

COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

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Last data extracted on 12.06.2018

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-dicarboximide

CAS number: 103361-09-7

EC number: 600-425-7

Dossier submitter: Czech Republic

GENERAL COMMENTS

Date	Country	Organisation	Type of Organisation	Comment number
07.06.2018	Belgium		MemberState	1
Comment received				
<p>BE CA would like to thank the Czech CA for this new CLH report of Flumioxazin in order to address uncertainties raised by RAC through new studies.</p> <p>Indeed, first CLH report was submitted in 2013 and RAC concluded in the June 2014 meeting (RAC-29) that the doubts with regard to human relevance were not sufficiently convincing to warrant classification as Repr. 2 or no classification and that the current classification for flumioxazin as Repr. 1B should be retained. During the first public consultation, BE CA submit a comment where we did not support the Repr. 1B classification removal for flumioxazin. However, at this moment, we didn't had the opportunity to take note of two new studies that were submitted during the same public consultation (Kawamura 2013a and 2013b).</p> <p>Five new mechanistical studies are presented in the new CLH report (Hosokawa, 2015 ; Abe, 2014 ; Kawamura 2015a, 2015b and 2015c) and brief summaries are available for previous studies in this report, including the studies submitted during the last public consultation.</p> <p>We were surprised to learn from the industry in charge of this CLH proposal that they will send new data during the present public consultation and even after this consultation directly to the RAC. Majority of those new studies were available before this public consultation was launched. According to article 5 and 37 of CLP Regulation, and as explained in the "Guidance on the preparation of dossiers for harmonised classification and labelling" sections 5.2.2 and 5.2.5, all relevant information shall be taken into consideration in order to determine the appropriate classification of a substance. We therefore regret that the procedure was "infringed" in this case and that the MS do not have a chance to look carefully at all the known existing data.</p>				

TOXICITY TO REPRODUCTION

Date	Country	Organisation	Type of Organisation	Comment number
01.06.2018	Japan	Sumitomo Chemical	Company-Manufacturer	2

Comment received
Sumitomo fully support the proposed classification presented in the CLH report. New data are provided here to confirm further the unlikely relevance of the MoA to human.
ECHA note – An attachment was submitted with the comment above. Refer to public attachment open.zip
ECHA note – An attachment was submitted with the comment above. Refer to confidential attachment confidential.zip

Date	Country	Organisation	Type of Organisation	Comment number
01.06.2018	United Kingdom		Individual	3

Comment received
<p>We have more than forty years of professional experience in the field of developmental and reproductive toxicity. We have reviewed the CLH dossiers, study reports for the mechanistic investigations and related documentation for flumioxazin, as independent consultants for Sumitomo Chemical Agro Europe S.A.S. The evaluation below represents our own opinion on the data reviewed.</p> <p>Flumioxazin is currently classified by ECHA as Category Repro 1B, based upon the occurrence in rats of foetal growth retardation, oedema, death in utero and foetal abnormalities, in particular enlarged heart, interventricular septal defect (IVSD) and wavy ribs at non-maternally toxic doses. No adverse foetal effects were seen in rabbit foetuses, even at maternally toxic doses that were x100 those used in the rat studies. In 2013 mechanistic data regarding the mode of action were submitted to ECHA with a view to revising the classification to Category Repro 2, but the RAC members felt that there were still a number of questions to be answered. To address these, a further programme of mechanistic studies has been completed and these form part of the current submission to ECHA.</p> <p>A new in vivo study in rats (Hosokawa 2015; study no. SBT-0129) has confirmed that the foetal abnormalities produced by single high doses of flumioxazin (≥ 400 mg/kg bwt on gestation day 12) can be reproduced by much lower repeated doses (≥ 30 mg/kg/bwt/day). The proposed mechanism underlying the foetal abnormalities appears to be the same in both cases, namely the induction of foetal anaemia as a consequence of inhibition of protoporphyrinogen oxidase (PPO), leading to the accumulation of protoporphyrin IX (PPIX) outside the mitochondria and interference with haem synthesis. In order to overcome the hypoxia due to the anaemia, the stroke volume of the foetal heart would increase, leading to enlargement of the ventricles, thinning of the ventricle walls and IVSDs. Rib abnormalities were likely to be a consequence of foetal hypoxia resulting from the foetal anaemia (Kast 1994).</p> <p>Because of the limited information in the public domain, it has not been possible to give an explanation for the apparent absence of IVSDs in foetuses exposed to other PPO inhibitors that caused maternal anaemia. Sumitomo has suggested that this might be due to the method by which the foetal hearts were examined and have demonstrated that the transverse sectioning method employed routinely by them is a much more sensitive method of identifying IVSDs than the fresh dissection method used by many other laboratories (Fujii 2016, Study number STB-0136). Our experience confirms that this is the case; however, without knowing the methods of evaluation used to examine the foetuses from studies with other PPO inhibitors, no conclusion can be drawn. Also, with no information as to whether foetal anaemia was induced in these studies, it is not possible to speculate whether IVSDs should have been present. However, as similar foetal abnormalities in rats have been recorded following maternal treatment with the antimalarial drugs artemisinins, which induce foetal anaemia but by a different mechanism from flumioxazin (White et al. 2008; Clark et al. 2018) it is considered that it is likely the foetal anaemia that is responsible for</p>

