Committee for Risk Assessment

RAC

Annex 1

Background document

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

4 vinylcyclohexene (VCH)

EC number: 202-848-9
CAS number: 100-40-3

ECHA/RAC/CLH-O-0000002966-62-01/A1

Adopted

14 September 2012

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Part A.

Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***4-vinylcyclohexene***
EC number:	***202-848-9***
CAS number:	***100-40-3***
Annex VI Index number:	
Degree of purity:	***95% min (for technical product) and 99% (research)***
Impurities:	***1,5-cyclooctadiene*** ***tert-butylcatechol*** ***para-tert-butylcatechol*** ***1,5,9-cyclododecatriene*** ***1,2-divinylcyclobutane*** ***water***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation	**Directive 67/548/EEC (Dangerous Substances Directive; DSD)**
Current entry in Annex VI, CLP Regulation	-	-
Proposal submitted for consideration by RAC	Carc. 1B – H 350	Carc. Cat. 2; R45
RAC opinion for resulting harmonised classification (future	Carc. 2 – H 351	Carc. Cat. 3; R40

entry in Annex VI, CLP Regulation)		

1.3 Dossier submitter's proposal for harmonised classification and labelling based on CLP Regulation and/or DSD criteria

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives	None		None	Not evaluated
2.2.	Flammable gases	None		None	Not evaluated
2.3.	Flammable aerosols	None		None	Not evaluated
2.4.	Oxidising gases	None		None	Not evaluated
2.5.	Gases under pressure	None		None	Not evaluated
2.6.	Flammable liquids	None		None	Not evaluated
2.7.	Flammable solids	None		None	Not evaluated
2.8.	Self-reactive substances and mixtures	None		None	Not evaluated
2.9.	Pyrophoric liquids	None		None	Not evaluated
2.10.	Pyrophoric solids	None		None	Not evaluated
2.11.	Self-heating substances and mixtures	None		None	Not evaluated
2.12.	Substances and mixtures which in contact with water emit flammable gases	None		None	Not evaluated
2.13.	Oxidising liquids	None		None	Not evaluated
2.14.	Oxidising solids	None		None	Not evaluated
2.15.	Organic peroxides	None		None	Not evaluated
2.16.	Substance and mixtures corrosive to metals	None		None	Not evaluated
3.1.	Acute toxicity - oral	None		None	Not evaluated
	Acute toxicity - dermal	None		None	Not evaluated
	Acute toxicity - inhalation	None		None	Not evaluated
3.2.	Skin corrosion / irritation	None		None	Not evaluated
3.3.	Serious eye damage / eye irritation	None		None	Not evaluated
3.4.	Respiratory sensitisation	None		None	Not evaluated
3.4.	Skin sensitisation	None		None	Not evaluated
3.5.	Germ cell mutagenicity	None		None	Not evaluated
3.6.	Carcinogenicity	H350: May cause cancer	None	None	
3.7.	Reproductive toxicity	None		None	Not evaluated
3.8.	Specific target organ toxicity –single exposure	None		None	Not evaluated
3.9.	Specific target organ toxicity – repeated exposure	None		None	Not evaluated

3.10.	Aspiration hazard	None		None	Not evaluated
4.1.	Hazardous to the aquatic environment	None		None	Not evaluated
5.1.	Hazardous to the ozone layer	None		None	Not evaluated

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Signal word: Danger
Hazard statements: H350
Precautionary statements: not harmonised

Proposed notes assigned to an entry:

Table 4: Proposed classification according to DSD

Hazardous property	Proposed classification	Proposed SCLs	Current classification [1)]	Reason for no classification [2)]
Explosiveness	None		None	Not evaluated
Oxidising properties	None		None	Not evaluated
Flammability	None		None	Not evaluated
Other physico-chemical properties *[Add rows when relevant]*	None		None	Not evaluated
Thermal stability	None		None	Not evaluated
Acute toxicity	None		None	Not evaluated
Acute toxicity – irreversible damage after single exposure	None		None	Not evaluated
Repeated dose toxicity	None		None	Not evaluated
Irritation / Corrosion	None		None	Not evaluated
Sensitisation	None		None	Not evaluated
Carcinogenicity	Carc. Cat. 2; R45 May cause cancer	None	None	
Mutagenicity – Genetic toxicity	None		None	Not evaluated
Toxicity to reproduction – fertility	None		None	Not evaluated
Toxicity to reproduction – development	None		None	Not evaluated
Toxicity to reproduction – breastfed babies. Effects on or via lactation	None		None	Not evaluated
Environment	None		None	Not evaluated

[1)] Including SCLs
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling: Indication of danger: T
R-phrases: R45
S-phrases: S53

2 BACKGROUND TO THE CLH PROPOSAL

2.1 History of the previous classification and labelling

VCH was not listed in Annex I of the 67/548/EC Directive.

2.2 Short summary of the scientific justification for the dossier submitter's CLH proposal

VCH is currently not classified according to Annex VI of CLP. However, IARC classifies it as Group 2B: The agent (mixture) is possibly carcinogenic to humans since 1994. Based on the increased incidence of ovarian tumors in female mice exposed orally by gavage to VCH for two years, and considering the fact that the mode of action described for this effect is plausible in humans, according to the dossier submitter, classification as Carc. Cat. 2; R45; May cause cancer, or Carc. 1B; H350: May cause cancer, is warranted.

2.3 Current harmonised classification and labelling

No current harmonised classification in Annex VI of CLP.

