Committee for Risk Assessment

RAC

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

20 September 2019

CLH-O-0000001412-86-301/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **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**

The proposal was submitted by the **Netherlands** and received by RAC on **7 June 2018.**

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

PROCESS FOR ADOPTION OF THE OPINION

The Netherlands has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **20 August 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **19 October 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Veda Varnai**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **20 September 2019** by **consensus**.

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

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	603-066-00-4	1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	Carc. 2 Acute Tox. 3* Acute Tox. 3* Acute Tox. 3*	H351 H331 H311 H301	GHS08 GHS06 Dgr	H351 H331 H311 H301			
Dossier submitters proposal	603-066-00-4	7-oxa-3-oxiranylbicyclo[4.1.0]heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	**Add** Repr. 1B **Modify** Carc. 1B Acute Tox. 4 Acute Tox. 3 **Remove** Acute Tox. 3	**Retain** H311 **Add** H360F **Modify** H350 H332 **Remove** H301	**Retain** GHS08 GHS06 Dgr	**Retain** H311 **Add** H360F **Modify** H350 H332 **Remove** H301		**Add:** inhalation: ATE=4.656 mg/L dermal: ATE=680 mg/kg bw	
RAC opinion	603-066-00-4	7-oxa-3-oxiranylbicyclo[4.1.0]heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	**Add** Muta. 2 Repr. 1B **Modify** Carc. 1B Acute Tox. 3 Acute Tox. 4 **Remove** Acute Tox. 3	**Retain** H331 **Add** H341 H360F **Modify** H350 H302 **Remove** H311	**Retain** GHS08 GHS06 Dgr	**Retain** H331 **Add** H341 H360F **Modify** H350 H302 **Remove** H311		**Add:** inhalation: ATE= 0.5 mg/L (dusts and mists) oral: ATE=1847 mg/kg bw	
Resulting Annex VI entry if agreed by COM	603-066-00-4	7-oxa-3-oxiranylbicyclo[4.1.0]heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	Carc. 1B Muta. 2 Repr. 1B Acute Tox. 3 Acute Tox. 4	H350 H341 H360F H331 H302	GHS08 GHS06 Dgr	H350 H341 H360F H331 H302		inhalation: ATE= 0.5 mg/L (dusts and mists) oral: ATE=1847 mg/kg bw	

GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

4-vinylcyclohexene diepoxide (VCD) has an existing harmonised classification in Annex VI to Regulation (EC) 1272/2008 (CLP Regulation) as Carc. 2; H351 (Suspected of causing cancer), agreed by the technical committee for classification and labelling (TC C&L) under the Dangerous Substance Directive.

However, in 1976 IARC identified VCD as carcinogenic in mice by skin application (producing squamous cell skin carcinomas), and in 1994, also classified VCD as possibly carcinogenic to humans (Group 2B), based on sufficient evidence in experimental animals of carcinogenicity. In 2008, the Dutch Expert Committee on Occupational Standards DECOS, a committee of the Health Council of the Netherlands, concluded that VCD should be regarded as a genotoxic carcinogen to humans.

The DS's proposal was based on an updated report of the Health Council of the Netherlands in 2016, which contains a re-evaluation of the mutagenic and carcinogenic properties of VCD.

It is used as a chemical intermediate and a diluent for other diepoxides and for epoxy resins derived from bisphenol A and epichlorohydrin (Cancer, 1994; Netherlands, 2008), and is accepted by the US EPA as a rodenticide.

VCD is a metabolite of the industrial chemical 4-vinylcyclohexene, for which there is a RAC opinion (2012) concluding on classification as Carc. 2 (H351), and this classification is included in Annex VI of the CLP Regulation.

VCD is a clear, colourless or pale yellow liquid at standard temperature and pressure (20 °C and 101.3 kPa), volatile at room temperature, but not extensively (vapour pressure 13 Pa at 20 °C), and of high water solubility (35.2 g/L at 25 °C), with partition coefficient n-octanol/water of 0.44 Log P_{ow}. Boiling point is stated to be 227 °C.

Toxicokinetic data in rodents indicate that VCD is absorbed via oral, dermal and inhalation exposure routes (Weil *et al*., 1963 in National Toxicology Program (NTP), 1989). The preliminary results in the NTP indicate that 30 % of the dose applied to the skin is absorbed over a 24-hour period, both in rats and mice (NTP, 1989). 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. Toxicokinetic data for the fate after oral administration, however, were not available in the CLH report.

In vitro studies (rabbit liver microsomal preparations) indicate that VCD can be metabolised by epoxide hydrolase to monoepoxymono glycols (1,2-hydroxy-4-vinylcyclohexane oxide and 4-(1',2'-dihydroxyethyl)-1-cyclohexane oxide) or could be conjugated with glutathione (Watabe and Sawahata, 1976, Watabe *et al*., 1980, and Giannarini *et al*., 1981, in NTP, 1989).

Impurities of 4-vinylcyclohexene diepoxide, that would be relevant for the classification of the substance, are unknown according to the CLH report.

Only selected hazards classes were assessed in the report: acute toxicity via oral, dermal and inhalation route, germ cell mutagenicity, carcinogenicity and reproductive toxicity.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute oral toxicity

The available data on acute oral toxicity is limited. The Weil *et al*. (1963) study indicates an LD_{50} of 3 110 mg/kg bw in rats (calculated from LD_{50} of 2.83 mL/kg bw), but without further details. This value is supported by a secondary reference (from a review by Dhillon and Von Burg, 1996), which indicates an LD_{50} of 2 130 mg/kg bw in rats.

Since both references suggest an oral LD_{50} value above 2 000 mg/kg bw, indicating no requirement for classification, and since it is unknown on which data the current classification in Category 3 is based, the Dossier submitter (DS) proposed to remove the classification for acute oral toxicity.

Acute dermal toxicity

One acute dermal toxicity study is available (Weil *et al*., 1963) in which an LD_{50} of 0.62 mL/kg bw was established in rabbits, without further details given.

Using a density of 1.1 g/mL (Lide, 1992), the DS calculated an LD_{50} value of 680 mg/kg bw. This value falls within the range of Category 3 (200 < ATE < 1 000 mg/kg bw), which is the same category as the existing minimum classification in Annex VI of CLP. The DS hence proposed to retain the current classification in Category 3, with an ATE value of 680 mg/kg bw.

Acute inhalation toxicity

Data on acute inhalation toxicity of VCD originate from two poorly reported studies.

In the study by Weil *et al*. (1963), no mortality occurred after an 8-h exposure to the concentrated vapours of VCD. There was no information on the actual test concentration of VCD, or on any other study conditions. Nevertheless, based on vapour pressure of 0.13 hPa at 20 °C and 101.3 kPa, the DS calculated a saturated vapour concentration of 748 mg/m^3 (0.75 mg/L) or 128 ppm.

Information from the second study (Shell Chemical Company, 1995) is available only as a secondary source, provided as a brief review of available literature on VCD (by Dhillon and Von Burg; 1996). In this review, it is only stated that "the 4-h LC_{50} was determined to be 800 ppm in rats", that observed symptoms included vasodilation and unsteady gait, and that death occurred during or soon after exposure. It is not known whether the substance was in a form of vapours or mists. However, since 128 ppm was calculated by the DS as a saturated vapour concentration of VCD (at 20 °C and 101.3 kPa), the DS considered that the tested concentration of 800 ppm was probably in the form of a mist.

The DS also considered that the absence of mortality after an 8-h exposure (Weil *et al*., 1963) to the concentrated vapours, which is assumed to be the saturated vapour concentration of 128 ppm, is in line with the LC_{50} of 800 ppm reported by Dhillon and Von Burg (1996).

Since the only available LC_{50} value of 4.6 mg/L (800 ppm) would result in classification in category 4 (1.0 < ATE ≤ 5.0 for mists or dusts), and it is unknown on which data the current classification in category 3 is based, the DS proposed classification in category 4 with an ATE of approximately 4.6 mg/L.

Comments received during public consultation

One MSCA expressed their concern about the reliability of the acute toxicity studies, which cannot be assessed due to lack of information, and proposed to reject them if more information cannot be obtained. In case their reliability can be confirmed, the MSCA agreed that they supported classification as Acute Tox. 3 for dermal toxicity, and Acute Tox. 4 for inhalation toxicity, and removal of the current entry in the Annex VI as Acute Tox. 3 for oral toxicity.

Another MSCA supported the removal of the classification for acute oral toxicity.