the abnormalities and not any direct action of flumioxazin per se on the developing heart. From investigations in vivo, in vitro and in silico, a body of evidence has been amassed which demonstrates that the adverse effects seen in the rat are unlikely to be of relevance to humans. Arakawa et al. (2017a, b) have investigated differences in the binding affinity between PPO and three ligands, namely, flumioxazin which is teratogenic in the rat but not the rabbit, and two structurally related N-phenylimides, S-23121, which is also teratogenic in the rat but not the rabbit, and S-23031, which is not teratogenic in either species. The findings demonstrated that rat PPO showed the greatest binding affinity for flumioxazin, human PPO showed considerably less affinity, whilst rabbit PPO showed the least binding affinity. These data are in agreement with the inhibitory potencies (pIC50) of flumioxazin determined experimentally in vitro using rat, human and rabbit hepatic mitochondria. The binding affinity of the three ligands showed a positive correlation with the teratogenic potential in the rat and rabbit, with flumioxazin showing the greatest affinity and S-23031 showing the least affinity.

The global and local dynamics of the flumioxazin binding "pockets" of the PPO molecule were also investigated. It was shown that variants in the amino acids sequence in the 107-120 loop sequence of PPO were responsible for the differences in binding characteristics of the three species, by changing Arg97 dynamics. In the case of the human PPO/flumioxazin complex, a change in shape of the "pocket" led to reduction in the van der Waal force, whereas, with the rabbit PPO/flumioxazin complex, the weakened binding affinity was due to a positional and orientational shift that reduced the Coulomb force.

Because it is not possible to investigate the effects of flumioxazin administered directly to humans, a series of studies have been performed in vitro with different human cell lines to investigate the human relevance of the foetal anaemia produced by flumioxazin in the rat. Following the 2013 submission, ECHA had expressed concern regarding the suitability of the K562 cells of neoplastic origin (Kawamura 2012, study number SBT-0119) and the CD36+ cells of non-neoplastic foetal origin (Kawamura 2013, study number SBT-0126) to model the normal human embryonic primitive erythroid cell. Therefore, a third cell line derived from human pluripotent stem cells (hiPSC) has also been investigated (study number SBT-0152). Quantitative mRNA expression analysis has shown that, in hiPSC-derived erythroid cells, over 60% of the total beta-like globins mRNA was embryonic ϵ -globin; the remaining 28-38% was γ -globin which is fetal globin but also expressed in primitive erythroid cells. In K562-derived erythroid cells approximately 25-30% was ϵ -globin and the remainder γ -globin. Thus it can be deduced that the erythroblasts differentiated from hiPSC cells are more representative of embryonic erythroblasts than those derived from K562 cells.

Each of the human cell lines was chemically induced to differentiate into erythroblasts, and was then exposed to flumioxazin at concentrations up to 5.0 μM , i.e. x90 greater than the concentration of 0.056 μM found in rat embryos from dams treated at 30 mg/kg bwt/day during organogenesis, a dose level that resulted in foetal anaemia and IVSDs. Whilst accumulation of PPIX was recorded for each cell line there was no effect upon haem synthesis or cell proliferation at concentrations up to 5.0 μM .

Thus, despite the differences in erythroblast origin and in globin content, the similarity of the response of erythroblasts derived from all three human cell types to flumioxazin exposure (accumulation of PPIX, no effect upon haem synthesis) suggests that the enzymes involved in haem synthesis are conserved between the cell types. On this basis it is not unreasonable to assume that human erythroblasts of embryonic origin would also show no reduction of haem synthesis if exposed to flumioxazin.