2.4 Current self-classification and labelling

No data available.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

VCH has a CMR property (carcinogenicity). Harmonised classification and labelling for CMR and respiratory sensitisation is a Community-wide action under article 115 of REACH and article 36(1) of CLP. VCH is currently not classified according to Annex VI of CLP.
Repeated-dose toxicity and genotoxicity data are presented for information as they may provide relevant data for assessment of carcinogenicity but no classification is discussed and proposed for these endpoints.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

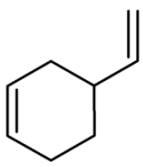
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	202-848-9
EC name:	4-vinylcyclohexene
CAS number (EC inventory):	100-40-3
CAS number:	
CAS name:	Cyclohexene, 4-ethenyl-
IUPAC name:	4-vinylcyclohexene
CLP Annex VI Index number:	
Molecular formula:	C_8H_{12}
Molecular weight range:	108,18 g/mol

Structural formula:



1.2 Composition of the substance

Table 6: Constituents (non-confidential information)

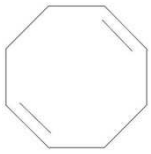
Constituent	Typical concentration	Concentration range	Remarks
4-vinylcyclohexene		95% - 99%	

Current Annex VI entry: no harmonised classification

Table 7: Impurities (non-confidential information)

Impurity	Typical concentration	Concentration range	Remarks
cycloocta-1,5-diene		3% max	
water		200 ppm max	
tert-butylpyrocatechol		25-200 ppm	
para-tert-butylpyrocatechol	50 ppm		
1,5,9-cyclododecatriene		traces	
1,2-divinylcyclobutane		traces	

Chemical Name: 1,5-cyclooctadiene
EC Number: 203-907-1
CAS Number: 111-78-4
IUPAC Name: cycloocta-1,5-diene
Molecular Formula: C_8H_{12}
Structural Formula:



Molecular Weight: 108.18 g/mol
Typical concentration (% w/w):
Concentration range (% w/w): 3 % max

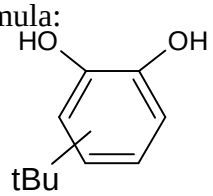
Classification: No harmonised classification

Chemical Name: water
EC Number: 231-791-2
CAS Number: 7732-18-5

IUPAC Name: water
Molecular Formula: H_2O
Structural Formula: H-O-H
Molecular Weight: 18.02 g/mol
Typical concentration (% w/w):
Concentration range (% w/w): 200ppm max

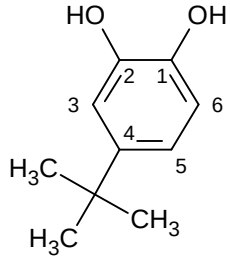
Classification: No harmonised classification

Chemical Name: tert-butylpyrocatechol
EC Number: 248-325-9
CAS Number: 27213-78-1
IUPAC Name: tert- Butylbenzene-1,2-diol
Molecular Formula: $C_{10}H_{14}O_2$
Structural Formula:



Molecular Weight: 166.22 g/mol
Typical concentration (% w/w):
Concentration range (% w/w): 25-200 ppm
Classification: No harmonised classification

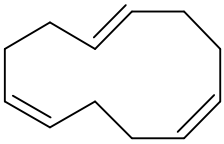
Chemical Name: para-tert-butylpyrocatechol
EC Number: 202-653-9
CAS Number: 98-29-3
IUPAC Name: 4-tert-Butylbenzene-1,2-diol
Molecular Formula: $C_{10}H_{14}O_2$
Structural Formula:



Molecular Weight: 166.22 g/mol
Typical concentration (% w/w): 50 ppm
Concentration range (% w/w): Information not available

Classification: No harmonised classification

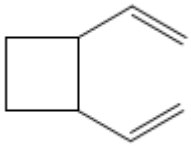
Chemical Name: 1,5,9-cyclododecatriene
EC Number: 220-437-2
CAS Number: 2765-29-9
IUPAC Name: trans,cis,cis-1,5,9-cyclododecatriene
Molecular Formula: $C_{12}H_{18}$
Structural Formula:



Molecular Weight: 162.27g/mol
Typical concentration (% w/w): traces
Concentration range (% w/w): Information not available

Classification: No harmonised classification

Chemical Name: 1,2-divinylcyclobutane
EC Number:
CAS Number: 2422-85-7
IUPAC Name: 1,2-divinylcyclobutane
Molecular Formula: C_8H_{12}
Structural Formula:



Molecular Weight: 108.18g/mol
Typical concentration (% w/w): traces
Concentration range (% w/w): Information not available

Classification : No harmonised classification

Table 8: Additives (non-confidential information)

Additive	Function	Typical concentration	Concentration range	Remarks
No data concerning the additives of VCH are available.				

1.2.1 Composition of test material

VCH used in the rat and mouse carcinogenicity studies from NTP had a purity of 98%. Impurities in two lots of test chemical included 0.01 % butylated hydroxytoluene in one and 0.005% tert-butylcatechol in the other, which had been added as inhibitors of peroxide formation

1.3 Physico-chemical properties

Table 9: Summary of physico - chemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
State of the substance at 20°C and 101,3 kPa	Colourless liquid	NTP Technical Report on the toxicology and carcinogenesis studies of 4-VCH in F344/N rats and B6C3F1 mice, US National Toxicology Program, 1986	
Melting/freezing point	-108,9°C	Handbook of chemistry and physics 2005-2006 National Library of Medecine HSDB Database	
Boiling point	128°C	Handbook of chemistry and physics 2005-2006 National Library of Medecine HSDB Database	
Relative density	0,8299 at 20°C	Handbook of chemistry and physics 2005-2006 National Library of Medecine HSDB Database	
Vapour pressure	2,09 kPa at 25°C	NLM - ChemIDplus Lite National Library of Medecine HSDB Database	
Surface tension	Not available		
Water solubility	50mg/L at 25°C Not water soluble	NLM - ChemIDplus Lite National Library of Medecine HSDB Database Handbook of chemistry and physics 2005-2006	
Partition coefficient n-	Log Pow = 3,93	International	

