Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

4 vinylcyclohexene (VCH)

EC Number: 202-848-9

CAS Number: 100-40-3

ECHA/RAC/CLH-O-0000002966-62-01/F

Adopted

14 September 2012

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14 September 2012
CLH-O-0000002966-62-01/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 (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:

Substance Name: **4-vinylcyclohexene (VCH)**

EC Number: **202-848-9**

CAS Number: **100-40-3**

The proposal was submitted by **France** and received by RAC on **30 May 2011.**

In this opinion, all classifications are given firstly in the form of CLP hazard classes and/or categories, the majority of which are consistent with the Globally Harmonised System (GHS) and secondly, according to the notation of 67/548/EEC, the Dangerous Substances Directive (DSD).

The proposed harmonised classification

	CLP	**DSD**
Current entry in Annex VI of CLP Regulation (EC) No 1272/2008	-	-
Proposal by dossier submitter for consideration by RAC	Carc. 1B – H350	Carc. Cat. 2; R45
Resulting harmonised classification (future entry in Annex VI of CLP Regulation) as proposed by dossier submitter	Carc. 1B – H350	Carc. Cat. 2; R45

PROCESS FOR ADOPTION OF THE OPINION

France 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 **30 May 2011**. Parties concerned and Member-State Competent Authorities (MS-CA) were invited to submit comments and contributions by **14 July 2011**.

ADOPTION

Rapporteur, appointed by RAC: **Marianne van der Hagen**
Co-rapporteur, appointed by RAC: **Karen van Malderen (till 30 June 2012)**

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

The RAC opinion of the RAC on the proposed harmonised classification and labelling has been reached on **14 September 2012***,* in accordance with Article 37 (4) of the CLP Regulation, giving parties concerned the opportunity to comment. Comments received are compiled in Annex 2.

The opinion of the RAC was adopted by **consensus**.

OPINION OF RAC

The RAC adopted the opinion that **4-vinylcyclohexene (VCH)** should be classified and labelled as follows[1]:

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

Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
				Hazard Class and Category Code(s)	Hazard state-ment Code(s)	Pictogram, Signal Word Code(s)	Hazard state ment Code(s)	Suppl. Hazard statement Code(s)		
601-088-00-9	**4-vinylcyclohexene (VCH)**	**202-848-9**	**100-40-3**	**Carc. 2**	**H351**	**Wng**	**H351**			

Classification and labelling in accordance with the criteria of the DSD, 67/548/EEC

Index No	International Chemical Identification	EC No	CAS No	Classification	Labelling	Concentration Limits	Notes
601-088-00-9	**4-vinylcyclohexene (VCH)**	**202-848-9**	**100-40-3**	**Carc. Cat. 3; R40**	**Xn** **R40** **S36/37**		

[1] Note that not all hazard classes have been evaluated

SCIENTIFIC GROUNDS FOR THE OPINION

The opinion relates only to those hazard classes that have been reviewed on the basis of the available scientific data as contained in the proposal for harmonised classification and labelling submitted by France.

HUMAN HEALTH HAZARD ASSESSMENT

Carcinogenicity

Toxicokinetics (absorption, metabolism, distribution and elimination)

Dossier submitter

Data from mice and rats demonstrate rapid uptake after oral dosing and elimination mainly via the urine but also via expired air. There is abundant data on the metabolism and the interspecies differences in the metabolism of 4-vinylcyclohexene (VCH).

In hepatic microsomes, VCH is first metabolised to VCH-epoxides (see figure 1). These and/or their reaction products are considered to contribute to the carcinogenicity of VCH. This bioactivation is also seen in other organs in vitro. Although the cytochromes (CYPs) known to participate in VCH bioactivation are present in different cell types of the ovaries, most studies show that liver is the main organ for metabolism and bioactivation of VCH.

CYP 2A and CYP 2B are the main cytochromes involved in VCH metabolism in rodents. VCH promotes its own metabolism by increasing expression of these cytochromes. The differences in VCH metabolism in rats and mice correlate with the expression of these two cytochromes in these species: VCH is poorly metabolised in rats due to the fact that CYP2A is not present and CYP2B is poorly expressed. Consequently, after dosing, rats accumulate more VCH (or equivalent) than mice, whereas mice metabolise VCH to VCH-1,2-epoxide or VCH-7,8-epoxide more rapidly and more efficiently than rats. Available rates for this metabolism step are given in Table 1. In a second step, the epoxides can be catalyzed into the carcinogenic metabolite vinylcyclohexene diepoxide (VCD) and the corresponding diols (see figure 1). VCD is presumed to be the carcinogen producing ovotoxicity and ovary tumours in mice. The available data indicate qualitatively no differences in this step in any of the species investigated (see Table 1). In the final step of the metabolic pathway, VCD is hydrolyzed.

The study of Keller et al. (1997) indicates that the mouse has significant greater capacity to metabolize VCH to reactive species than does the rat, and that the mouse hydrolyzes epoxides less efficiently than the rat. In conclusion, the balance of activation versus detoxification reactions in rats and mice suggests that the mouse may be more susceptible to 4-VCH toxicity because of the generation of higher levels of epoxide metabolites.

