Committee for Risk Assessment

RAC

Annex 2

Response to comments document (RCOM)

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

7-oxa-3-oxiranylbicyclo[4.1.0]heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide

EC Number: 203-437-7
CAS Number: 106-87-6

CLH-O-0000001412-86-301/F

Adopted

20 September 2019

ANNEX 2 - COMMENTS AND RESPONSE TO COMMENTS ON CLH PROPOSAL ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION

Comments provided during public consultation are made available in the table below as submitted through the web form. Any attachments received are referred to in this table and listed underneath, or have been copied directly into the table.

All comments and attachments including confidential information received during the public consultation have been provided in full to the dossier submitter (Member State Competent Authority), the Committees and to the European Commission. Non-confidential attachments that have not been copied into the table directly are published after the public consultation and are also published together with the opinion (after adoption) on ECHA's website. Dossier submitters who are manufacturers, importers or downstream users, will only receive the comments and non-confidential attachments, and not the confidential information received from other parties.

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Substance name: 7-oxa-3-oxiranylbicyclo[4.1.0]heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide
EC number: 203-437-7
CAS number: 106-87-6
Dossier submitter: The Netherlands

GENERAL COMMENTS

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2018	Belgium		MemberState	1
Comment received				
The BE CA thanks the RIVM for this proposal of harmonized classification of 4-vinylcyclohexene diepoxide. Overall, we would appreciate some details on repeated-dose toxicity studies to support the endpoints open for discussion in this CLH report.				
Dossier Submitter's Response				
Thank you for you comments. Please view our response to comments below. An assessment of the repeated dose studies (focusing on the reproductive effects) was included in section 10.11 on reproductive toxicity. Also see our response to comment number 7 about the effects seen in male animals in the reproductive and repeated dose studies.				
RAC's response				
Thank you for your comment. RAC agrees with the Dossier Submitter's (DS) response, and also points out that 16-d and 13-week NTP gavage studies have been assessed in the RAC opinion, related to reproductive toxicity.				

Date	Country	Organisation	Type of Organisation	Comment number
08.10.2018	Germany		MemberState	2
Comment received				
Based on the information given in the CLH-report the classification proposal of the dossier submitter is justified.				
Dossier Submitter's Response				
Thank you for your comment and support.				

RAC's response
Thank you for your comment. Please note that RAC agrees with the DS's proposal for classification for carcinogenicity and reproductive toxicity, while RAC's opinion differs regarding acute toxicity and mutagenicity (please see RAC opinion for justification).

CARCINOGENICITY

Date	Country	Organisation	Type of Organisation	Comment number
19.10.2018	France		MemberState	3
Comment received				
Based on the compiled factors to be taken into consideration in the hazard assessment as indicated in table 17 p 20, FR agrees with the conclusion of the classification for the 4-vinylcyclohexene diepoxide as Carc 1B based on skin tumors observed in both sexes in rat and mice.				
Dossier Submitter's Response				
Thank you for your comment and support.				
RAC's response				
Thank you for your comment. RAC agrees with the DS's proposal for classification in Category 1B for carcinogenicity.				

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2018	Belgium		MemberState	4
Comment received				
The BE CA supports the proposal to classify 4-vinylcyclohexene diepoxide as Carc. 1B. The evidences of carcinogenicity are seen as strong and relevant to human. After dermal exposure, mice and rats showed mainly skin tumours, including squamous cell carcinomas and papillomas, basal cell adenomas, mixed cell carcinomas. Findings were consistent between numerous studies and species and included different types of skin tumours (cell types and malignancy). Moreover, in some of these studies neoplastic tumours included benign and malign ovary tumour and lung alveolar/bronchiolar adenomas or carcinomas. This classification proposal is also based on the very high mutagenic potential of 4-vinyl cyclohexene diepoxide. Considering that the compound is known to be absorbed and widely distributed after oral and inhalatory exposure and the lack of oral and inhalatory studies, we are of the opinion that no specific route of exposure should be specified.				
Dossier Submitter's Response				
Thank you for your support and shared considerations also regarding the route of exposure.				
RAC's response				
Thank you for your comment. RAC agrees with the DS's proposal for classification in Category 1B for carcinogenicity, without specifying route of exposure.				

Date	Country	Organisation	Type of Organisation	Comment number
08.10.2018	Germany		MemberState	5
Comment received				
Based on the available data the proposal to modify the classification for VCD as Carc. 1B is supported.				

After dermal application an increase in dermal tumours at the site of application was observed in mice in both sexes. In addition, tumours extended at least to a second site (ovaries).
For the evaluation of the transgenic studies it would be helpful if data on the frequency of applications und the treatment duration was included.
The evidence in rat is a bit more limited than in mice. In the NTP study described there is a clear dose dependent increase in skin squamous cell carcinoma, but survival rates especially in male rats are very low. It is worth to know whether tumour-bearing animals died because of their tumours.
The summary (table 17) should include that the mode of action is not only limited to local mutagenicity but also possibly to systemic mutagenicity leading to ovarian tumours.

Dossier Submitter's Response

Thank you for your support and and shared considerations.