octanol/water		Chemical Safety Card, NIOSH, 1995 National Library of Medecine HSDB Database	
Flash point	15.9°C (open cup) 16°C (closed cup)	IUCLID Data Set 2006 National Library of Medecine HSDB Database	
Flammability	Flammable, dangerous fire risk. Dangerous fire hazard when exposed to heat or flame OSHA flammability Class: IB	National Library of Medecine HSDB Database MSDS OHS30250, 2009	
Explosive properties	Vapor/air mixtures are explosive	International Chemical Safety Card, NIOSH, 1995	
Self-ignition temperature	269°C	International Chemical Safety Card, NIOSH, 1995 National Library of Medecine HSDB Database	
Oxidising properties	Can react with oxidizers Upon contact with oxygen, VCH undergoes auto-oxydation to produce vinylcyclohene hydroperoxide	National Library of Medecine HSDB Database	
Granulometry	Non applicable		
Stability in organic solvents and identity of relevant degradation products	Miscible with methanol Soluble in ether, benzene, petroleum ether	National Library of Medecine HSDB Database	
Dissociation constant	Not available		
Viscosity	Not available		
Reactivity towards container material	Incompatible materials: halogens, oxidizing materials Closed containers may rupture violently. Containers may rupture or explode if exposed to heat.	MSDS OHS30250, 2009	
Thermal stability	May polymerise at temperatures above	IARC monographs	

	26,6°C and prolonged exposure to oxygen	volume 60	
Stability/shelf life	Oxidizes in air to form hydroperoxide	National Library of Medecine HSDB Database	
Henry's law constant	0.045 atm.m^3/mol at 25°C	NLM - ChemIDplus Lite	

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant for this dossier

2.2 Identified uses

VCH is found in industrial processes involving 1,3-butadiene, including the manufacture of lauric acid. VCH formed may be sold as-is or converted to 4-vinylcyclohexene diepoxide. It has been used as a chemical intermediate for production of flame retardants, flavours and fragrances, in the manufacture of polyolefins, as a solvent and in the manufacture of its diepoxide. Low levels of occupational exposure have been measured during the production and use of 1,3-butadiene.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated in this dossier

4 HUMAN HEALTH HAZARD ASSESSMENT

Publications reported in this proposal and not reviewed in the IARC monograph on VCH (IARC, 1994) are indicated in the section: 7 References.

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

4.1.1 Non-human information

4-vinylcyclohexene (VCH) has numerous data regarding its metabolism and its interspecies difference. Data are summarised below and grouped by paragraphs.

Smith and co-workers compared the disposition and the *in vitro* metabolism of VCH in female mice and rats (Smith *et al.*, 1990a). Female B6C3F1 mice and Fisher 344 rats were exposed to a single

dose of 400 mg/kg bw [^{14}C]VCH in corn oil by gavage. By 24h, mice and rats eliminated 97 and 88% of the dose, respectively. In both species, urine was the major route of excretion with 50-60% of the administered dose eliminated by this route. The second major route of elimination was expired air, as expired organics (1/3 of the absorbed dose in mice and rats). There were no differences between the rat and the mouse in the disposition of [^{14}C]VCH equivalents in the ovary. When the data were expressed as the percentages of total radioactivity administered the amount distributed to the ovaries was negligible. However, when the data were expressed as the concentrations of radioactivity present in the ovaries these values were comparable to those in liver. The tissue with the highest concentration of VCH in both species was adipose tissue. The highest concentration of VCH in the adipose tissue of mice was found between 1 and 2 h after VCH treatment, whereas rat adipose tissue continued to accumulate VCH until at least 8 h after dosing. Tissue concentrations in other tissues were slightly higher in the tissues of rats than the corresponding values in mouse tissues over the times studied (6 h in mice, and 8 h in rats).

After an i.p. administration of VCH 800 mg/kg bw, the highest concentration of VCH-1,2-epoxide (41 nmol/mL) was found in blood of mice, 2 h after dosing. VCH-7,8-epoxide was not detected in mice. These monoepoxides were not detected in rats up to 6 h after dosing (less than 2.5 nmol/mL).

The rate of epoxidation of VCH to VCH-1,2-epoxide is 6.5-fold greater in female mice liver microsomes compared to those of female rats. The blood concentration of VCH-1,2-epoxide was present in the blood of mice with the highest concentration at 2 h (41 nmol/mL) whereas the blood concentration of this monoepoxide in rats was < 2.5 nmol/mL up to 8 h after exposure. VCH-7,8-epoxide was not present in the blood of either species at the level of detection (level of sensitivity for VCH epoxide = 2.5 nmol/mL). Mouse hepatic microsomes formed 4 times more of VCH-1,2-epoxide.

Giannarini and co-workers administered 500 mg/kg VCH or VCH-1,2-epoxide or VCH diepoxide i.p. in corn oil to male albino Swiss mice (Giannarini *et al.*, 1981). VCH and VCH-1,2-epoxide were able to enhance the activities of certain xenobiotic transforming enzymes. CYP, cytochrome *b5*, NADPH-cytochrome *c* reductase, aminopyrine-*N*-demethylase and epoxide hydrolase were induced by VCH or VCH-1,2-epoxide (results from four separate tissue pools, each pool consisting of livers from 5-8 mice). On the contrary, p-nitroanisole-O-demethylase activity was not significantly induced. Hepatic glutathione levels were rapidly depleted after exposure to VCH, VCH-1,2-epoxide or VCH diepoxide, suggesting that glutathione is probably involved in the metabolism of VCH. VCH-1,2-epoxide and VCH diepoxide were more active than VCH, maybe because of the conjugation of the monoepoxide and the diepoxide with GSH.