In humans, as in mice and rats, VCH-1,2-epoxide is the major monoepoxide of VCH formed by hepatic microsomes and human CYP "supersomes". The rate of VCH epoxidation in human hepatic microsomes *in vitro* was lower than in hepatic microsomes from female mice and was comparable to the VCH epoxidation rates with hepatic microsomes obtained from female rats (Smith and Sipes, 1991). Out of eight human hepatic CYP isoforms tested, CYP2E1 and CYP2B6 were the only isoforms significantly catalyzing VCH epoxidation (Fontaine et al., 2001a). The Dossier Submitter underlines that the species difference in terms of metabolism is not obvious, as the metabolism in

humans might have a high variability, e.g. from CYP induction in humans due to ethanol consumption given the role of CYP 2E1 in human VCH epoxidation. Moreover, the published rates of formation of VCH monoepoxides in rat liver microsomes compared to mouse liver microsomes vary considerably from study to study (see Table 1). In the view of the Dossier Submitter, no firm conclusion on the rate of formation of VCH monoepoxides in humans relative to rodents can be drawn.

Regarding the second step of the metabolic pathway, Fontaine et al. (2001a) showed that isolated human CYPs were capable of significantly converting monoepoxides into VCD. Information regarding the rate of epoxidation of VCH monoepoxides into VCD in human hepatic microsomes is lacking and no human data are available on the final hydrolysis step of VCD.

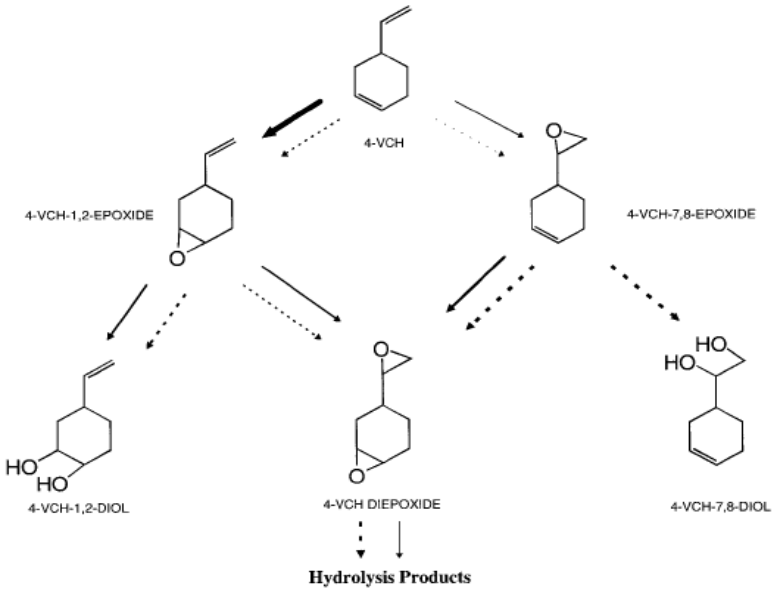

Figure 1 . Metabolic pathway for 4-vinylcyclohexene. All of the reactions shown were studied as was the hydrolysis of 4-vinylcyclohexene diepoxide. The thickness of the lines indicates the relative velocity of the reactions in liver, compared to other reactions in liver. Solid lines indicates the reaction rate for mouse liver, dashed line indicates the reaction rate for rat liver (Keller *et al*., 1997)

Table 1. Summary of the rate of formation of VCH mono-and diepoxides in liver microsomes from rats, mice and human liver microsomes (in nmol/mg protein/min)

Metabolism STEP		**Mouse**	**Rat**	**Human**
1	**4-VCH to 4 VCH-1,2 epoxide**	9.1 (B6C3F1, F) (Smith et al, 1990a) 11.1 (B6C3F1, F) (Keller et al, 1997)	1.4 (F344, F) (Smith et al, 1990a) 0.20 (Crl:CD BR, F) (Keller et	0.67 (Smith and Sipes, 1991) (n=12) Range: M: 0.23-0.85 (n=6) **F: 0.3-1.25** (n=5) one value with gender unkown:

		0.9 (B6C3F1, F) (Fontaine et al, 2001a)*	al,1997) 0.47 (F344, F) (Fontaine et al, 2001a) 0.49 (Wistar, M) (Watabe et al, 1981)**	1.14
1	**4-VCH to 4 VCH-7,8 epoxide**	0.91 (B6C3F1, F) (Keller et al, 1997) 0.61 (B6C3F1, F) (Fontaine et al, 2001a)	0.007 (Crl:CD BR, F) (Keller et al,1997) 0.37 (F344, F) (Fontaine et al, 2001a)	0.08 (Smith and Sipes, 1991) (n=12) Range: M: <0.01-0.11 (n=6) **F: 0.06-0.21** (n=5) one value with gender unkown: 0.20
2	**4 VCH-1,2 epoxide to VCD**	5.35 (B6C3F1, F) (Keller et al, 1997)	3.69 (Crl:CD BR, F) (Keller et al,1997)	No data
2	**4 VCH-7,8 epoxide to VCD**	8.83 (B6C3F1, F) (Keller et al, 1997)	9.45 (Crl:CD BR, F) (Keller et al,1997)	No data

F: female; M: male

*Please note that Fontaine et al (2001a) reported rates per 60 minutes, but for comparison the values in Table 1 have been converted to rates per minute.

**In the Watabe paper the corresponding rate for formation of 4 VCH-7,8 epoxide is 0.12 (reported as 4VCH-1´,2´-epoxide), (this data was not included in the DS CLH report)

Comments received in the public consultation

Comments were received from three industry representatives, all underlining the species differences in the rates of formation or activation of the epoxide metabolites of VCH, as well as in the rate of detoxification; they state that the mouse may be more susceptible than the rat, and that the rat would be the more appropriate animal model for extrapolation to humans. A comment from a fourth industry representative stated that species and tissue differences in activation and detoxication, as well as differences in tissue affinity and distribution, appear relevant to differences in susceptibility of rats and mice to 4-VCH-induced ovarian toxicity and neoplasia. Comments from a fifth insutry representative also supported the view that "humans seem to have a rat-like toxicokinetic behaviour". They state that the toxicokinetic species differences are

attributed to the creation of the corresponding monoepoxides, and that these rates are comparable in rats and humans.