The flumioxazin results using human cell lines were different from the findings using rat erythroblasts differentiated from REL cells in that, in addition to accumulation of PPIX, haem synthesis was inhibited at a dose of 0.1 μM . Using a PBPK model based upon a comparison of rat and human parameters, it has been calculated that if a pregnant woman were to be exposed to a dose of 1000 mg/kg the foetal exposure would be 1.92 μM . This is well below the 5.0 μM to which the erythroblasts derived from human cells were exposed in vitro without

affecting haem synthesis and thus it may be predicted that foetal anaemia is unlikely result from exposure during human pregnancy even following exposure as high as 1000 mg/kg, equivalent to x20,000 the ARfD of 0.05 mg/kg bwt/day or x111,000 the ADI of 0.009 mg/kg bwt/day. With regard to accumulation of PPIX, in the experience of an independent expert, patients with VP have not shown any complications of pregnancy nor have foetuses presented with anaemia or cardiac malformation (see annex 3 of 2017 CLH dossier). On a weight of evidence basis, therefore, whilst it is recognised that a clear adverse effect upon development in utero has been demonstrated in the rat following administration of flumioxazin during the period of organogenesis, no adverse effects were seen in the rabbit at doses levels x100 those which resulted in adverse effects in the rat. It has been demonstrated that the mechanism by which the abnormalities are induced in the rat (foetal anaemia) is unlikely to be of relevance to the human foetus.

Taking into account the recent investigations, it is considered that a classification of Category Repro 2 would be more appropriate for flumioxazin than the current classification of Category Repro 1B.

References:

Arakawa A., Otani M., Iwashita K., Yamakazi K. (2017a): Molecular dynamics mechanism to generate species differences in inhibition of protoporphyrin oxidase by flumioxazin. *Computational Toxicol.* 1 pp.12-21.

Arakawa A., Otani M., Iwashita K., Yamakazi K. (2017b). Corrigendum to "Molecular dynamics mechanism to generate species differences in inhibition of protoporphyrinogen oxidase by flumioxazin". [*Comput. Toxicol.* 1 (2017) 12–21]. In press: *Comput. Toxicol.* (2017), <http://dx.doi.org/10.1016/j.comtox.2017.06.001>

Clark R.L., Edwards T.L., Longo M., Kinney J., Walker D.K., Rhodes J., Clode S.A., Rückle T., Wells T., Andermatten N., Huber A.C. (2018): Improved safety margin for the new endoperoxide artefenomel (OZ439) as compared to artesunate. *Birth Defects Res.* 110(7) pp. 553-578

Kast A. (1994): "Wavy ribs". A reversible pathologic finding in rat fetuses. *Exp Toxicol Pathol.* 46(3) pp. 203-10.

White T.E.K., Bushdid P.B., Ritter S., Laffan S.B., Clark R.L. (2006): Artesunate-induced depletion of embryonic erythroblasts precede embryoletality and teratogenicity in vivo. *Birth Defects res. (Part B)* 77 pp. 413-429

Date	Country	Organisation	Type of Organisation	Comment number
29.05.2018	Netherlands		MemberState	4

Comment received

Thank you for your efforts and classification proposal.

In 2014, RAC concluded that a reduction/removal of cat 1B classification could not be justified because of some remaining concerns:

1. Only single high exposure was used to explore pathogenesis and the critical window. Since the repeated dose developmental toxicity studies had lower internal concentrations but at least similar developmental effects, the critical events were not sufficiently demonstrated for the low dose level of 30 mg/kg bw.
2. The difference between PPO inhibition in vitro between rat and human hepatocytes was used to demonstrate higher sensitivity for rat cells. However, the difference was small (<3x) and relevance uncertain. Further, the difference between adult and foetal rats with respect to PPO-inhibition was limited (similar IC50 values), whereas the foetus was far more sensitive to flumioxazin damage. In contrast, PPIX was demonstrated to accumulate in the rat foetus at very high levels compared to adult liver, which could be due to rapid excretion into bile and faeces in the adult.
3. Investigation of subsequent inhibition of haem production revealed that in K62 and CD36+ derived erythroblasts (human) no inhibition of haem occurred after flumioxazin

exposure while this was the case when REL derived erythroblasts were exposed to flumioxazin. RAC expressed the concern that there was uncertainty regarding the extrapolation of results obtained from in vitro erythroblasts to fetal erythroblasts during development.

4. There is certainly no doubt the rat embryo is very sensitive to flumioxazin exposure. The proposed cause was not considered sufficiently proven (synchronous maturation in rat embryos during a short window while in humans erythroblast populations are more heterogenous over a longer time period). Additionally RAC considered it likely based on this hypothesis that some damage should occur in human embryos but the nature and recovery are uncertain.