- As for the evaluation of the transgenic studies you requested the data on the frequency of applications and the treatment duration. It is acknowledged that this information is not stated in Table 13 (page 14). We would like to point to page 19 where the information on frequency of application and treatment duration is stated:

  "…After dermal application of 4-vinylcyclohexene diepoxide at 12.5 or 25 mg/animal, two times per week for 24 weeks, treated transgenic mice developed the same type of squamous cell tumours at the application site as did normal mice in the two-year dermal carcinogenicity study of the NTP2…." (Tennant et al. (1995, 1996))

  And:

  …."4-Vinylcyclohexene diepoxide was applied to the dorsal skin of the transgenic (Tg) and non-transgenic mice (non-Tg mice) at 5 or 10 mg/kg body weight, five times per week for 24 weeks"... (Yamamoto et al., 1998)

- With regard to carcinogenicity, we agree that the evidence in rat is a bit more limited than in mice. And it is indeed worth to know whether tumour-bearing male rats died because of their tumours or not. The 2-year bioassay conducted by NTP showed that skin application of 4-vinylcyclohexene diepoxide produced squamous cell neoplasms (predominantly carcinomas) and basal cell neoplasms (adenomas and carcinomas) in male and female rats, in all dose groups. We would like to point to page 19 where it is stated:

  "…Survival in male rats was very low for all groups (8/50 and 4/50 for the low and high dose group), controls (7/50) included, but showed no significant differences between dosed males and controls (Table 16)…."

  Additionally, the NTP study report (1989) presented Kaplan-Meier survival curves for male and female rats; these are also presented on page 58 of our CLH-dossier. These curves do not provide any evidence of reduced time to death in the dosed (and tumour-bearing) males. So it seems plausible that tumour-bearing male rats did not die because of their tumours.

- With regard to your suggestion to include also possibily systemic mutagenicity leading to ovarian tumours as part of the mode of action in Table 17: The exact mechanism of ovarian tumours is not identified. Mutagenicity may have contributed to the neoplastic

effects in the ovaria. In addition, non-neoplastic effects on the ovaria (which included follicular atrophy) was shown to be related to VCD directly interacting with membrane-bound KIT and its downstream signaling pathway in the oocyte to cause follicular destruction (see page 44-46 of the CLH report). Also see our reponse to comment number 6 with respect to mutagenicity.
RAC's response
Thank you for your comment. RAC supports the DS's response. Discussion regarding potential mechanisms of VCD carcinogenicity is included in the RAC opinion.

MUTAGENICITY

Date	Country	Organisation	Type of Organisation	Comment number
19.10.2018	France		MemberState	6
Comment received				
According to CLP guidance criteria (section 3.5 germ cell mutagenicity):"Classification as a germ cell mutagen (Category 1A, 1B, and 2) classifies for the hazard heritable genetic damage as well as providing an indication that the substance could be carcinogenic ». FR agrees that the available information indicates that both local irritation and probable local mutagenicity have contributed to the increase in dermal tumors (table 17 p 20 of CLH report). There is no in vivo data on germ cells with the epoxide. However the positive results of the mammalian mutagenicity assays in vitro supported by chemical structure activity relationship as epoxide to known germ cell mutagens and the classification of the parent cyclo 4-vinylcyclohexene as Carc. 2 H351 provide a probable genotoxic mechanism for the ovarian tumors observed in female mice by dermal route. According guidance CLP criteria (Annex I: 3.5.2.2., Table 3.5.1., "Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens"), the substance could be therefore proposed in Category 2 mutagens. FR therefore proposes the classification Muta. Cat. 2 for 4-vinylcyclohexene diepoxide.				
Dossier Submitter's Response				
Thank you for your considerations. We agree that Muta1B is not an option since there are no in vivo data on germ cells with VCD (see also our response to comment 7). According to the criteria, classification for Muta 2 is possible when there is a positive in vitro mammalian mutagenicity assay that is supported by chemical structure activity relationship to known germ cell mutagens. With respect to a possible Muta 2 for VCD, we have sympathy for your comments. For VCD, positive in vitro mammalian mutagenicity data are available, including gene mutation and chromosome aberration. VCD is a diepoxide compound. However, the argument of structural similarity alone might not be sufficient. For this, a weight of evidence approach should be applied with a complete picture of the data, which also includes the data of the analogue chemical. At this moment, information/data is lacking where the mutagenicity of VCD may be predicted from data of a known source substance. As far as known to the Dossier Submitter, Annex VI of the CLP does not include any (di)epoxide with a Muta classification. And ofcourse, epoxides are the toxic metabolites of parent compounds, being formed endogenously, whereas, the parent compounds are the chemicals being primarily evaluated (and included in Annex VI). Therefore, due to lack of data, it might be considered that the read-across approach does not provide a reliable basis for classification for mutagenicity.				
RAC's response				
Thank you for your comment. RAC also considers that Muta. 2 is justified for VCD, based on weight-of-evidence approach. Namely, for VCD there are *in vivo* somatic cell genotoxicity				

tests (*in vivo* DNA adducts formation in mouse skin), supported by positive results from *in vitro* mutagenicity assays, which could trigger Category 2 classification according to the CLP (*i.e*. the classification in Category 2 could be based on other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays).

Although available *in vivo* genotoxicity studies on DNA adducts formation (Randerath and Mabon, 1996; Mabon and Randerath, 1996) are open literature studies with some limitations, they are considered reliable enough to serve at least as supportive evidence. Positive findings in these studies are supported by the carcinogenic potential of VCD, regarding both local (skin) and systemic (ovary) tumorigenesis, which is considered to be, at least partially, a consequence of its mutagenicity. Direct ovarian toxicity of VCD indicates that VCD reaches mammalian germ cells.