RAC assessment

The toxicokinetics of VCH in rodents is well described in the available literature. There is a species difference in the liver metabolism of VCH, which appears to be reflected in species differences in susceptibility to VCH-induced ovarian tumours. The balance of activation versus detoxification reactions in rats and mice suggests that the mouse may be more susceptible to 4-VCH toxicity because of the generation of higher levels of epoxide metabolites in the liver and the reduced capacity to detoxify them, resulting in a higher level of the active metabolite VCD reaching the target organ - the ovaries - in mice than in rats. Human CYP "Supersomes" have been shown to be able to metabolise VCH into VCD (Fontaine et al., 2001a). Smith and Sipes (1991) indicate significant epoxide hydrolase activity in human microsomes *in vitro,* to explain that an epoxide hydrolase inhibitor was required to detect the appearance of VCH epoxides. No firm conclusion on the rate of formation of epoxide metabolites in humans relative to rodents can be drawn. However in a comparison by the above authors of the rate of formation of VCH-1,2-epoxide from VCH by hepatic microsomes of female mouse, rat and human samples, the *in vitro* rates for human are the lowest as shown in Figure 2 below

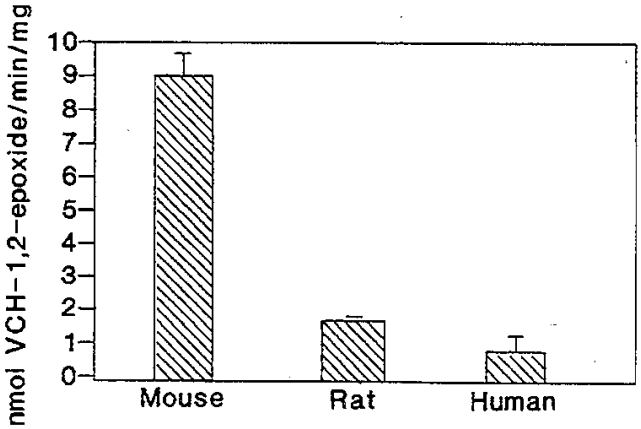

Figure 2. Comparison of the rate of formation of VCH-1,2-epoxide from VCH by hepatic microsomes (Fig. 3 in Smith and Sipes, 1991)

RAC noted that the data from mouse and rat were carried out in a previous study and were replotted from Smith et al., 1990a. The rate of *in vitro* hydrolysis of VCD in rat and mouse liver was 5.51 and 0.63 nmol/min/mg protein, respectively (Keller et al., 1997). The rates for the major pathway of hydrolysis of the monoepoxides were similar in rats and mice liver (hydrolysis of 4-VCH-1,2epoxide), with rates of 6.53 and 5.76, but hydrolysis of 4-VCH-7,8-epoxide via the minor pathway was only detected in rats. Corresponding rates of hydrolysis of 4-VCH-mono- and diepoxides and formation of diols in human cells are not available.

Mode of action (MoA) for ovarian carcinogenicity

Dossier submitter

Following public consultation, the dossier submitter further elaborated this issue. After epoxidation of VCH into mono-epoxides and diepoxide (VCD), mainly in the liver, VCD enters the systemic circulation and is distributed throughout the body. Upon reaching the ovary, VCD selectively destroys the primordial and primary follicles. VCD is assumed to produce ovarian atresia in follicles through a mechanism involving programmed cell

death, apoptosis. Since 17β-estradiol and inhibin are no longer produced from the primordial and primary follicles in the ovary, loss of the negative feedback inhibition of FSH release from the hypothalamus and pituitary occurs, leading to high plasma levels of FSH. Increased plasma levels of FSH results in turn in the initiation and/or promotion of ovarian tumors. The mode of action described for ovarian tumors is plausible in humans.

Information received in the public consultation

Comments were received from industry representatives stating that the conclusion from the MoA assessment is that VCH acts via a non-genotoxic, threshold mechanism. They also submitted the mode of action (MoA) proposed by the Sapphire group as it was presented in a draft document from the Texas Commission on Environmental Quality from 2011. This is identical to the MoA as presented in the next paragraphparagraph. According to this MoA VCD, the metabolite of VCH, is selectively cytotoxic to oocytes in the ovary resulting in premature menopause, and as a consequence results in increased plasma levels of FSH which acts as a tumour promoter in ovaries. IND points out that a significant amount of research on the metabolism, ovotoxicity and species differences in toxicity of VCH has been carried out at the University of Arizona, and that this group in a recent comprehensive review of the toxicity of VCH (Hoyer, 2007) described the mouse as being uniquely susceptible to VCH and concluded that the rat was as a better model than the mouse to predict VCH-induced ovarian toxicity in humans.