Acute dermal toxicity

In the NTP technical report (NTP, 1989), in addition to carcinogenicity studies described in the CLH report, acute dermal toxicity was tested in both rats and mice.

Groups of five rats of each sex were administered a single dermal application of 198, 388, 773, or 1 568 mg/kg of VCD (97 % purity) in acetone and 3074 mg/kg neat, in a volume of 3 × 0.1 mL, to the clipped dorsal interscapular region.

Groups of five mice of each sex were administered 338.3, 671.6, 1 378, or 2 741 mg/kg of VCD (in acetone) and 5 487 mg/kg (neat), in a volume of 0.1 mL, on the same schedule.

Animals were observed two times daily, and necropsy was performed on all animals.

However, the skin application area is not stated, it is not known whether it was covered, and if it was, for how long. According to OECD TG 402, the test substance should be applied uniformly and retained for 24 h with a gauze dressing over an area which is approximately 10 % of the total body surface area.

In rats, there was no mortality during a 14-d observation period, and no lesions were observed at necropsy. Decreased activity, which was considered a compound-related clinical sign, was observed in the 773, 1 568, and 3 074 mg/kg bw groups.

Data on survival and LD_{50} value in *mice* are presented in the table below (from the NTP report).

TABLE 11. SURVIVAL OF MICE IN THE SINGLE-ADMINISTRATION DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE

Dose (mg/kg)	Survival	
	Male	Female (a)
338.3	5/5	5/5
671.6	5/5	(b) 4/5
1,378	5/5	5/5
2,741	5/5	(c) 4/5
5,487	(d) 0/5	(d) 0/5

(a) LD_{50} by probit analysis: 3,216 mg/kg (95% confidence interval 1,766-10,501 mg/kg)
(b) Day of death: 8
(c) Day of death: 2
(d) All deaths occurred within 8 hours of dosing.

Clinical signs included decreased activity, rapid respiration, and irritation of the skin at the dermal application site. However, no lesions were observed at necropsy.

Assessment and comparison with the classification criteria

Acute oral toxicity

VCD is presently classified as Acute Tox. 3; H301 (Toxic if swallowed). According to the CLH Report, it is not known upon which data the current classification is based.

The DS proposed to remove the current classification, based on oral LD_{50} values above 2 000 mg/kg bw stated in two references (Weil et al. study, 1963; Dhillon and Von Burg review, 1996), indicating no requirement for classification.

In RAC's opinion, however, data from these two references are too limited to justify removal of the current classification.

On the other hand, well reported NTP data show values in the range of the CLP criteria for Category 4:

- LD_{50} of 1 847 mg/kg bw for female rats; and
- LD_{50} of 1 862 mg/kg bw for male mice.

Although the LD_{50} for female mice was above 2 000 mg/kg bw (2 358 mg/kg bw), and the LD_{50} for male rats was not calculated, RAC considers that data for female rats and male mice warrant classification as **Acute Tox. 4; H302 (Harmful if swallowed)**.

An **ATE of 1 847 mg/kg bw** is proposed (LD_{50} for female rats), as the lowest ATE available, tested in the appropriate species. Also, according to the CLP Regulation, rat is the preferred species for evaluation of oral toxicity via the oral route.

This is supported by the data from 16-d oral studies in rats and mice (NTP, 1989). In rats dosed at 2 000 mg/kg bw, four out of five males and females died within the first 72 h after the beginning of VCD treatment. In mice, one out of 5 males dosed at 1 000 mg/kg bw died the second day after the beginning of treatment, and at 2 000 mg/kg bw all five males and females died within the first 72 h after the beginning of VCD treatment. According to the Guidance on the application of the CLP criteria (CLP guidance, ECHA, 2017), "mortalities during the first 72 h after first treatment (in a repeated dose study) may also be considered for the assessment of acute toxicity".

Acute dermal toxicity

VCD is currently classified as Acute Tox.; H311 (Toxic in contact with skin). According to the CLH report, it is not known on which data the current classification is based.

The DS proposed to retain the existing classification, based on dermal LD_{50} value of 680 mg/kg bw in rabbits, stated in one reference without further details (Weil *et al*., 1963), with an ATE of 680 mg/kg bw.

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 indicate no requirement for 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). Hence, the data are not considered reliable enough to change the classification from Acute Tox. 3 to no classification. It can also be noted that acute toxicity via the dermal route is expected to be lower than via the oral route, where an LD_{50} of 1 847 mg/kg bw was reported.

RAC considers the database on acute dermal toxicity conflicting and too limited to classify. Hence, the Committee concluded that VCD **should not be classified for acute dermal toxicity**, due to insufficient evidence.

Acute inhalation toxicity

VCD is currently classified as Acute Tox. 3; H331 (Toxic if inhaled). According to the CLH report, it is not known on which data the current classification is based.

The DS proposed to classify VCD as Acute Tox. 4 with an ATE of approximately 4.6 mg/L, based on the LC_{50} of 800 ppm reported by Dhillon and Von Burg (1996). The DS considered that the tested concentration of 800 ppm was probably in the form of a mist.

In RAC's opinion, the uncertainties related to the data from Weil *et al*. (1963) study and a secondary reference from the review article by Dhillon and Von Burg (1996) are too high to justify changing the classification from Category 3 to Category 4, as proposed by the DS.

The secondary reference from the review article by Dhillon and Von Burg (1996), does not state whether the 4-h LC_{50} of 800 ppm in rats refers to vapours or mists. Since 128 ppm was calculated by the DS as a saturated vapour concentration of VCD (at 20 °C and 101.3 kPa), the DS considered that a LC_{50} concentration of 800 ppm (4.6 mg/L) was probably in the form of a mist, which would result in classification in Category 4 ($1.0 < ATE \leq 5.0$ for mists and dusts).

However, based on the available information, it is not possible to rule out that the LC_{50} of 800 ppm (4.6 mg/L) in the review of Dhillon and Von Burg (1996) was not at least partially in the form of vapour, since it is not known whether the substance was heated during the experiment. In that case, classification for acute inhalation toxicity as Acute Tox. Category 3 ($2.0 < ATE \leq 10.0$ for vapours) could be justified.

It is possible that a temperature needed to obtain 800 ppm in a form of vapour is too high to be safely applied to animals (i.e. causing burns), and that 800 ppm was indeed in the form of mist. RAC, however, cannot further assess this issue since information on vapour pressure-temperature relationship for VCD is not available to either RAC or the DS (the only data available is for vapour pressure at 20 °C).

Due to significant limitations in the reporting of the available studies (*i.e.*, no further details on methodology apart from the information stated above), RAC proposes to retain **Acute Tox. 3**; **H331 (Toxic if inhaled)**.

RAC proposes to apply the default **ATE value** for dusts or mists for Acute Tox. 3 (**0.5 mg/L**) since this value is lower than a default ATE value for vapours for the same category (3 mg/L).

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

VCD was found to be mutagenic in various strains of *Salmonella typhimurium*, in the presence and absence of an exogenous metabolic system. In addition, it showed positive results in *in vitro* mammalian cells in NTP studies (NTP, 1989) (gene mutation study in L5175Y mouse cells in the absence of metabolic activation; chromosome aberration test and sister chromatid exchange test in Chinese hamster ovary cells in the presence and absence of metabolic activation). Other studies were considered not adequate for genotoxicity assessment because of deficiencies in design and reporting.

The DS concluded that VCD is mutagenic *in vitro*, causing gene mutations and chromosomal aberrations.

Also, in both *in vitro* study in calf thymus DNA and in *in vivo* study in skin, of topically treated mice, VCD produced DNA adducts (Mabon and Randerath, 1996; Randerath and Mabon, 1996). These are the only *in vivo* studies available.

In an *ex vivo* human skin tissue comet assay, VCD significantly increased percentage of tail DNA at non-cytotoxic dose levels (Reus *et al*., 2012).

The DS concluded that due to lack of data on germ cell and somatic cell mutagenicity *in vivo*, there is no evidence that VCD has the potential to cause mutations to germ cells, and classification as a germ cell mutagen was not proposed.

Comments received during public consultation

One MSCA supported no classification. Two MSCAs expressed their concern about not classifying VCD as a germ cell mutagen. The question on using possible chemical structure activity relationship with known germ cell mutagens to support classification was raised, and classification in Category 2 and even Category 1B for germ cell mutagenicity was proposed, based on evidence of VCD reaching germ cells (VCD ovarian toxicity).

