither anemia nor iron overload. Disruption of the C-terminal region of ALAS2 leads to markedly increased ALAS2 activity and the production of protoporphyrin in excess of the amount required for hemoglobinization. These findings suggest that the rate of ALA formation is increased to such an extent that insertion of Fe into PP by FECH becomes rate limiting for heme synthesis.

These clinical findings demonstrate that PPO activity in human erythroid cells is much higher than FECH or ALAS2 activity, which is rate-limiting in heme biosynthetic pathway in human erythroids. It is therefore unlikely that PPO deficiency would induce anemia or disturbances of heme synthesis in human erythroid cells. In contrast, the results of toxicity studies in rats suggest that in rat erythroid cells, PPO activity is close to a rate-limiting enzyme activity. Therefore decreased PPO activity becomes rate-limiting in porphyrin production in erythroids resulting in PPIX accumulation, iron deposit, and anemia.

Enzymatic activities from various tissues are presented in Table. Although the data are derived from non-erythroid tissues, we present them to illustrate relative activities in human and rat tissues. In humans PPO activity could be higher than other enzymes. In rats

FECH activity varies and PPO activity is not necessarily higher than FECH.

Table Activities of enzymes in the heme synthetic pathway (nmol/h/mg protein)

		ALAS	CPO	PPO	FECH
Human	Fibroblast	0.003 - 0.005	-	2.12	0.032
	Liver	-	0.6	10.8	3.72
	Leucocyte	-	-	8.73	0.24
rat	Liver (mitochondria)	-	-	3	87
	Liver (homogenate)	-	1.2	10.2	3.18 - 4.2
	Liver (mitochondria)	0.61	2.7	8.5	8.0

- : not reported

3)

Sumitomo further conducted a study with human CD36+ cells, which are isolated from human cord blood, and the report of the study was posted during the period of public comment (see Comment number 6). CD36+ Cells are a precursor of erythroblasts, which can be differentiated into heme-synthesizing cells, and are more relevant to physiological maturation of human fetal erythroblasts, hence better addressing effects on heme biosynthesis in human fetal erythroid cells. The results of the study demonstrated that there were no effects on heme content and cell number of human heme-synthesizing cells treated with flumioxazin at 5 μM.

Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0126.

4)

The proposed mechanism was based on a series of studies designed to elucidate the bases of the species specific developmental toxicity produced by flumioxazin in rats but not in rabbits. These studies were evaluated during the previous review of flumioxazin for Annex 1 inclusion and were summarized in the DAR. The proposed mode of action was endorsed by the Scientific Committee on Plants (Opinion SCP/FLUMIO/002-Final 23 May 2001).

The proposed mode of action was as follows;

a) Flumioxazin inhibits protoporphyrinogen oxidase (PPO). Its inhibition results in degeneration of fetal erythroblasts leading to anemia.

b) Severe fetal anemia leads to the fetal death.

c) Surviving fetuses are growth-retarded as indicated by a decrease in body weight. They compensate for this anemia by pumping a greater volume of blood which leads to observed enlargement of the heart just prior to closure of the interventricular foramen. This result in delayed closure of the foramen represented as ventricular septal defects (VSD) in the term fetus due to mechanical distortion of the heart or abnormal blood flow.

d) Concurrently serum protein is decreased in the fetus resulting in wavy ribs.

As summary it has been demonstrated:

a) A strong correlation exists between protoporphyrin XI (PPIX) accumulation, considered to result from PPO inhibition, and developmental toxicity. Evidence for this correlation is based on differences between rats and rabbits, the critical period of sensitivity to developmental effects in rats, and compound-specific differences between chemicals of the N-phenylimide family. Protoporphyrin IX accumulates in rat fetuses in response to flumioxazin but not in rabbit fetuses (SBT-0061). Protoporphyrin IX accumulation is observed when rat fetuses are treated with developmentally toxic compounds that cause significant PPO inhibition (SBT-0062). The peak period of PPIX accumulation in rat fetuses corresponds to its developmental effects (SBT-0063, SBT-30-0044). This correlation demonstrates a close link between PPO inhibition and developmental abnormality.

b) Histological examination of rat fetuses at light and electron microscopic levels after oral administration of flumioxazin (1000 mg/kg) to dams on gestational day 12 (the day of greatest sensitivity) demonstrated mitochondrial lesions. These included abnormal iron deposits, probably due to inhibition of heme biosynthesis, in polychromatophilic erythroblasts that were observed as early as 6 hours after treatment. Subsequent degeneration of these erythroblasts was indicative of fetal anemia (SBT-0064). Histological examination of hearts from exposed embryos revealed thinning of the ventricular wall by 36 hours after treatment. This may reflect compensation for the loss of embryonic blood cells. Therefore, the VSD caused by flumioxazin appears to result from inhibition of heme biosynthesis rather than from direct injury to embryonic heart tissue. No effects were observed in rabbits treated in the same manner as rats (SBT-0064). The observed difference in histological changes between rats and rabbits completely corresponds to those of developmental toxicity and PPIX accumulation caused by exposure to flumioxazin.

c) Observations in the pathogenesis of developmental effects of flumioxazin in rat fetuses included: anemia, reduced serum protein, enlarged heart, edema, delayed closure of the interventricular foramen, and incomplete/delayed ossification of the ribs. Severe fetal anemia is observed up to gestational day 16 following treatment with flumioxazin (400 mg/kg) on gestational day 12. Fetal death occurs by gestational day 15 as additional lethality is not observed from gestational day 15 through 20. Enlarged heart is seen among surviving fetuses in concurrence with severe fetal anemia. This suggests that enlarged heart results from pumping greater volumes of blood in compensation for fetal anemia. Enlargement of the heart precedes interventricular foramen closure. Therefore, the VSD caused by flumioxazin is due to failure of heart closure resulting from mechanical distortion of the heart or abnormal blood flow rather than from direct toxic effects of flumioxazin on cardiac tissue. Concurrently, decreased serum protein is observed in the fetus, presumably due to reduced production in the liver in response to hypoxia. The resulting osmotic imbalance causes edema. Reduction of fetal serum protein leads to incomplete/delayed ossification of the ribs and the wavy ribs seen at term (SBT-0065).

d) Species- and compound-related differences were observed in *in vitro* inhibition of PPO and *in vitro* inhibition corresponded closely with PPIX accumulation and teratogenicity. Thus, PPO inhibition is considered to be the primary cause of developmental toxicity in rats. Sensitivity of PPO activity extracted from adult female liver was found to be comparable to that of embryonic PPO, suggesting that inhibition of adult liver PPO is indicative of embryonic PPO inhibition. Based on the relative sensitivity to inhibition of adult liver PPO in the three species tested (rat>human>rabbit), risk assessments using the NOAEL for studies in the rat protect humans more than adequately (SBT-31-0045, SBT-0060).

Other modes of action were evaluated as follows.

<u>Accumulated PPIX</u>
Because PPIX accumulation corresponded to the developmental toxicity of flumioxazin, it might be assumed that developmental toxicity of flumioxazin is mediated through the same mode of action as the herbicidal one. If photodynamic action is a cause of developmental toxicity, a photodynamic dye should be a developmental toxicant. However a photodynamic dye, rose bengal, exhibited neither embryolethality nor teratogenicity in rats (1). The light would not sufficiently reach embryos through the maternal body wall to induce photodynamic action of accumulated PPIX in embryos.
Protoporphyrin IX is assumed to be an endogenous ligand to the peripheral benzodiazepine receptor on mitochondria. Presumably acting through the receptor, PPIX suppressed DNA

replication in mouse spleen lymphocytes *in vitro* (2). Nevertheless many of the benzodiazepoines such as diazepam, lorazepam, clonazepam, and oxazepam failed to exhibit teratogenicity in rats (3). Accumulated PPIX is considered to be indicative of PPO inhibition rather than a causative factor in teratogenicity.

Form of anemia

Flumioxazin caused hypochromic microcytic anemia that generally occurs as a consequence of impaired hemoglobin synthesis (4). Impairment of hemoglobin synthesis is caused by iron-deficiency or defective porphyrin metabolism resulting in abnormal iron accumulation in erythroblasts termed sideroblasts. As indicated in mechanistic studies, iron deposition in mitochondria was an initial histological change and increased sideroblasts were observed. Flumioxazin induced anemia is due to inhibition of porphyrin metabolism rather than iron deficiency.

Relationship between fetal death and malformation

In some cases fetal death is attributable to malformation. Beck and Lloyde investigated the relationship between fetal deaths and fetal malformation by treating rats with trypan blue at day 8.5 and examining the uteri and fetuses on days 11.5, 14.5 and 20.5 of gestation (5). Because the incidence of fetal malformations fell with a corresponding rise in fetal deaths as pregnancy proceeded, the authors concluded that fetal death was a result of pre-existing fetal malformation in the majority of cases. In the flumioxazin studies, most of the dead fetuses were observed by day 15 while VSD can be diagnosed following completion of closure of the interventricular foramen on day 16. Consequently, fetuses were dead prior to closure of the interventricular septum indicating that VSD is not the direct cause of conceptal death but rather occurs in some surviving fetuses. This supports fetal anemia as the cause of embryonic deaths rather than deaths from malformations.

VSD

Initial histological changes observed in rat embryos were iron deposits in mitochondria and dilatation of mitochondrial matrix space in polychromatophilic erythroblasts. Following the mitochondrial lesions, affected erythroblasts degenerated in the embryonic circulation and were engulfed by macrophages. Treatment-related changes in the embryonic cardiovascular system and liver accompanied the appearance of erythroblastic lesions. Thinning of the ventricular walls of the heart is indicative of dilatation of the ventricles, and reflects a compensatory reaction to embryonic anemia since enlargement of heart was observed corresponding to decreased hemoglobin content and reduced red blood cell number. Increased stroke volume in the heart is an important reaction to anemia (6). No treatment-related changes in myocardial cells were observed at the electron microscopic level. As noted previously, the interventricular foramen closes from day 15 to day 16. In our studies, exposed hearts were enlarged from day 14 and completion of ventricular septa formation was delayed.

Clark has proposed five pathogenic modes of actions for some congenital cardiac malformations based on mechanism rather than anatomic anomaly. They are ectomesencymal tissue migration abnormalities, abnormal intracardiac blood flow (cardiac hemodynamics), cell death, extracellular matrix, and abnormal targeted growth (7). Clark stated that perimembranous ventricular septal defect may represent abnormal fusion of the muscular, inflow and outflow septa, and that deviation of the septal components by abnormal blood flow pattern may lead to defects in this region of the heart (8).

A comparison of sensitive periods for development of VSD between flumioxazin and several other agents shows considerable differences in peak sensitivity. X-ray irradiation (9) and nimustine (10), an alkylating agent, or bisdiamnie (11), which acts on the proliferation or migration of mesencyme, probably produce VSD by direct injurious effects (cell damage) on the fetal heart. The peak of sensitivity to these agents occurs between days 8 – 10, while the most sensitive day for flumioxazin-induced-VSD is gestational day 12.

Earlier studies by Haring (12) and Clemmer and Telford (13) support the proposed

mechanism by showing that prenatal hypoxia produces cardiovascular abnormalities including VSD in rats. Jaffee stated that, when hypoxia was used as a teratogenic agent following the onset of circulation, distortion of the form of the heart tube was a primary lesion (14).
Thus, overall it is concluded that VSD caused by flumioxazin is attributed to fetal anemia and not to any other direct injurious effect on the heart.

Wavy ribs

Wavy ribs are induced in the later stages of rib chondrification and ossification, and may be indicative fetal pathology as opposed to malformations. It may be possible that many agents produce wavy ribs through several mechanisms leading to two final common effects including inhibition of mineralization and increased uterine tone. Renal loop diuretics and beta-stimulants have been studied in detail because they are associated with a high incidence of wavy ribs. Maternal serum chloride was decreased after treatment with furosemide, a renal loop diuretics. Co-administration of a muscular relaxant reduced the incidence of wavy ribs after furosemide exposure. Decreased fetal serum alkaline phosphatase and total protein were reported following exposure of fenoterol, a beta-stimulant (15). Flumioxazin decreased fetal serum protein and increased incomplete/delayed ossification of the ribs. The increased incidence of wavy ribs is more likely to be associated with these changes rather than being caused by a different mechanism.

Link between fetal anemia and developmental toxicity

A link between fetal anemia and developmental effects observed in the flumioxazin teratogenicity study is also demonstrated in recent studies with artesunate, an anti-malarial drug. Artesunate induces developmental abnormalities consisting of fetal death, growth retardation and anomalies such as VSD, rib abnormalities and bent long bones (16). Embryonic erythroblasts are the primary target of artesunate toxicity and consequent embryonic anemia resulted in developmental toxicity similar to that produced by flumioxazin (17).

Species difference in metabolism between rats and rabbits

When pregnant rats and rabbits received oral administration of ^{14}C-flumioxazin at 30 mg/kg for seven consecutive days, no clear pattern of absorption, distribution, metabolism or excretion was seen that could account for the species specific developmental toxicity in rats. After initial dose, Cmax/min of ^{14}C concentration in plasma ranged from 4.49 to 0.70 in rats and from 4.14 to 1.02 in rabbits. In both species most of the previous dose of ^{14}C was excreted before the next dose, and the metabolic profiles of flumioxazin were similar (18).