Comments were received from an interested expert in this field, referring to multiple publications including his own abstract (Bevan et al., 2009) which now is a manuscript in preparation on the mode of action for VCH mouse ovarian tumours. This expert stated that following exposure and uptake, VCH is metabolized, primarily in the liver, to VCH-1,2-epoxide or VCH-7,8-epoxide, which are further metabolized to VCH-diepoxide[1]. VCH-diepoxide enters the blood and circulates through the body. Upon reaching the ovary, VCH-diepoxide selectively destroys the primordial and primary follicles through a mechanism involving programmed cell death or apoptosis. Repeated exposures to VCH ultimately result in premature ovarian failure, due to complete follicular loss. Since 17β-estradiol and inhibin are no longer produced from the primordial and primary follicles in the ovary, loss of the negative feedback inhibition of follicle-stimulating hormone (FSH) release from the hypothalamus and pituitary occurs, leading to high plasma levels of FSH. Increased plasma levels of FSH result in the initiation and/or promotion of ovarian tumors.

Industry representatives and the aforementioned expert (identical assessments) presented an assessment of the weight of evidence for the proposed mode of action using the modified Bradford Hill criteria for causality in the IPCS Human Relevance Framework. These criteria were originally developed for use in epidemiology (Bradford Hill, 1965). They conclude that in summary there is strong evidence for the proposed MoA for mouse ovarian tumours by a non-genotoxic, threshold mechanism. They also conclude that VCH may be metabolized to VCH diepoxide in humans but at low levels when compared to mice, so that no ovotoxicity or ovarian tumours would be expected. This view was also expressed by an undisclosed commenting party from Germany (anonymous).

RAC opinion

The RAC agrees that the proposed mode of action for ovarian carcinogenicity presented by the dossier submitter in the revised CLH report/BD is plausible but doubts that elevated FSH levels contribute to the initiation of ovary tumours. However RAC is of the view that VCH/VCD contribute to tumour promotion in mouse ovaries via the hypothalamic-pituitary-gonad axis. On the other hand it cannot be excluded that a

[1] synonym VCD

genotoxic, non-threshold mechanism could be responsible for the observed tumours, or that both mechanisms act at the same time.

Repeated Dose Toxicity studies

Dossier submitter
The dossier submitter has summarized data from repeated dose toxicity studies of VCH and VCD for information. Ovaries were target organs in both rodent species in the 13-week inhalation study, but not in the 13-week oral toxicity study, where only mice were affected.

Two 13 weeks studies of subchronic toxicity in rats and mice are available (by gavage in NTP, 1986; by inhalation in Bevan et al., 1996). In these studies precursors to the neoplastic lesions seen in the cancer study were observed in mice as ovarian atrophy and ovary follicle effects. Ovarian atrophy was also observed in two female rats after inhalation of VCH, but the atrophy in these cases was morphologically distinct from that seen in mice. In a 30 days intra-peritoneal (i.p.) administration study in mice the oocyte count was reduced. The dose which reduced the small oocyte count to 50% of the control (=ED50) was 2.7 mmol VCH/kg bw (Smith et al., 1990b). VCD, the ultimate metabolite of VCH, is a very potent ovotoxicant both in rats and mice after i.p. administration. Moreover, based on the dose which reduced the small oocyte count to 50% that of control (=ED50), the potency of VCD to destroy small oocytes is similar in rats and mice through i.p. administration It is assumed that the ovotoxicity of VCH is attributed to that of VCD and that the epoxidation of VCH into VCD is required to affect the ovary.

Carcinogenicity studies

Dossier submitter
VCH: An oral carcinogenicity study (by gavage in corn oil) was performed with rats and mice (NTP, 1986; Collins et al., 1987). Rats (Fischer 344) and mice (B6C3F1) received 0, 200 or 400 mg/kg bw/d for 103 weeks, 5 days per week. There were 50 animals/sex/group.

Mortality was high at the end of the study (103 weeks) and is given below:

- Mortality in rats:
    - males: control 17/50, low-dose 37/50*, high-dose 45/50*
    - females: control 10/50; low-dose 22/50; high-dose 36/50*

    - Survival of high dosed male/female rats was significantly lowered compared to controls after week 5/3.
    - Survival of low dosed male/female rats was significantly lowered compared to controls after week 88/102.

- Mortality in mice:
    - males: control 13/50; low-dose 11/50; high-dose 43/50*
    - females: control 10/50; low-dose 11/50; high-dose 33/50*

    - Survival of high dosed male/female mice was significantly lowered compared to controls after week 29/32.
    - Survival of low dosed male/female mice was not lowered compared to controls.

Neither gross observations nor histopathological evaluations revealed a specific cause of death in any of the dosed animal groups. Survival was poor in rats and male mice, but

the results observed in female mice were considered especially valid. The most pronounced effect is the significant treatment-related increased incidence of granulosa-cell tumours or carcinoma of the ovary (overall rates: control, 2.0%; low-dose, 21%*; high-dose 28%*, (NTP historical incidence: 0.2%)) and of mixed tumours composed of epithelial and granulosa cells of the ovary (0%, 52%*, 23%*, respectively). The incidence of uncommon ovarian neoplasms in NTP historical corn oil control female B6C3F1 mice is: 1.2% = 12/1028 animals. Despite significant mortality in high-dose female mice, ovary tumors could not have been confounded by the excessive toxicity since the incidence is statistically significant even at the low dose at which mortality was similar to the control. In female mice, there was a slight increase of adrenal gland adenoma in the high-dose group (overall rates: control, 0; low-dose, 6%; high-dose; 8%* (NTP historical incidence: 0.7%).