Rats accumulate more VCH (or equivalent) than mice but mice metabolise VCH to VCH-1,2-epoxide or VCH-7,8-epoxide more rapidly and more efficiently than rats.

Cytochrome involvement during VCH metabolism

Fontaine and co-workers investigated the induction of CYP involved in VCH bioactivation in mice and rats after repeated exposure (Fontaine *et al.*, 2001a). B6C3F1 mice and Fischer-344 rats (28-38 days of age) were dosed with either VCH (7.5 mmol/kg i.p. for 10 days), VCH-1,2-epoxide (1.75 mmol/kg/i.p. for 10 days), vinylcyclohexene diepoxide (VCD)[1] (0.4 mmol/kg i.p. for 10 days), or phenobarbital (80 mg/kg i.p. for 5 days). In each treatment group, microsomes were prepared from

[1] Vinylcyclohexene diepoxide (VCD) is the ultimate ovotoxic metabolite of VCH (Doerr-Stevens *et al.*, 1999). Its toxicity is described in sections 4.7.1.6 and 4.10.3.

four individual rats or were pooled from four mice per group (16 mice total). Hepatic microsomes prepared from mice or rats treated repeatedly with VCH demonstrated significantly increased VCH bioactivation *in vitro*, as assessed by increased formation of VCH-1,2-epoxide (3.8- and 2.0-fold in mice and rats, respectively) and VCH-7,8-epoxide, the highest amounts being formed in mice. Although incubations were conducted for up to 60 min to allow possible VCD formation, VCD was only detected in mice pre-treated with either phenobarbital or VCH, but not in control mice or in control or pre-treated rats.

Mice and rats were then dosed with VCH, VCH-1,2-epoxide, or VCD for 10 days and measured for increases in hepatic microsomal CYP levels or activities. Total hepatic CYP levels were elevated only in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. Immunoblotting analysis of microsomes from VCH-treated rodents revealed elevated levels of CYP2A and CYP2B in mice but not in rats. VCH-1,2-epoxide pretreatment also increased CYP2B levels in the mouse. Activities toward specific substrates for CYP2A and CYP2B (coumarin and pentoxyresorufin, respectively) confirmed that VCH and VCH-1,2-epoxide pretreatments resulted in increased catalytic activities of CYP2A and CYP2B in the mouse but not the rat. Pretreatment with phenobarbital, a known inducer of CYP2A and CYP2B, increased VCH bioactivation in both species. Interestingly, metabolism studies with human CYP "Supersomes" (human CYP + P450 reductase + cytochrome b5) reveal that, of eight isoforms tested (CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11 and aromatase), only human CYP2E1 and CYP2B6 were capable of significantly catalyzing VCH epoxidation, whereas CYP2B6, CYP2A6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of the monoepoxides (Fontaine et al., 2001a).

Smith and co-workers investigated the biochemical basis for the different susceptibility to 4-vinylcyclohexene -induced ovarian toxicity and carcinogenesis in mice and rats (Smith *et al*., 1990c). Cytochromes P450 (CYP) involved in the epoxidation of 4-vinylcyclohexene (VCH) in liver microsomes from female B6C3F1 and 129/J (deficient in constitutive expression of CYP2B forms) mice and F344 rats were determined.

In mice liver microsomes, mouse CYP2A forms were involved in VCH epoxidation. No protein immunochemically related to mouse CYP2A was detected in female rat hepatic microsomes. However, VCH epoxidation was catalysed by CYP2B1 in microsomes from both species. But the level of constitutive expression of CYP2B forms is lower in rats compared with mice (0.07 nmol/min/mg protein in rats vs 0.26 nmol/min/mg protein in mice). A high rate of VCH epoxidation was observed in Phenobarbital-treated female rat liver microsomes, suggesting that pre-treatment with Phenobarbital, which induces mainly CYP2B isoforms, could increase the blood level of VCH-1,2-epoxide in VCH-treated rats and increase susceptibility to VCH-induced ovarian carcinogenicity. The constitutive expression in mouse liver of CYP2A and 2B forms, which catalyse ca. 80% of VCH epoxidation by hepatic microsomes from B6C3F1 mice, partially explains the susceptibility of mice to VCH-induced ovarian carcinogenicity. Although CYP2B forms of female Fischer 344 rats can also catalyze VCH epoxidation into VCH-1,2-epoxide, the expression of these isozymes in untreated animals probably does not occur at levels comparable to mice. Other CYPs are involved in VCH epoxidation in female rat hepatic microsomes, but they are probably expressed at very low levels and / or have poor catalytic activity toward VCH.

The effect of repeated exposure of VCH (7.5 mmol/kg/day) on mouse liver microsomal activities and VCH epoxidation was determined by Doerr-Stevens and co-workers (Doerr-Stevens *et al*., 1999). CYP2B and CYP2A, principle isoforms involved in the bioactivation of VCH, as well as CYP2E1 and CYP3A were evaluated. VCH exposure increased total CYP content (35-83% above control levels) after either 5, 10, or 15 days of treatment. Western blot analysis revealed an

induction of CYP2A, CYP2B, and CYP2E1 at day 10. Elevated levels of CYP2A and CYP2B correlated with marker androstenedione and testosterone 16alpha- and 16beta-hydroxylase activities. Microsomes prepared from mice pretreated with VCH for 10 days demonstrated an increase (≥ 2-fold) in the rate of VCH monoepoxide and diepoxide formation. Microsomal VCH epoxidation was increased to a similar extent by phenobarbital, acetone, and dexamethasone treatment. An increase in cytosolic glutathione S-transferase activity was observed after repeated VCH treatment, an enzyme potentially involved in detoxification of the VCH epoxides. Interestingly, preliminary studies indicated that circulating levels of the monoepoxide (VCH-1,2-epoxide) and diepoxide metabolites of VCH were elevated after repeated dosing of VCH. Overall, the results indicate that repeated exposure of VCH in mice induces CYP-dependent activities, and in turn induction of its metabolism.