PPO inhibitory activity of metabolites

Oral doses of [phenyl-^{14}C] flumioxazin (30 mg/kg) administered to pregnant rats from gestational days 6 through 12 cross the placenta and reach the rat fetus. Major metabolites in the fetus included 3OH-flumioxazin, 4OH-flumioxazin, and APF (18). In order to determine the active form that inhibits PPO, we employed *in vitro* PPO inhibition assays using liver extracts prepared from adult, female livers. Experiments were conducted with flumioxazin and its three major metabolites (19). The results showed that flumioxazin was the strongest PPO inhibitor. There was no metabolite that could account for the species-specific developmental toxicity in rats based on the degree of PPO inhibition.
The possibility of a direct effect of metabolites on developmental toxicity is also considered. APF was detected at higher concentrations in rat fetuses compared with other metabolites. It is a benzoxazinone moiety formed from cleavage of the amide linkage. There is convincing evidence that embryolethality and VSD are attributed to the consequences of fetal anemia and that the fetal malformations are not the causative factors in embryonic deaths. The spectrum of developmental effects associated with flumioxazin is consistent with a single mode of action. Therefore, it is considered very unlikely that a metabolite would be a direct acting teratogen causing VSD and skeletal anomalies by a mechanism that

was independent of fetal anemia and embryolethality. Furthermore, the main metabolite APF was also detected in rabbit fetuses and no developmental toxicity was seen in rabbits at a dose level 100 times higher than that causing developmental toxicity in the rat. Fetal concentrations of metabolites at this dose level in the rabbit would be much higher than those in the rat.
In conclusion there is no compelling evidence for any other MOA for the developmental toxicity of flumioxazin in the rat.
The available data, in concert with published study results, shows that the available evidence is robust to support the proposed mode of action and that the key event is fetal anaemia. Based on PBPK model prediction, it is implausible that a fetal concentration of flumioxazin could be achieved in humans following exposure that would result in toxic insult to the fetal erythroblasts.
Effects on activity of enzymes involved in the heme biosynthesis
A strong correlation exists between protoporphyrin IX (PPIX) accumulation, considered to result from protoporphyrinogen oxidase (PPO) inhibition, and developmental toxicity. Evidence for this correlation is based on differences between rats and rabbits, the critical period of sensitivity to developmental effects in rats, and compound-specific differences between two chemicals of the N-phenylimide family. The latter are structurally related to flumioxazin: one (S-23121) that produces developmental effects in rats and one (S-23031) that does not (PPT-00-0023, SAT-11-0024). Protoporphyrin IX accumulates in rat fetuses in response to flumioxazin but not in rabbit fetuses (SBT-0061). Protoporphyrin IX accumulation is observed when rat fetuses are treated with the developmentally toxic compounds (flumioxazin and S-23121) that cause significant PPO inhibition (SBT-0062). The peak period of PPIX accumulation in rat fetuses corresponds to its developmental effects (SBT-0063, SBT-30-0044). Species- and compound-related differences were observed in *in vitro* inhibition of PPO, and *in vitro* inhibition also corresponded closely with PPIX accumulation and teratogenicity. These correlations demonstrate a close link between PPO inhibition and developmental abnormality.
In addition, flumioxazin inhibits PPO at very low concentration, suggesting high specificity to PPO. Thus, PPO inhibition is considered to be the primary cause of developmental toxicity in rats.
Flumioxazin did not affect normal heme synthesis in human cells. Therefore flumioxazin did not alter activities of any enzymes including PPO involved in human heme biosynthesis to such a degree that normal heme biosynthesis was interfered and anemia was induced. Sumitomo conducted studies with CD36+ and K562 cells. Human erythroblasts are considered to be non-susceptible to flumioxazin when treated at concentrations as high as 5 µM. The concentration is expected to far exceed those attained in human embryos following flumioxazin exposure, since a developed physiologically based pharmacokinetic human pregnant model for flumioxazin demonstrated that flumioxazin concentration in the human fetus at dose of 1000 mg/kg po was 1.92µM (SBM-0089). In the studies significant inhibition of any enzymes in heme biosynthesis can be detected as reduced heme content or decreased cell number, and no effects were observed on heme content and cell number in human cells treated with flumioxazin. Flumioxazin is considered not to cause inhibition of any enzymes in human heme biosynthesis, which interferes with normal heme biosynthesis and leads to anemia, although inhibition of each enzyme in heme biosynthetic pathway was not investigated.
Herbicidal mode of action of N-phenylimide herbicides is similar to that of diphenyl ether herbicides. Diphenyl ether herbicides inhibit PPO, but not ferrochelatase in corn, potato, mouse and yeast (Matringe M et al. (1989) Protoporphyrin oxdase as a molecular taget for diphenyl ether herbicides. Biochem. J. 260)

A link between fetal anemia and developmental effects observed in the flumioxazin

teratogenicity study is also demonstrated in recent studies with artesunate, an anti-malarial drug. Artesunate induces developmental abnormalities consisting of fetal death, growth retardation and anomalies such as VSD, rib abnormalities and bent long bones (Clark RL et al, 2008. Developmental toxicity of artesunate in the rat: Comparison to other artemisinins, comparison of embryotoxicity and kinetics by oral and intravenous routes, and relationship to maternal reticulocyte count. Birth Defects Research (Part B) 83:397-406.). Embryonic erythroblasts are the primary target of artesunate toxicity and consequent embryonic anemia resulted in developmental toxicity similar to that produced by flumioxazin (White TEK et al, 2006. Artesunate-induced depletion of embryonic erythroblasts precedes embryolethality and teratogenicity in vivo. Birth Defects Research (Part B) 77:413-429.).

Variegate Porphyria (VP) is hepatic porphyria. Porphyrias can be classified as either hepatic or erythropoietic, depending on the principal site of expression of the specific enzymatic defects. Symptoms of VP are due to hepatic PPO inhibition. Patients present after puberty with skin lesions or with acute nuerovisceral crisis, or with both together. Clinically silent VP is at least five times commoner than overt disease. Hepatic porphyrias usually do not accompany anemia or haematological problems. This fact suggests that a defective enzymatic activity that results in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells. In reviews, neurovisceral symptoms and photosensitivity are referred as major types of clinical features of VP. In a recent article results of hematological analysis of VP were reported and no anemia was observed.

Other toxicities

Flumioxazin can lead to accumulation of toxic porphyrin metabolites. Toxic porphyrin metabolites are associated with phototoxicity in humans. The protoporphyrin molecule absorbs light radiation in a range of wavelength from 320 to 595 nm (Lecha M et al., 2009). In a rat chronic / carcinogenicity study, there were no abnormal skin regions even in the highest dose group (1000 ppm (36.5 mg/kg/day (male), 43.6 mg/kg/day (female)), in which a decrease in haemoglobin concentration (caused by PPO inhibition) was observed (SBT-30-0040). Although this study was conducted in room lighting condition, it is reported that standard fluorescent light with a 12h- light / 12h- dark cycle could induce phototoxic ear lesion in mice (Jonker JW et al., 2002). In addition, a medical surveillance report conducted on manufacturing plant personnel (SBT-0116) revealed no evidence of haematotoxicity, or other adverse health effects in workers (n=15) who have been involved in the manufacture of flumioxazin for the last decade. This is considered to demonstrate not only effective uses of personal protective equipment, but also the intrinsic low toxicity of flumioxazin. In conclusion, there is no evidence that flumioxazin exposure is associated with phototoxicity.

Lecha, M et al. 2009. Erythropoietic protoporphyria, Orphanet J Rare Dis, 4:19-28.
Jonker, JW et al. 2002. The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria, Proc Natl Acad Sci U S A, 99:15649-15654.

Toxic porphyrin metabolites are also associated with neurological manifestations in humans. Sumitomo is submitting acute and subchronic neurotoxicity studies (SBT-0112 & SBT-0115) conducted for the US EPA. Acute and subchronic neurotoxicity studies in the rat demonstrate that flumioxazin does not have any neurotoxic potential and there was no evidence of neuronal developmental effects in the reproductive toxicity and developmental toxicity studies.

5)

Table 32 (p. 43) It says in one of the table column headings "Time after administration (h)" – shouldn't this be "day of exposure"?

<Answer/response> The indication is correct.

6)

"Selected" means specific day of treatment. Day 12 of gestation was selected as the day of administration for both species on the basis of data showing that rats were the most sensitive to flumioxazin on day 12 and that rabbit embryos on day 12 were similar to rat embryos in respective to the development.

RAC's response

The additional discussion by the applicant and RMS of the evidence and proposed mechanism is noted. RAC agrees with the MS that the described MOA is plausible and that the evidence is supportive of different sensitivity between species. However, the MOA is likely to operate in humans.

The reason for differences in PPO sensitivity between species is not known. The enzyme appears to be closely conserved and there is no relevant information in the literature about isoenzymes etc. The appendix 2 reference SBT-o122 evaluates other possible MOA, and concludes that none are feasible. RAC notes the notifiers additional discussion of other possible modes of action. The discussion of chemicals causing similar effects on the foetal cardiac system and the possibility of other toxic effects etc is noted.

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely ?

This question from the applicant raises the central element of the rationale for removing the classification as Repr. 1B. It is not denied that the hazard potentially exists for humans also, notwithstanding the possible interspecies differences in PPO sensitivity and the particular sensitivity of the rat foetus to induction of anaemia through this route. The issue is, should the element of risk be considered relevant to classification and if so, has this risk been proven to be negligible?

The applicant contends that the argument for no classification is supported by the criteria…..

… *If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified.*"

RAC does not agree that the mechanism has no relevance to humans.

RAC does not believe that the differences identified in the data set are 'toxicokinetic' other than the outcome of the PBPK modelling which suggests that the low levels arising in the foetus at a maternal dose of 1000 mg/kg would not cause foetal anaemia.

The reason for the apparent interspecies differences in PPO sensitivity demonstrated in hepatocytes *in vitro* is not known. The difference between humans and rats (approx. 2.4 - fold) is also not considered so marked that any potential risk is reduced to negligible levels. The main argument concerning the relative susceptibility of rat foetus to anaemia due to its synchronously differentiating erythroblasts is also not a toxicokinetic difference and while plausible, it is not proven that no significant damage will occur in the human foetus. While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Germany		MemberState	11

Comment received

Based on the submitted data, the German CA does not support the proposal to delete the classification for developmental toxicity of flumioxazin without further clarification of some aspects.
In our view clarification of the following issues is necessary before discussing the human relevance of the developmental effects induced by flumioxazin.
1. Is a fetal anaemia detectable at a dose of 30 mg/kg bw in rats, wherein the developmental effects have been observed?
2. The discussed relation between fetal anaemia and the formation of malformations is not convincing and has to be proved in more detail. Is there any evidence of other substances, which cause fetal anaemia and induce malformations like ventricular septal defects and scapular curvature?

Dossier Submitter's Response

1)No haematological data are available on foetuses of rats administered fluioxazin in a daily oral dose of 30 mg/kg bw from GD5 to Gd 16. On the other hand, significant changes in the haematopoiesis have been described in repeated dose study in adult female rats of the 300 ppm diet group (≈20 mg/kg bw per day): moderate decrease in haemoglobin concentration, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and hematocrit values.
The developmental effects in foetuses of rats administered single dose of 400 mg/kg bw flumioxazin on GD 12 included: severe anaemia (decreased red blood cell count to 1/3 and 1/2 of values in controls on days 14 and 16, respectively), enlarged heart (by 60% on days 14 and 15), oedema, delayed closure of the interventricular foramen (0% compared to 75% in controls on day 16), reduced serum protein and incomplete/delayed ossification of the ribs. As shown in the study by Ihara (2011), in rats are erythroblasts produced in a single wave and synchronized differentiation of erythroblasts in rat embryos does not alow for an effective compensation of haem synthesis inhibition in citical gestational days 11 to 14.

Flumioxazin	Gest. Day			13	14	15	16	17		20
0	Ery count	10^6/µL		0.35	0.6	0.6	0.8	1.4		2.3
+	Ery count	10^6/µL		0.15	0.2	0.3	0.35	1.0		2.2
+	Heart size	% contr.		100	160	161	125	110		100
0	IVF closure	% foetuses		0	0	0	72	86		95
+	IVF closure	% foetuses		0	0	0	0	21		55

2) Preclinical animal studies have shown similar relationship between foetal anaemia, retarded ossification and morphological anomalies of heart incl. delay of timely IVF occlusion in rats administered antimalaric drugs (e.g. Schmuck et al.) in doses used in pregnant women without adverse developmental effects. Higher doses caused severe foetal anaemia and late resorptions of litters in rats. (Schmuck G, Klaus AM, Krötlinger F, Langewische FW Developmental and reproductive toxicity studies on artemisone. Birth Defects Res B Dev Reprod Toxicol. 2009 Apr;86(2):131-43.)
Hematopoiesis has been suspected and confirmed as a potential target for developmental toxicity of drugs in the rat, and specially the role of the foetal anemia induced by drugs in the critical period of gestation (GD 11 - GD14) in adverse developmental outcome (eg. Shuey DL, Zucker RM, Elstein KH, Rogers JM.: Fetal anemia following maternal exposure to

5-fluorouracil in the rat. Teratology. 1994 Apr;49(4):311-9).

Classification of flumioxazin for developmental toxicity.
The teratogenic effect in rats at doses not toxic to mother animals is clearcut, but their relevance for humans is not.
Applicant assessed them as not relevant for humans. RMS agrees that they probably are not relevant..
According to Regulation (EC) No 1272/2008:

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

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely ?
Regulation (EC) No 1272/2008, subpoint 3.7.2.3.2: admits not to classify such substances:

„ . . If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."