Overall, RAC concluded in 2014 that the MoA was plausible, but not convincingly demonstrated and human relevance could not be excluded.

In the updated CLH proposal, the dossier submitter provided new studies to address these concerns:

- Inhibition of PPO by flumioxazin and its major metabolites
- 3 in vitro studies assessing the comparative effects flumioxazin and dihydroartemisinin (DHA) (antimalarial drug also reducing haem synthesis but with no known developmental effects in humans) on the haem pathway in rat erythroleukemia cells, K562 and CD35+ cells.
- Additional in vivo mechanistic study evaluating the potential of flumioxazin to cause developmental toxicity in rats.

1. The new in vivo mechanistic study (SBT-0129) used low repeated exposure to address the first concern but no single low dose was given during the proposed critical window. Therefore this study answers the question whether the same pathogenesis occurs (by dissection of half of the animals on GD14) at this low repeated dose but does not answer if it occurs after a single relevant (low) dose during the proposed critical window. Comparing the effects observed after a single dose during the proposed critical window and with those observed after a similar dose given outside the critical window (which should then yield no developmental effects) would provide more evidence for the critical window and hence the proposed single mode of action. There are positive effects seen after dosing during the critical window, but evidence showing negative effects after dosing outside the proposed critical window is lacking for flumioxazin (while it has been shown with DHA).

2/3. In the understanding of the NL-CA, concerns no. 2 and 3 are in part addressed with the comparative in vitro studies using the human antimalarial drug DHA (SBT-0130/0131/0132). The proposed MoA for flumioxazin is partly the same as for DHA. Both flumioxazin and DHA cause fetal anaemia which leads to a similar pattern of developmental toxicity in the rat characterized by ventricular septal defects and embryofetal death (SBT-00-0012, and Qigui and Weina 2010, respectively). Fetal anaemia from exposure to flumioxazin is caused by inhibition of haem synthesis resulting from inhibition of PPO. On the other hand, several hypotheses for the mechanism of fetal anaemia by DHA are reported. The critical window is supposedly not present or not as tight during human embryonic development according to the DS. In support, no developmental effects are seen in humans after exposure to DHA since its application (>20 years). The dossier submitter considers this likely holds true for flumioxazin as well.

The NL-CA notices the following: the new in vitro studies indicate that DHA inhibits haem synthesis like flumioxazin (although via a different pathway) and it does so in both the rat and the human cell lines. In contrast, flumioxazin exerts this effect only in a rat cell line and not in human cell lines. This suggests the mode of action of DHA is more applicable across species in contrast to flumioxazin. The assays indicate that flumioxazin does not inhibit haem synthesis in human cell lines while it does in rat cell lines. However, the mechanism by which both substances inhibit haem synthesis is different (i.e. flumioxazin: via PPO-inhibition, DHA: several hypotheses for the mechanism of fetal anaemia are reported). The

reduced haem synthesis is considered linked to a reduction in viable blood cells and therefore to anemia. The NL-CA considers this valuable supporting evidence for the proposed mode of action. Concern 2/3 included also uncertainty regarding the small species differences in PPO inhibition and haem synthesis reduction as well as the small difference between adult and foetal PPO-inhibition. These concerns do not seem to be addressed completely. The relevance of the in vitro findings for in vivo erythroblasts in developing embryos will remain uncertain but should not be a leading argument against declassification if most of evidence (woe approach) indicates the effects seen in rats are not applicable to humans.

4. Concern 4 is not specifically addressed using new data, but the plausibility of the MoA is increased further by the comparison of DHA and flumioxazin. However, there still remains some uncertainty regarding the relevance and possible damage to human embryos. With DHA, no human developmental effects have been noted. However, this may be a cause of exposure regime rather than intrinsic hazard.

The in vitro study including major metabolites indicating that the parent flumioxazin is the most potent PPO inhibitor (SBT-0128) does not seem to be very informative regarding the concerns of the RAC. Perhaps the notifier wanted to use this data to support their PBPK model that estimates small concentrations in the human embryo after high external exposure. However, uncertainties mentioned in the previous RAC opinion remain including those related to the quantitative relevance of the in vitro (transformed erythroblast or erythrocytes) dose-response compared to in vivo. Additionally, it is noted that the biologically effective dose relevant for internal in vivo exposure was not measured in the in vitro assays. The free concentration, being more related to the biologically effective dose, may be lower compared to the added nominal concentration (because of sorption to proteins, glass/plastic labware etc). For quantitative extrapolation from in vitro to in vivo this is an aspect that has to be taken into account. Since flumioxazin has a LogP of above 2 (Pubchem), binding to various in vitro components is likely to occur with resultant reduction of the bioavailable concentration by over 50% (Armitage et al., 2014, Fischer et al., 2017, Gulden and Seibert., 2003).