The data presented above show that, CYP2A and 2B are the main cytochromes involved in VCH metabolism in rodents. VCH promotes its own metabolism by increasing expression of these cytochromes. Inter-rodent difference in VCH metabolism is correlated to the expression of these 2 cytochromes: VCH is poorly metabolised in rats due to the fact that CYP2A is not present and CYP2B is poorly expressed in rats.

Presence of VCH metabolites in various tissues

It has to be noted that VCD is classified in Annex I of the 67/548/EC Directive as Carc. Cat. 3; R40 - T; R23/24/25.

Keller and co-workers studied the metabolism of VCH in microsomes from Crl:CD BR rat and B6C3F1 mouse liver, lung, and ovary (Keller *et al*., 1997). Tissue samples were incubated with the test chemicals for 15 min at 37°C, and were tested for their ability to catalyze the following reactions:

- 4-VCH to VCH-1,2-epoxide and VCH-7,8-epoxide
- VCH-1,2-epoxide to VCD and VCH 1,2-diol
- VCH-7,8-epoxide to VCD and VCH-7,8-diol
- Hydrolysis of VCD.

3,3,3-trichloropropene oxide was added for reactions in which an epoxide metabolite was expected.

The reaction 4-VCH to VCH-1,2-epoxide was detected in liver and lung of rat and mouse . Mouse liver had a Vmax value 56-fold higher than that for rat liver. In lung tissue, the reaction proceeded only 2.5- to 3.8 fold faster in mice than in rats.

The reaction 4-VCH to VCH-7,8-epoxide was detected in rat and mouse liver and in mouse lung. The Vmax was lower in rat liver compared to mouse liver (0.007 nmol/min/mg protein in rats vs 0.91 nmol/min/mg protein in mice), and it was 12-fold lower than that for metabolism to the VCH-1,2-epoxide.

Rat and mouse livers were very similar in their Km and Vmax values for the reaction VCH-1,2-epoxide to VCD and also for VCH-7,8-epoxide to VCD.

VCH-1,2-epoxide to VCH 1,2-diol was only detectable in liver tissue from rats and mice and was similar. The hydrolysis of VCH-7,8-epoxide to VCH-7,8-diol was only detectable in rat liver. (Vmax = 135.8 nmol/min/mg protein). Hydrolysis of VCD was detectable in rat and mouse liver

and lung and in rat ovary. Rat liver had a Vmax nine-fold higher than did the mouse liver. For lung and ovary, the reactions seemed to be saturated even at the lowest concentrations where substrate is detectable. Maximum activity is approximately 14-fold lower in rat lung than in rat liver, and is approximately equal in mouse liver and lung. The mouse ovary had activity less than or equal to the rat ovary, and in both species the ovarian activity was low (no control).

In conclusion, this study 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.

Metabolism of VCH in mice ovaries or ovarian fractions

The expression of CYP2E1, CYP2A, and CYP2B isoforms was investigated in isolated ovarian fractions from mice exposed to VCH or to its ovotoxic metabolite, VCD (Cannady *et al*., 2003). B6C3F1 mice were administered i.p. daily for 15 days with VCH (7.4 mmol/kg bw/d) or VCD (0.57 mmol/kg bw/d). Ovaries were removed and either isolated into specific ovarian compartments for mRNA analysis, fixed for immunohistochemistry, or prepared for enzymatic assays. mRNA and protein for all isoforms were expressed/distributed in all ovarian fractions from vehicle-treated mice. In small preantral follicles (= F1 follicles) which are specifically destroyed by VCH or VCD, VCH or VCD dosing increased ($p < 0.05$) mRNA encoding CYP2E1 (645 ± 14% in VCH above control; 582 ± 16% in VCD above control), CYP2A (689 ± 8% in VCH above control; 730 ± 22% in VCD above control), and CYP2B (246 ± 7% in VCH above control). VCH dosing altered ($p < 0.05$) mRNA encoding CYP2E1 in non targeted F3 follicles (168 ± 7%) and CYP2A in interstitial cells (207 ± 19%) above control. Immunohistochemical analysis revealed the greatest staining intensity for all CYP isoforms in the interstitial cells. VCH dosing altered ($p < 0.05$) staining intensity in interstitial cells for CYP2E1 (19 ± 2.4% below control) and CYP2A (39 ± 5% above control). Staining intensity for CYP2B was increased ($p < 0.05$) above control in granulosa cells of small preantral (187 ± 42%) and antral (63 ± 8%) follicles. Catalytic assays in ovarian homogenates revealed that CYP2E1 and CYP2B were functional. Only CYP2E1 activity was increased (149 ± 12% above control; $p < 0.05$) by VCH dosing. The results demonstrate that mRNA and protein for CYP isoforms known to bioactivate VCH are expressed in the mouse ovary and are modulated by *in vivo* exposure to VCH and VCD. Interestingly, there is high expression of these isoforms in the interstitial cells. Thus, the ovary may contribute to ovotoxicity by promoting bioactivation of VCH to the toxic metabolite, VCD.