The developmental effects of flumioxazin in rat foetuses were observed at maternal dose of 30 mg/kg bw per day (systemic dose of 15 mg/kg bw). The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect. RMS agrees that difference in sensitivity of protoporphyrinogen oxidase in human and rat hepatocytes to inhibition by flumioxazin doesn't appear to be very large, namely 2.4. For a systemic dose of 15 mg/kg bw, systemic dose predicted to cause similar PPO inhibition in human liver would be 15x2.4 = 36 mg/kg bw and respective oral dose 108 mg/kg bw per day (taking into account decrease in absorption rate with oral dose): that means, for instance, pregnant woman receiving orally more than 6000 mg of flumioxazin daily (> 6000 ADI) for two first months of pregnancy. Moreover, this calculation does not consider two more influential differences between prenatal development in man and in rat:
1) Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5µM, corresponding to doses > 1000 mg/kg bw.
2) Much longer time between the end of yolk sac haematopoiesis and final foetal stages in humans (from 2 to 9 months) compared to rats (from GD 16 to GD 20) allows for a complete compensation of delays - if any - in the morphological development of human foetus (such as development of the interventricular septum).
All these one-direction points of dissimilarity raise doubt about the relevance of this hazard for humans.

1)
Developmental toxicity induced by a single administration of flumioxazin during the sensitive period was identical to that caused by a repeated administration during the period of major fetal organogenesis. This fact indicates that underlying mode of action is identical between single and repeated administration studies. The single administration studies

demonstrated that key biological event to cause developmental effects is fetal anemia.
2)
A link between fetal anemia and developmental effects observed in the flumioxazin teratogenicity study is also demonstrated in recent studies with artesunate, an anti-malarial drug. Artesunate induces developmental abnormalities consisting of fetal death, growth retardation and anomalies such as VSD, rib abnormalities and bent long bones (Clark RL et al, 2008. Developmental toxicity of artesunate in the rat: Comparison to other artemisinins, comparison of embryotoxicity and kinetics by oral and intravenous routes, and relationship to maternal reticulocyte count. Birth Defects Research (Part B) 83:397-406.). Embryonic erythroblasts are the primary target of artesunate toxicity and consequent embryonic anemia resulted in developmental toxicity similar to that produced by flumioxazin (White TEK et al, 2006. Artesunate-induced depletion of embryonic erythroblasts precedes embryolethality and teratogenicity in vivo. Birth Defects Research (Part B) 77:413-429.).

RAC's response

RAC notes the additional clarification provided by the applicant and by the the RMS. RAC agrees that the mechanistic data raise doubts about the relevance of the observed effects to man on the basis of 1. relative PPO sensitivity and 2. the difference in importance of PPO activity to haem production between man and rats and 3. the issue of synchronous *vs* non-synchronous erythrocyte differentiation. There is evidence to support the possible reduction of the classification from Repr. 1B to Repr. 2. However, the doubts are not considered sufficient grounds for non-classification as Repr. 2 and that the current classification of Repr. 1 should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Belgium		MemberState	12

Comment received

The BE CA would like to thank the Dossier Submitter for detailed overview of various studies presented in the CLH dossier of flumioxazin. However, despite the strong arguments presented by the Czech CA, we have decided not to support the proposal for removal of the classification as Repr. Tox. 1B (H360D: May harm the unborn child). Our argumentation can be found below.
First of all we would like to underline that indeed flumioxazin was proven to cause embryo lethality, teratogenicity (mainly VSD and wavy ribs), and growth retardation in rats at 30 mg/kg in absence of maternal toxicity. We also agree that this effect was not seen in rabbits even at the maternal toxic level of 3000 mg/kg bw.
The Dossier Submitter argues that on basis of a weight of evidence approach a single mode of action causing the developmental toxicity in rat has been demonstrated. The mechanism postulated for the observed developmental effects is the inhibition of one of the key enzymes in the heme synthesis: the protoporphirinogen oxidase (PPO), which is leading to anemia. This mechanism would not appear in rabbit because of differences in sensitivity of this key enzyme to Flumioxazin, as demonstrated in vitro. This is also supported by the lack of evidence of PPIX accumulation in rabbit embryo, compared to the rat embryo where a pick of PPIX is observed during the critical period for sensitivity to the developmental effects. Nevertheless, it has to be kept in mind that placental transfer of flumioxazin and its metabolites was also proven.
The Dossier Submitter concluded also that humans are considered to unlikely develop anemia from PPO inhibition and listed following source of information to support their conclusions:
- the clinical findings, which according to the Dossier Submitter are proving that no signs of anemia were found in PPO deficient patients with Variegate Porphyria;
- the experimental evidence that flumioxazin and its metabolites do not reduce heme

production in K562 cells, which are derived from human erythroleukemia; and
- the current knowledge suggesting that humans are less sensitive to PPO inhibition than rats.
The argument of the absence of anemia in PPO deficient patients seems us too weak because it is well known that in genetic diseases, the expression of the genetic aberration can vary in the different tissues and for Variegate Porphyria patients, the liver is the major if not sole site of expression of the biochemical abnormality.
Can the absence of reduction in heme production in K562 cells be sufficient to exclude such a mechanism in vivo? Is the test validated for such extrapolation?
It is true that in vitro studies have shown that human hepatocytes are less sensitive to PPO inhibition than rat hepatocytes but rabbit hepatocytes are much more less sensitive than human hepatocytes (rat > human >> rabbit). We think that this argument could be used for risk assessment but not for hazard assessment. This mechanism is still relevant to human. Despite the very detailed analysis of arguments presented by the Czech CA, we found it still difficult to make a conclusion on the removal of the classification as Repr. Tox. 1B, since from our point of view the evidence is not sufficient to exclude the relevance of the mode of action in human. Additionally, we would like to ask the Dossier Submitter for a more detailed (and quantitative) presentation of human clinical data available. The possibility of reviewing available clinical data is essential in supporting of the proposed mode of action and species difference.

We understand that the proposal focuses on the change related to reproductive toxicity but we would like to ask to the Dossier Submitter if the hematotoxicity will be also reconsidered according to the CLP criteria. This effect was probably not considered sufficient for a classification as R48 under the 67/548/EEC because of the reversibility of the effect but according to the new criteria under the CLP regulation, both reversible and irreversible effects, if they are significant and can impair function need to be taken into consideration. The emphasis on the reversibility of the effects is less important under CLP.

Additionally, we have the following comments/ questions concerning the setting of some NOAELs:
1. Effects on developmental toxicity oral route: study by Hoberman (1991)
From the data presented in table 25 pg. 32 it seems that the NOAEL for maternal toxicity should be set at 300 mg/kg/d, since at 1000 mg/kg/d clear decrease of body weight gain (-17.6%) and food consumption (-9%) in comparison to the current controls was found. Can the Dossier Submitter provide data on any other parameters to justify the proposed conclusion?
2. Additional data on developmental toxicity: studies by Kawamura (1990b), Hoberman (1990), Lemen (1991a,b) (pg. 42-47)
Since no quantitative data have been included in the dossier, conclusions on the set NOAELs cannot be reviewed at the moment. Could the Dossier Submitter provide more detailed data regarding above mentioned studies?

Dossier Submitter's Response

The teratogenic effect in rats at doses not toxic to mother animals is indisputable. The relevance of this hazard for humans is not. To contribute to discussion of species specificity and broader relevance, following text incorporating also data from two new *in vitro* studies and quantitative details in some parts of the document is proposed.

<u>Species specificity and hazard relevance</u>
In the two-generation study, P_1female rats receiving 300 ppm diet both body weight and body weight gain were reduced and there was an increase in litter resorptions.

Flumioxazin dose of 30 mg/kg bw caused evident developmental toxicity in rats. No such effects have been observed in rabbits at doses per kg bw by two orders of magnitude higher. The interspecies differences in flumioxazin absorption, distribution and elimination were small: After repeated oral administration of the same dose per kg bw the concentration in blood, maternal tissues and fetus were lower in rabbits than in rats by about one third 2 hours after the first dose and by more than 50% 24 hours after the first dose and after repeated dosing. The concentration of flumioxazin in foetuses of rabbits was half that in rat foetuses. The developmental effects in foetuses of rats administered 400 mg/kg bw flumioxazin on GD 12 included: severe anaemia (decreased red blood cell count to approx. 1/3 and 1/2 of values in controls on gestation days 14 and 16, respectively), enlarged heart (by 60% on days 14 and 15), oedema, delayed closure of the interventricular foramen (0% compared to 72% in controls on day 16, see table), reduced serum protein (up to GD 16) and incomplete/delayed ossification.

Flumioxazin	Gest. Day			13	14	15	16	17		20
0	Ery count	10^6/µL		0.35	0.6	0.6	0.8	1.4		2.3
+	Ery count	10^6/µL		0.15	0.2	0.3	0.35	1.0		2.2
+	Heart size	% contr.		100	160	161	125	110		100
0	IVF closure	% foetuses		0	0	0	72	86		95
+	IVF closure	% foetuses		0	0	0	0	21		55

Similar association between foetal anaemia, retarded ossification and morphological anomalies of heart incl. delay of timely IVF occlusion in rats has been confirmed, e.g in preclinical studies of antimalaric drugs (e.g. Schmuck et al.) in doses used in pregnant women without adverse developmental effects. Higher doses caused even more severe foetal anaemia and late resorptions of rat foetuses. (Schmuck G, Klaus AM, Krötlinger F, Langewische FW Developmental and reproductive toxicity studies on artemisone. Birth Defects Res B Dev Reprod Toxicol. 2009 Apr;86(2):131-43.)

At this developmental stage, yolk sac erythropoiesis is the primary source of new circulating fetal erythrocytes. As shown in the study by Ihara (2011), in rats are erythroblasts produced in a single wave: GD 11: more than 95% of blood cells were basophilic erythyroblasts; GD12: predominant cell type were polychromatophilic erythroblasts; GD13: polychromatophilic erythroblasts constituted about 95% of embryonic blood cells; GD 14: orthochromatophilic erythroblast population (postmitotic cells) became the predominant cell type. The primary erythroid cell population affected by flumioxazin is the population of polychromatophilic erythroblasts.
A characteristic of hemopoiesis in yolk sac in rat embryos is that erythroid cells undergo synchronous maturation as a relatively homogeneous population. Synchronized differentiation of erythroblasts in rat embryos does not alow for an effective compensation of haem synthesis inhibition in citical gestational days 12 to 14. Fresh blood cells would not be supplied until haemopoiesis shifts from the yolk sac to the liver: lower output of mature erythrocytes in flumioxazin rats has been completely compensated and no anemia has been observed on GD 20.
Such a single wave of erythroblast formation in a short period coinciding with rapid organogenesis does not occur in humans. In humans, erythroblast formation in yolk sac is characteristic for embryonal days 20 – 50 and is extended over a period of several weeks; haematopoiesis then shifts to liver and finally to bone marrow. Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at an exposure equivalent to a maternal dose exceeding 1000 mg/kg/day.

Direct comparison with human subjects in *in vivo* experiments is not feasible. *In vitro* studies confirm substantial differences (see table). Two new studies of this type have

been recently conducted. CD36+ cells, derived from human cord blood, are precursor of erythroblasts which can be stimulated to differentiate into haem-synthesizing cells under appropriate culture conditions, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells. The results of the study demonstrated that there were no effects on haem content and cell number of human haem-synthesizing cells at up to 5 µM flumioxazin although PPIX concentration in them increased more than ten times. Similarly, rat erythroleukemia cells can also differentiate into haem-synthesizing cells and may be more convincingly compared to human K562 cells: flumioxazin at 0.1µM and above reduced haem production in them, whereas no such effect was observed at 5.0 µM in human K562 cells. Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM (corresponding to doses > 1000 mg/kg bw).

Flumioxazin effect in rat and man compared *in vitro*

Dose of flumioxazin	Tissue concentr.	PPIX liver – rat	PPIX liver – man	PPIX ELR - rat	Haem ELR rat	PPIX K562 - man	Haem K562+ man	PPIX CD36+ man	Haem CD36+ man
mg/kg bw *	µM	pg /mg protein	pg /mg protein	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6 cells	ng/ 10^6cells
0	0	293	180	0.56	127	0.19	208	1.18	1777
0.018 +	0.00007 +								
4	0.01			0.58	116	0.19	202	1.77	1706
15	0.03	370	190						
30 repeated	0.06 mean								
50	0.1			0.80	91	0.18	186	1.59	2198
30 repeated	0.2 peak								
200	0.3	1200	400	0.95	85				
	1.0	3000	800	2.67	60	0.44	224	2.82	1882
1000	2.4/1.9**								
>1000	5.0			8.32	47	3.0	213	14.0	1535

+ AOEL; absorption rate for oral dose levels ≤ 0.01 mg/kg bw is presumed to be almost 100%
*Flumioxazin oral dose X) corresponding to average tissue concentration. Systemic dose (Y) = X x (oral absorption rate); tissue concentration = Y / MW. Measured oral absorption rates were 89, 50, 35 and 12.4% for oral doses of 1, 30, 100 and 1000 mg/kg bw, respectively. Decrease in absorption rate with dose is represented by regression equation: Y = 130 x (1 – EXP(-0.003xX)) ; dose in the last line is estimated for absorption rate ≤ 10% (≤0.1)
**peak concentration in maternal blood /foetal tissue predicted by PBPK model (Takaku, 2012c)

<u>Classification of flumioxazin for developmental toxicity</u>.
The teratogenic effect in rats at doses not toxic to mother animals is clearcut, but their relevance for humans is not.
Applicant assessed them as not relevant for humans. RMS agrees that they probably are not relevant..
According to Regulation (EC) No 1272/2008:
„The classification of a substance in Category 1B is largely based on data from animal

studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate."