Assessment and comparison with the classification criteria

Data on genotoxicity presented in the CLH report are available from:

- nine bacterial reverse mutation tests, out of which four had Klimisch score 2 (*i.e.* they were considered by the DS as reliable with restrictions; Mortelmans *et al*., 1986 (NTP, 1989); Simmon and Baden, 1980; Frantz and Sinsheimer, 1981; El-Tantawy and Hammock, 1980);
- two *in vitro* studies using *Saccharomyces cerevisiae*, which were considered by the DS as not adequate for genotoxicity assessment because of deficiencies in design and reporting;
- five *in vitro* studies in mammalian cells, out of which three NTP studies were assigned Klimisch Score 2 (McGregor *et al*., 1988; chromosomal aberration study and sister chromatid exchange study described in NTP report, 1989);
- one *ex vivo* comet assay, considered by the DS as not adequate for genotoxicity assessment because it was not validated; and
- *in vitro* and *in vivo* studies on DNA adducts (Mabon and Randerath, 1996; Randerath and Mabon, 1996) considered by the DS as reliable for assessment.

In vitro studies

All available bacterial reverse mutation tests had limitations: only one bacterial species, *i.e. S. typhimurium*, with less than five bacterial strains tested; in 4 studies designated by the DS with Klimisch Score 2 only the strains with GC base pair at the primary reversion site were tested; in Klimisch 3 studies there were significant limitations in methodology and reporting.

However, VCD was positive in all 9 studies, in at least one strain per study (i.e. in TA100, TA1535, TA98), both in the presence and absence of metabolic activation, and at concentration levels that were not cytotoxic (Table 11, CLH report).

Summary results for Klimisch Score 2 studies are presented below:

Reference	*S. typhimurium* strains	Concentration range/metabolic activation/cytotoxicity	Results
Mortelmans *et al*., 1986 (NTP, 1989)	TA98 TA100 TA1535 TA1537	0.10 - 10 mg/plate +/- S9 No cytotoxicity observed	**Positive** in TA98, TA100 and TA1535, with and without S9 TA1537 without S9 – **equivocal** TA1537 with S9 – **positive** in 1st trial, **equivocal** in 2nd trial
Simmon and Baden, 1980	TA98 TA100 TA1535	0.01 - 0.5 mL/9 liter desiccator (closed chamber used for volatile chemicals) +/- S9 No cytotoxicity observed	**Positive** in TA98, TA100 and TA1535, with and without S9
Frantz and Sinsheimer, 1981	TA100 TA1535	15 - 60 µmoL/plate Growth inhibition ≥ 45 µmol/plate	**Positive** in TA100 and TA1535, probably without S9 (not stated)
El-Tantawy and Hammock, 1980	TA1535, TA100, TA1537, TA98	0.06 - 2 mg/plate Without S9 Cytotoxicity: 2 mg/plate	**Positive** in TA100 and TA1535, without S9 **Negative** in TA1537 and TA98

RAC concludes that VCD is positive in bacterial reverse mutation tests, based on four studies that have limitations in methodology and reporting, but are considered sufficiently reliable for assessment. Other available studies showed similar results, but they were of inadequate reliability and not further assessed by RAC.

In vitro studies in *Saccharomyces cerevisiae* showed positive result for reverse mutation, mitotic gene conversion and mitotic cross over (Bronzetti *et al*., 1980). RAC, however, agrees with the DS that the limitations in methodology and reporting (*i.e.* limited information on design and results, no metabolic activation used, no information on compound purity, no information on potential solvent used, no information on what has been used as negative control, no data on positive control) render the studies unreliable for genotoxicity assessment.

VCD was clearly positive in three NTP *in vitro* studies in mammalian cells assessing gene mutations, chromosome aberrations and sister chromatid exchange (see table below).

Reference	Method/cell type	Concentration range/cytotoxicity	Results
McGregor *et al*., 1988	Gene mutation / mouse lymphoma L5178Y tk+/ tk- cells No metabolic activation	25-200 µg/mL (Trial 2) Cytotoxicity: ≥50 µg/mL	**Positive** – highly significant, dose-dependent increase in mean mutant frequency already at dose levels below cytotoxic
NTP report, 1989	Chromosome aberration / Chinese hamster ovary cells	-S9: 37.8, 50.3, and 62.9 µg/mL +S9: 447, 503, and 548 µg/mL Some cell cycle delay was observed	**Positive** – highly significant, dose-dependent increase in percentage of cells with aberrations, already at the lowest dose, with and without metabolic activation, at dose levels without overt cytotoxicity
NTP report, 1989	Sister chromatid exchange / Chinese hamster ovary cells	-S9: 1.12, 3.73, and 11.2 µg/mL +S9: 37.3, 112, and 373 µg/mL Some cell cycle delay was observed	**Positive** – highly significant, dose-dependent increase in SCE, already at the lowest dose, with and without metabolic activation, at dose levels without overt cytotoxicity

RAC concludes that VCD is positive for gene mutations and chromosomal aberrations in *in vitro* studies in mammalian cells, based on three NTP studies. Although not all methodological details are provided in the NTP report, and there are some deviations (e.g. no metabolic activation in gene mutation test; incubation times in chromosome aberration test differed from those recommended by TG 473), it seems that protocols of these studies are very similar to those recommended by OECD test guidelines (OECD TG 473, 476 and 479). They are thus considered sufficiently reliable for assessment.

Other available *in vitro* studies also showed positive results for gene mutation and chromosome aberrations, although the result of micronucleus test was negative (Turchi *et al*., 1981), but RAC agrees with the DS that they are of inadequate reliability, and they were not further assessed by RAC.

***Ex vivo* studies**

One *ex vivo* comet assay in human skin is described (Reus *et al*., 2012). VCD significantly increased percentage of tail DNA at non-cytotoxic dose levels in human skin membrane prepared from a single donor (a Caucasian female), obtained after breast or abdominal surgery. Although this is a well-described experiment, RAC agrees with the DS that it is a non-guideline, non-validated study, which could be used as supporting evidence only.

***In vivo* studies**

Although there are no available *in vivo* mutagenicity studies, RAC recognises positive *in vivo* genotoxicity studies, and considers them reliable enough for assessment. The studies showed the formation of DNA adducts by VCD *in vitro* (calf thymus DNA) and *in vivo* in mice, after topical exposure. In the *in vivo* experiment, female ICR mice received, for 3 days, topical applications

of acetone (control), VCD or 1,3-butadiene diepoxide (BDE) in acetone at 17, 51 or 153 µmoL/mouse (Mabon and Randerath, 1996), or acetone or VCD at 14.4, 36, 90 or 225 µmoL/mouse (Randerath and Mabon, 1996). Skin DNA was isolated from five mice per group, 5 h after the third treatment, and DNA adducts were measured by the monophosphate ^{32}P-postlabeling assay. The results showed dose-dependent increases in DNA-adduct formation (Table 1 from the Mabon and Randerath (1996) article, presented in *Supplemental information - In depth analyses by RAC in Background document*).

Although typical positive control, such as benzo[a]pyrene or other polynuclear aromatics (Schurdak *et al*., 1989), was not applied, a group of animals in the experiment of Mabon and Randerath (1996) was exposed to BDE, a substance with harmonised classification as Muta. 1B and Carc. 1B, and which is known to be DNA-reactive, forming DNA adducts (e.g. Goggin *et al*., 2011). There is no information on an adherence to GLP, and the adduct levels were at least two orders of magnitude lower that those formed with comparable doses of potent carcinogens (as explained by the study authors). However, the studies were well conducted and described, and are considered by RAC as reliable enough to serve as at least supportive evidence.

A formation of DNA adducts was tested only topically, in mice skin. Nevertheless, ovarian toxicity of VCD, which has been shown to be a consequence of direct action of VCD on ovarian tissue (Kappeler and Hoyer, 2012), indicates that VCD reaches mammalian germ cells and could, probably, react with their DNA. Increased incidence of ovarian tumours in mice following dermal exposure to VCD supports this assumption.

According to the CLP criteria, "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".

Although VCD, as a diepoxide, triggers several alerts for mutagenicity (see reports from ToxTree in *Supplemental information - In depth analyses by RAC*: Figures 1-3), 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 in the RCOM, Annex VI of the CLP does not include any (di)epoxide with a germ cell mutagenicity classification (regarding two structural analogues with similar *in vitro* mutagenicity profile that are stated in the NTP Report 1989, please see *Supplemental information - In depth analyses by RAC*). It could therefore be concluded, that VCD currently 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 a supportive evidence for the mutagenicity potential of VCD.

Conclusion

In standard, test guideline assays, VCD was clearly positive for gene mutations in bacterial assays and for gene mutations and chromosomal aberrations in mammalian cells *in vitro*.