In high dose male mice, there were increased incidences of malignant lymphomas (terminal rate 8%, 13%, 57%; overall rate 8%, 14%, 10% in controls, low and high dose groups). In male mice, there was an increased incidence of alveolar/bronchiolar adenomas or carcinomas (combined) of the lung seen in the males surviving to the end of the study (terminal rates: 8%, 23%*, 43%*; overall rates: 8%, 22%, 8% in controls, low-dose, and high-dose group (NTP historical incidence: 14.3%). The extensive mortality seen in the high-dose male mice confounded the interpretation of these incidences.

In addition, the incidence of adenomas or squamous-cell carcinomas (combined) of the clitoral gland was slightly increased in low-dose female rats (5 cases) for which the survival was significantly different from that of control only after week 102 (overall rates: control, 2%; low-dose, 10%; high-dose; (0%*)). This effect was not found in the high dose group, probably due to the high mortality in that group (NTP historical incidence: 2.1% (range: 0-8%)). One case of clitoral gland tumour was observed in the control group.

There was a slightly increased incidence of squamous-cell papillomas or carcinomas (combined) of the skin in high-dose male rats (terminal rates: control, 0/33; low-dose, 0/13; high-dose 1/5 (20%); and overall rates: control, 0/50; low-dose, 1/50 (2%); high-dose, 4/50* (8%)) (NTP historical incidence: 1.9% (range: 0-10%)). Due to the high incidence of mortality in low- and high-dose male rats, it is not possible to relate the increased incidence of squamous-cell papillomas or carcinomas (combined) of the skin to exposure to VCH.

The classification of VCH proposed by the dossier submitter is as Carc. 1B; H350 (DSD: Carc. cat. 2; R45)

Information received in the public consultation

The proposed classification as Carc. 1B (CLP) was supported by four member States. One member State considered that Carc. 2 could be more appropriate, due to apparent lack of genotoxicity and uncertainties surrounding the proposed mode of action. One Member State questioned the dosing causing premature mortality in the carcinogenicity studies and asked for more details in the chapter on comparison with criteria, which were provided later on by the dossier submitter in the revised report. One member State proposed to include other cancer findings as well, which was taken care of by the dossier submitter in the revision of the report for types of tumours not masked by excessive mortality. Comments were received from industry representatives stating that there is only one cancer study that is reliable and that is the one carried out with female mice. This study showed a clear, statistically and biologically significant increase in tumours of the ovaries only. They also comment that subchronic studies with female mice have demonstrated lesions that can be considered precursors to the neoplastic lesions seen in

the cancer study. Similar studies in female rats have not demonstrated such precursor treatment-related changes.

In comments from an industry representative a dermal study in mice was mentioned, demonstrating an increased number of benign squamous cell papillomas in male Swiss mice after exposure to VCH (Van Duuren et al., 1963). Another industry representative commented that VCH was carcinogenic in female mice due to the toxicokinetics of VCH in mice, but since humans were more comparable to rats regarding the toxicokinetics, VCH should be placed in carcinogen category 2. The conclusion in the comments from a group of three industry representatives is that according to their comparison of the findings against the CLP criteria, there is only limited evidence of a carcinogenic effect and therefore a classification as Carc. 2 rather than Carc. 1B would be most appropriate. This view was also expressed by an undisclosed commenting party from Germany (anonymous).

RAC opinion on the carcinogenicity of VCH

Tumour type and background incidence
As pointed out by the dossier submitter in the RCOM, the critical point of the classification of VCH is the relevance of the VCH-induced ovary tumours seen in mice to human.

A clear treatment-related increased incidence of granulosa-cell tumours of the ovary and of mixed tumours composed of epithelial and granulosa cells of the ovary was observed in the NTP study in female mice. These tumour types are traditionally not among the ones classified as irrelevant to humans, and are therefore considered relevant (see also Mode of action).

The incidence of uncommon ovarian neoplasms[2] in NTP historical corn oil control female B6C3F1 mice is: 1.2% = 12/1028 animals (data as of 16 March 1983). Mice were exposed up to 29 October 1982 in the 2 year NTP study. The historical control for granulosa cell carcinoma[3] showed 0.1% (=1/1028x100) and for granulosa cell tumors, 0.2% (=2/1028x100)). Therefore, RAC considers the increase in ovary tumours in mice as an unusually high incidence of a rare tumour type. Also the ovotoxicity from VCH observed in a subchronic study in a species other than mice (rats, 13 weeks) adds somewhat to the weight of evidence regarding ovaries as target organs (Bevan, 1996). However, the ovotoxicity in rats was morphologically different from findings in ovaries of mice, and considered by the author to be spurious and not compound related.

Progression of lesions to malignancy
Increased incidence of granulosa-cell hyperplasia and tubular-cell hyperplasia of the ovary, mixed benign tumours, granulosa cell tumours, and benign or malignant granulosa cell tumours (combined) occurred in female mice. The malignant neoplasms designated granulosa cell carcinoma by NTP (1 in low-dose, 2 in high-dose) had replaced the entire ovary and were described grossly as cystic and hemorrhagic; all had metastasized to the lungs. The granulosa cell lesions were a continuum of hyperplastic to benign and malignant neoplastic proliferations (NTP, 1986). Benign tumours that are considered to have the potential to progress to malignant tumours are generally considered along with malignant tumours (CLP Guidance).

[2] Including mixed benign tumours, granulosa cell tumours, and granulosa-cell tumours or carcinomas (combined).
[3] Please note that a carcinoma is a malignant tumor of epithelial origin. RAC notes that granulosa cells are stromal cells, and thus that malignant granulosa cell tumours do not arise from epithelial cells. Hence these tumours should not be termed carcinomas. RAC prefers to use the term malignant granulosa cell tumours instead.