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely ?
Regulation (EC) No 1272/2008, subpoint 3.7.2.3.2: admits not to classify such substances:

„ . . If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."

The developmental effects of flumioxazin in rat foetuses were observed at maternal dose of 30 mg/kg bw per day (systemic dose of 15 mg/kg bw). The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect. RMS agrees that difference in sensitivity of protoporphyrinogen oxidase in human and rat hepatocytes to inhibition by flumioxazin doesn't appear to be very large, namely 2.4. For a systemic dose of 15 mg/kg bw, systemic dose predicted to cause similar PPO inhibition in human liver would be 15x2.4 = 36 mg/kg bw and respective oral dose 108 mg/kg bw per day (taking into account decrease in absorption rate with oral dose): that means, for instance, pregnant woman receiving orally more than 6000 mg of flumioxazin daily (> 6000 ADI) for two first months of pregnancy. Moreover, this calculation does not consider two more influential differences between prenatal development in man and in rat:
1) Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM, corresponding to doses > 1000 mg/kg bw.
2) Much longer time between the end of yolk sac haematopoiesis and final foetal stages in humans (from 2 to 9 months) compared to rats (from GD 16 to GD 20) allows for a complete compensation of delays - if any - in the morphological development of human foetus (such as development of the interventricular septum).
All these one-direction points of dissimilarity raise doubt about the relevance of this hazard for humans.

We are not aware of any cases of human poisoning with flumioxazin. On the other hand, clinical experience with porphyrias - incl. Porphyria variegata - is extensive. Short text on variagate porphyria reproduced below is proposed for assessment report and may be added also to CLH document.
„In the last decades, multiple genetic studies identified about 80 mutations in the protoporphyrinogen oxidase gene, resulting in a complete (rarely) or in a partial loss of enzyme activity. For instance, in Finland, one type of mutation in the protoporphyrinogen oxidase gene results in a complete loss of activity of the enzyme produced by the mutated allele whereas in other mutations 9.5–25% of the wild type activity is preserved ; the heterozygotic variagate porphyria patients have, therefore, at least 50% total activity of protoporphyrinogen oxidase. The reduced rate of haem synthesis is compensated by an increase in synthesis of delta aminolevulinic acid in porphyria patients. Anaemia is not a component of the clinical picture that is – in porphyric crisis - dominated by symptoms and

signs of toxic injury of porphyrin metabolites to liver, skin and nervous system. As with other porphyrias, manifestation of disease (porphyric crisis) in heterozygotes requires precipitating factors, such as some drugs, chronic exposure to alcohol, hepatotropic viruses, excess iron intake or storage, oral estrogen use etc. Unlike the mostly latent course of heterozygous porphyria variegata, the extremely rare homozygous variant – only 11 cases have been described before 2006 - is characterized by severe PPO deficiency, high concentration of porphyrins in erythrocytes, onset of photosensitization by porphyrins in early childhood, skeletal abnormalities of the hand, and, less constantly, short stature, mental retardation, and convulsions. Exceptionally, a very low activity of PPO (5 – 15%) may be assotiated with hypochromic anaemia in some of these homozygotic carriers.. Lowering of activity of porphyrinogen oxidase has no role in other types of human porphyric disease. Manifest hypochromic anaemia is not found neither in latent state nor in porphyric crises, when accumulation of toxic porphyrin metabolites impairs organ functions."

Response to additional comments:
1)Dividing line between LOAEL and NOAEL for maternal toxicity in the study by Hoberman (1991) is based on statistical significance of intergroup differences of food consumption and weight gain data. Lowered consumption of food and contingent reduction in weigt gain are usual artifacts of high-dose diet experiments; as isolated effects they are ascribed to lowered food palatability and not to maternal toxicity of the substance.
2)Studies by Kawamura (1990b), Hoberman (1990) and Lemen (1991a,b) compare developmental toxicity of two other members of the N-phenylimidases family; the differences were discussed in the first EU evaluation and in the original DAR from 1997 as cited below.
„Three chemically related compounds (flumioxazin, V-23121 and V-23031 have been tested in standard FIFRA guideline developmental toxicity studies in rat and rabbits.
Both flumioxazin and V-23121 were found to produce the same pattern of developmental toxicity in rats and were negative when tested in rabbits. V-23031, however, did not produce developmental toxicity even when tested at 1500 mg/kg/day in rats, a dose well above the test limit dose of 1000 mg/kg/day or at 800 mg/kg/day in rabbits, a dose which produced maternal toxicity.
To investigate whether a compound difference in PPIX accumulation was present in rat embryos, pregnant rats were administered 1000 mg/kg of each compound late on day 12 of gestation and PPIX accumulation was measured 14 hours later. The 14 hour time period approximates the peak PPIX accumulation time point reported earlier. Both flumioxazin and V-23121 induced remarkable and similar amounts of PPIX accumulation in rat embryos, about 250 times that of control foetuses. The PPIX concentration in embryos of V-23121 was approximately three times that observed in livers of control animals. The PPIX concentration in embryos of V-23031 treated rats was similar to the value for control embryos, while the concentration in maternal livers was similar or slightly higher than control. Thus, there is a strong correlation between PPIX accumulation in embryos and the chemicals which were identified as developmental toxicants."

Applicant will provide more details on these studies.

1)
As discussed in "An update of a discussion on human relevance of the developmental effects induced by flumioxazin in rats" (SBT-0122), Sumitomo has investigated whether or not PPO inhibition in erythroblasts can cause anemia in humans. Porphyrias are disorders in which the activities of the enzymes of the heme biosynthetic pathway, including PPO, are deficient. They can be classified as either hepatic or erythropoietic, depending on the

principal site of expression of the specific enzymatic defect. The tissue-specific expression of porphyrias is largely due to the tissue-specific control of heme pathway gene expression, especially at the level of aminolevulinate synthase (ALAS), the first and rate-limiting enzyme of heme biosynthesis. In liver, hemoprotein enzymes are rapidly turned over in response to current metabolic needs. The activity of ALAS1, the housekeeping isoenzyme of ALAS, in normal liver is the lowest among all enzymes in the heme biosynthetic pathway. In erythroid cells, the activity of ALAS2 (the erythroid-specific isoenzyme of ALAS) is induced only during the period of active heme synthesis, and is regulated by the amount of free iron present.

Variegate porphyria (VP) is a disease associated with PPO deficiency. VP is categorized as hepatic porphyria and the main symptoms are neuronal manifestation and dermal inflammation. Hepatic porphyrias usually do not include anemia or hematological problems. Anemia was not found in hepatic porphyrias attributable to marked deficiency of delta aminolevulinic acid dehydratase (ALAD), coproporphyrinogen oxidase (CPO), or PPO. This suggests that defective enzymatic activity resulting in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells. Variegate porphyria is associated with reduced PPO content and ALAD activity in erythrocytes. Erythrocytes counts were not affected by VP and hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin in VP were slightly higher than their controls. The low rate of heme production in VP is enough to generate the same, or even greater, quantity of hemoglobin as control women.

In contrast to VP, erythropoietic protoporphyria (EPP) resulting from deficiency of ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway, sometimes includes mild anemia with hypochromia and microcytosis or mild anemia with reticulocytosis. Microcytic anemia occurs in 20% to 60% of patients. Erythropoiesis was impaired in most patients with dominant EPP from the UK and France and all had a downward shift in hemoglobin. FECH deficiency in EPP results in the accumulation of protoporphyrin almost exclusively in erythroid tissue, even though FECH is deficient in all other tissues in these patients. This finding suggests that FECH activity can become rate limiting in erythroid cells, but not in other tissues when the enzyme itself or its substrate, iron, is partially deficient.

Recently families with X-linked, dominant protoporphyria (XLDPP) have been described. Patients with this disorder have normal FECH activities, indicating that protoporphyrin accumulation is not caused by FECH deficiency. Patients showed neither anemia nor iron overload. Disruption of the C-terminal region of ALAS2 leads to markedly increased ALAS2 activity and the production of protoporphyrin in excess of the amount required for hemoglobinization. These findings suggest that the rate of ALA formation is increased to such an extent that insertion of Fe into PP by FECH becomes rate limiting for heme synthesis.

These clinical findings demonstrate that PPO activity in human erythroid cells is much higher than FECH or ALAS2 activity, which is rate-limiting in heme biosynthetic pathway in human erythroids. It is therefore unlikely that PPO deficiency would induce anemia or disturbances of heme synthesis in human erythroid cells. In contrast, the results of toxicity studies in rats suggest that in rat erythroid cells, PPO activity is close to a rate-limiting enzyme activity. Therefore decreased PPO activity becomes rate-limiting in porphyrin production in erythroids resulting in PPIX accumulation, iron deposit, and anemia.

Enzymatic activities from various tissues are presented in Table. Although the data are derived from non-erythroid tissues, we present them to illustrate relative activities in human and rat tissues. In humans PPO activity could be higher than other enzymes. In rats FECH activity varies and PPO activity is not necessarily higher than FECH.

Table Activities of enzymes in the heme synthetic pathway (nmol/h/mg protein)

		ALAS	CPO	PPO	FECH
Human	Fibroblast	0.003 - 0.005	-	2.12	0.032
	Liver	-	0.6	10.8	3.72
	Leucocyte	-	-	8.73	0.24
rat	Liver (mitochondria)	-	-	3	87
	Liver (homogenate)	-	1.2	10.2	3.18 - 4.2
	Liver (mitochondria)	0.61	2.7	8.5	8.0

- : not reported

2)

Sumitomo further conducted a study with human CD36+ cells, which are isolated from human cord blood, and the report of the study was posted during the period of public comment (see Comment number 6). CD36+ Cells are a precursor of erythroblasts, which can be differentiated into heme-synthesizing cells, and are more relevant to physiological maturation of human fetal erythroblasts, hence better addressing effects on heme biosynthesis in human fetal erythroid cells. The results of the study demonstrated that there were no effects on heme content and cell number of human heme-synthesizing cells treated with flumioxazin at 5 μM. The developed human pregnant PBPK model demonstrated that flumioxazin concentration in the human fetus at dose of 1000 mg/kg po was 1.92μM. This concentration is lower than the maximum no effect concentration of 5 μM in K562 and CD36+ cells, supporting the view that humans would not be susceptible to anemia and the developmental effects of flumioxazin.

Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0126.

Sumitomo has also conducted a study with rat erythroleukemia (REL) cells which can be differentiated into heme-synthesizing cells by treatment with inducers. REL cells correspond to human K562 cells. Flumioxazin at 0.1μM and above reduced heme production in the rat heme-synthesizing cells. The results demonstrated that reactions of heme-synthesizing cells derived from erythroleukemia cells to flumioxazin exposure reflect that of normal erythroid cells.

Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0125.

Sumitomo believes the results provide further evidence that human fetuses would not be affected by exposure to maternal doses as high as 1000 mg/kg.

3)

Porphyrias can be classified as either hepatic or erythropoietic (1), depending on the principal site of expression of the specific enzymatic defects. Variegate Porphyria (VP) is hepatic porphyria. Symptoms of VP are due to hepatic PPO inhibition. Patients present after puberty with skin lesions or with acute nuerovisceral crisis, or with both together. Clinically silent VP is at least five times commoner than overt disease (2).

Hepatic porphyrias usually do not accompany anemia or haematological problems. For example, anemia was not found in ALA dehydratase porphyrias, homozygous variants of hereditary coproporphyria or VP that can occur in childhood and are attributable to marked deficiency of ALA dehydratase, coproporphyrinogen oxidase, or PPO, respectively (3). This fact suggests that a defective enzymatic activity that results in disturbances in heme biosynthesis in liver does not necessarily limit heme synthesis in erythroid cells. In reviews (1, 4, 5), neurovisceral symptoms and photosensitivity are referred as major types of clinical features of VP. In a recent article (6) results of hematological analysis of VP were reported and no anemia was observed.

In heterozygotes of VP, PPO activity is decreased by 50%. Homozygous VP is a rare

recessive disease, presenting with residual PPO levels between 5 and 20%, and to date only a few cases have been reported. In heterozygotes the disease dose not usually present before puberty and homozygous patients develop severe symptoms in early infancy, presumably as a consequence of the profound enzyme deficiency (7). Short stature and neurological defects are often present in homozygous variants of acute intermittent porphyria and hereditary coproporphyria, suggesting that certain early stages of development may be critically depend on heme biosynthesis (2). The effects in VP patients, therefore, are considered to occur after birth and to result from PPO inhibition.
Poblete-Gutierrez et al. (8) stated that only 11 homozygous VP patients have been published. Results of hematological investigation in four patients out of 11 homozygous VP were described in the text portions, although data were not shown (9, 10, 11, 12). Three patients with 5~15% of control PPO activity had normal haemoglobin contents (9, 10, 11). One patient with 12% of control PPO activity showed dyserythropoietic anaemia by bone marrow examination without changes in peripheral blood picture (12).
At least one of mutations in PPO must preserve substantial activity, otherwise heme biosynthesis would not occur, which is incompatible with life.
Some types of porphyrias are classified as erythropoietic, in which sign of anemia can be seen. Congenital erythropoietic porphyria (uroporphyrinogen synthase deficient) and hepatoerythropoietic porphyria (uroporphyrinogen decarboxylase deficient) include hemolytic anemia, and erythropoietic protoporphyria (ferrochelatase deficient) patients sometimes show mild anemia (3). Flumioxazin caused sideroblastic anemia, not haemolytic anemia (SBT-0059). Mode of action of N-phenylimide herbicides is similar to that of diphenyl ether herbicides. Diphenyl ether herbicides inhibit PPO, but not ferrochelatase in corn, potato, mouse and yeast (13).
As discussed earlier, no effects were observed on heme content and cell number in human cells treated with flumioxazin. Flumioxazin is considered not to cause inhibition of any enzymes in human heme biosynthesis, which interferes with normal heme biosynthesis and leads to anemia,

Variegate porphyria is a disease associated with PPO deficiency. Variegate porphyria is, therefore, relevant to flumioxazin induced effects. The discussion of human relevance of the developmental toxicity findings in the rat was summarised in B 6.6.2.4 (Kawamura, 2012b). Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at exposure equivalent to a maternal dose exceeding 1000 mg/kg/day, thus demonstrating the large species difference in sensitivity. Although the mode of action could occur in humans, taking into account kinetic and dynamic factors it is not plausible in humans. In other words human exposure could not possibly be envisaged to reach the levels that would produce the toxicological effect. Thus the proposed reference doses for flumioxazin are conservative and appropriate (B 6.10.3).