Overall, in the opinion of the NL-CA, concern 1 has been answered to limited extent, concerns 2 and 3 may be considered largely addressed while concern 4 was not addressed. The NL-CA concludes that the mode of action is plausible but a few uncertainties remain including a definite confirmation of the MoA and concern 4 as described above.

It is difficult to assess whether the newly provided data sufficiently reduces the concerns to allow a downscale of the classification to category 2. According to the CLP regulation, Category 2 is more appropriate when mechanistic information raises doubt about the relevance of the effect for humans. Clearly, the available information raises doubt regarding the relevance for humans but the mechanistic information consists of a hypothesis that is not ultimately proven.

Additional references.

Armitage, J.M., Wania, F. & Arnot, J.A. 2014. Application of mass balance models and the chemical activity concept to facilitate the use of in vitro toxicity data for risk assessment. *Environmental Science and Technology*, 48, 9770-9779.

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K.-U. & Esscher, B. I. 2017. Modeling Exposure in the Tox21 in Vitro Bioassays. *Chemical Research in Toxicology*, 30, 1197-1208.

Gulden, M., Seibert, H., 2003. In vitro-in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. *Toxicology*, 189, 211-222

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Date	Country	Organisation	Type of Organisation	Comment number
07.06.2018	United States		Individual	5

Comment received

I agree with the evaluation by the Rapporteur 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 and the evaluation of its human relevance.

ECHA note – An attachment was submitted with the comment above. Refer to public attachment Cohen_CLH Report Response - rev 6-6-18.docx

Date	Country	Organisation	Type of Organisation	Comment number
07.06.2018	Belgium		MemberState	6

Comment received

Developmental toxicity

Flumioxazin induced embryoletality and teratogenicity in the rat following dosing via both the oral (at 30 mg/kg bw/day) and dermal (at 300 mg/kg bw/day) routes. Adverse effects on rat fetuses included cardiac ventral septal defect, wavy ribs, reduced ossification of sacrococcygeal vertebral bodies and growth retardation, without maternal toxicity. These observations were supported by the occurrence of reduced litter size and reduced pup weight seen in the 2-generation study.

The substance has a harmonized classification for reproductive toxicity in category 1B (H360D). The current CLH proposal aims to modify the classification in category Repr. 2 (H360d) based on the justification that the effects seen in the rat would not be relevant for humans due to toxicokinetic differences between the two species.

The proposed mode of action is the following : flumioxazin induces the inhibition of protoporphyrinogen oxidase (PPO), a key enzyme in the normal haem synthesis in mitochondria, resulting in protoporphyrin IX (PPIX) accumulation in the plasma and potential anaemia. BE CA is of the opinion that this mode of action is likely and considered as relevant for both rat and human. In rat, the reported developmental effects might be explained by the induction of fetal anaemia which further leads to tissues hypoxia followed by suppressed liver function and decrease in protein synthesis. The latter might explain wavy ribs and oedema observed in the fetus, and also growth retardation and fetal death. The fetus compensation for the anaemia might cause heart enlargement, which might be considered as the cause of cardiac ventral septal defect.

In rat, the critical period for sensitivity to the pre-natal developmental effects of flumioxazin has been shown to be on gestation day 12 and the adverse effects were seen at 30 mg/kg bw/day in the rat developmental oral study. It has been proposed that rat embryos are particularly sensitive to haematotoxic effects of flumioxazin due to a "synchronized" differentiation of erythroblasts, whereas a relatively heterogenous population is reported to occur in human embryos during primitive haematopoiesis. However no clear demonstration has been made that the rat erythroblast differentiation is really synchronized, leading to a single population, and not just happening faster than in human, due to a shorter gestation length (21 days vs 280 days). In contrary, results of the Ihara study show that although a vast majority of the cells present the same morphology, erythroblasts are indeed observed

as a heterogeneous population. BE CA would therefore prefer not to use the term "synchronized" to describe erythroblast differentiation in rat because this term suggests that the rat erythroblast differentiation pattern is dissimilar to the human one.