Rajapaksa and coworkers evaluated the role of ovarian CYP2E1 in VCH-induced ovotoxicity (Rajapaksa *et al*., 2007). Ovaries from B6C3F1 mice, CYP2E1 wild-type (+/+) mice and null (−/−) mice were sampled at postnatal day 4. Then they were cultured for 15 days with VCD (30 μM), or VCH-1,2-epoxide (125-1000 μM). Female CYP2E1 +/+ and −/− mice (28 days of age) were administered i.p. daily for 15 days with VCH, VCH-1,2-epoxide, or VCD. Following culture or *in vivo* dosing, ovaries were histologically evaluated. In the *in vitro* study, VCD decreased ($p<0.05$) primordial and primary follicles in ovaries from all three groups of mice. VCH-1,2-epoxide decreased ($p<0.05$) primordial follicles in B6C3F1 and CYP2E1 +/+ ovaries, but not in CYP2E1 −/− ovaries in culture. VCH-1,2-epoxide did not affect primary follicles in any group of mouse ovaries. However, after *in vivo* exposure, primordial and primary follicles were reduced ($p<0.05$) by VCD and VCH-1,2-epoxide in CYP2E1 +/+ and −/−. VCH reduced significantly primordial and primary follicles in CYP2E1 +/+ mice and in CYP2E1 +/+ and −/− mice, respectively. The data demonstrate that, whereas *in vitro* ovarian bioactivation of VCH or VCH-1,2-epoxide requires

CYP2E1 enzyme, *in vivo* CYP2E1 plays a minimal role. Thus, the findings support that hepatic metabolism dominates the contribution made by the ovary in bioactivation of VCH and VCH-1,2-epoxide to the ovotoxic metabolite, VCD.

Although the CYPs known to participate in VCH bioactivation are present in different cell types of the ovaries, potentially contributing to ovotoxicity by promoting bioactivation of VCH to the toxic metabolite, VCD, other studies tend to show that liver is the main organ for metabolism and bioactivation of VCH.

4.1.2 Human information

Smith and Sipes investigated the epoxidation of VCH in human hepatic microsomes (Smith and Sipes, 1991). Microsomes were prepared from livers from organ donors dying in traumatic accidents and from patients undergoing surgical resection of liver tumors (male and female). Twelve human liver microsomal samples (1.0 mg/mL microsomal protein) were incubated with VCH (1 mM) and 3,3,3-trichloropropene oxide, an inhibitor of epoxide hydrolase for 20 min. The major microsomal metabolite of VCH was VCH-1,2-epoxide. The rate of production of this metabolite ranged from 0.13 to 1.25 nmol/mg microsomal protein/min. VCH-7,8-epoxide was formed at rates approximately 6-fold lower than VCH-1,2-epoxide (< 0.01-0.21 nmol/mg microsomal protein/min). No differences between males and females were observed in the rate of hepatic microsomal VCH-1,2-epoxide formation. This was not investigated for the monoepoxide VCH-7,8-epoxide. The rate of VCH epoxidation by humans was lower than that from female mice and comparable to the VCH epoxidation rates with hepatic microsomes obtained from female rats (see results from Smith *et al*., 1990c). These studies indicate that VCH-1,2-epoxide is the major epoxide of VCH formed by human hepatic microsomes, as in mice and rats. However, the metabolism of VCH proceeds at a slower rate by human microsomes compared with hepatic microsomes obtained from mice or rats. VCH-7,8-epoxide were not quantified in mice and rats in the previous study of Smith and coworkers, probably because of the shorter incubation time (5 min vs 20 min). Epoxide hydrolase activity toward VCH epoxides was present in human liver microsomes since an epoxide hydrolase inhibitor (3,3,3 trichloropropene oxide) was needed to prevent hydrolysis of epoxides in the system (also used in mice and rats microsomes in the previous study of Smith).

Direct comparison of these rates could be misleading, especially given the variation of the rate of formation of VCH monoepoxides reported in the literature. Indeed, the difference in the rate of formation of VCH monoepoxides (i.e. the critical step thought to account for the higher mouse sensitivity towards VCH-induced ovotoxicity) in rat liver microsomes compared to mouse liver microsomes varies from study to study [rate of formation of VCH-1,2-epoxide in mice / rate of formation of VCH-1,2-epoxide in rats = 1.9 to 55.5; and rate of formation of VCH-7,8-epoxide in mice / rate of formation of VCH-7,8-epoxide in rats = 1.6 to 13, depending on the publication (Fontaine *et al*., 2001a; Keller *et al*., 1997 respectively)]. Taking into account the results from the study of Fontaine *et al*. (2001a), the mean rate of formation of VCH-1,2-epoxide in human liver microsomes is only 1.3-fold lower than in mice and even 1.4-fold higher than in rats (when comparing the highest rate of VCH-1,2-epoxide formation in women, it is even 1.4 and 2.7 higher than in mice and rats, respectively).

Moreover, induction of VCH epoxidation has been demonstrated in both rats and mice (Fontaine *et al*., 2001a). CYP2E1 which catalyzes the epoxidation of VCH into VCH monoepoxides in human supersomes (Fontaine *et al*., 2001a) can be induced in people who consume e.g. alcohol beverages regularly. Therefore, it cannot be discarded that metabolism of VCH in human might suffer from a

high variability. Consequently no firm conclusion on the rate of formation of VCH monoepoxides in human relative to rodents can be drawn.

Additionally, Fontaine and co-workers have demonstrated that, using human CYP "Supersomes" [human CYP (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, CYP3A4, CYP4A11, or Aromatase) + P450 reductase + cytochrome b5 + recycling NADPH system], CYP2E1 and CYP2B6 were capable of significantly converting VCH into VCH monoepoxide, whereas CYP2B6, CYP2A6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of the monoepoxides into the ultimate metabolite VCD (Fontaine *et al*., 2001a).