1) Philips JD and Anderson KE. (2010) The porphyrias, Chapter 57 in Williams Hematology, 8th edition, pp839-863..
2) Roberts AG et al. (1998) Molecular characterization of homozyous variegate porphyria. Human Molecular Genetics 1921-1925
3) Sassa S (2000) Hematological aspects of the porphyrias. Int J Hematol 71:1-17
4) Nordmann Y and Puy H (2002) Human hereditary hepatic porphyrias. Clinica Chimica Acta 325 17-37
5) Gross U, Hffmann GF, and Doss MO (2000) Erythropoietic and hepatic porphyrias. J Inherit Metab Dis 23:641-661
6) Ferrer MD et al. (2009) Enzyme antioxidant defences and oxidative damage in red blood cells of variegate porphyria patients. Redox Report 14:69-74
7) Frank J et al. (1998) Homozygous variegate porphyria: Identification of mutations on

both alleles of the protoporphyrinogen oxidase gene in a severely affected proband. J Invest Dermatol 110:452-455.

8) Poblete-Gutierrez P et al. (2006) A Chilean boy with severe photosensitivity and finger shortening: the first case of homozygous variegate porphyria in South America. Br J Dermatol 154:368-371

9) Norris PG et al. (1990) Homozygous variegate porphyria: a case report. Br J Dermatol 122:253-257.

10) Mustajoki P et al. (1987) Homozygous variegate porphyria. Clin Genet 32:300-305.

11) Murphy GM et al. (1986) Homozygous variegate porphyria: two similar cases in unrelated families. J R Soc Med 79:361-363

12) Korda V et al. (1984) Homozygous variegate porphyria. Lancet 14:851

13) Matringe M et al. (1989) Protoporphyrin oxdase as a molecular taget for diphenyl ether herbicides. Biochem. J. 260

4)

Body weight on gestation day 7 and 19 are added to the Table. It is generally known that bodyweight change in gestation period in rabbit is smaller than that in rat. The mean maternal body weights on Day 19 in the control and 1000 mg/kg were 3.91 and 3.88 kg, respectively. The slight difference (-0.8% to the control value) is usually thought to be toxicological meaningless. In fact, there are no statistically differences in the bodyweight gain, food consumption, and actual body weight between the control and 1000 mg/kg groups. It is concluded that the maternal NOAEL is 1000 mg/kg/day.

Table Effects on Maternal Animals (SBT-0017)

Parameter	Dose level (mg/kg/d)			
	0	300	1000	3000
Body weight (kg) Gestation day 7	3.73	3.71	3.74	3.71
Body weight (kg) Gestation day 19	3.91	3.89	3.88	3.76
Body weight gain (kg) Gestation day 7-19	0.17	0.18	0.14	0.05*
Food consumption (g/d) Gestation day 7-19	165.1	160.2	150.3	135.1*

*: Statistical significance, $p<0.05$

5)

Although the studies are not related to the risk assessment for flumioxazin directly, Sumitomo has added detail data related to the endpoints of each study to the summary tables as follows.

Report:	Kawamura, S. (1990b). Teratology study of S-23121 administered orally to rats. Sumitomo Chemical Co Ltd. Unpublished report no.: PPT-00-0023
Guidelines:	EPA FIFRA 83-3
GLP:	Yes (certified laboratory)
Summary	S-23121(a chemically related herbicide similar to flumioxazin) was administered orally via gavage to groups of pregnant female rats at concentrations of 0, 1, 3, 10 and 20 mg/kg/day from GD 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined.

Maternal signs of toxicity were limited to high dose group dams, and included dark reddish material around the vagina. Necropsy data confirmed dark reddish material in vagina and uterus. These findings were considered to be related to the deaths of the litters. Decreases in body weight and body weight gain at term were limited again to high dose group animals; whilst not reaching statistical significance these decreases were due to reductions in gravid uterine weight which was considered to be attributable to the deaths of the litter and the decrease in fetal body weight.

High incidence of mortality of embryos and decrease of fetal body weights were limited to the high dose groups. There however was no treatment related effects on the number of implantations or sex ratio. External abnormalities found were considered not to be treatment-related. The incidence of foetuses with cardiovascular abnormalities, primarily VSDs were increased in the 20 mg/kg/d group ($p < 0.01$). Test material related increase in wavy ribs (minor anomaly) was observed, this however did not reach statistical significance. Test material related decreases in ossified sacrococcygeal vertebral bodies were also observed. Whilst a reduction in the number of ossified sacrococcygeal vertebral bodies was observed, this was considered to be related to the decreased fetal body weights.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be 10 mg/kg/d, based on increased incidence of cardiac VSD, growth retardation and embryo lethality. No signs of maternal toxicity were observed, therefore the maternal NOAEL for considered to be greater than 20 mg/kg/d.

Maternal observations

Parameters	Dose level (mg/kg/d)				
	0	1	3	10	20
Body weight (g) Gestation day 20	367	367	369	368	323**
Body weight gain (g) Gestation day 6-20	103	102	106	102	59**
Mean gravid uterine weight (g)	67	67	67	65	13**

Fetal observations

Parameters	Dose level (mg/kg/d)				
	0	1	3	10	20
Embryonic death (%)	8.4	8.6	7.6	2.7*	91.9**
Fetal body weight (upper, M; lower, F; g)	3.47 3.27	3.55 3.39	3.49 3.31	3.46 3.25	2.78** 2.32**
VSD (%)	0.0	1.4	1.4	0.0	63.6**
Wavy ribs (%)	0.7	0.0	1.4	0.0	21.4
Ossified	8.9	9.1	8.9	8.9	8.3

sacrococcygeal vertebral bodies (upper, M; lower, F; n)	8.8	9.0	8.8	8.8	7.7**

**: Statistical significance p<0.01
*: Statistical significance p<0.05

Report: Hoberman, A.M. (1990). Teratology study in rabbits with S-23121. Sumitomo Chemical Co Ltd. Unpublished report no.: PPT-01-0020

Guidelines: EPA FIFRA 83-3

GLP: Yes (certified laboratory)

Summary S-23121 was administered orally via gavage to groups of pregnant female rabbits at concentrations of 0, 2, 4, 8 or 15 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and foetuses were removed by caesarean section and examined.

Maternal signs of toxicity included adverse clinical signs of toxicity, decreases in maternal body weight and body weight gains and feed consumption values during the dosage period in the 4 mg/kg dose group and higher. The 15 mg/kg dosage also caused the death of one dam.

No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be greater than 15 mg/kg/d (the highest dose tested). The maternal NOAEL was considered to be 2 mg/kg/d based on clinical signs, reductions in maternal body weight and body weight gains and relative and absolute food consumption.

Maternal observations

Parameters	Dose level (mg/kg/d)				
	0	2	4	8	15
Body weight (kg) Gestation day 19	3.98	3.92	3.82	3.68	3.43**
Body weight gain (kg) Gestation day 7-19	0.17	0.13	0.02*	-0.13**	-0.34**
Food consumption (g/d) Gestation day 7-19	154.0	146.6	112.6**	90.4**	58.9**

**: Statistical significance p<0.01
*: Statistical significance p<0.05

Report: Lemen, J.K. (1991a). Rat teratology study with S-23031.

	Sumitomo Chemical Co Ltd. Unpublished report no.: SAT-11-0024
Guidelines:	EPA FIFRA 83-3
GLP:	Yes (certified laboratory)
Summary	S-23031 (a chemically similar herbicide to flumioxazin) was administered orally via gavage to groups of pregnant female rats at concentrations of 0, 50, 500 and 1500 mg/kg/day from gestation day 6 to 15. Dams were culled on GD 20 and fetuses were removed by caesarean section and examined. No maternal signs of toxicity were observed, with food consumption, body weights or clinical signs of toxicity in treated animals being comparable to the concurrent control group. No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control. Based on the result of this study, the NOAEL for both developmental and maternal toxicity was considered to be greater than 1500 mg/kg/d (the highest dose tests) based on no signs of maternal or developmental toxicity observed.
Report:	Lemen, J.K. (1991b). Rabbit teratology study with S-23031. Sumitomo Chemical Co Ltd. Unpublished report no.: SAT-11-0025
Guidelines:	EPA FIFRA 83-3
GLP:	Yes (certified laboratory)
Summary	S-23031 was administered orally via gavage to groups of pregnant female rabbits at concentrations of 0, 100, 200, 400 and 800 mg/kg/day from gestation day 7 to 19. Dams were culled on GD 29 and foetuses were removed by caesarean section and examined. Four dams in the high dose group were found dead during the study, with a 5th animal from this group aborting (which was subsequently sacrificed). Clinical signs of toxicity recorded during the study were not indicative of a treatment related effect. Whilst not reaching statistical significance (due to high variability), body weight loss in the high dose group was notably greater than the control and was considered to be treatment related with high dose group animals not gaining weight during the dosing period. Mean group body weight gain for these animals was17% lower than the control for the period of GD 7 to 29. No significant difference in pre or post implantation loss or early / late resorptions were observed. No test material related fetal changes were observed, with external / visceral and skeletal malformations / variations in test material treated animals being comparable to the concurrent control.

Based on the result of this study, the NOAEL for developmental toxicity was considered to be greater than 800 mg/kg/d (the highest dose tested). The maternal NOAEL was considered to be 400 mg/kg/d based on reductions in maternal body weight gains and maternal mortality.

Maternal observations

Parameters	Dose level (mg/kg/d)				
	0	100	200	400	800
Body weight (g) Gestation day 19	3827.0	3760.2	3908.8	3831.5	3694.1
Body weight gain (g) Gestation day 7-19	-6.67	81.00	113.60	91.31	-123.38
Body weight gain (g) Gestation day 7-29	225.36	324.45	306.86	307.00	187.00

RAC's response

RAC agrees with the MS (BE CA) and notes the additional detailed information and discussion provided by the applicant.

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	United Kingdom		Individual	13

Comment received

The studies on flumioxazin conducted since 2002 have provided further evidence that the primary action of flumioxazin is to inhibit the activity of the enzyme protoporphyrinogen oxidase (PPO) thereby reducing haem synthesis and causing anaemia in the rat fetus. The action is primarily on the developing erythroblasts in the rat embryo yolk sac and, since maturation of these erythroblasts is synchronised in the rat, this results in a large loss of erythroblast cells in the fetus causing severe anaemia in and fetal death, or in cardiovascular defects in surviving fetuses. Strong evidence is presented that the failure of closure of the ventricular septum resulting in permanent ventricular septal defects (VSD), is secondary to the effects induced on blood cell formation, and is not due to a direct toxic action on the heart.

The species specificity of the actions of flumioxazin has also been confirmed and extended by the in vitro human cell culture studies to show that even at very high concentrations of flumioxazin there is no effect on cell division or haem synthesis in human cells.

Kinetic modelling data (Takaku, 2012) suggest that even an oral dose of 1000 mg/kg of flumioxazin in humans would not lead to a teratogenic concentration in the fetus. However, more important is the action of flumioxazin on the yolk sac erythroblasts since this is an embryonic system which differs significantly between rats and humans. In this regard, the similarity of the actions of flumioxazin with those of the antimalarial drugs, the artemisinins, should be considered; this is an important comparison since artemisinins are embryolethal in rodents at dose levels similar to those used clinically in the treatment of malaria.

In rats, the primary action of artemisinins is on primitive embryonic red blood cells,

erythroblasts, which are especially sensitive to the cytotoxic effects of this class of chemicals (Finaurini et al., 2012). The production of erythroblasts is unique in that this population is generated in a single wave in the yolk sac from progenitors that cannot self-renew, rather than being produced semi-continuously from self-renewing progenitors, as is the case at later stages of erythropoiesis. The window of sensitivity to artesunate in rats, GD 10-12, matches closely with this erythroblast phase. The consequence is a very severe anaemia in the rat embryo causing fetal death. At lower doses of artemisinins, fetal malformations can be observed in rats, including VSD. Such a single wave of erythroblast formation in a short period is not thought to occur in humans, but rather erythroblast formation extends over a longer period of time in humans. Although there has been considerable concern about treatment with artemisinins in pregnancy, studies on several thousand women given artemisinins during the second and third trimesters, and on several hundred women given artemisinins in the first trimester, have not shown any increase in either fetal death or malformations (WHO, 2007; WHO, 2010; Li & Weina, 2009; Mayando et al., 2012).
From the above and the evidence available on flumioxazin, which seems to have a similar mode of action to the artemisinins, the effects are unlikely to be relevant for humans, and should not lead to classification for toxicity to reproduction.