Moreover, DS argued that in rat an acute exposure to flumioxazin during the critical period of sensitivity would lead to the loss of one particular population, leading to an impairment of blood production in fetuses and therefore anaemia. In humans, it has been suggested that the same exposure to flumioxazin might not lead to developmental toxicity because the heterogeneous population would compensate for the effects of flumioxazin. BECA believes that this justification might only apply if the exposure would happen over a short period of time. It cannot be excluded that a repeated exposure during human early erythropoiesis would possibly lead to fetal anaemia due to a repeated impairment of the same targeted population.

Additional in vitro studies using cord blood derived and erythroleukemia rat and human cell lines were conducted to support the proposal that human erythroid cells are less sensitive to the effects of flumioxazin on haem production than rat erythroid cells. These findings are based on cell lines that need to be differentiated in vitro, with all the associated inherent uncertainties. For example :

- In vitro derived cells are erythroblasts-like cells and might therefore not reflect normal erythroblast cells, although their profiles might be close. This uncertainty is particularly critical if one considers that a specific population might be targeted, as discussed above ;
- The differentiation-inducing cocktail is different between the various investigated cell lines, which suggest that it might be inappropriate to compare these cell lines ;
- K562 and REL cell lines are derived from cancerous cell lines.

Moreover, the maximum tested dose is comparable in human and rat cell lines (up to 5 μ M), without observation on cell proliferation in both. Taking into consideration the demonstrated lower sensitivity of human liver enzymes to PPO inhibition, the experimentation should have been carried with higher dose-ranges in human cell lines. For example, a slight decrease in haem production associated with substantial accumulation of PPIX are observed in human CD36+ cells only at the highest dose of 5 μ M. Finally, BE CA also noted that no purity is stated in the Kawamura studies before 2015.

Therefore, although these in vitro studies suggest that modelled human erythroblasts might be less sensitive to flumioxazin than rat erythroblasts, these results should only be used as supportive because of their inherent uncertainties.

The DS proposed that rats would be more sensitive than humans, which would in turn be more sensitive to rabbits. Indeed, no developmental effects have been reported in rabbit at doses up to 3000 mg/kg bw/day. Thanks to the mechanistic studies provided by the DS, this observation can be explained. Various studies demonstrated that rabbit liver enzymes showed less sensitivity to inhibition by Flumioxazin than the rat enzymes. In particular, a different sensitivity to adult liver-derived PPO inhibition has been shown between rat, human and rabbit :

IC₅₀ rat = 7,15 nM ; IC₅₀ human = 17,3 nM and IC₅₀ rabbit = 138 nM.

Overall, IC₅₀ results for the three species suggest that the sensitivity of human liver PPO is closer to rat liver PPO than rabbit liver PPO. Therefore, BE CA believe that, although the potency of flumioxazin might be higher in rat than human, rat remains a better model than rabbit for assessing the developmental toxicity of flumioxazin to humans.

DS also argued that patients with Variegate Porphyria (VP), a genetic disease characterized by a deficiency of PPO activity, show no signs of anaemia and no cardiac malformations, either for themselves or their babies. In humans, haem synthesis is carried out by erythropoietic cells for 75% and by liver parenchymal cells for 25% and there is significant

tissue-specific regulation for enzymes in the haem biosynthetic pathway. Therefore, porphyrias are metabolic diseases that can be divided into three different categories according to the impaired tissue-specific haem synthesis enzyme : erythropoietic, hepatic or mixed. Anaemias are only observed in erythropoietic or mixed porphyrias because haem is mostly produced by bone marrow, as discussed above. On the opposite, VP is an autosomal dominant hepatic porphyria and in most of the cases, the enzyme inactivation is partial because only one allele is affected. It is therefore not expected to observe anaemia or developmental effects induced by fetal anaemia in VP patients, as the organism may compensate the slight impairment in haem production.

Finally, it was pointed out that no evidence of haematotoxicity or other adverse health effects in workers were reported in a manufacturing plant of Flumioxazin. DS concluded on this basis of the intrinsic low toxicity of flumioxazin. BE CA is of the opinion that such statement is inappropriate because personal protective equipment was applied and the number of workers involved was very low (n=15).

As a general conclusion, considering that :

- The observed effects, without maternal toxicity, warrant a classification in Cat 1 and that the substance is already classified Repr. 1B ;
- The mode of action is considered relevant to humans ;
- It cannot be concluded that the toxicokinetics differences between rat and human are so marked that it is certain that the hazardous effects will not be expressed in humans ;

BE CA is of the opinion that the current Repr 1B (H360D) classification should be maintained.