Regarding structural alerts triggered by VCD, although this substance, as a diepoxide, triggers several alerts for mutagenicity (e.g. for DNA covalent binding, *in vivo* micronucleus assay in rodents, and structural alert for genotoxic carcinogenicity in ToxTree v.3.1.0), there are no structurally highly similar analogues for VCD in OECD QSAR Toolbox and ToxTree for *in vivo* mutagenicity endpoints. In addition, as pointed out by the DS, Annex VI of the CLP does not include any (di)epoxide with a germ cell mutagenicity classification. It could be concluded, therefore, that currently VCD has no adequate analogues for a reliable read across for *in vivo* mutagenicity prediction. Nevertheless, although VCD-triggered structural alerts cannot fulfil a criterion required by the CLP stated above, they are supportive evidence for the mutagenicity potential of VCD.

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2018	Belgium		MemberState	7
Comment received				
4-Vinylcyclohexene diepoxide contains two epoxide functional groups. These chemical structures are well known to induce DNA damages and therefore raise QSAR alerts. As expected, gene mutations with or without preliminary metabolic activation, were reported in S. typhimurium TA100, TA1535 and TA98 strains after exposure to 4-vinylcyclohexene diepoxide. In mammalian cells, this compound also demonstrated its capacity to induce gene mutation in mouse lymphoma and Chinese hamster cells. Finally chromosome aberrations with and without metabolic activation were seen in Chinese hamster ovary cells without indication of micronuclei. Supportive studies included indications of gene mutation and mitotic conversion in S. cerevisiae and exchange of sister chromatids in Chinese ovary cells. Overall, 16 in vitro studies are available in the CLH report, all showing positive results. On the contrary, very few in vivo studies are available for this compound. Only two mice dermal genotoxicity studies from the same laboratory were available in the CLH report. Nevertheless, in vivo results showed that 4-vinylcyclohexene diepoxide is also able to produce DNA-adducts in female ICR mice after topical application. These findings were further supported by a positive Comet assay in human skin biopsy. No relevant genotoxicity studies in somatic cells after oral exposure or in germ cells were found. We express our surprise and concern about the lack of such studies. On the basis of the chemical structure, the in vitro and available in vivo genotoxicity studies, the BE CA is of the opinion that the evidence indicating the mutagenic potential of 4-vinylcyclohexene diepoxide is high. Nevertheless, to warrant a CLP classification for mutagenicity, there is a need to demonstrate that the compound is able to induce DNA damage on germ cells.				

Toxicokinetic studies showed that 4-vinyl cyclohexene diepoxide is absorbed by rodents exposed dermally, orally or by inhalation and is widely distributed after dermal exposure in rats and mice. Considering the very high mutagenic potential of 4-vinyl cyclohexene diepoxide, the BE CA is of the opinion that even at very low concentration, the indication that this compound is distributed in reproductive tissues is of the highest concern. The BE CA would therefore appreciate further details on this point.
Findings in numerous reproductive toxicity studies clearly support this concern. In females, reductions in primordial and primary follicles have been undoubtfully demonstrated in mice and rats after exposure to 4-vinylcyclohexene diepoxide. This finding indicates that the compound is able to affect germ cells.
In males, few details are provided in the CLH report. Nevertheless, degeneration of the germinal epithelium in testis at dose levels of ≥ 250 mg/kg was seen in mice in a 13-week gavage exposure study whereas a greatly reduced number of spermatozoa in the adluminal space was reported in rats. According to the CLP criteria's, mutagenic effects in sperm cells, e.g. sperm aneuploidy, are considered as a positive evidence to classify for germ cell mutagenicity. We would greatly appreciate further discussion about effects seen in sperm in the reproductive and repeated exposure studies.
Overall, the BE CA is of the opinion that the clear evidence of mutagenicity in mammalian cells and the capacity of 4-vinylcyclohexene diepoxide to affect the germinal epithelium of testis an ovaries in rodents should be seen as sufficient to classify for germ cells mutagenicity on a weight-of-evidence basis. A Muta. 1B classification for 4-vinylcyclohexene diepoxide might be considered depending on further information as discussed.

Dossier Submitter's Response

Thank you for your shared considerations.

- You indicated to appreciate further details on the indication that this compound is distributed in reproductive tissues which is of the highest concern. We would like to refer to the CLH dossier on information about distribution of the compound on page 7-8.

  "4-Vinylcyclohexene diepoxide is absorbed by rodents exposed dermally, orally, or by inhalation (Weil et al., 1963 in NTP) (National Toxicology 1989). The National Toxicology Program (NTP) has studied the fate of a single dermal application of [14C] 4-vinylcyclohexene diepoxide in female F344/N rats and B6C3F1 mice. These studies were conducted to determine if there were differences in disposition which could explain the differences in toxicity observed in rats and mice. Rats and mice received 0.1 ml and 0.001 ml, respectively, of solutions containing 500 mg/ml (200 pC/ml) [ethylene-^{14}C]4-vinylcyclohexene diepoxide in acetone. The preliminary results indicate that 30% of the dose applied to the skin is absorbed over a 24-hour period for both rats and mice; only 1%-3% of the dose remained on the skin at the site of application. By 24 hours, 70%-80% of the absorbed dose had been eliminated from the body, virtually all in the urine. The radioactivity remaining in the body was distributed over a number of tissues, with no tissue containing more than 1% of the applied dose.4 The liver, muscle, and adipose tissue, however, contained 0.5%-1.6% and 1.2%-2.9% of the absorbed dose in rat and mouse tissue, respectively. Tissue to blood ratios ranged from 0.3 to 1.5 in rats and from 0.8 to 2.8 in mice (NTP unpublished data in NTP 1998) (program 1989)."
  Details concerning potential distribution to the reproductive tissues have not been presented in the NTP 1989 report. The study focusing on the fate upon a single dermal application concerns a secondary reference mentioned in the NTP-report (i.e. Snipes et al. Chemical Disposition in Mammals: Preliminary Report on 4-Vinylcyclohexene

Diepoxide. NIEHS Contract No. N01-ES-3-5031. Tucson: University of Arizona). The study report is however not available to the Dossier Submitter.
No further information was found in literature on the distribution of the compound to reproductive tissues.
Nevertheless, VCD exerts toxic effect on the reproductive organs.