In addition, these authors showed that VCD was not produced at detectable levels by "supersomes", containing CYP2E1, CYP2B6, CYP2A6, or CYP3A4, incubated with VCH (Fontaine *et al*., 2001b). Similarly, VCD was not detected in mouse or rat microsomes incubated with VCH when rodents were not previously exposed to VCH (Fontaine *et al*., 2001a). This demonstrates that whatever the species, VCD is not directly formed by microsomes incubated with VCH if those were not pre-activated.

Therefore the species difference in terms of metabolism is not obvious. In addition, these results could demonstrate that a combination of different isozymes is likely needed to convert VCH into VCD.

4.1.3 Summary and discussion on toxicokinetics

The major route of excretion of VCH in mice and rats is urine (50-60% of the dose), the second route being expired air as expired organics. Elimination of VCH is slower in rats than in mice. The tissue with the highest concentration of VCH in both species was adipose tissue. VCH or its metabolites were found similarly in ovary from mice and rats.

The major monoepoxide VCH-1,2-epoxide was found in mice blood but not in rats. This could be explained by a higher rate of formation of this metabolite in mice, and/or by a more efficiently detoxification by e.g. epoxide hydrolases in rats compared to mice. Monoepoxide metabolites are further epoxided in VCD, a more potent ovary toxicant. Mono- and/or diepoxide metabolites are probably formed in the liver and are distributed in the ovary via circulating blood to exert their toxicities. However, CYP isoforms present in the mouse ovary are able to bioactivate VCH or the ovotoxic metabolite VCD.

After a repeated exposure, VCH is able to induce CYP involved in its own bioactivation in mice and rats. CYP (particularly, CYP2A and CYP2B) are involved in the epoxidation of VCH in liver microsomes from female mice. VCH epoxidation into VCH-1,2-epoxide can be catalyzed, by CYP2B forms in female Fischer 344 rats, but at a lower degree than in mice. Detoxication of VCH epoxides can be mediated by glutathione conjugation and/or hydrolysis by epoxide hydrolase.

Interestingly, it was demonstrated that human hepatic microsomes and human CYP "supersomes" were able to catalyze VCH epoxidation into monoepoxides and to epoxide these latter in VCD, the ultimate metabolite, like rodent hepatocytes. CYP2E1 and CYP2B6 were found to significantly catalyze VCH into VCH monoepoxide; epoxidation of the monoepoxides into VCD was mediated by CYP2B6, CYP2A6, CYP2E1, and CYP3A4. CYP2E1 induction in human liver can be easily achieved by regular ethanol consumption. Regular and heavy drinkers might be considered as population with increased potential to activate VCH to carcinogenic metabolites.

Female human microsomes demonstrated epoxidation of VCH at rates 13-fold and 2-fold less than those in mice and rats, respectively (Smith and Sipes, 1991), and total hepatic CYP per milligram of protein is significantly lower in humans than in rodents (Imaoka *et al*., 1991; Shimada *et al*., 1994). However, taking into account the results from the study of Fontaine *et al*. (2001a), the mean rate of formation of VCH-1,2-epoxide in human liver microsomes is only 1.3-fold lower than in mice and even 1.4-fold higher than in rats (when comparing the highest rate of VCH-1,2-epoxide formation in women, it is even 1.4 and 2.7 higher than in mice and rats, respectively) (Table 10). Consequently no firm conclusion on the rate of formation of VCH monoepoxides in human relative to rodents can be drawn.

Table 10: Summary of the rate of formation of VCH mono- and diepoxides in liver, lung, and ovary microsomes from rats and mice, and in human liver microsomes (in nmol/mg protein/min)

Conversion	Liver			Lung		Ovary		References
	Mouse	Rat	Human	Mouse	Rat	Mouse	Rat	
4-VCH to 4 VCH-1,2 epoxide	9.1 (B6C3F1, F)	1.4 (F344, F)	-	-	-	-	-	Smith *et al*., 1990a
	-	0.49 (Wistar, M)	-	-	-	-	-	Watabe *et al*., 1981
	-	-	0.67 (n =12) Range : M: 0.23-0.85 (n=6) F: 0.36-1.25 (n=5) (one value with gender unknown: 1.14)	-	-	-	-	Smith and Sipes, 1991
	11.1 (B6C3F1, F)	0.20 (Crl:CD BR, F)	-	3.49 (B6C3F1)	1.39 (Crl:CD BR)	ND	ND	Keller *et al*., 1997
	0.9* (B6C3F1, F)	0.47 (F344, F)	-	-	-	-	-	Fontaine *et al*., 2001a
4 VCH to 4 VCH-7,8 epoxide			0.08[a] (n =12) Range : M: <0.01-0.11 (n=6) F: 0.06-0.21 (n=5) (one value with gender unknown: 0.20)	-	-	-	-	Smith and Sipes, 1991
	0.91 (B6C3F1, F)	0.007 (Crl:CD BR, F)	-	1.83	ND	ND	ND	Keller *et al*., 1997

	0.61 (B6C3F1, F)	0.37 (F344, F)						Fontaine *et al*., 2001a
4 VCH-1,2 epoxide to VCD	5.35 (B6C3F1, F)	3.69 (Crl:CD BR, F)	-	2.70	2.06	ND	ND	Keller *et al*., 1997
4 VCH-7,8 epoxide To VCD	9.45 (B6C3F1, F)	8.83 (Crl:CD BR, F)	-	11.8	1.35	ND	ND	Keller *et al*., 1997

-; not evaluated
ND; not detected
a; formation of VCH-7,8-epoxide was not detected in liver microsomes from three donors. The mean value of the rate of formation of the monoepoxide was calculated on the basis of the limit of detection which leaded to a rate of formation of 0.01 nmol/mg protein/min.
F:female; M: male
*Please note that Fontaine et al (2001a) reported rates per 60 minutes, but for comparison the values in this table have been converted to rates per minute.