References :

Fujita H, Molecular Mechanism of Haem Biosynthesis. *Exp. Med.*, 1997, 183, 83-99
 Sassa S, Modern diagnosis and management of the porphyrias. *British Journal of Haematology*, 135, 281-292

Date	Country	Organisation	Type of Organisation	Comment number
07.06.2018	United States	Sumitomo Chemical	Company-Manufacturer	7

Comment received

I am a consultant with more than 40 years of experience in the areas of reproductive and developmental toxicology. I have reviewed the statement of the Committee on Risk Assessment concerning the potential developmental and reproductive effects of flumioxazin and other documents in the flumioxazin dossier. I have also reviewed new studies performed by Sumitomo Chemical Company, which were provided to me by Sumitomo. The following is my objective interpretation of the data regarding the potential reproductive and developmental toxicity of flumioxazin and my independent opinion regarding its potential risk to human reproductive health.

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 not 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 embryonic and fetal blood stream. This is potentially catastrophic to rat embryos because their yolk sac-derived erythroblasts develop synchronously and entire

segments of erythroblasts would be lost, leading to anemia. The resulting embryonic and fetal anemia and hypoxia are the cause of developmental toxicity seen in rat fetuses. 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. It is my opinion that embryonic and fetal hypoxia and changes in serum proteins in rat embryos may exacerbate tissue fixation issues associated with the Nishimura fetal dissection method. Because tissue fixation makes thin tissues (such as the membranous ventricular septum) brittle and subject to breakage during dissection, the low incidence of cardiac ventricular septal defects (VSDs) reported mainly among fetuses from treated dams in the rat embryo-fetal development study in rats may be artifacts of the Nishimura fixation method.

Previously available evidence, including the Sponsor's mechanistic data combined with literature studies, was adequate to support the proposed mode of action in the rat. A new study has also demonstrated that the mode of action previously demonstrated at a single high dose of flumioxazin also operates at the lowest dose (30 mg/kg bw/day). The evidence supporting this mode of action is bolstered by additional new experiments. The first experiment compared the impact of flumioxazin on in vitro embryonic erythropoiesis in rat versus human erythroid cell lines. This experiment demonstrated interference with cellular proliferation and heme synthesis in rat, but not human, erythroid cells. In a second experiment, the in vitro IC50 for PPO inhibition by flumioxazin was highest for rats compared to rabbits and humans. A final set of experiments used molecular dynamics tools to correlate the IC50's of rat, rabbit and human PPO with the changes in free energy associated with molecular docking and to measure the species differences in the local dynamics of the flumioxazin binding pockets. The data demonstrate that flumioxazin has a higher binding potency against rat PPO compared to rabbit or human PPO (22% and 21% lower binding affinities, respectively, based on $\Delta G_{\text{binding}}$), providing evidence that humans are less susceptible than rats to the PPO-inhibiting effects of flumioxazin. Taken together, the available information is sufficient to establish that the developmental effects seen in the rat are not relevant to humans because human yolk sac-derived erythroblasts do not develop synchronously and therefore any loss of erythroblasts in humans would be small and would cause neither anemia nor hypoxia in embryos, which is the underlying mechanism of developmental toxicity for flumioxazin.

In conclusion, based on the totality of the toxicological data supported by these new mechanistic data, it is my opinion that flumioxazin should not be classified as a human reproductive hazard.

Date	Country	Organisation	Type of Organisation	Comment number
05.06.2018	United States		Individual	8
Comment received				
<p>I am a toxicological consultant with substantial expertise in classification under CLP. I have been able to review the data for flumioxazin in detail, including new data which I understand will be submitted for this consultation, with my time funded by the Industry data owner. My comments however represent my own independent opinion; they are not submitted on behalf of the company and the company has no influence on my comments. After review of the data and reasoning, I agree the statement (p 62 of the CLH Report) that "the MoA in the rat is unlikely to be relevant to (hu)man".</p> <p>To my interpretation, new data demonstrate that: the crucial foetal anaemia in rats occurs at the same low doses as the low effect level for developmental toxicity including for VSD; and that there are structural amino acid differences at the flumioxazin PPO binding site between rat, rabbit and human. Most crucially, there is a marked qualitative difference in</p>				