- You mention the reduced number of spermatozoa in the adluminal space was reported in rats. It must be noted that this effect was observed in the study of Witmer 2017 and this study should be regarded as less relevant since it did not use the single compound VCD (see response to comment number 11).

  Discussion of additional 'male' data: You refer to effects with male mice in a 13-week gavage study. Indeed, in a 13-week gavage study (NTP), multifocal to diffuse testicular degeneration was present in 8/10 male mice receiving 250 mg/kg, 8/10 receiving 500 mg/kg, and 9/10 receiving 1,000 mg/kg. In a 13-week gavage study (NTP) with rats, smaller than normal testes in males were seen in rats administered 500 or 1,000 mg/kg. One rat that received 1,000 mg/kg had degeneration of the tubular epithelium of the testis.

  For a discussion of the other male effects in the other studies we would like to refer to page 43 of the CLH report.

  According to the CLP criteria, mutagenic effects in sperm cells, e.g. sperm aneuploidy, are considered as a positive evidence to classify for germ cell mutagenicity, íf these effects are seen in humans. This is however not the case for VCD.

- Reaction on Muta. 1B classification:
  Our opinion is that Muta. 1B is not an option. According to the CLP criteria's, muta 1B classification is based on:
  - *positive result(s) from in vivo heritable germ cell mutagenicity tests in mammals;*→ We do not have positive in vivo germ cell assays.
  - *positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells. It is possible to derive this supporting evidence from mutagenicity /genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells*;→ We do not have positive in vivo somatic cell mutagenicity assays and in addition, no direct data on distribution of VCD to the reproductive organs. There is no evidence that VCD is genotoxic in germ cells. Overall, due to lack of data on germ cell mutagenicity, no evidence exists that VCD has the potential to cause mutations to germ cells.
  - *positive results from tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny; for example, an increase in the frequency of aneuploidy in sperm cells of exposed people.* → We do not have these data.

  Therefore, based on the current information, classification for muta 1B is not an option according to these CLP criteria. We would also like to refer to response to comment number 6 about mutagenicity. This endpoint needs further discussion at the meeting.

RAC's response

Thank you for your comment. RAC agrees that VCD should be classified for mutagenicity and proposes Muta. 2; H341. For details, please see RAC's response to comment #6 as well as the RAC opinion.

Date	Country	Organisation	Type of Organisation	Comment number
08.10.2018	Germany		MemberState	8
Comment received				
There is limited data available on germ cell mutagenicity (mainly from in vitro studies) which is not considered to be sufficient for classification.				
Dossier Submitter's Response				
Thank you for your comment and support. See also our response to comment numbers 6 and 7. This endpoint needs some discussion at the meeting.				
RAC's response				
Thank you for your comment. RAC agrees that VCD should be classified for mutagenicity and proposes Muta. 2; H341. For details, please see RAC's response to comment #6 as well as the RAC opinion.				

TOXICITY TO REPRODUCTION

Date	Country	Organisation	Type of Organisation	Comment number
19.10.2018	France		MemberState	9
Comment received				
Based on the ovotoxicity effects (decrease in number of follicles, atrophy,..) observed in several animal species (monkey, rat, mice, hamster females), the clear effects on testis (reduced testicular weights and testicular and epididymal dysfunction) observed in male rats and the absence of maternal toxicity, a classification for reproduction should be proposed. Concerning the category to be proposed for this endpoint, there is insufficient evidence to justify a category 1B. Indeed <u>there is no information regarding effects on male fertility. There is no evidence by experimental genotoxic results that the germ cells are damaged by a treatment with 4-vinylclohexene diepoxide. There is also no available toxicokinetic information whether the substance could reach the reproductive organs</u>. Therefore, FR is of the opinion that a classification in category 2 should be warranted for the reprotoxicity endpoint.				
Dossier Submitter's Response				
Thank you for your comments and considerations. In our opinion, this has no effect on classification for Repro1B. According to the CLP criteria, the classification of a substance for Repro1B 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. It is indisputable that VCD induces ovotoxicity, reducing consistently the number of primordial and primary ovarian follicles after intraperitoneal and intramuscular injection in rat, mice and hamsters as well as after intramuscular injection in nonhuman primates. This is considered a clear adverse effect on sexual function and fertility. Similar results were found in mice after dermal exposure. Ovotoxicity was also observed after oral exposure (Abolaji 2016) (see also response to comment number 11) ; following oral VCD administration to rats, the histopathology of the ovary revealed large cystic follicles and scanty number of follicles which is suggestive of ovotoxicity and corroborates existing literature that VCD depletes follicular number in rats. You mention that there is no available toxicokinetic information whether the substance could reach the reproductive organs. This is true, but, it is also known that VCD directly targets primordial and primary follicles. VCD interacts directly with membrane-bound KIT and its downstream signaling pathway in the oocyte to cause follicular destruction. In				

addition the presence of KITL and c-kit in the adult human ovary has been demonstrated throughout follicle development, in addition to showing the presence of each isoform. This suggests that the KITL/c-kit system which is a target for VCD in animals is also involved in human folliculogenesis and supports that the ovotoxicity effect is also relevant to humans.
(see also chapter on mechanistic information on page 44-46).