Additionally, VCD was positive in a non-validated *ex vivo* comet assay in human skin, and able to form DNA adducts in calf thymus DNA in an *in vitro* test, and in mice skin in an *in vivo* test.

Human data as well as standard *in vivo* genotoxicity studies, which would assess the genotoxic endpoint(s) following systemic exposure to VCD, are not available.

Comparison with the CLP criteria:

- Since there is no data from human population on mutagenicity of VCD, Category 1A is not warranted;
- Also, there is no *in vivo* animal data for heritable germ cell mutagenicity or for somatic cell mutagenicity, therefore Category 1B does not appear justified;

- On the other hand, 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.

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 (Please see the discussion in "*Assessment and comparison with the classification criteria*" in the sections "*RAC evaluation of carcinogenicity*" and "*RAC evaluation of reproductive toxicity*"). Structural alerts for VCD mutagenicity add to this conclusion. Direct ovarian toxicity of VCD indicates that VCD reaches mammalian germ cells.

Based on these data, RAC proposes that, based on the weight of the evidence, VCD should be classified as **Muta. 2; H341 (Suspected of causing genetic defects)**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The carcinogenicity studies consist of seven dermal studies (in mice and rats), two intraperitoneal studies in rats, and one study in mice using an unspecified administration route. No long-term oral and inhalation studies are available.

Four dermal studies are assigned with a Klimisch score of 2: one in rats and three in mice (two NTP studies and two open literature studies in transgenic mice: Tennant *et al*., 1995, and Van Duuren *et al*., 1963). All other available carcinogenicity studies were assigned Klimisch score 3 (not reliable) or 4 (not assignable, *i.e*. insufficient experimental details, only listed in short abstracts or secondary literature) due to significant limitations in methodology or reporting, and were not considered reliable enough for carcinogenicity assessment.

The DS considered that the NTP studies (one in rats and one in mice) were well performed and reported, and therefore suitable for assessing the carcinogenic potential of VCD. In these studies, VCD was carcinogenic for F344/N rats and B6C3F1 mice of both sexes, causing *dermal tumours at the application site*: skin squamous cell neoplasms (predominantly carcinomas) and basal cell neoplasms (adenomas and carcinomas) in rats and skin squamous cell carcinomas in mice. VCD exposure was also related to ovarian neoplasms (benign or malignant granulosa cell tumours, benign mixed tumours), and an increase in lung neoplasms (alveolar/bronchiolar adenomas or carcinomas) in female mice.

Although the design of two dermal studies in transgenic mice (Tennant *et al*., 1995, and Van Duuren *et al*., 1963) differs considerably from that of a conventional two-year rodent carcinogenicity study, the DS considered that these studies provide supportive evidence for VCD carcinogenicity. In addition, p53-deficient C57BL/6 mice developed the same type of skin tumours at the application site as did normal mice in the two-year mouse study by the NTP. In rasH2 (CB6F1) mice, VCD induced skin tumours, and also forestomach papilloma, thymic lymphoma, lung adenoma, squamous cell carcinoma and spleen hemangiosarcoma.

Regarding lung tumours found in female mice in the NTP study, the DS pointed out that an increase in the incidence of lung tumours in a mouse carcinogenicity study is generally considered to have little relevance to man, and that in the NTP study it was observed only in females. Therefore, the DS considered that this finding was not unequivocally related to treatment.

On the other hand, the DS considered the dermal and ovarian tumours found in NTP studies as relevant for humans. Overall, the DS proposed to classify VCD in Category 1B, based on the following considerations:

- consistent substance-related increase in skin tumours was observed in both rats and mice of both genders, and tumours progressed to malignancy;
- no skin tumours were observed in control animals;
- contribution of local skin toxicity on formation of dermal tumours is likely limited;
- the increase in tumours was limited to the site of exposure in rats but is extended to at least the ovaries in mice;
- the ovary tumours in female mice were partly malignant;
- there are no human data available regarding human exposure to VCD leading to carcinogenicity, so category 1A is not justified.

Regarding the relevance of skin tumours, the DS discussed that local skin toxicity due to the irritating properties may have contributed to the formation of tumours, especially in mice. Nevertheless, local effects in rats were limited to acanthosis and sebaceous gland hypertrophy and there was no necrosis or inflammation, indicating no excessive toxicity. Also, based on the available data, the substance is expected to be genotoxic and mutagenic in the skin. The DS concluded that both local irritation and probable local mutagenicity may have contributed to the increase in dermal tumours. Since both mechanisms are considered relevant to humans, the dermal route was considered relevant for classification by the DS.

As no information on the carcinogenicity by other routes is available but also systemic tumours are observed in mice, the DS did not propose to limit the classification to a single route of exposure.

The DS did not consider that the available information on potency justifies SCL derivation.

Comments received during public consultation

Three MSCAs supported classification in Category 1B for carcinogenicity as proposed by the DS.

Assessment and comparison with the classification criteria

Out of ten carcinogenicity studies available, only two dermal NTP studies, one in rats and one in mice, were performed and reported in line with the OECD TG recommendations, although with some deviations from OECD TG 451 and limitations. For example, two instead of three dose levels of VCD were tested in rats; exposed skin area is not reported, and it is not known whether it was ensured that VCD is in contact with the skin, and if it was, for how long.

Among the other eight available studies, two dermal studies in transgenic mice were considered by the DS as reliable enough to serve as supportive evidence (Tennant *et al*., 1995, and Van Duuren *et al*., 1963).

RAC agrees with the DS that the methodology and reporting in six other studies (three dermal studies in mice, two intraperitoneal studies in rats, and one study in mice using an unspecified administration route) is too limited to be used in the assessment of carcinogenic properties of VCD. For example, there were no quantitative data on doses, no data on general toxicity, intraperitoneal route of exposure, no data for control group, only 12 months exposure period, low number of animals per group (e.g. 10-20 per group) and only one gender tested.

NTP study in rats

In an NTP study in 344/N rat (60 animals/sex/dose), VCD (97 % pure, in acetone) was dermally applied for 5 days per week, for 105 weeks, at 0 (vehicle control), 15 (low dose) or 30 (high dose) mg/rat/d[1]. Ten animals from each group were killed and examined during month 15 for toxicological evaluation. Statistical analysis of tumour incidences included life table tests, logistic regression (with adjustment for inter-current mortality), Cochran-Armitage trend test, and Fisher exact test.

The table in the BD (*Supplemental information - In depth analyses by RAC)* includes the main findings regarding survival, general toxicity and neoplastic changes in the study, as well as available historical control data (HCD; NTP historical control database, Haseman *et al*., 1984, 1985) for the period when this study was performed (1982-1984).

General toxicity

While 100 % survival in both genders was noted at the 15-month evaluation, two-year survival in males was very low in all groups (8-16 %), including controls. It was higher in females (54 % and 46 % in control and low dose group, respectively), except for the high dose group in which the survival rate (30 %) was significantly lower compared to control.

Body weight at the 15-month evaluation was 10 % lower in males and 7 % lower in females in the high dose group, compared to respective controls, and relative organ weights (organ weight to body weight ratio) and haematological parameters were not affected by VCD treatment. Terminal body weights (at the end of the 2-year study), were 11 % lower in males and 14 % lower in females in the high dose group, compared to controls, and the only clinical sign described in the NTP report was discoloured hair at the site of application.

Neoplastic changes

In both genders, the incidences of skin tumours at the application site (primarily basal cell adenomas and carcinomas, and squamous cell papillomas and carcinomas) were significantly higher than in controls and in non-application skin areas in exposed animals. While skin tumour incidences in the control group were within NTP historical control ranges from the relevant time period, incidences in exposed animals were above the historical control data (HCD) range, especially for squamous cell carcinoma.

For almost all tumour types, there was a clear dose-response pattern. Increased squamous cell carcinoma incidence (2 out of 10 necropsied animals) was observed in male rats already at the 15-month evaluation. Skin squamous cell carcinomas were metastatic (to the lung and/or multiple organs in four low dose and three high dose males and in one high dose female). Available general toxicity data described above suggest that increased incidence of skin tumours occurred in absence of marked general toxicity (e.g. increased incidence in squamous cell carcinoma was observed in both genders already at low dose at which survival and body weight gain were comparable with control values). Local toxicity at the application site also did not seem to be excessive. Namely, only acanthosis (thickening of the epidermis) and hyperkeratosis were found, without epithelial necrosis or ulceration. At the 15-month evaluation, only mild acanthosis was observed at the high dose, and it was minimal at the low dose. Hyperkeratosis was observed only at the high dose. The latent period for development of skin neoplasms was shorter at the high dose compared to the low dose (e.g. the day of first observation of squamous cell carcinoma

[1] In this NTP study doses are expressed as mg/rat/day. Same applies for the other studies where it is indicated like this.

in the skin was day 596 at low dose and day 543 at high dose in males; Table A3 and B3 in NTP Report, 1989).