Dossier Submitter's Response

Agreed

RAC's response

The species sensitivity of the actions of flumioxazin is well supported by the data provided. The mechanism of action (inhibition of PPO leading to anaemia) is not species specific however, but rather a quantitative difference in sensitivity. The difference between rat and human embryos in erythroblast production could be the basis for a species specific difference in response, but it is not proven that the human embryo will be entirely unaffected by flumioxazin during the critical period in embryogenesis.
The comparison of the effects seen with flumioxazin to that observed with the antimalarial drugs (artemisinins) is noted.
The conclusion that the effects are unlikely to be relevant to humans is not sufficient for declassification without direct proof. While all arguments related to relative sensitivity of the rat are taken into account, significant doubt still exists as to the actual sensitivity of the human foetus to PPO inhibition during a sensitive period of erythrocyte maturation. The mechanism of flumioxazin induced developmental toxicity is considered relevant to man, although it is acknowledged that significant differences between rat and man may exist with regard to sensitivity to this mechanism for the reasons outlined above. The RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	United States		Individual	14

Comment received

Clear species-specific difference between rat and rabbit establishes doubt as to relevance for human. New mechanistic data show a basis for the difference, and fulfil the criterion "raises doubt about the relevance for humans" (Table 3.7.1 (a) of the CLP Directive). Repr 1B is not appropriate.

(ECHA note: The following attachment was provided:
"Flumioxazin: Classification for Developmental Toxicity" [Attachment 1])

Dossier Submitter's Response

Agreed
RAC's response
RAC agrees that the mechanistic data raises doubts as to the human relevance of the observed effects and considers that classification in Cat 2 could be discussed in this context. However, on balance, RAC concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2013	Spain		MemberState	15
Comment received				
Effects on developmental toxicity Flumioxazin was included in Annex I of Commission Directive 2001/59/EEC adapting to technical progress for the 28th time Directive 67/548/EEC with the classification of Category 2; R61, based on effects observed in the rat developmental studies. Since the initial inclusion of flumioxazin further mechanistic work has been undertaken to demonstrate that the effects observed in rat (embryolethality and teratogenicity, mainly ventricular septal defects and wavy ribs) are species specific and not considered relevant for humans. After assessing the new information, the dossier submitter proposes the removal of the current reproductive toxicity classification [Category 2; R61 (DSD); Repr. 1B H360D (CLP)]. Rats are particularly sensitive to protoporphyrinogen oxidase activity (PPO) inhibition induced by flumioxazin, resulting in fetal anaemia and consequent developmental toxicity. The effects reported in the rat developmental study were observed in the absence of maternal toxicity. Regarding the difference in sensitivity between the adult female livers and fetus in rats, irrespective of the fact that the degree of inhibition of PPO was equal in adult and fetus, treatment of pregnant rat with flumioxazin on day 12 of gestation, the most sensitive day, resulted in the accumulation of protoporphyrin IX (PPIX) in both the whole embryos and maternal livers. The extent of accumulation in embryos was greater than that observed in maternal livers, with the increase of PPIX in embryos up to 290-fold greater than the control value. PPIX concentration in maternal liver was 0.679 µg/g, which was only 2.87 times greater than the control value (Kawamura, 1996b and Kawamura, 1993d). Studies in the rabbit (where clear evidence of maternal toxicity was observed) revealed no significant inhibition of PPO by flumioxazin and there was no evidence of fetal anaemia, accumulation of PPIX or developmental toxicity in the rabbit fetus, even though there was evidence of placental transfer of flumioxazin and its metabolites. Concerning human sensitivity to PPO inhibition by flumioxazin, in relation to the animal data, the human sensitivity might be intermediate to that of the very sensitive rat and the non-sensitive rabbit. There is in vitro experimental evidence that, using human erythroleukemia cells, flumioxazin doesn't reduce the production of hemo, even when an increase of PPIX in erythroblast cells was shown. However, having into account that no comparable studies have been done using erythroleukemia cells in rat, it can not be concluded that this will be				

the situation in vivo.

In the additional mechanistic study (Abe 2011b) effects of flumioxazin on the accumulation ratio of PPIX in rat (10-fold) and human hepatocytes (6.2-fold in the HIE batch) don't seem to be so different. Besides, basal PPIX levels in the LMP batch of human hepatocytes was comparable to the basal concentration in rat. In another study (Green & Dabbs 1996) the IC50 for the inhibition of PPO after incubation with flumioxazin in livers from rats was only 2.4 fold lower than the IC50 value in livers from humans.

On overall, it may be reasonably assumed that humans are less sensitive to the elucidated mechanism behind the anaemia. However, it cannot be concluded that this mode of action and the resulting findings could no occur in man. Flumioxazin can be expected to have an intrinsic possibility to also cause in humans similar developmental toxicity observed in rat, but with some reservation for expected lower sensitivity of humans than of rats. Besides, fetus might have a special sensitivity.

Therefore, Spanish CA considers that classification and labelling for developmental toxicity is needed and it is not appropriate the removal of the current classification.

Dossier Submitter's Response

The teratogenic effect in rats at doses not toxic to mother animals is indisputable. The relevance of this hazard for humans is not. To contribute to discussion of species specificity and broader relevance, following text incorporating also data from two new *in vitro* studies and quantitative details in some parts of the document is proposed.

<u>Species specificity and hazard relevance</u>

In the two-generation study, P_1female rats receiving 300 ppm diet both body weight and body weight gain were reduced and there was an increase in litter resorptions.

Flumioxazin dose of 30 mg/kg bw caused evident developmental toxicity in rats. No such effects have been observed in rabbits at doses per kg bw by two orders of magnitude higher. The interspecies differences in flumioxazin absorption, distribution and elimination were small: After repeated oral administration of the same dose per kg bw the concentration in blood, maternal tissues and fetus were lower in rabbits than in rats by about one third 2 hours after the first dose and by more than 50% 24 hours after the first dose and after repeated dosing. The concentration of flumioxazin in foetuses of rabbits was half that in rat foetuses. The developmental effects in foetuses of rats administered 400 mg/kg bw flumioxazin on GD 12 included: severe anaemia (decreased red blood cell count to approx. 1/3 and 1/2 of values in controls on gestation days 14 and 16, respectively), enlarged heart (by 60% on days 14 and 15), oedema, delayed closure of the interventricular foramen (0% compared to 72% in controls on day 16, see table), reduced serum protein (up to GD 16) and incomplete/delayed ossification. The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect.

Flumioxazin	Gest. Day			13	14	15	16	17		20
0	Ery count	10^6/µL		0.35	0.6	0.6	0.8	1.4		2.3
+	Ery count	10^6/µL		0.15	0.2	0.3	0.35	1.0		2.2
+	Heart size	% contr.		100	160	161	125	110		100
0	IVF closure	% foetuses		0	0	0	72	86		95
+	IVF closure	% foetuses		0	0	0	0	21		55

Similar association between foetal anaemia, retarded ossification and morphological anomalies of heart incl. delay of timely IVF occlusion in rats has been confirmed, e.g in

preclinical studies of antimalaric drugs (e.g. Schmuck et al.) in doses used in pregnant women without adverse developmental effects. Higher doses caused even more severe foetal anaemia and late resorptions of rat foetuses. (Schmuck G, Klaus AM, Krötlinger F, Langewische FW Developmental and reproductive toxicity studies on artemisone. Birth Defects Res B Dev Reprod Toxicol. 2009 Apr;86(2):131-43.)

At this developmental stage, yolk sac erythropoiesis is the primary source of new circulating fetal erythrocytes. As shown in the study by Ihara (2011), in rats are erythroblasts produced in a single wave: GD 11: more than 95% of blood cells were basophilic erythyroblasts; GD12: predominant cell type were polychromatophilic erythroblasts; GD13: polychromatophilic erythroblasts constituted about 95% of embryonic blood cells; GD 14: orthochromatophilic erythroblast population (postmitotic cells) became the predominant cell type. The primary erythroid cell population affected by flumioxazin is the population of polychromatophilic erythroblasts.
A characteristic of hemopoiesis in yolk sac in rat embryos is that erythroid cells undergo synchronous maturation as a relatively homogeneous population. Synchronized differentiation of erythroblasts in rat embryos does not alow for an effective compensation of haem synthesis inhibition in citical gestational days 12 to 14. Fresh blood cells would not be supplied until haemopoiesis shifts from the yolk sac to the liver: lower output of mature erythrocytes in flumioxazin rats has been completely compensated and no anemia has been observed on GD 20.
Such a single wave of erythroblast formation in a short period coinciding with rapid organogenesis does not occur in humans. In humans, erythroblast formation in yolk sac is characteristic for embryonal days 20 – 50 and is extended over a period of several weeks; haematopoiesis then shifts to liver and finally to bone marrow. Pharmacokinetic modelling in the rat and the human predicts that human erythroblasts would be insusceptible to flumioxazin at an exposure equivalent to a maternal dose exceeding 1000 mg/kg/day.

Direct comparison with human subjects in *in vivo* experiments is not feasible. *In vitro* studies confirm substantial differences (see table). Two new studies of this type have been recently conducted. CD36+ cells, derived from human cord blood, are precursor of erythroblasts which can be stimulated to differentiate into haem-synthesizing cells under appropriate culture conditions, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells. The results of the study demonstrated that there were no effects on haem content and cell number of human haem-synthesizing cells at up to 5 µM flumioxazin although PPIX concentration in them increased more than ten times. Similarly, rat erythroleukemia cells can also differentiate into haem-synthesizing cells and may be more convincingly compared to human K562 cells: flumioxazin at 0.1µM and above reduced haem production in them, whereas no such effect was observed at 5.0 µM in human K562 cells. Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM (corresponding to doses > 1000 mg/kg bw).

Flumioxazin effect in rat and man compared *in vitro*

Dose of flumioxazin	Tissue concentr.	PPIX liver – rat	PPIX liver – man	PPIX ELR - rat	Haem ELR rat	PPIX K562 - man	Haem K562+ man	PPIX CD36+ man	Haem CD36+ man
mg/kg bw *	µM	pg /mg protein	pg /mg protein	ng/ 10^6cells	ng/ 10^6	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6 cells	ng/ 10^6cells

					cells				
0	0	293	180	0.56	127	0.19	208	1.18	1777
0.018 +	0.00007 +								
4	0.01			0.58	116	0.19	202	1.77	1706
15	0.03	370	190						
30 repeated	0.06 mean								
50	0.1			0.80	91	0.18	186	1.59	2198
30 repeated	0.2 peak								
200	0.3	1200	400	0.95	85				
	1.0	3000	800	2.67	60	0.44	224	2.82	1882
1000	2.4/1.9**								
>1000	5.0			8.32	47	3.0	213	14.0	1535

+ AOEL; absorption rate for oral dose levels ≤ 0.01 mg/kg bw is presumed to be almost 100%
*Flumioxazin oral dose X) corresponding to average tissue concentration. Systemic dose (Y) = X x (oral absorption rate); tissue concentration = Y / MW. Measured oral absorption rates were 89, 50, 35 and 12.4% for oral doses of 1, 30, 100 and 1000 mg/kg bw, respectively. Decrease in absorption rate with dose is represented by regression equation: Y = 130 x (1 – EXP(-0.003xX)); dose in the last line is estimated for absorption rate ≤ 10% (≤0.1)
**peak concentration in maternal blood /foetal tissue predicted by PBPK model (Takaku, 2012c)

<u>Classification of flumioxazin for developmental toxicity</u>.
The teratogenic effect in rats at doses not toxic to mother animals is clearcut, but their relevance for humans is not.
Applicant assessed them as not relevant for humans. RMS agrees that they probably are not relevant..
According to Regulation (EC) No 1272/2008:

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

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely ?
Regulation (EC) No 1272/2008, subpoint 3.7.2.3.2: admits not to classify such substances:

> *„ . . If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans <u>or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans</u> then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."*

The developmental effects of flumioxazin in rat foetuses were observed at maternal dose of 30 mg/kg bw per day (systemic dose of 15 mg/kg bw). The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect. RMS agrees that difference in sensitivity of protoporphyrinogen oxidase in human and rat hepatocytes to inhibition by flumioxazin doesn't appear to be very large, namely 2.4. For a

systemic dose of 15 mg/kg bw, systemic dose predicted to cause similar PPO inhibition in human liver would be 15x2.4 = 36 mg/kg bw and respective oral dose 108 mg/kg bw per day (taking into account decrease in absorption rate with oral dose): that means, for instance, pregnant woman receiving orally more than 6000 mg of flumioxazin daily (> 6000 ADI) for two first months of pregnancy. Moreover, this calculation does not consider two more influential differences between prenatal development in man and in rat:
1) Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM, corresponding to doses > 1000 mg/kg bw.
2) Much longer time between the end of yolk sac haematopoiesis and final foetal stages in humans (from 2 to 9 months) compared to rats (from GD 16 to GD 20) allows for a complete compensation of delays - if any - in the morphological development of human foetus (such as development of the interventricular septum).
All these one-direction points of dissimilarity raise doubt about the relevance of this hazard for humans.