In summary, since effects observed in different species, with highly severe potential outcomes, classification as repro. 1B H360F is warranted. Further, we would like to refer to the CLH report page 46.

RAC's response

Thank you for your comment. RAC agrees with the DS's response, and adds that in the studies that used standard routes of exposure (oral and dermal NTP studies) ovarian, uterine and testicular toxicity was observed, at dose levels without or with only limited general toxicity. Although in these studies functional fertility parameters were not directly evaluated, evidence of toxic effects of VCD on ovaries, uteri and testes in rodents was clear, especially regarding ovarian toxicity (follicle depletion) in mice. These findings, according to CLP Regulation[1] and ECHA CLP Guidance[2], justify classification for reproductive fertility. As a supporting evidence, intraperitoneal study in mice showed decreased fertility index at dose level without marked general toxicity (Haas *et al*., 2007).

(1) CLP Regulation, paragraph 3.7.1.3. Adverse effects on sexual function and fertility: "Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, … premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems".
(2) ECHA CLP Guidance 2017: "Use of data from standard repeat dose tests, Fertility effects: Toxicological effects, including marked effects, observed in a standard repeat dose study could be considered valid for the pre-mating phase for adult females and the pre- and post-mating phase for adult males".

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2018	Belgium		MemberState	10

Comment received

Fertility
We acknowledge the good work done to report all these studies relative to reproductive toxicity. We can truly agree with the DS and support its conclusions regarding the fertility endpoint. It is incontestable that there is 4-vinylcyclohexene diepoxide-induced ovotoxicity. Indeed, significant reduction in primordial, primary and secondary ovarian follicles was consistently observed. It is of course reasonable to hypothesize that this adverse effect would result in lower number of pups in the offspring. This significant effect was reported in 4 different species: mouse, rat, hamster and nonhuman primate) after dermal application or intramuscular or intraperitoneal injection. The route of administration can be questioned regarding human relevance, but considering the consistent effects observed in different species, with highly severe potential outcomes, we are in the opinion that a classification as repro. 1B H360F is warranted.

Development
BECA agrees with the dossier submitter that there is no data available showing developmental effects on the offspring. Therefore, no classification is warranted.

Dossier Submitter's Response
Thank you for your support and shared considerations.
RAC's response
Thank you for your comment. RAC also points out that in the studies that used standard routes of exposure (oral and dermal NTP studies) ovarian, uterine and testicular toxicity was also observed, at dose level without or with only limited general toxicity.

Date	Country	Organisation	Type of Organisation	Comment number
08.10.2018	Germany		MemberState	11
Comment received				
Based on the available data, the proposal for VCD as Repr. 1B, H360F for adverse effects on sexual function and fertility in more than one species is supported. There is a clear toxic effect of VCD on the ovaries, reducing the number of primordial and primary follicles after intraperitoneal and intramuscular injection in rat, mice and hamsters as well as after intramuscular injection in nonhuman primates. Similar results were found in mice after dermal exposure which is considered the more relevant route of exposure to humans. Limited evidence also showed an effect on fertility, mainly reduction in pregnancies, an increase in preimplantation loss and a decrease in the number of implanted embryos. The studies on carcinogenicity are clearly arranged into different sections (dermal and intraperitoneal injection). A similar structure could be helpful for the reproductive toxicity studies shown as well. There is only limited data on development. Therefore, classification is not possible. Furthermore some aspects should be clarified to improve the rationale: chapter 10.11.1 In table 18 (p. 30) an oral rat study (Witmer 2017) is mentioned, which is further discussed only on page 43 as to effects on male gonades (testes, epididymis) and hormone levels. It appears that rats were exposed via drinking water to a mixture of VCD (0.109%) and triptolide (0.001%), not only VCD as stated in the CLH-report. Triptolide is a minor component in this mixture, but is also described to target ovarian function. The relevance of this study should be clarified. In addition pronounced effects on fertility parameters after mating were described in the publication, which are not mentioned in the CLH-report. On page 42 it is stated: "Effectiviness of orally delivered VCD on the fertility of male rats and mice has been examined (Hooser, DeMerell et al. 1995, Schmuki 2009, Burd 2014)." The study from Hooser, DeMerell et al. 1995 is an i. p.-study, not oral. No further information can be found in the CLH-report about the study of Burd (2014) which seems to be a doctoral (PhD) thesis. If the study is worth mentioning some more information should be given. chapter 10.11.2 It should be added in this summarizing chapter that ovotoxicity was also observed after oral exposure (Abolaji 2016). The results of the study of Witmer (2017) could be mentioned, if the study is considered to be relevant after an in-depth analysis. It is stated in chapter 10.11.2: "Exposure of rats to VCD induces testicular and epididymal dysfunctions via endocrine suppression, disruption of antioxidant enzymes activities,				

increase in biomarkers of oxidative stress, inflammation and apoptosis in rats." The route of exposure is missing here.
In the same chapter the following sentence can be found. "No effects on reproductive function of treated males were reported." Again the route of exposure (likely oral, Schmuki 2009) should be added.

chapter 10.11.3
It is stated: "As the effect in female animals is specifically targeted to the ovaries, after dermal, intramuscular and intraperitoneal exposure, it is considered an effect on sexual function and fertility." Effects were also observed after oral exposure (see above).
Moreover it is stated: "Although there are no data on the effect of the ovotoxicity on the resulting fertility via relevant routes of exposure, the observed ovotoxicity is considered to result in a reduction of the number of offspring." The results of the study of Witmer (2017) could be mentioned, if the study is considered to be relevant after an in-depth analysis.
It is stated: "Further, there is clear evidence for effects on the testis but no information regarding effects on male fertility." The study of Schmuki (2009) is not described in detail, but it seems that it included mating of animals with no effect on reproduction function.