Regarding other tumour sites and types, squamous cell carcinoma of forestomach was found in 1 out of 10 females necropsied at 15 month. However, HCD were not provided for this tumour type, and it was not observed later in the remaining 50 female rats in which exposure continued for up to 2 years. The authors of the NTP report also pointed out an observation of single small papilloma of the transitional cell epithelium of urinary bladder in 2/50 low dose females, which is above the NTP HCD range (0/50 to 1/49). Absence of dose-response does not necessarily invalidate this finding, due to 70 % mortality rate at the high dose (i.e. lowering the chance for tumour development in high dose group). Nevertheless, the papillomas were not accompanied with hyperplasia of the urinary bladder transitional epithelium, and the incidence was slightly above the HCD range. RAC, therefore, considers that the significance of these findings for carcinogenicity classification of VCD is uncertain. Incidences of neoplastic changes in other organs and tissues did not indicate substance-related effect (Appendix B in NTP report, 1989).

Non-neoplastic changes

Regarding non-neoplastic, non-dermal changes, increased incidence of ovarian cyst was found (8 % in controls, 10 % at low dose, and 18 % at high dose).

NTP study in mice

In an NTP study in B6C3F1 mice (60 animals/sex/dose), VCD (97 % pure, in acetone) was dermally applied for 5 days per week, for 103 weeks, at 0 (vehicle control), 2.5 (low dose), 5 (mid dose) or 10 (high dose) mg/mouse/d. Ten animals from each group were killed and examined during month 15 for toxicological evaluation. Statistical analysis of tumour incidences included tests already stated for the rat study.

See the table in *Supplemental information - In depth analyses by RAC* that presents the main findings regarding survival, general toxicity and neoplastic changes in the study, as well as available HCD (NTP historical control database, Haseman *et al*., 1984, 1985) for the period when this study was performed (1982-1984).

General toxicity

At the 15-month evaluation, no mortality was observed. Only in the high dose males (10 mg/mouse/d) body weight was 13 % lower compared to controls. However, all male mice receiving the high dose died by week 83, and the surviving high dose group female mice were killed during week 85. After the 15th month of treatment, in both genders survival of the mid (after day 543 in males and day 666 in females) and the high (after day 451 in males and day 474 in females) dose groups was significantly lower compared to control. Survival was above 50 % in control and low dose group. Reductions in body weights were observed in mid and high dose groups. Crusts, scales, and ulcers were seen at the site of application, while there is no information about systemic clinical signs.

Neoplastic changes

In both genders, the incidence of skin squamous cell carcinomas at the application site was significantly higher than in controls and in non-application skin areas in exposed animals. In exposed groups, they were above the HCD range (NTP HCD ranges from relevant time period). Increased incidence occurred already after 15-month exposure, and was dose-dependent. In many animals, carcinomas metastasised to lymph nodes or visceral organs. Squamous cell carcinomas occurred at an incidence slightly above the HCD range also in skin areas away from the site of VCD application. The study authors considered that this could be due to inadvertent

spread of the study material away from the application site, but also due to metastasising process (via subcutaneous lymph nodes) from skin carcinoma at the application site. The latent period for development of skin neoplasms was shorter at higher doses compared to lower doses. For example, the day of first observation of squamous cell carcinoma in the skin was day 525, 411 and 376 at low, mid and high dose, respectively, in males, and day 642, 402 and 376 at low, mid and high dose, respectively, in females (Table C3 and D3 in NTP, 1989).

Although increased incidence of squamous cell carcinomas was observed already at the low dose, at which marked general toxicity was not present (no significant effects on survival or body weight), local toxicity at the application site was present in a form of acanthosis, hyperkeratosis, and necrotising inflammation. Nevertheless, severe local toxicity in the form of necrotising inflammation was present in 4 males and 5 females out of 50 necropsied low-dose males and female mice (see table in *Supplemental information - In depth analyses by RAC*), while squamous cell carcinoma was present in 15 male and 6 female mice at this dose level, indicating that carcinomatous changes were present also in the absence of severe local toxicity.

In mice, not only tumours at the application site, but primary tumours at a distant site were recorded. In ovaries, increased incidence of benign or malignant tumours (*i.e.* luteoma, granulosa cell tumour, benign mixed tumour, or malignant granulosa cell tumour) was observed at mid and high dose level, i.e. dose levels with pronounced systemic toxicity. However, increased incidence of granulosa cell tumours was observed already during the first 15 months of treatment at 10 mg/mouse/d (2/9 females compared to 0/10 incidence in controls and to upper HCD range of 3/47 for 2-year study), when there were still no adverse effects on survival or body weight gain in female mice.

Lung tumours (alveolar/bronchiolar adenoma or carcinoma) incidence increased at mid dose level in female mice. Although low survival rate could be a cause for the lack of effect in the high dose group, RAC agrees with the DS that lung tumours are of limited significance for the assessment of VCD carcinogenicity. Namely, an incidence of 11 out of 50 female mice is not markedly above the upper limit of the HCD range (8/50), and it was similar to the incidence observed in control males (10/50).

Non-neoplastic changes

In the ovaries of exposed mice, already at the low dose, increased incidence of ovarian atrophy (characterised by a complete absence of follicles and corpora lutea) and tubular hyperplasia were observed. In addition, at the 15-month evaluation, uterine relative weight at mid and high dose levels were significantly lower than in controls.

Increased incidence of epididymis subacute inflammation was observed at mid and high dose, and haematopoietic cell proliferation in the spleen, expressed primarily as myeloid hyperplasia, was found. Study authors considered this change as a response to necrotising inflammation and neoplasms of the skin.

Non-NTP studies assigned by the DS with Klimisch score 2

Tennant *et al*. (1995, 1996) used VCD as model compound to examine the potential of transgenic mouse models (p53-deficient C57BL/6) to differentiate carcinogens and non-carcinogens. It is known that p53-deficient C57BL/6 mice are susceptible to tumour development due to reduced expression of the p53 tumour suppressor gene. Dermal application of VCD at 12.5 or 25 mg/animal, two times per week for 24 weeks, induced, in transgenic mice of both genders, the same type of squamous cell tumours at the application site as in non-transgenic mice (B6C3F1 mice) in a 2-year dermal NTP study. Due to use of a non-standard animal model and study protocol for a carcinogenicity assay, this study is considered by RAC as supportive evidence only.

The article by van Duuren *et al*. (1963) describes a dermal study in male Swiss ICR/Ha mice, which were exposed to ca. 100 mg of VCD solution/application in benzene, three times per week for life. Skin carcinogenicity in exposed animals was observed, but VCD purity was unspecified and the carcinogenic potential of the vehicle (benzene) introduces an uncertainty into the interpretation of the results (IARC, 1976). This study was, therefore, not further assessed by RAC.

Literature data and NTP studies indicate that VCD has a direct irritant effect at the site of contact, and it is possible that irritancy contributes to VCD's carcinogenic properties. Nevertheless, as pointed out by the DS, excessive local toxicity was not found in rats at a dose level which already lead to increased incidence in malignant skin tumours (15 mg/animal/d).

RAC also wants to point out that skin tumour incidences at all dose levels in mice were higher than incidences of pronounced local toxicity (necrotising inflammation). RAC agrees with the DS that both local irritation and probable local mutagenicity (indicated by *ex vivo* and *in vivo* mutagenicity studies in human and mice skin; Reus *et al*., 2012; Mabon and Randerath, 1996) could contribute to the increase in dermal tumours. Since both mechanisms are considered relevant to humans, skin tumours related to dermal exposure to VCD are regarded as relevant for carcinogenicity classification.

The carcinogenic mechanism of VCD-related ovarian carcinogenicity in mice is unclear. Due to uncertainties related to VCD mutagenicity, a direct genotoxic mechanisms cannot be ruled out. Also, the NTP report (1989) proposed that elevated levels of gonadotropins in response to oocyte depletion could act as tumour promotors. Nevertheless, this hypothesis is not uniformly supported by either experimental results (Hoyer and Sipes, 1996) or epidemiological data (Jamieson and Fuller, 2012).