Comparison of absorption, distribution, metabolism and excretion between test animals and humans
VCH is bioactivated to a number of mutagenic metabolites and the diepoxide VCD is especially of concern with regard to carcinogenicity. Bioactivation to VCD has been observed in *in vitro* and *in vivo* studies with test animals, showing variability in the rates of formation and detoxification of VCH epoxides in rats and mice. The rates of these metabolisation steps in humans are largely unknown but there are no indications that the observations in rodents, and the proposed carcinogenic mechanism would not occur in humans. On the contrary, *in vitro* studies using human microsomes or supersomes demonstrated monoepoxide formation from VCH and further bioactivation of the monoepoxides into VCD. Moreover, VCD has been shown to selectively deplete primordial and primary follicles in the ovaries of nonhuman primates (*Macaca fascicularis*; Appt et al., 2006). Therefore RAC is of the opinion that the mode of action for ovarian carcinogenicity should be considered as relevant to humans (cf. section on Mode of action above). RAC notes however that information on the potential for VCD detoxification via hydrolysis in humans is lacking.

Multi-site response
Administration of VCH predominantly induced tumours in the ovaries of female mice but other benign and malignant tumour types and preneoplastic lesions in both sexes of rats and mice were also found. The interpretation of tumours in organs other than the ovaries and of pre-neoplastic findings were complicated and possibly underestimated by the high mortality in the 2 year study. The findings suggested a carcinogenic potential of VCH in such organs, e.g. in skin and in the clitoral gland of rats, and in the adrenal gland, hematopoietic system and lung of mice. See the Appendix to the Background Document with analyses of primary tumours in rats and mice, which is an excerpt of Appendix E of NTP (1986).

RAC notes that the authors of the NTP 2 year study regarded the results in male and female rats and male mice to be inadequate for determining the presence or absence of a carcinogenic response (NTP, 1986; Collins, 1987). Furthermore, these authors concluded that the increased incidence of adrenal-gland adenomas in high-dose female mice may have been compound-related. RAC notes that there is no equivalent gland in humans to the clitoral gland in rodents (Treuting and Dintzis, 2012). In RACs view the tumour pattern seen is not consistent between rats and mice, or (excluding gender specific findings), between male and female animals.

Confounding effects of excessive toxicity
High mortality occurred among male and female rats (low and high dose groups) and male and female mice (high dose group only) in the NTP study. However, there is no indication for a specific cause of death in any of the dose groups. RAC acknowledges that the high mortality confounded the interpretation of the results but is of the opinion that it cannot be excluded that the high mortality may have hidden the increase of some type of tumours. This is also indicated by the authors of the NTP study.

Evidence from the Van Duuren et al. (1963) study
The study from Van Duuren et al. (1963) which was cited in the comments/IUCLID-information from the 4-VCH Group had some shortcomings. VCH was dissolved in benzene, and the compound tested probably contained a minute amount of the hydroperoxide formed by auto-oxidation. This was later described by the author Van Duuren in a life-long dermal male mouse study from 1965 (page 282) where it is stated that no carcinogenic response is obtained when hydroperoxides and oxygen were excluded in the purification. As a result of this RAC considers that the positive results from the Van Duuren study from 1963 should not be included in the Weight of Evidence analysis.

Evidence from structurally-related compounds
A dermal carcinogenic study with the ultimate metabolite VCD was performed in mice and rats (NTP, 1989). VCD induced benign and malignant skin tumours at the site of application in both species. Furthermore, in female mice a significant increase in benign and malignant ovarian tumours was observed and an increased incidence of lung tumours may have been treatment-related. VCD is listed in CLP annex VI as a category 2 carcinogen. The documentation for this classification was not available for the Committee to examine. The RAC furthermore notes that another structurally related substance - 1,3-butadiene - also induces benign tumours and carcinomas of the ovary in mice, as well as tumours in other organs including lymphomas and lung adenomas and carcinomas (IARC, 2008). VCH can be viewed as a dimer of 1,3-butadiene and the latter is classified as Carc. 1A, Muta. 1B.

CLP criteria for carcinogenicity
According to the criteria, classification as a carcinogen is warranted for VCH based on the findings of treatment-related tumours in rodents administered VCH orally by gavage for 2 years. Classification as Carc. 1A is not warranted because there are no data on the carcinogenicity of VCH in humans.

Classification in category Carc. 1B based on animal studies would normally require sufficient evidence of carcinogenicity demonstrated in either a) two or more species, or b) two or more independent studies in one species, or increased incidence of tumours in both sexes of a single species. However, a single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regards to i.a. incidence, site, and type of tumour. The finding of uncommon ovarian neoplasms in mice is the starting point for the classification of VCH.

The RAC regards the design of the 1986 two year NTP carcinogenicity studies of VCH in mice and rats as well intended. Dose selection rationale was based on the results of the oral 13 weeks studies in both species, so the high mortality observed in the carcinogenicity studies was unexpected. Due to the high mortality among most dose groups in these studies, the results for tumours other than the ovary tumours in mice were difficult to interpret, making the classification of VCH a borderline case between Carc. 1B and Carc. 2.

The mortality in low dose female mice in the two year NTP study was no higher than in the control group, and showed a clear finding of ovarian cancer, an effect which was also observed in the high dose group.