1)
Low concentrations of PPIX in maternal livers would result from its rapid excretion into feces. A study of oxadiazon, which induces porphyria in rats and mice probably due to PPO inhibition, demonstrated that PPIX was excreted into bile and feces quickly.
Krijt et al., 1992. Experimental hepatic porphyria induced by oxadiazon in male mice and rats. Pestic Biochem Physiol 42: 180-187
2)
Sumitomo has conducted a study with rat erythroleukemia (REL) cells which can be differentiated into heme-synthesizing cells by treatment with inducers. REL cells correspond to human K562 cells. Flumioxazin at 0.1µM and above reduced heme production in the rat heme-synthesizing cells. The results demonstrated that reactions of heme-synthesizing cells derived from erythroleukemia cells to flumioxazin exposure reflect that of normal erythroid cells.
Kawamura S, 2013. Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0125.

RAC's response

RAC agrees with theMS (ES) that declassification is not considered an appropriate measure and also agrees with their remarks with regard to human relevance and strength of evidence. A case could be made to reduce the classification on the basis of the doubts raised on human relevance by the DS. RAC notes the further discussion of the data by the applicant and the RMS but concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

Date	Country	Organisation	Type of Organisation	Comment number
04.10.2013	Netherlands		MemberState	16

Comment received

Flumioxazin induces developmental effects including heart malformations and reduced litter size in the rat but not in the rabbit. The proposed mechanism for the flumioxazin-induced developmental toxicity is via inhibition of protoporphyrinogen oxidase (PPO). PPO is an enzyme responsible for catalyzing the seventh step in heme (the portion of hemoglobin that carries oxygen in the blood from the lungs to rest of the body) production, and it is responsible for removing hydrogen atoms from protoporphyrogen IX (the product of the

sixth step in heme production) to form protoporphyrin IX (PPIX). Mechanistic studies demonstrate that flumioxazin has a strong inhibitory activity against PPO (p.35, 48-49). Two in vitro studies investigating human K562 cell differentiation into erythroid cells in the presence of flumioxazin were presented: one showed the accumulation PPIX in K562 cells at concentrations higher than 1µM, with no effect on cell proliferation or heam synthesis at the highest concentration (5 µM), while the other showed increases in PPIX at 5 µM. The accumulation of PPIX is also observed in vivo in rat embryos up to 12 h post dosing while PPIX levels in rabbits remained low; albeit both effects at 1000 mg/kg (p.48). Altogether, the inhibition of haem biosynthesis or PPIX accumulation as a result of flumioxazin treatment might be responsible for the observed ventricular septal defects (VSD), wavy ribs and growth retardation observed in rat embryos via a reduction in fetal erythrocytes, reduced oxygen supply and compensatory growth of the heart. Therefore, the mechanism for VSD is sufficiently well established.

It was concluded that the mechanism for rats was also relevant for humans but that there are quantitative differences between rats and humans and therefore humans are unlikely to develop anaemia from PPO inhibition. Several arguments were provided:

1. Clinical findings that PPO deficient patients with Variegate Porphyria show no signs of anaemia (pg. 54)

We do not agree that Variegate Porphyria (VP) patients are deficient in PPO. As described on page 135 of the proposal, VP patients have a reduced PPO content in erythrocytes. As this is an autosomal dominant disease, the reduction is only partly and could be compensated by increased enzyme production. Further, PPO content in erythrocytes may not be a good indicator for PPO activity in erythroblasts because erythrocytes have no nucleus meaning that no new PPO can be produced. The absence of anemia in VP patients is therefore not considered to be evidence that humans are not or less sensitive to the effects of flumioxazin.

2. Experimental evidence that flumioxazin and its metabolites do not reduce heme production in K562 cells, which are derived from human erythroleukemia (pg.54)

In vitro evidence shows that flumioxazin increases PPIX in a human erythroblast cell line but does not reduce heam production and cell proliferation. However, no comparable studies in rat erythroblast cell line were provided. It cannot be excluded that the absence of an effect on heam production and proliferation is due to the specific conditions in this in vitro experiment. The PPIX concentration was increased approximately 10-fold in this study (Table 29, pg. 35) whereas in vivo the PPIX concentration in rats increased more than 100-fold (Table 34, pg. 50). This difference could be related to the possibly daily refreshing of the cell culture suspension. Therefore, these studies are not considered determinative to show a difference between rats and humans.

3. Humans are less sensitive to PPO inhibition than rats (pg. 54)

Species differences investigating the accumulation ratio of PPIX in primary hepatocytes from rats, rabbits, monkeys and humans by flumioxazin were 10.3, 1.1, 1.4, and 4.4-fold (pg.35). These results suggest that the difference between rats and humans is ~2.3-fold, while the difference between rats and rabbits was ~9.4-fold. Another study investigating the IC50 for the inhibition of PPO after 20 minute incubation with flumioxazin in livers from rats, rabbits and humans showed that the IC50 from rats was only ~2.4-fold lower than that from humans and ~ 19-fold lower than that from rabbits (pg.48). Overall, although

species differences exist between rats and rabbits, the response in rats is only about 2-fold higher than that of humans, indicative that the teratogenic effects observed in rats might also occur in humans.

In addition it was stated that PBPK modeling predicts that human erythroblasts would be insusceptible to flumioxazin. However, this is based on the same studies with K562 cells as in point 2. Further, the level of detail on this model is too limited to assess. The dossier submitter is requested to provide information on the major differences between the PBPK model for humans and for the rat that would justify the claimed large species differences.

Finally, the statement on the absence of effects in the manufacturing plant (pg. 51) does not affect the conclusion on classification because classification is based on hazard whereas the presence of effects is based on hazard and exposure.

Overall, there is good evidence for the mechanism for inducing VSD in rats and there is no evidence that this mechanism is qualitatively not relevant to humans. There are some small quantitative differences between rats and humans indicating that humans may be less sensitive but this only affects the potency and not the hazard. Therefore, the removal of Repr. 1B H360D is not warranted based on the provided data.

Dossier Submitter's Response

1)

In *in vitro* experiments, 50% inhibition of liver cell PPO corresponds to a flumioxazin concentration of 0.006 mg/L. Not even in rats has been anaemia observed up to a concentration by one order of magnitude higher. Absence of anaemia in variagate porphyria patients only confirms this expected resistance of humans to anaemia due to PPO inhibition. In the last decades, multiple genetic studies identified about 80 mutations in the protoporphyrinogen oxidase gene, resulting in a complete (rarely) or in a partial loss of enzyme activity. For instance, in Finland, one type of mutation in the protoporphyrinogen oxidase gene results in a complete loss of activity of the enzyme produced by the mutated allele whereas in other mutations 9.5–25% of the wild type activity is preserved ; the heterozygotic variagate porphyria patients have, therefore, at least 50% total activity of protoporphyrinogen oxidase. The reduced rate of haem synthesis is in addition compensated by an increase in synthesis of delta aminolevulinic acid in porphyria patients. Anaemia is not a component of the clinical picture that is – in porphyric crisis - dominated by symptoms and signs of toxic injury of porphyrin metabolites to liver, skin and nervous system. Unlike the mostly latent course of heterozygous porphyria variegata, the extremely rare homozygous variant is characterized by severe PPO deficiency, high concentration of porphyrins in erythrocytes, onset of photosensitization by porphyrins in early childhood, skeletal abnormalities of the hand, and, less constantly, short stature, mental retardation, and convulsions. Exceptionally, a very low activity of PPO (5 – 15%) may be associated with hypochromic anaemia in some of these homozygotic carriers.
In a recent article (Ferrer et al., 2009) results of hematological analysis of VP were reported and no anemia was observed although PPO content was significantly reduced in erythrocytes. As discussed in the article, this might be the first article to report decreased PPO content in erythroblasts, however, reduced PPO activity have been reported in VP patients. Hepatic porphyrias usually do not accompany anemia or haematological problems. For example, anemia was not found in ALA dehydratase porphyrias, homozygous variants of hereditary coproporphyria or Variegate Porphyria (VP) that can occur in childhood and they are attributable to marked deficiency of ALA dehydratase, coproporphyrinogen oxidase, and PPO, respectively (Sassa, 2000). This fact suggests that a defective enzymatic activity that results in disturbances in haem biosynthesis in liver does not necessarily limit haem

synthesis in erythroid cells. In reviews (Philips & Anderson, 2010; Nordmann & Puy, 2002; Gross et al., 2000), neurovisceral symptoms and photosensitivity are referred as major types of clinical features of VP.

Ferrer MD et al. (2009) Enzyme antioxidant defences and oxidative damage in red blood cells of variegate porphyria patients. Redox Report 14:69-74

Gross U, Hffmann GF, and Doss MO (2000) Erythropoietic and hepatic porphyrias. J Inherit Metab Dis 23:641-661

Nordmann Y and Puy H (2002) Human hereditary hepatic porphyrias. Clinica Chimica Acta 325 17-37

Philips JD and Anderson KE. (2010) The porphyrias, Chapter 57 in Williams Hematology, 8th edition, pp839-86

Sassa S (2000) Hematological aspects of the porphyrias. Int J Hematol 71:1-17

2)

Direct comparison of rats with human subjects in *in vivo* experiments is not feasible. *In vitro* studies confirm substantial differences (see table). Two new studies of this type have been recently conducted. CD36+ cells, derived from human cord blood, are precursor of erythroblasts which can be stimulated to differentiate into haem-synthesizing cells under appropriate culture conditions, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells. The results of the study demonstrated that there were no effects on haem content and cell number of human haem-synthesizing cells at up to 5 µM flumioxazin although PPIX concentration in them increased more than ten times. Similarly, rat erythroleukemia cells can also differentiate into heme-synthesizing cells and may be more convincingly compared to human K562 cells: flumioxazin at 0.1µM and above reduced haem production in them, whereas no such effect was observed at 5 µM in human K562 cells..

Flumioxazin effect in rat and man compared *in vitro*

Dose of flumioxazin	Tissue concentr.	PPIX liver – rat	PPIX liver – man	PPIX ELR - rat	Haem ELR rat	PPIX K562 - man	Haem K562+ man	PPIX CD36+ man	Haem CD36+ man
mg/kg bw *	µM	pg /mg protein	pg /mg protein	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6cells	ng/ 10^6 cells	ng/ 10^6 cells	ng/ 10^6cells
0	0	293	180	0.56	127	0.19	208	1.18	1777
0.018 +	0.00007 +								
4	0.01			0.58	116	0.19	202	1.77	1706
15	0.03	370	190						
30 repeated	0.06 mean								
50	0.1			0.80	91	0.18	186	1.59	2198
30 repeated	0.2 peak								
200	0.3	1200	400	0.95	85				
	1.0	3000	800	2.67	60	0.44	224	2.82	1882

1000	2.4/1.9**								
>1000	5.0			8.32	47	3.0	213	14.0	1535

+ AOEL; absorption rate for oral dose levels ≤ 0.01 mg/kg bw is presumed to be almost 100%
*Flumioxazin oral dose X) corresponding to average tissue concentration. Systemic dose (Y) = X x (oral absorption rate); tissue concentration = Y / MW. Measured oral absorption rates were 89, 50, 35 and 12.4% for oral doses of 1, 30, 100 and 1000 mg/kg bw, respectively. Decrease in absorption rate with dose is represented by regression equation: Y = 130 x (1 – EXP(-0.003xX)) ; dose in the last line is estimated for absorption rate ≤ 10% (≤0.1)
**peak concentration in maternal blood /foetal tissue predicted by PBPK model (Takaku, 2012c)

A study (Kawamura, 2013a) with rat erythroleukemia (REL) cells, corresponding to K562 cells, has been submitted to the public consultation of ECHA in 18 Oct 2013. The objective of this study was to investigate the effect of flumioxazin on the heam synthetic pathway in rat erythroid cells; whereby REL cells were induced to differentiate into erythroid cells using hexamethylenebisacetamide (HMBA), followed by treatment with flumioxazin at concentrations of ranging from 0.01 to 5.0 µM (which is higher than expected concentrations in human embryos whose mother is exposed to flumioxazin at 1000 mg/kg). The result revealed that PPIX was accumulated and heam synthesis was inhibited in REL cells at 0.1 µM and above in a dose dependent manner although heam synthesis was not inhibited in K562 derived from human erythroleukemia at highest dose of 5.0 µM.

A study (Kawamura, 2013b) with human CD36+ cells has also been submitted to the public consultation of ECHA. CD36+ Cells are a precursor of erythroblasts, which can be differentiated into haem-synthesizing cells, and are more relevant to physiological maturation of human fetal erythroblasts than K562 human erythroleukemia cells, hence better addressing effects on haem biosynthesis in human fetal erythroid cells. PPXI was accumulated in human CD36+ cells at 1.0 µM and above in a dose dependent manner. However, there was no effect on cell proliferation and haem synthesis at the highest dose of 5.0 µM in CD36+ cells as well as K562 cells.

In conclusion, the results demonstrated that there are clear species differences in inhibition of haem biosynthesis by flumioxazin between human and rat cells.

Kawamura S, 2013a. Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0125.

Kawamura S, 2013b. Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells. Sumitomo Chemical Co., Ltd. Report No. SBT-0126.

3)
The teratogenic effect in rats at doses not toxic to mother animals is clearcut, but their relevance for humans is not.
Applicant assessed them as not relevant for humans. RMS agrees that they probably are not relevant..
According to Regulation (EC) No 1272/2008:

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

Consequential avoidance of the quantitative aspect in criteria of hazard (for man) may be justified from the procedural point of view. On the other hand, when the risk for humans is negligible, should hazard identification and characterisation neglect this fact completely ?
Regulation (EC) No 1272/2008, subpoint 3.7.2.3.2: admits not to classify such substances:

„ . . If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expresssed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."