In the NTP Report (1989), the immunotoxicity studies in mice showed that VCD in the 10 mg/mouse/d group, and to a lesser extent in the 5 mg/mouse/d group, produced immunosuppression. This was characterised by a decrease in the lymphoproliferative response to phytohemagglutinin and concanavalin A, and suppression of the antibody plaque-forming-cell responses. Immunosuppression was, therefore, proposed as another factor potentially contributing to skin and ovarian carcinogenesis in mice.

Conclusions

RAC bases its opinion on the carcinogenic potential of VCD observed in two well conducted and well reported dermal NTP carcinogenicity studies, one in rats and one in mice.

In both rodent species, a dose-related increase in incidence of benign and malignant skin tumours was observed, especially of squamous cell carcinoma. The increase was observed in both genders, in the absence of marked general toxicity. In mice, dermal exposure to VCD was also related to increased incidence of ovarian tumours, already in the absence of pronounced general toxicity.

Latent period for development of skin and ovarian neoplasms was shorter at higher doses compared to lower doses, and the tumours showed metastasising potential.

There is no mechanistic data indicating that these tumours are not relevant for humans.

Due to the fact that:

- there are no data on carcinogenetic effects of VCD in humans, which would warrant classification in Category 1A, while
- there are animal experiments showing sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen):
    - dose-dependent, significant increase in the incidence of benign and malignant skin tumours was observed in two rodent species (rats and mice), and in both genders,

- tumours in extra-dermal sites, away from the site of exposure, were also observed (ovarian tumours in mice),
- latent period for tumour development followed inverse dose-response relationship,
- tumours were observed already at dose levels which did not induce significant general or skin toxicity,

RAC agrees with the DS that VCD should be classified as Carc. 1B; H350 (May cause cancer).

Since in addition to skin tumours, systemic tumours were also observed (ovarian tumours in mice), and there is no information on the carcinogenicity by other exposure routes (oral and inhalation), **a specific exposure route is not proposed**.

No Specific Concentration Limit (SCL) is proposed since only dermal studies are available for carcinogenicity assessment. According to the EC Guidelines for setting specific concentration limits for carcinogens in Annex I of Directive 67/548/EEC (1999) "the data for calculating T25 should preferentially be from lifetime oral (feed or gavage) or inhalation studies in mammals" since "experience in obtaining T25 is only available for substances administered by oral administration or inhalation".

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

The DS assessed the available data on reproductive toxicity, including animal studies with dermal, oral, intraperitoneal and intramuscular route of exposure, while noting that intraperitoneal and intramuscular exposure routes are less relevant for human exposure.

The DS summarised that in these studies, VCD was toxic to ovaries in rats, mice, hamsters and non-human primates, *i.e.* all species tested. VCD was also ovotoxic following dermal, intraperitoneal and intramuscular route, inducing a significant loss of primordial and primary follicles in exposed females at dose levels without or with only limited general toxicity. Some studies also showed a reduction in the number of offspring. Potential mechanisms of ovotoxicity were discussed by the DS, and they concluded that it is likely that the effects are relevant to humans.

Exposure to VCD also had an effect on male animals, inducing reversible testicular and epididymal dysfunctions, testicular damage, and reduced testicular weights in rodents. However, no effects on reproductive function of treated males were reported.

The DS concluded that since:

- there are no human data on the effects on sexual function and fertility by VCD, classification in Category 1A is not warranted;
- ovotoxicity was observed in four species exposed via different routes of exposure, including oral and dermal route;
- it is considered that the observed ovotoxicity is a direct effect of VCD and not secondary to the general toxicity, and that the proposed mechanisms are relevant to humans;
- although there are no data on the effect of the ovotoxicity on the resulting fertility via relevant routes of exposure (oral, dermal or inhalation), the observed ovotoxicity is considered to result in a reduction of the number of offspring in intraperitoneal studies,

classification in Category 1B; H360F (May damage fertility) is justified.

Developmental toxicity and Effects on or via lactation

There are no human data on the developmental toxicity of VCD, and data from animal studies are too limited to conclude on developmental toxicity. For example, only the intraperitoneal route was assessed, there was exposure only during the pre-mating period, and adverse effects on implantation were not statistically significant and were considered to be a consequence of ovotoxicity (Haas *et al*., 2007; Kodama *et al*., 2009). Therefore, the DS proposed no classification due to absence of data. The same applies for effects on or via lactation.

Comments received during public consultation

One MSCA considered that there is insufficient evidence to justify classification in Category 1B, since there is no information regarding effects on male fertility, no evidence by experimental genotoxic results that the germ cells are damaged by VCD treatment, and no available toxicokinetic information on whether the substance could reach the reproductive organs. Classification in Category 2 was therefore proposed.

Two MSCA supported classification as Repr. 1B; H360F, of which one specified that they also supported no classification for developmental toxicity. In addition, some corrections in the presentation and interpretation of data in the CLH report were given.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

In the CLH Report, adverse effects on fertility were evaluated from:

- four oral studies – three in rats and one 13-week NTP study in mice,
- four dermal NTP studies – two in rats and two in mice (13-week and 2-year studies),
- 16 intraperitoneal studies – seven in rats, six in mice, one in rats and mice, and two in hamsters,
- one intramuscular study in Cynomolgus monkeys, and
- one study in Cynomolgus monkeys in which VCD incorporated into biodegradable fibre was applied next to ovary.

RAC is also aware of a 16-d oral NTP study in rats and mice, and a 13-week oral NTP study in rats (NTP, 1989), in which reproductive organs were examined.

Out of the above listed studies, only oral and dermal NTP studies (in mice and rats) were performed according to protocols very similar to those recommended by OECD test guidelines (repeat dose studies and long-term toxicity/carcinogenicity studies), and are well reported. Although these studies were not specifically designed for fertility assessment, histopathological evaluation of reproductive organs is available.

Other studies are non-guideline studies reported in peer-reviewed journals, and in a majority of them, non-standard routes of exposure (for regulatory purposes) were applied (e.g. intraperitoneal or intramuscular route). Additionally, in many of these studies general toxicity was not reported. Nevertheless, they could serve as supporting evidence, as well as to provide information on mechanisms of ovarian toxicity of VCD. Ovarian toxicity of VCD has been extensively studied, including experiments in which VCD was used as a model to study menopausal effects, or as a substance for pest control.

Oral Studies

16-day oral NTP studies in rats and mice

Male and female F344/N rats and male and female B6C3F1 mice, 5 per group and gender, were administered 0, 125, 250, 500, 1 000, or 2 000 mg/kg bw/d VCD in corn oil by gavage, 5 days per week, in 12 doses over 16 days. Necropsy was performed on all animals, and histological examinations were performed on all vehicle controls, on all animals in the 500 and 1 000 mg/kg bw/d groups, and all rats receiving 2 000 mg/kg bw/d.

In rats, adverse effects on reproductive organs were not reported.

In mice, degeneration of the testis was seen in 4 out of 5 mice that received 1 000 mg/kg bw/d (see table in *Supplemental information - In depth analyses by RAC*). Nevertheless, at this dose 2 out of 5 male mice died, which was considered compound-related, and in these animals hyperplasia, hyperkeratosis, and/or ulcers were seen in the forestomach. At 2 000 mg/kg bw/d clinical signs of toxicity were observed (hyperpnea, burrowing behaviour, and half-closed eyelids), and all animals died by day 2 or 3 of the experiment.

13-week oral NTP studies in rats and mice

Male and female F344/N rats and male and female B6C3F1 mice, 10 animals per group and gender, were administered 0, 62.5, 125, 250, 500, or 1 000 mg/kg bw/d VCD in corn oil by gavage, 5 days per week, for 13 weeks. Histopathologic examinations were performed on all control and 1 000 mg/kg bw/d animals, 500 mg/kg bw/d rats, and all animals that died before the end of the studies. Testes, ovaries, and uterus of mice that received 250 and 500 mg/kg bw/d were examined microscopically.

In rats, at 500 or 1 000 mg/kg bw/d, smaller than normal testes in males and smaller uterine horns in females were observed (see table in *Supplemental information - In depth analyses by RAC*). In addition, at the top dose (1 000 mg/kg bw/d) degeneration of the tubular epithelium of the testis was noted in one male. Although marked systemic toxicity was observed at this dose level, at which 3 males and 6 females died and the animals had 20-23 % lower body weight compared to controls, general toxicity was not significant at 500 mg/kg bw/d. At 500 mg/kg bw/d there were no fatalities, and body weight was only 6-7 % lower compared to controls. No effects on ovaries were reported in this study.