The ovary tumours induced by VCH were mostly benign, and only a few malignant neoplasms were identified. These tumours may, however, represent one stage in the continuous process of tumour development from hyperplastic changes, via benign to malignant tumours. The mechanism for carcinogenicity is assumed to be mainly via formation of the active diepoxide metabolite VCD. The information on VCH regarding its genotoxicity is limited, with generally negative findings, and the mechanism for the ovary tumours is assumed to require a threshold level of VCD inducing apoptosis of ovarian primary/primordial follicles, successively resulting in ovarian follicle atresia and ovary tumours due to disruption of systemic hormonal feedback. Due to liver bioactivation of VCH to VCD possibly some liver tumours would be expected after exposure to VCH, but the number of liver tumours in exposed mice and rats (either sex) were not higher than those in the corresponding control group. Apart from the clear finding of ovary tumours in mice, there were some findings of other tumours in mice and rats. These findings suggested carcinogenic potential of VCH in e.g. skin and in the clitoral gland of rats, and in the adrenal gland, hematopoietic system and lungs of mice. However, because of the high mortality in these dose groups, the reliability of these studies was compromised, thereby not providing adequate evidence to draw conclusions on these findings. The five

cases of clitoral gland tumours in female low dosed rats were statistically significant relative to the controls (where one case was found), and survival in this group was only lowered significantly at the end of the study (week 102). No clitoral gland tumours were seen in the high dose female rats, but survival in this group was significantly lowered already from week 3. No clitoral gland tumours were seen in mice and no ovary tumours were seen in the two year rat NTP study.

Based on the strength of evidence observed in the causal relationship between oral exposure to VCH and the incidence of ovary tumours in mice and taking additional considerations in the weight-of-evidence analysis into account, the RAC considers that there is evidence demonstrating animal carcinogenicity of VCH. In the weight-of-evidence analysis, RAC regards the well-described MoA in animals as relevant for humans, and also gives weight to the additional considerations discussed above. Because the reliability of the rat studies were compromised, it was not possible to conclude with sufficient confidence that the findings of tumours in these studies resulted from exposure to VCH, and are relevant to humans. From available *in vitro* studies, RAC concluded that qualitatively similar metabolic pathways probably occur *in vivo* in mice, rats and humans for the metabolism of VCH to its carcinogenic diepoxide metabolite, VCD. However, there was also evidence indicating that there are quantitative differences among the species in the activity of these pathways, which could be interpreted as indicating a lower carcinogenic potential of VCH in humans than in mice. In particular, quantitative information on the VCD detoxification potential in humans via epoxide hydrolase is lacking. In conclusion, and based on a weight of evidence analysis, RAC disagrees with the dossier submitter´s proposal that VCH should be classified as Carc. 1B, and recommends that VCH is classified as Carc. 2 (suspected of causing cancer in humans).

It is important to point out however that there were also some valid arguments in favour of 1B as follows, i.e. that when taking the available rat and mouse studies as they are, including their limitations and recognizing that the high mortality may even have obscured a more outspoken increase in tumour incidences, increased tumour incidences in two sexes of two species were seen at multi-site, albeit not in a consistent pattern. The strength of evidence for a causal link with VCH exposure is the highest for the ovary tumours in mice, but for some of the other tumours the tumour rates, when adjusted for intercurrent mortality also show a dose response relationship. These comments are reflected here in full to illustrate the complexity of the decision on this particular classification.

In RACs view there is limited evidence of carcinogenic potential of VCH from findings other than ovary tumours in the NTP carcinogenicity studies, due to the high mortality that occurred without identified causes, which complicates the interpretation of these studies. In a weight of evidence analysis RAC considered the overall carcinogenicity findings in combination with the low concern for VCH related mutagenicity *in vivo*, as well as the species differences in metabolism all to weigh in favour of Carc. 2 under CLH.

Mutagenicity

Dossier submitter

The mutagenicity database for VCH is so limited that no firm conclusion can be drawn about the genotoxic potential of VCH. VCH was negative in the bacterial reverse mutation assay (Ames test) (NTP, 1986). In a poorly reported mouse lymphoma assay VCH induced mutagenicity in one out of three experiments with metabolic activation with S9, the results were equivocal in the other two experiments while no mutations were induced without S9 (NTP, undated). VCH did not induce micronuclei in rats or mice (Bevan et al., 2001). In the Bevan study only 1000 polychromatic erythrocytes (PCE) were scored and no historical control data were presented (at least 2000 immature erythrocytes per animal should be scored for the incidence of micronucleated immature erythrocytes, according to the OECD Test Guideline 474). Positive *in vitro* results are reported for VCD.

Information received in the public consultation
Four industry representatives and an undisclosed commenting party commented that VCH is neither mutagenic or genotoxic. Comments were received from an interested expert stating that 1,3-butadiene was used as a positive control for the mouse studies.

RAC opinion
The available study results indicate low concern for mutagenicity from VCH. However bioactivation into the metabolite VCD is a reason for concern, as several positive *in vitro* mutagenicity studies have been reported for VCD. No *in vivo* data on mutagenicity is available for VCD.