The developmental effects of flumioxazin in rat foetuses were observed at maternal dose of 30 mg/kg bw per day (systemic dose of 15 mg/kg bw). The systemic dose-response for these key events has proved to be very steep: half-dose has been without any effect. RMS agrees that difference in sensitivity of protoporphyrinogen oxidase in human and rat hepatocytes to inhibition by flumioxazin doesn't appear to be very large, namely 2.4. For a systemic dose of 15 mg/kg bw, systemic dose predicted to cause similar PPO inhibition in human liver would be 15x2.4 = 36 mg/kg bw and respective oral dose 108 mg/kg bw per day (taking into account decrease in absorption rate with oral dose): that means, for instance, pregnant woman receiving orally more than 6000 mg of flumioxazin daily (> 6000 ADI) for two first months of pregnancy. Moreover, this calculation does not consider two more influential differences between prenatal development in man and in rat:
1) Haem synthesis reduction and severe anaemia in rats at flumioxazin blood concentration of 0.06 µM (corresponding to oral dose of 30 mg/kg bw) contrasts with no haem reduction in human K562 cells or in human CD36+ cells at concentrations 5.0 µM, corresponding to doses > 1000 mg/kg bw.
2) Much longer time between the end of yolk sac haematopoiesis and final foetal stages in humans (from 2 to 9 months) compared to rats (from GD 16 to GD 20) allows for a complete compensation of delays - if any - in the morphological development of human foetus (such as development of the interventricular septum).
All these one-direction points of dissimilarity raise doubt about the relevance of this hazard for humans.

The same PBPK model (USEPA , Godin et al., 2010) predicts time course of flumioxazin concentration in maternal blood and in foetal tissue for oral doses of 30 mg/kg bw (in rats) and for 1000 mg/kg bw (in humans), the predicted concentrations for human subjects is only 10 times higher than concentration measured and predicted in rats administered 30 time higher oral dose; this result is in good agreement with oral absorption rate decreasing from 50% to 12.4%.

Model- predicted peak concentration of flumioxazin in human foetuses (1.92 µM) is lower than concentration inhibiting haem synthesis in human haem synthetising cells (see table summarising results of in vitro comparative studies).

The data of the study demonstrated that rats are more sensitive to flumioxazin treatment than other three species, including humans. On the other hand, flumioxazin did not affect erythrocyte development in human cells (K562 and CD36+ cells), although PPXI accumulation was observed (Kawamura, 2013a & 2013b).

In addition, the developed human pregnant PBPK model demonstrates that the human fetal exposure level to flumioxazin following a maternal oral dose of 1000 mg/kg would be relatively lower than the concentration level observed haem synthesis inhibition in rats. The developed PBPK model for human foetus demonstrated that the expected concentration of the human foetus whose mother is exposed to flumioxazin at 1000 mg/kg would be lower

than the no-observed-effect-concentration for heam synthesis in the human K562 and CD36+ cells studies.

4)

PBPK model is recommended to clarify the difference of pharmacokinetics between animals and humans in the guideline of OECD (OECD, 2008). Since it is impossible to reveal the concentration of flumioxazin in the human fetus experimentally, the prediction using PBPK model is needed to elucidate the concentration. Unfortunately, there are no harmonized PBPK models in EU level at the present moment. Therefore, we selected the PBPK model developed by USEPA (Godin et al., 2010).

OECD GUIDELINE FOR THE TESTING OF CHEMICALS Draft proposal for a revised TG 417: Toxicokinetics, 2008.

Godin SJ, DeVito MJ, Hughes MF, Ross DG, Scollon EJ, Starr JM, Setzer RW, Conolly RB, Tornero-Velez R. Physiologically based pharmacokinetic modeling of deltamethrin: development of a rat and human diffusion-limited model. Toxicol Sci. 115, 330-343, 2010.

5)

It is very important information that no evidence of haematotoxicity, or other adverse health effects in workers who have been involved in the manufacture of flumioxazin for the last decade because 1) the information is very useful in considering a hazard of flumioxazin to humans who are expected higher expose to flumioxazin and 2) hematotoxicity is one of the most representative indicators of the flumioxazin toxicity. In fact, the information demonstrated that manufactory workers who are expected the highest exposure to flumioxazin are not shown anemia.

RAC's response

RAC agrees with the MS (NL) that declassification is not considered an appropriate measure and with their remarks with regard to human relevance and strength of evidence. However, a case has been made to reduce the classification on the basis of the doubts raised on human relevance by the DS. RAC notes the further discussion of the data by the applicant and the RMS but concludes that the doubts with regard to human relevance are not sufficient to warrant classification as Repr. 2 and that the current classification of Repr. 1B should be retained.

OTHER HAZARDS AND ENDPOINTS – Specific Target Organ Toxicity Repeated Exposure

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Belgium		MemberState	17

Comment received

We understand that the proposal focuses on the change related to reproductive toxicity but we would like to ask to the Dossier Submitter if the hematotoxicity will be also reconsidered according to the CLP criteria. This effect was probably not considered sufficient for a classification as R48 under the 67/548/EEC because of the reversibility of the effect but according to the new criteria under the CLP regulation, both reversible and irreversible effects, if they are significant and can impair function need to be taken into consideration. The emphasis on the reversibility of the effects is less important under CLP.

Dossier Submitter's Response

Original proposal of RMS to classify flumioxazin as toxic for specific target organs – repeated exposure has been based on a mild liver injury in dogs exposed orally to daily

doses of 100 mg/kg bw in a 90-day study; still lower doses induced similar liver injury and in addition anaemia in rats. Liver injury – but not anaemia - has been considered relevant to human toxicity. Liver, skin and central nervous system are regularly affected in porphyria patients incl. secondary porphyrias caused by disruptors of porphyrine metabolism. Accumulation of porphyrine metabolism toxic by-products in tissues is common mechanism across animal species, including man. Anaemia has been observed only in rats and not in mice, rabbits or dogs.
Determined effective doses correspond (marginally) to classification STOT RE 2. and hazard statement H373 – "May cause damage to liver through repeated exposure." Oral, dermal and inhalatory routs may all contribute to total systemic dose.
Co-RMS FR commented: According to the CLP criteria, STOT-RE is assigned on the basis of findings of "significant" or "severe" toxicity. In the 90-day dog study, it is questionable if the observed liver effects (biochemical change only) are significant enough at the dose level of 100 mg/kg bw/d to classified flumioxazin. CZ agreed and proposal of STOT RE classification has been withdrawn.

Anemia induced by flumioxazin leads to reproductive toxicities observed in rats. Anemia is observed in only in rats but not in other experimental animals. A series of studies demonstrated human is not likely to cause anemia. We believe R48 is not necessary.

RAC's response

RAC believes that the MS intended that the haematological findings should be considered in the context of classification with STOT RE (blood). Comparison of the data from the rat 90-day and 105 week study indicate that the criteria may be met in one of the two 90-day studies and the anaemia seen in the 1 year study is above the cut-off value. The case for classification is borderline when considering that the relative sensitivity of the rat to this effect is well documented in the data base. On balance, classification is not required as the criteria are not consistently met.

OTHER HAZARDS AND ENDPOINTS – Hazardous to the Aquatic Environment

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Germany		MemberState	18

Comment received

The use of data from aquatic plant tests instead of algae tests is usual for classification and labeling purposes. As a general remark we suggest to use for classification and labeling of acute effects of the substance EC50 values at day 7 (if available) instead of data at day 14 from aquatic plant toxicity tests.
page 105: Justification for the proposal
The study with Navicula pelliculosa (Hoberg, 1996a) gives only a NOEC < 0.000042 mg/L, we would prefer to use for classification and labeling the calculated value of EC5 = 0.000041 mg/L for aquatic chronic category 1 and chronic M-factor of 1000.

Dossier Submitter's Response

The 7-day EC50 is not available from the study on Lemna.
Regarding the study on Navicula pelliculosa, using of EC5 = 0.000041 mg/L would influence neither the classification of the substance nor a chronic M-factor.

RAC's response

According to the DS, RAC noted that in the CLH report there is not the concentration value at day 7. The day 6 concentration, on the other hand, is much more higher than the EC50

at day 14.

RAC agrees with Dossier Submitter that the most sensitive species are Lemna gibba and Navicula pelliculosa as Flumioxazin is an herbicide. Therefore, the test result on Navicula pelliculosa, the EC5 value (=0.000041 mg/L) seen with the EPA 122-2,123-2 method, could be used to define the chronic M factor, instead of the result suggested in the CLH report, that is not adequate for classification because it is reported as a less than (<) value (page 23, ECHA Guidance R7b).

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Belgium		MemberState	19

Comment received

The lowest available acute toxicity value (most sensitive species : Lemna gibba, 14dEC50 = 0.00035 mg/l) results in a classification of Aquatic Acute 1, H400 and a M-factor =1000 (0.0001<LC50≤0.001mg/l). However these results are based on the initial measured concentration. Following approach is given for unstable substances (non-hydrolytically stable substance) in the guidance on CLP :
If measured data are available for the start and the end of the test, the LC50 may be calculated on the geometric mean concentration of the start and end of the test.
Furthermore the OECD test guideline 221 recommends a test duration of 7 days. It would be preferable to recalculate the EC50 to 7d, as this will probably not change the classification of the substance but may have an impact on the determination of the M-factor.

Based on the chronic toxicity of the most sensitive species : Navicula pelliculosa with 5dNOEC<0.000042mg/l, flumioxazin should be classified as Aquatic chronic 1, H410
M-factor : 1000 (0.00001mg/l <NOEC <0.0001 mg/l)
However no indication is given on the analytical measured conc. at the end of the test. Results are based on the initial measured concentration which can give an overestimation of the EC50/NOEC.
Moreover the EC50 and NOEC were measured after 5d while in OECD test guideline 201 for freshwater algae the exponentially growing test organisms are exposed to the test substance over a period of normally 72 hours.

As mentioned above, toxicity studies show that algae are the most sensitive species. However the EC50 for Lemna gibba and the NOEC for Navicula pelliculosa are based on the initial mean measured concentration, while it would have been more appropriate to determine those values on the geometric mean concentration of the start and the end of the test. This will however not influence the classification of the substance as it is already classified as aquatic acute 1 and aquatic chronic 1, but can have an influence on the setting of the M-factor.

Dossier Submitter's Response

According to SANCO 3268/2001/rev.4, endpoints based on initial measured concentrations are considered relevant when effect data are obtained from the test performed under static conditions. It should be noticed that this issue will be discussed in the Pesticides Peer Review Meeting on Ecotoxicology in February 2014.

RAC's response

RAC agrees that the LC50 should be based on the geometric mean, instead of the initial measured concentration, because the concentration is not within the range ± 20% of the

nominal/initial concentration. Flumioxazin, on the other hand, is rapidly hydrolysed and it is necessary to refer to geometric mean concentration during exposure.

Regarding the EC50 recalculation, see the response to comment 18.

RAC noted that EC50 and NOEC were measured after 5d while in OECD test guideline 201 for freshwater algae the exponentially growing test organisms are normally exposed to the test substance over a period of 72 hours.

Date	Country	Organisation	Type of Organisation	Comment number
21.10.2013	Sweden		MemberState	20

Comment received

The Swedish CA supports the environmental classification of Flumioxazin (CAS 103361-09-7) as specified in the proposal. SE agrees with the rationale for classification into the proposed hazard classes and differentiation.

CLP- Aquatic acute hazards

Aquatic acute category 1 (H400) follows from the acute toxicity of the active substance to Lemna gibba: EC50 < 1 mg a.s./L (EC50 = 0.00035 mg a.s./L, Hoberg, 1996b). A M-factor of 1000 is applicable based on 0.0001 < LC50 ≤0.001 mg a.s./l.

CLP-Aquatic chronic hazards

Aquatic chronic category 1 (H410) follows from the chronic toxicity of the active substance to Navicula pelliculosa: NOEC ≤ 1 mg a.s./L (NOEC < 0.000042 mg/L, Hoberg, 1996a) and the fact that the active substance is not readily biodegradable and not rapidly biodegradable. A M-factor of 1000 is applicable based on 0.00001 < NOEC ≤ 0.0001 mg/l.

Pictogram is required for 'Aquatic acute 1' and 'Aquatic chronic 1' category substance.

Dossier Submitter's Response

Noted.
The mistake has been revealed in the dossier submitted: the trigger value for aquatic chronic category 1 was erroneously given as NOEC ≤1 mg a.s./L, it should be NOEC ≤0.1 mg a.s./L.

RAC's response

Noted.

ATTACHMENTS RECEIVED

1. **Flumioxazin: Classification for Developmental Toxicity** [Filename: Flumioxazin - Simon Warren statement] Submitted by an Individual on 18.10.2013. *[Please refer to comment 4 and 14]*

CONFIDENTIAL ATTACHMENTS

2. ***Effects of flumioxazin on heme synthetic pathway and cell proliferation in human CD36+ cells****,* Kawamura S., 2013. [Filename: Effects of flumioxazin in CD36+ cells] Submitted by Sumitomo Chemical on 18.10.2013 *[Please refer to comment 6]*

3. ***Effects of flumioxazin on heme synthetic pathway and cell proliferation in rat erythroleukemia cells****,* Kawamura S., 2013. [Filename: Effects of flumioxazin in REL cells] Submitted by Sumitomo Chemical on 18.10.2013 *[Please refer to comment 6]*