Acute toxicity:
The proposed classification is supported.
The ATE = 680 mg/kg bw dermal and the ATE = 4.656 mg/L inhalation lie within the range of the proposed classification for Cat. 3 and Cat. 4 respectively. The removal of the classification for acute oral toxicity is supported as the available data indicate a LD50 value of > 2000 mg/kg bw.
Please note on acute dermal toxicity, there is an inconsistency regarding the species between the table and the text.

Dossier Submitter's Response

Thank you for your support and your comments regarding improving the rationale. The inconsistency regarding the species between the table and text for acute dermal toxicity is noted, the species should be rabbits. You mention that the reproductive toxicity studies in the CLH report could be arranged into different sections (for example dermal and intraperitoneal studies). It is true that this could have been helpfull since there are a lot of studies. Unfortunately, the CLH-report cannot be adapted at this stage of the CLH-process, but below we will address the various issues raised by MSCA.

Chapter 10.11.1, Table 18 (Witmer 2017).
Indeed rats were treated not only with VCD (0.109%) but with a mixture of VCD (0.109%) with triptolide (0.001%). This study is therefore considered less relevant. VCD has a selective effect on primordial follicles, whereas triptolide is known to damage larger ovarian pre-antral and antral follicles. Triptolide targets also the ovarian function in female rats and impairs spermatogenesis in males.

Chapter 10.11.1 (Hooser, DeMerell et al. 1995, Schmuki 2009, Burd 2014).
Thanks for pointing us towards the mistake. The study from Hooser, DeMerell et al. 1995 is indeed an i. p.-study, not an oral study. You mention that no further information can be found in the CLH report about the study of Burd (2014). This is indeed a doctoral (PhD) thesis. A short summary of the content is shown below:

In this doctoral thesis of Burd, the effects are examined of orally adminstered VCD on female possum ovarian follicle populations. Orally delivered VCD had no effect on the primordial follicles of adult female possums (See figure 4.2 below). The thesis also describes the uptake and metabolism of orally administered VCD in female possums and rats in vivo. VCD concentration in the blood of rats was significantly greater than in possums while concentrations of VCD in the stomach were comparable between species (See table 5.1 and 5.2 below). VCD dosing did not alter pH of stomach contents of possums while that of rats was increased and sustained for 6 hours (see table 5.3 below). VCD-induced reductions in ovarian and liver glutathione levels were observed in the rat with no effects in the possum (See figure 5.9 and 5.10 below). It was determined that the highly acidic environment of the stomach of possums poses an initial barrier for orally delivered VCD.

At last, the thesis examined the fate of VCD when exposed to acidic environments and stomach contents and the effects of VCD on liver metabolism *in vitro* in possums and rats. VCD hydrolysis in stomach contents was slower in possums compared with rats suggesting that possum stomach contents are able to retain VCD longer, thus potentially modulating VCD toxicity. GSH levels in possum liver tissue were less affected when incubated with VCD compared with rats, suggesting an increased detoxifying capacity of possums.

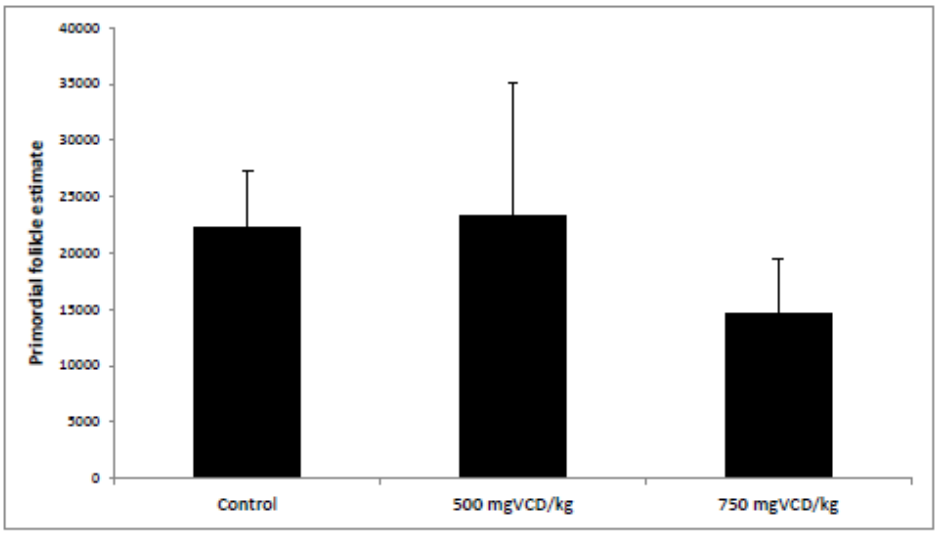

Figure 4.2 Effect of daily oral administration (13 days; Study 1) of VCD on mean total primordial ovarian follicle estimates in wild-caught female brushtail possums. Counts were recorded from the left ovary of each animal and total counts estimated using a correction factor formula (Gougeon and Chainy, 1987). Control (oil only), n = 6; 500 mg VCD/kg, n = 6; 750 mg VCD/kg, n = 7. Vertical bars represent + SEM.