Reproductive toxicity

Dossier submitter
Due to the lack of OECD guideline studies, classification for this hazard class was not proposed by the dossier submitter, who reported data from studies of toxicity to reproduction to support ovotoxicity of VCH in mice (see section on *Repeated Dose Toxicity)*. Only one reprotoxicity study is available in the literature (Grizzle et al., 1994): Reproductive assessment by continuous breeding (RACB) were assessed in Swiss mice exposed to VCH. In this study 11 weeks old mice in F0 and 22 days old mice in F1 (CD-1 (ICR) BR outbred Swiss albino) received 0, 100, 250 or 500 mg VCH/kg bw (by gavage, in corn oil). There were 20 animals/sex/group in the dose groups, and 40 animals/sex/group in the control group. F0 and F1 males were exposed for 14 weeks. Females were exposed for 20 weeks in the F0 generation and for 16 weeks in the F1 generation. The treatment did not induce changes in mortality, feed/water consumption or clinical signs in the treated parental generation, but a slightly decreased postpartum weight was observed in dam treated with 500 mg/kg bw/day VCH. In F1 mice, VCH did not affect mortality, clinical signs or water consumption at any dose. However, the high-dose group displayed a decreased mean body weight from PND77-117, an increased relative liver weight and an increased feed consumption. In the presence of a slight toxicity (decreased body weight of dams (8%) and increased relative liver weight in F1 males and females), the following were decreased, although reproductive capacity was not altered in F0 and in F1:

- sperm count (85.5% vs 68.9% in control group, but within the historical control range) and
- number of oocytes
    - decreased mean number of primordial oocytes/follicles (140.6 vs 208.9 per ovary),
    - growing follicles (23.2 vs 51.2 per ovary) and
    - antral follicles (4.95 vs 7.40 per ovary)].

Information received in the public consultation
A member State agreed that the screening study showed effects on testicular sperm concentration and oocyte/follicles without apparently impacting fertility, and that it is important that more data is gathered for evaluation of the reproductive effects of VCH. An interested expert submitted a description of the key events in the proposed Mode of Action in great detail, as described elsewhere in this document. VCH is metabolised to VCD which in turn is selectively cytotoxic to oocytes in the ovary resulting in premature menopause, and as a consequence results in increased plasma levels of FSH which acts as a tumour promoter in ovaries (RCOM, annex 2).

RAC opinion
RAC agrees with the dossier submitter that no classification for this hazard class can be proposed unless more information is gathered.

Appendix:

Analyses of primary tumours in rats and mice (Excerpt of Appendix E in NTP, 1986)

ANNEXES:

Annex 1 Background Document (BD)[4]
Annex 2 Comments received on the CLH report, response to comments provided by the dossier submitter and RAC (excl. confidential information)

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[4] The Background Document (BD) supporting the opinion contains scientific justifications for the CLH proposal. The BD is based on the CLH report prepared by a dossier submitter.

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Appendix: Analyses of primary tumours in rats and mice. Only **statistically significant tumour** findings using the Incidental Tumour Tests are shown. (Excerpt from Appendix E in NTP, 1986)

Male rats Skin: Squamous cell papilloma or carcinoma	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	0/50 (0%)	1/50 (2%)	4/50 (8%)
Adjusted rates	0%	3.6%	37.5%
Terminal rates	0/33 (0%)	0/13 (0%)	1/5 (20%)
Incidental Tumour Tests	**P=0.024**	P=0.718	P=0.070
Female rats Clitoral gland: Adenoma or Squamous Cell Carcinoma	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	1/50 (2%)	5/50 (10%)	0/49 (0%)
Adjusted rates	2.5%	17.9%	0.0%
Terminal rates	1/40 (3%)	5/28 (18%)	0/13 (0%)
Incidental Tumour Tests	P=0.387	**P=0.040**	P=0.723N
Male mice Lung: Alveolar/Bronchiolar Adenoma or Carcinoma	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	4/49 (8%)	11/50 (22%)	4/50 (8%)
Adjusted rates	10.4%	26.5%	44.7%
Terminal rates	3/37 (8%)	9/39 (23%)	3/7 (43%)
Incidental Tumour Tests	**P=0.047**	P=0.068	P=0.065
Male mice Hematopoietic System: Lymphoma, All Malignant	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	4/50 (8%)	7/50 (14%)	5/50 (10%)
Adjusted rates	10.5%	16.7%	62.5%
Terminal rates	3/37 (8%)	5/39 (13%)	4/7 (57%)
Incidental Tumour Tests	**P=0.013**	P=0.340	**P=0.001**
Female mice Adrenal Gland Capsule or Cortex: Adenoma	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	0/50 (0%)	3/49 (6%)	4/48 (8%)
Adjusted rates	0%	7.7%	18.3%
Terminal rates	0/40 (0%)	3/39 (8%)	2/17 (12%)
Incidental Tumour Tests	**P=0.027**	P=0.117	P=0.056

Female mice Ovary: Mixed Tumor, Benign	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	0/49 (0%)	25/48 (52%)	11/47 (23%)
Adjusted rates	0.0%	64.1%	43.3%
Terminal rates	0/39 (0%)	24/38 (63%)	4/16 (25%)
Incidental Tumour Tests	**P<0.001**	**P<0.001**	**P<0.001**
Female mice Ovary: Granulosa Cell Tumor	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	1/49 (2%)	9/48 (19%)	11/47 (23%)
Adjusted rates	2.6%	23.7%	47.3%
Terminal rates	1/39 (3%)	9/38 (24%)	6/16 (38%)
Incidental Tumour Tests	**P<0.001**	**P=0.008**	**P<0.001**
Female mice Ovary: Granulosa Cell Tumor or Carcinoma	Vehicle control	200 mg/kg	400 mg/kg
Overall rates	1/49 (2%)	10/48 (21%)	13/47 (28%)
Adjusted rates	2.6%	25.5%	54.9%
Terminal rates	1/39 (3%)	9/38 (24%)	7/16 (44%)
Incidental Tumour Tests	**P<0.001**	**P=0.006**	**P<0.001**