In *male mice*, dose-dependent, multifocal to diffuse testicular degeneration (a decrease in the number of germinal epithelial cells within the seminiferous tubules) was present at 250, 500 and 1 000 mg/kg bw/d (see table in *Supplemental information - In depth analyses by RAC*). Final mean body weights of mice that received 500 or 1 000 mg/kg bw/d were 13 % or 15 % lower, respectively, compared to controls. There was no compound-related mortality.

In *female mice*, a dose-dependent increase in the incidence of diffuse ovarian atrophy was observed at 250, 500 and 1 000 mg/kg bw/d. Increased incidence of uterine atrophy was present at the top dose (1 000 mg/kg bw/d). No compound-related deaths occurred in females, and the final mean body weights of mice that received 500 or 1 000 mg/kg bw/d were only 3 % or 6 % lower, respectively, compared to controls. An accident that happened during the experiment, when 9 females (one at 62.5 mg/kg bw/d, two at 125 mg/kg bw/d, 4 at 250 mg/kg bw/d, and 2 at 1 000 mg/kg bw/d) delivered litters during week 4 of the study, after they escaped from their cages, is not expected to invalidate the study results.

Non-NTP oral studies

In the study of Abolaji *et al*. (2016), adult female Wistar rats, 7 per group, were exposed orally to 0, 100, 250 and 500 mg/kg bw/d of VCD (96 % pure, procured from Sigma Chemical Co.) in corn oil (presumably by gavage), for 28 days, with the aim to study the mechanisms of ovarian toxicity of VCD.

In VCD-exposed rats, large cystic and scanty number of follicles were found in the ovary, and the uterus showed gradual loss of the endometrial cells. Grading of these changes was not performed, and there is no information on systemic toxicity in the animals.

Regarding the mechanisms of VCD ovotoxicity, increased ovarian and uterine levels of malondialdehyde (MDA, an index of lipid peroxidation), observed changes in the activities of ovarian and uterine antioxidant enzymes (catalase, glutathione peroxidase (GPx), glutathione S-transferase (GST) and superoxide dismutase (SOD)) and depletion of ovarian glutathione, already at the lowest applied VCD dose (100 mg/kg bw/d), indicate the role of VCD-induced oxidative damage in these organs. Additionally, based on an increased expressions of ovarian inflammatory markers (inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)) and pro-apoptotic proteins (caspase-9 and caspase-3), it seems that inflammation and apoptosis are also involved in VCD-induced ovarian toxicity.

Despite the limitations in study reporting (no data on general toxicity, no quantification of ovarian and uterine morphological changes), the results of this study support the findings of ovarian and uterine toxicity of VCD in the NTP oral studies. Uterine toxicity could be secondary to depletion of ovarian follicles, but it could also be, at least partially, caused directly by VCD since indices of oxidative damage were found in uterine tissue. Hormonal changes observed at the highest dose of 500 mg/kg bw/d was decreased progesterone and oestrogen levels as well as increased luteinizing hormone (LH) and follicle stimulating hormone (FSH) levels, compared to controls (Table 1 from Abolaji *et al*., 2016, see *Supplemental information - In depth analyses by RAC*). These are probably a consequence of ovarian toxicity (decreased progesterone and oestrogen production caused by follicle depletion, which increased serum LH and FSH levels). Changes in prolactin levels did not follow a dose-response relationship, and are difficult to interpret.

The study by Berger and Horner (2003) aimed to evaluate whether oocyte fertilising ability was sensitive to exposure to some known toxicants, including VCD, and whether it was coupled with changes in other oocyte parameters, such as ovulation rate and oocyte fragility. Female rats (a Sprague-Dawley derived strain) received 0 %, 0.02 % or 0.04 % VCD in drinking water (estimated exposure of 20 and 40 mg/kg bw/d) for 2 weeks prior to oocyte recovery.

VCD treatment did not affect body weight, and had no effect on the percentage of females ovulating and the fertilising ability of oocytes. There was a slight, statistically non-significant effect ($p < 0.10$) on the fragility of oocytes (Table 4 from Berger and Horner (2003), presented in *Supplemental information - In depth analyses by RAC*).

Although in the study of Witmer *et al*. (2017), ovarian changes typical for VCD treatment were observed (reduced number of primordial, primary and secondary follicles in treated animals compared to unexposed control), this study was not considered by RAC as appropriate for reproductive toxicity assessment, and was not further evaluated. Namely, the animals (inbred SD rats and wild-caught Norway rats) were concomitantly exposed (in the same liquid bait) to VCD and another reproductive toxic substance, triptolide. Although triptolide was present as a minor component in the mixture (0.001 %), and it is considered to affect mainly larger ovarian preantral and antral follicles (while VCD is selective for small preantral follicles), triptolide has also been shown to affect primordial follicles in mice (Zeng *et al*., 2016), cause menstrual cycle disruptions in women, affect oestrous cycles in female rats, cause infertility in male rats (Witmer

et al., 2017), and induce mitochondrial damage in mouse Sertoli cells (Cheng *et al*., 2018). Therefore, possible interference with the VCD reproductive toxic effects cannot be excluded.

The study of Abedara *et al*. (2017) on male fertility in rats, showed marked decreased in the absolute testes and epididymis weight, as well as reduced sperm numbers and degeneration of the seminiferous tubules. Nevertheless, more than 20 % decrease in body weight gain (compared to controls) was observed already at the lowest VCD dose.

Dermal Studies

13-week dermal NTP studies in rats and mice

Male and female F344/N rats and male and female B6C3F1 mice, 10 animals per group and gender, were administered VCD in acetone by dermal application to the clipped dorsal interscapular region, 5 days per week for 13 weeks. Rats were dosed at 0, 3.75, 7.5, 15, 30, or 60 mg/animal/d, and mice at 0, 0.625, 1.25, 2.5, 5, or 10 mg/animal/d. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. Histopathologic examinations were performed on all controls, animals in the 60 mg/rat/d and 10 mg/mouse/d groups, all mice that died before the end of the studies, and on ovaries and uterus of the 2.5 and 5 mg/mouse/d groups.

In rats, although systemic toxicity was observed at the highest dose (60 mg/rat/d), adverse effects in reproductive organs were not reported in any exposed group of either gender.

In female mice, ovarian atrophy (decreased number of follicles) was observed in 4/10 females dosed at 5 mg/mouse, and 10/10 females dosed at 10 mg/mouse. The incidence in control group is not available, but in the 13-week oral NTP study in mice, ovarian and uterine atrophy was not found, and in the chronic dermal NTP study in mice, ovarian atrophy was found in 1/10 females at 15-month evaluation, and in 3/50 females during the remaining period of 2-year study (see table in *Supplemental information - In depth analyses by RAC*). Uterine atrophy was seen in 2/10 females dosed at 10 mg/mouse. These changes were present in the absence of marked systemic and dermal toxicity, namely, there were no compound-related deaths, the final mean body weights of exposed and control mice were comparable, and there were no necrotic skin changes. There was a dose-dependent increase in relative (organ to body) weights of liver and kidney in both genders. In female mice, relative liver and kidney weights were 16 % and 18 % higher at 10 mg/mouse dose level, and 11 % and 16 % higher at 5 mg/ mouse dose level, compared to controls. However, pathological changes in liver and kidney were not reported.

2-year dermal NTP studies in rats and mice

Male and female F344/N rats and male and female B6C3F1 mice, 60 animals per group and gender, were administered VCD in acetone by dermal application to the clipped dorsal interscapular region, 5 days per week. Rats were dosed at 0, 15, or 30 mg/animal/d for 15 months or 105 weeks, and mice at 0, 2.5, 5, or 10 mg/animal/d for 15 months or 103 weeks. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. Ten animals from each group were killed and examined during month 15 for toxicological evaluation. All male mice receiving 10 mg/animal/d died by week 83, while the surviving female mice receiving 10 mg/animal/d were killed during week 85. Tissues were examined histologically for controls and high dose groups at the 15-month kill, and for all animals after 2 years of exposure. Histological examination included reproductive organs in both genders.

In rats, general and dermal toxicity was not marked, as described in the section on carcinogenicity. The only adverse effect in reproductive organs, potentially related to VCD

exposure, was an increased incidence of ovarian cyst (8 % in controls, 10 % at 15 mg/rat/d, and 18 % at 30 mg/rat/d).