the in-vitro response of rat embryonic erythroblasts (REL cell line) and human embryonic erythroblast cell lines (K562, CD36+ and hiPSC): haemoglobin synthesis is significantly impaired in the rat cell line which reflects in-vivo findings in the rat embryo; but haemoglobin synthesis is not impaired in any of the three human cell lines. The sensitivity of haemoglobin synthesis to toxic insult in the in-vitro cell lines (rat and all three human lines) is demonstrated by a positive control, DHA, which is known to inhibit foetal haemoglobin synthesis by a different MoA. Use of three different and responsive human cell line models surely covers the sensitive stages of human embryo and foetal erythropoiesis: the presence of embryonic haemoglobin sub-types is demonstrated in these cell lines. These are strong data, indicating a clear qualitative species difference and that the human embryo and foetus would not be susceptible to the crucial anaemia seen in the rat; consequently that classification for human health on the basis of VSD and subsequent foetal loss in rats is not appropriate. As a result, I do not support the existing classification as Repr 1B.

Date	Country	Organisation	Type of Organisation	Comment number
02.06.2018	United States		Individual	9

Comment received

I have been provided by Sumitomo Chemical Agro Europe S.A.S. with studies bearing on possible reproductive and developmental effects of flumioxazin. I was asked to give an independent opinion on possible human reproductive or developmental hazard of flumioxazin. I reviewed the statement of the Committee for Risk Assessment (RAC) based on the RAC meeting of 6 June 2014. Since that meeting, additional studies have been performed that address the RAC opinion.

Flumioxazin is an herbicide that inhibits protoporphyrinogen oxidase (PPO), an enzyme involved in the synthesis of haem. As a consequence of this inhibition, there is a decrease in haemoglobin synthesis by the erythroblast in the rat fetus on about gestation day 12, resulting in fetal anemia with consequent fetal cardiac enlargement, ventricular septal defect, and embryofetal lethality. One concern of the RAC in 2014 was the lack of demonstration of this mode of action in the rat at a maternal dose level of 30 mg/kg/day, the lowest dose level at which a significant increase in ventricular septal defect has been observed in the rat fetus. The mode of action at this dose level has now been demonstrated in a study referenced as SBT-129 and shown to be the same as that previously demonstrated at a higher dose.

Impairment by flumioxazin of embryonic haemoglobin synthesis does not occur in cells modeling the human embryonic erythroblast at exposure levels in excess of that which could feasibly be achieved by human exposure to flumioxazin. The human cells that have been tested in this regard include a human erythroleukemia cell line (K562), an umbilical cord blood CD36+ cell line, and an erythroid stem cell induced from human pluripotent stem cells. These cells produce characteristic human embryonic and fetal haemoglobins and haem synthesis is not impaired at in vitro flumioxazin concentrations of up to 5 µM. By contrast, a rat erythroleukemia cell line shows impaired haem production at a flumioxazin concentration comparable to that estimated to be present in the rat embryo after administration of developmentally toxic dose levels of flumioxazin to the dam.

The basis for the difference in flumioxazin activity in the human versus the rat erythroid cells may be a difference in amino acid sequences in the PPO structure that results in energetically less favorable binding of flumioxazin to the human compared to the rat enzyme. Binding of flumioxazin to the human enzyme decreases the strength of van der Waals interactions, which tilts the energetics towards the unbound state.

Because the mode of action of flumioxazin developmental toxicity in the rat has been well characterized, it is appropriate to examine whether the steps in the mode of action pathway are possible in human embryos or fetuses. The molecular initiating event in the rat is

binding of flumioxazin to rat PPO. The molecular dynamics simulation suggests that if this initiating event occurs in human embryos, the weakened van der Waals forces decrease substantially the likelihood of stable binding. Indeed, the inhibition by flumioxazin of haemoglobin synthesis that has been documented in the rat embryo does not occur in three independent human cell lines modeling the human embryonic erythroblast. Because impairment of haem synthesis in the erythroblast is required in the mode of action of developmental toxicity in the rat, adverse outcome pathway analysis demonstrates that the rat data are not relevant to human hazard assessment.

Flumioxazin does not produce development toxicity in the rabbit, another species that is insensitive to PPO inhibition by flumioxazin. There is no evidence of developmental toxicity in the rat other than mediated by the PPO inhibition mode of action. There are, then, no relevant data suggesting that flumioxazin is a human developmental hazard, and this herbicide should not be classified with respect to reproductive and developmental toxicity.

PUBLIC ATTACHMENTS

1. Cohen_CLH Report Response - rev 6-6-18.docx [Please refer to comment No. 5]
2. open.zip [Please refer to comment No. 2]

CONFIDENTIAL ATTACHMENTS

1. confidential.zip [Please refer to comment No. 2]