Table 5.1 Change in VCD concentration in the stomach (mM) of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Statistical significance was set at $p < 0.05$. Data are means ± SEM; individual data are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment	Control treatment	VCD treatment
0	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1	-	-	-	-
3	-	-	0.0 ± 0.0 (n = 4)	82.7 ± 15.3 (n = 4)
5	-	-	0.0/0.0 (n = 2)	40.4/91.7 (n = 2)
15	0.0 ± 0.0 (n = 4)	90.7 ± 34.3 (n = 4)	0.0 ± 0.0 (n = 4)	58.4 ± 35.4 (n = 4)
30	-	-	0.0/0.0 (n = 2)	49.2/81.1 (n = 2)
60	-	-	0.0/0.0 (n = 2)	58.2/82.6 (n = 2)
120	-	-	0.0/0.0 (n = 2)	27.6/36.4 (n = 2)
180	-	-	0.0 ± 0.0 (n = 4)	29.2 ± 4.7 (n = 4)
360	-	-	0.0 ± 0.0 (n = 4)	23.4 ± 5.9 (n = 4)
1440 (24 hours)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	-	-

Table 5.2 Change in VCD concentration in the blood (µM) of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). * indicates difference ($p < 0.05$) between VCD-treated possums and VCD-treated rats. Data are means ± SEM; individual data are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment	Control treatment	VCD treatment
0	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1	0.0 ± 0.0 (n = 8)	221.7 ± 0.07 (n = 8)	-	-
3	0.0 ± 0.0 (n = 4)	192.0 ± 0.09 (n = 4)	0.0 ± 0.0 (n = 4)	*1048.7 ± 137.5 (n = 4)
5	0.0 ± 0.0 (n = 8)	178.7 ± 79.5 (n = 8)	0.0/0.0 (n = 2)	*480.5/*1412.6 (n = 2)
10	0.0 ± 0.0 (n = 8)	31.9 ± 1.02 (n = 8)	-	-
15	0.0 ± 0.0 (n = 8)	2.45 ± 1.38 (n = 8)	0.0 ± 0.0 (n = 4)	*385.1 ± 211.9 (n = 4)
30	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	*94.5/*115.5 (n = 2)
60	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	0.0/0.0 (n = 2)
120	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0/0.0 (n = 2)	0.0/0.0 (n = 2)
180	-	-	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
360	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)
1440 (24 hours)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 4)	-	-

Table 5.3 Gastric pH levels in the stomach contents of female brushtail possums and Norway rats following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Statistical significance was set at $p < 0.05$. Data are means ± SEM; actual values are listed for sample sizes ≤ 2.

Time (minutes)	Species			
	Possum		Rat	
	Control treatment	VCD treatment (n = 8)	Control treatment	VCD treatment
0	1.00 ± 0.2 (n = 4)	1.13 ± 0.13 (n = 4)	2.88 ± 0.13 (n = 4)	2.88 ± 0.32 (n = 4)
1	-	-	-	-
3	-	-	3.50 ± 0.50 (n = 4)	3.75 ± 0.25 (n = 4)
5	-	-	0.5/3.5 (n = 2)	4.0/5.0 (n = 2)
15	1.00 ± 0.0 (n = 4)	3.13 ± 1.05 (n = 4)	3.25 ± 0.25 (n = 4)	3.75 ± 0.25 (n = 4)
30	-	-	2.0/3.5 (n = 2)	4.0/5.0 (n = 2)
60	-	-	3.0/4.0 (n = 2)	4.0/5.0 (n = 2)
120	-	-	0.5/4.5 (n = 2)	4.0/6.0 (n = 2)
180	-	-	3.67 ± 0.33 (n = 4)	3.50 ± 0.50 (n = 4)
360	-	-	3.67 ± 0.60 (n = 4)	4.83 ± 0.17 (n = 4)
1440 (24 hours)	0.50 ± 0.29 (n = 4)	2.50 ± 1.66 (n = 4)	-	-

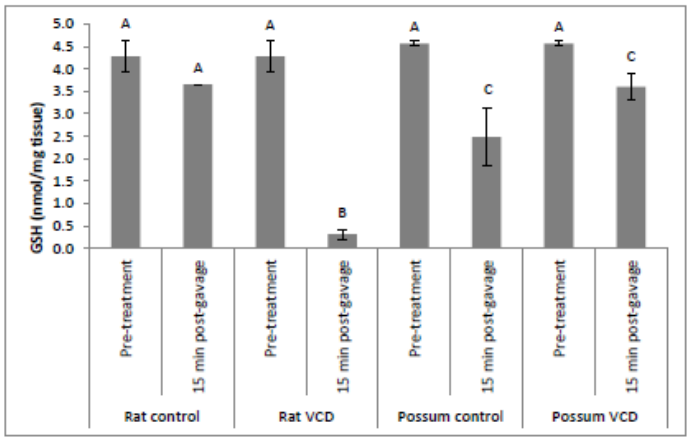

Figure 5.9 Hepatic GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

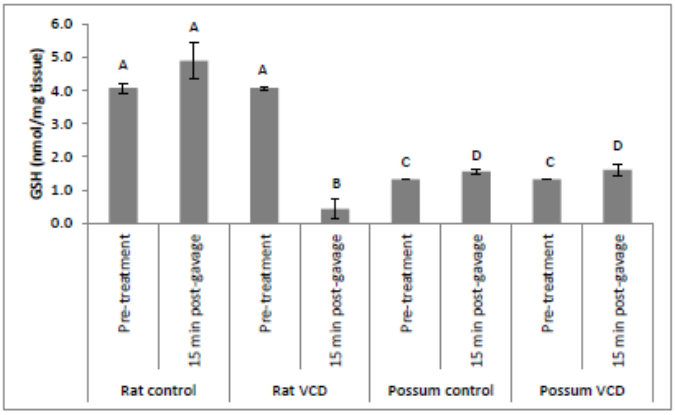

Figure 5.10 Ovarian GSH levels of wild-caught female brushtail possums and Norway rats during pre-treatment and 15 minutes following a single oral dose of oil or VCD (750 mg/kg) suspended in oil (1:3 v/v). Rats, n = 4/treatment/time point; possums, n = 4/treatment/time point. Values with different letters are significantly different ($p < 0.05$). Vertical bars represent ± SEM.