In *female mice*, as already described in the carcinogenicity section, increased incidence of ovarian atrophy (characterised by a complete absence of follicles and corpora lutea) was observed both at the 15-month and 2-year evaluation, already at 2.5 mg/mouse (see table in *Supplemental information - In depth analyses by RAC*). At the 15-month evaluation, relative uterine weights were significantly lower compared to controls at 5 mg/mouse/d (31 % of control values) and 10 mg/mouse/d dose levels (42 % of control values).

In *male mice*, increased incidence of epididymis subacute inflammation was observed at 5 and 10 mg/mouse/d dose levels at 2-year evaluation.

During the first, 15-month, study period, marked systemic toxicity or severe skin changes (such as necrotising inflammation) were not described in mice. At the highest dose (10 mg/mouse/d), body weight in males was 13 % lower compared to controls. During the second study period (after 15 months), marked general toxicity was observed in both genders at 5 and 10 mg/mouse/d dose levels, primarily reflected in significantly lower survival rates, as well as pronounced skin changes, such as necrotising inflammation. However, at the lower dose level, 2.5 mg/mouse/d, at which ovarian toxicity was already observed, survival and body weight did not differ from controls.

In conclusion, repeat dose oral and repeat dose and chronic dermal NTP studies showed VCD-related toxic effects on both male and female reproductive organs in mice and rats (ovary, uterus and testis), already at dose levels without or with only limited general toxicity. Mice appeared to be more sensitive to the toxic effects of VCD. As discussed by the DS and in the NTP report (1989), the observed difference in sensitivity between species could be due to more efficient metabolism of epoxides in rats compared to mice.

Studies with intraperitoneal, intramuscular or adjacent-to-ovary exposure

These studies, published in peer-reviewed journals, are summarised in Table 18 of the CLH report. The majority of these studies were performed using intraperitoneal exposure or even adjacent-to-ovary exposure (VCD incorporated into biodegradable fibre, applied next to monkey's ovary), which, as pointed out by the DS, allows direct contact of VCD with the ovaries, and are not representative for real-life exposure and regulatory assessment purposes.

Nevertheless, these and some other studies reviewed by Kappeler and Hoyer (2012), showed that in the absence of obvious systemic toxicity (e.g. Ito *et al*., 2009; Appt *et al*., 2006), VCD caused selective destruction of ovarian preantral follicles both in rodents and non-human primates; primordial and primary follicles in rats and mice, and primordial, intermediate, primary and secondary follicles in Cynomolgus macaques monkeys.

The mechanism seems to be a direct interaction of VCD with ovaries (*i.e.* not via hypothalamic-pituitary signalling) and includes an acceleration of the natural, apoptotic process of atresia (Kappeler and Hoyer, 2012), as well as oxidative damage (Abolaji *et al*., 2016). It seems that VCD directly interacts with the oocyte KIT receptor, impairing oocyte viability. Namely, the KIT receptor plays an important role in follicular survival during folliculogenesis, acting as an anti-apoptotic factor in oocytes of primordial follicles (Kappeler and Hoyer, 2012). As explained by the DS in the CLH report, the KIT signalling pathway seems to be relevant for human oocyte maturation as well. These findings are supported by the VCD ovotoxicity observed in non-human primates. Loss of the primordial follicle pool is of concern because it impairs fertility and could lead to early menopause.

Adverse effects on development of the offspring

There are no available guideline studies in which developmental toxicity was investigated, and non-guideline studies from open literature in which developmental parameters were studied, used intraperitoneal exposure route (Kodama *et al*., 2009; Haas *et al*., 2007). Their study protocols markedly deviated from those recommended by OECD TGs; e.g., only female animals were exposed, from two weeks prior to mating until gestation day 7, or only before mating.

Nevertheless, these studies showed that VCD increased pre-implantation loss in rats (Kodama *et al*., 2009) and resorptions in mice (Haas *et al*., 2007) at dose level at which ovarian toxicity was present, and in the absence of significant maternal toxicity.

Conclusions

Adverse effects on sexual function and fertility

RAC considers that the oral and dermal NTP studies represent the most reliable source of information on adverse effects of VCD on sexual function and fertility, since they used relevant routes of exposure and are well conducted and reported. Other studies published in peer-reviewed journals, in which oral or parenteral exposure routes were applied, are considered as supportive only, primarily providing information on mechanisms of VCD-induced ovarian and uterine toxicity.

Adverse effects on reproductive organs observed at higher VCD doses in the NTP studies are a continuation of the reproductive toxic effects observed at VCD doses that did not induce marked general toxicity. Also, it is questionable whether systemic toxicity could be related to the described effects on reproductive organs, especially ovaries, taking into account the proposed specific mechanisms of VCD ovotoxicity (direct toxic effects on oocytes, e.g. via interference with KIT signalling pathway). Therefore, RAC proposes classification for adverse effects on sexual function and fertility based on reproductive toxic effects observed at VCD dose levels without, or with only limited, general toxicity.

Adverse effects on fertility parameters *in females* at dose levels without or with only limited general toxicity were observed in the following NTP studies:

- 13-week oral NTP study in rats: smaller uterine horns (at 500 mg/kg bw/d);
- 13-week oral NTP study in mice: dose-dependent increase in the incidence of ovarian atrophy (at 250, 500 and 1 000 mg/kg bw/d), increased incidence of uterine atrophy (at 1 000 mg/kg bw/d);
- 13-week dermal NTP studies in mice: high incidence of ovarian atrophy (at 5 and 10 mg/mouse/d);
- 2-year dermal NTP studies in mice: increased incidence of ovarian atrophy (at 2.5, 5 and 10 mg/mouse/d at 15-month evaluation; at 2.5 mg/mouse/d at 2-year evaluation) and lower uterine weight (at 5 and 10 mg/mouse/d at 15-month evaluation).

Adverse effects on fertility parameters *in males* at dose levels without or with only limited general toxicity were observed in following NTP studies:

- 13-week oral NTP study in rats: smaller than normal testes (at 500 mg/kg bw/d);
- 13-week oral NTP study in mice: markedly increased incidence of testicular degeneration (at 250 mg/kg bw/d).

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 the CLP Regulation[1] and the CLP Guidance[2], justify classification for reproductive fertility. As supporting evidence, intraperitoneal study in mice showed decreased fertility index at dose level without marked general toxicity (Haas *et al*., 2007).

The findings described in NTP studies are supported by non-guideline studies from open literature, which provide evidence of ovarian toxicity of VCD in mice, rats, hamsters and non-human primates. There is no indication that mechanisms of VCD-related ovarian and uterine toxicity suggested by these studies (Kappeler and Hoyer, 2012; Abolaji *et al*., 2016) are not relevant for humans as well.

Since:

- there are no human data on the effects on sexual function and fertility by VCD, which would warrant classification in Category 1A,
- adverse effects on reproductive organs (ovaries, uteri and/or testes) were observed in two rodent species (mice and rats) after both oral and dermal exposure,
- these effects were observed at VCD dose levels without or with only limited general toxicity,
- even when these effects were observed at VCD doses with more pronounced general toxicity, they were not considered to be a secondary consequence of systemic toxicity, taking into account that they were continuation of reproductive toxic effects observed at lower VCD doses, and considering mechanism of VCD ovarian toxicity (direct toxic effects on oocytes), and
- there is no indication that mechanisms of VCD-related ovarian and uterine toxicity are not relevant for humans as well,

RAC agrees with the DS's proposal to classify VCD **Repr. 1B; H360F (May damage fertility).**

The exposure route is not proposed to be specified (effects on reproductive organs were observed in oral, dermal, intraperitoneal and intramuscular studies).

In addition, no SCL is proposed since oral NOAEL values in dermal studies were 125 mg/kg bw/d or higher (well above the limit value of 4 mg/kg bw/d for Group 1 – high potency), and extrapolation from dermal to oral exposure route could not be performed due to inadequate data on VCD kinetics, especially after oral exposure (CLP guidance 2017; ECHA Guidance on information requirements and chemical safety assessment. Chapter R.8: Characterisation of dose [concentration]-response for human health, Version: 2.1, 2012).

Adverse effects on development of the offspring

Since there are no available guideline studies in which developmental toxicity was assessed, and the available non-guideline studies from open literature used non-standard exposure route and study protocols which greatly differed from those recommended by OECD TGs, **RAC agrees with**

[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".

the DS that no classification for developmental toxicity of VCD is warranted due to lack of data.

Effects on or via lactation

Since there are no studies in which animals were exposed to VCD during postnatal development via the mother, **RAC agrees with the DS that no classification for adverse effects on or via lactation is warranted due to lack of data.**

Additional references

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ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).