Additions to chapter 10.11.2.

Thank you for pointing towards the addition of sentences about oral exposure (Abolaji 2016) to the summarising chapter (which are underlined and cursive below), and the absence of information on the route of exposure about 2 studies:

"Adequate studies on reproductive toxicity in experimental animals were available for the intraperitoneal and intramuscular route. In these studies 4-vinylcyclohexene diepoxide was ovotoxic in female rats and mice inducing a significant loss of primordial and primary follicles rats and mice. Ovotoxicity was also induced in non human primates after intramuscular injection where primordial and primary follicles were targeted selectively. However, these routes of exposure are less relevant for human exposure. Especially ip treatment could result in direct contact of VCD with the ovaria. However, also in dermal studies with mice, comparable effects on the ovaries were observed. *Ovotoxicity was also observed after oral exposure (Abolaji 2016); following oral VCD administration to rats, the histopathology of the ovary revealed large cystic follicles and scanty number of follicles which is suggestive of ovotoxicity and corroborates existing literature that VCD*

depletes follicular number in rats… The general toxicity in these studies were limited to local effects on the skin. Seen the limited general toxicity and the identified mechanism for the induction of ovotoxicity in rats and mice, it is considered that the observed ovotoxicity is a direct effect of VCD and not secondary to the general toxicity…"

It is stated in chapter 10.11.2: "Exposure of rats to VCD induces testicular and epidydimal dysfunctions via endocrine suppression, disruption of antioxidant enzymes activities, increase in biomarkers of oxidative stress, inflammation and apoptosis in rats." The route of exposure in this 28-day study was oral (Adedarra 2016).

It is stated in chapter 10.11.2: "No effects on reproductive function of treated males were reported." This was a 15-days oral exposure study (Schmuki 2009).

chapter 10.11.3
The study of Witmer (2017) is considered less relevant (see comment nr 11).
The reason that the study of Schmuki (2009) is not described in detail is that this study is a secondary source and not publicly available.

RAC's response

Thank you for your comments, which RAC took into consideration in the opinion, as well as the DS's response to your comments. For more information on the non-relevance of the Witmer *et al*. (2017) study due to fertility effects of triptolide, please see the RAC opinion.

Regarding acute toxicity, please note that RAC is of the opinion that the data from Weil *et al*. (1963) and a secondary reference from the review by Dhillon and Von Burg (1996) are too limited to justify removal of the current classification Acute Tox. 3 via oral route. On the other hand, well reported studies on acute oral toxicity from the NTP technical report (1989) indicate LD_{50} values in the range of the CLP criteria for Category 4 for acute oral toxicity, 300 < ATE ≤ 2 000). RAC, therefore, proposes Acute Tox. 4 via oral route.

Also, in RAC's opinion, the uncertainties related to the data from Weil *et al*. (1963) and Dhillon and Von Burg (1996), are too high to justify changing the current classification from Category 3 to Category 4 for acute inhalation toxicity, as proposed by the DS. RAC instead proposes to retain Acute Tox. 3 for inhalation toxicity. For details, please see RAC opinion.

Regarding acute dermal toxicity, RAC considers that the data for LD_{50} values in rabbits (from Weil *et al*. 1963, and Dhillon and Von Burg, 1996) are too limited to support classification as Acute Tox. 3. On the other hand, the NTP studies in rats and mice, which do not indicate classification for acute dermal toxicity, have significant deficiencies in reporting (e.g. it is not known whether it was ensured that VCD is in contact with the skin, and if it was, for how long), and the data are hence not considered reliable enough to change the classification from Acute Tox. 3 to no classification.

Due to conflicting evidence, and too limited database, RAC proposes not to classify VCD for acute dermal toxicity due to insufficient evidence for classification. See RAC opinion for further details.

OTHER HAZARDS AND ENDPOINTS – Acute Toxicity

Date	Country	Organisation	Type of Organisation	Comment number
18.10.2018	Belgium		MemberState	12
Comment received				
The BE CA would like to express their concern about the reliability of these studies. No information is given about the detailed protocol, the purity of the substance or the scientific rigor (GLP,…) behind these studies. Overall, we are of the opinion that these reports need to be deeply assessed and potentially rejected if they do not meet minimal standards. However, if if data reliability can be ensured, our conclusions are the following: Oral BECA supports the decision of the dossier submitter to remove the current entry in the Annex VI as Acute tox. 3, H301. Dermal BECA supports the DS in its assessment in determining an ATE of 680 mg/kg bw for dermal toxicity and agrees to its proposal to keep the classification as acute tox 3; H311 considering the ATE is within the range (200 ≤ ATE = 680 ≤ 2000 mg/kg bw) mentioned in the Guidance on the Application of the CPL Criteria. Inhalation According to the available data, BECA do not see any argument in favor of keeping the current classification as acute tox. Cat 3. Again, considering the poorly detailed studies, we support the calculated ATE and the resulting classification as acute tox. Cat 4.				
Dossier Submitter's Response				
Thank you for your comments and your support.				
RAC's response				
Thank you for your comments. Please see RAC's response to comment #11 as well as the RAC opinion.				