Interestingly, out of the eight human hepatic CYP isoforms tested in current studies (CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11 and aromatase), CYP2E1 and CYP2B6, were the only isoforms that significantly catalyzed the epoxidation of VCH.

Previous experiments focused on the role of CYP2E1 in the epoxidation of VCH because it has been reported to metabolize the structurally related compounds styrene and 1,3-butadiene (Lieber, 1997; Nieusma *et al*., 1998; Fontaine *et al*., 2001). Studies showed that, although hepatic microsomes from mice and rats pretreated with acetone showed increases in VCH-1,2-epoxide formation from VCH, hepatic microsomes from mice or rats pretreated with VCH for 5 or 10 days demonstrated no increases in CYP2E1 protein levels or activity (Fontaine *et al*., 2001). Those data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared with those of mice that do have CYP2E1, indicated that CYP2E1 is not an important isoform in the species-specific bioactivation of VCH. Current studies reconfirm this conclusion because neither VCH, VCH-1,2- epoxide, nor VCD pretreatment for 10 days affected CYP2E1 levels or activity in mice or rats.

Interestingly, although CYP2B6 and CYP2E1 were the only CYP isoforms that catalyzed VCH epoxidation in humans, CYP2B6, CYP2A6, CYP2E1, and CYP3A4 catalyzed the epoxidation of both monoepoxides to form the diepoxide. Although CYP3A4 is the major hepatic CYP isoform in humans, human liver CYP2A6 expression is relatively low (approximately 4%) (Cheng and Schenkman, 1982; Shimada *et al*., 1994). However, since the rate of formation of VCH 1,2-epoxide was shown to be significantly limited in humans compared with the mouse or rat (Smith and Sipes, 1991), the possibility of VCH-1,2-epoxide or VCH-7,8-epoxide bioactivation to VCD by these particular enzymes could be low in livers from humans exposed to VCH. However, information regarding the rate of epoxidation of VCH monoepoxides in VCD in human hepatic microsomes is missing.

Nevertheless, comparisons of VCH metabolism and hepatic CYP induction demonstrate that CYP2A and CYP2B are important CYP isoforms in the species-dependent bioactivation in rodents and, therefore, ovotoxicity of VCH. The increased expression of CYP2A and CYP2B seen exclusively in the mouse appears to be due to repeated treatment with VCH or VCH-1,2-epoxide.

This indicates that, with repeated exposure to VCH, the mouse is exposed to a greater concentration of the ovotoxic metabolites via enhanced bioactivation. The rat is resistant to the ovotoxicity of VCH, at least in part, because the increases in CYP levels/activities do not occur following repeated exposure to VCH. It is not known if exposure of humans to VCH would result in elevated levels of CYP isoforms. Perhaps studies with cultures of human hepatocytes could help address this question.

Available literature indicates that ovotoxicity of VCH is due to the formation of the ultimate ovotoxicant metabolite VCD, for which there is evidence of carcinogenicity in mice and rats (skin tumors in both species and ovary tumor in mice). Metabolism of VCH into mono- and di-epoxides metabolites is a critical step in the outcome of ovotoxicity and tumors. As long as human hepatic microsomes and human CYP "supersomes" have been demonstrated to be able to metabolise VCH into VCD and that a range of human CYPs are able to catalyse the epoxidation of VCH, an *in vivo* metabolisation (maybe slight) of VCH into VCD in humans cannot be ruled out. Moreover, no data are available to evaluate in which extent human CYPs levels/activities would be sensitive in VCH activation leading to increased VCD formation with chronic exposure to VCH, VCD or any other inducers of the specific CYPs involved in VCH metabolism in human.

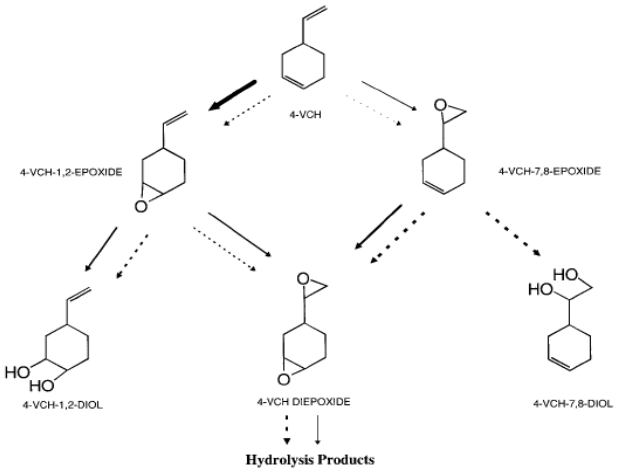


Figure 1 . Metabolic pathway for 4-vinylcyclohexene. All of the reactions shown were studied as was the hydrolysis of 4-vinylcyclohexene diepoxide. Thickness of lines indicates the relative velocity of the reactions in liver, compared to other reactions in liver. Solid lines indicates the reaction rate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

4.2 Acute toxicity

Not evaluated in this dossier.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated in this dossier.

4.4 Irritation

Not evaluated in this dossier.

4.5 Corrosivity

Not evaluated in this dossier.

4.6 Sensitisation

Not evaluated in this dossier.

4.7 Repeated dose toxicity

Table 11: Summary table of relevant repeated dose toxicity studies

Method	Results	Remarks	Reference
Rat (Fischer 344) (28 days old) 10/sex/group 0, 10, 40, 80 mg/kg (in corn oil) VCD 0, 100, 400, 800 mg/kg (in corn oil) VCH 0, 42.5, 170, 340 mg/kg (in corn oil) VCH-1,2-epoxide or VCH-7,8-epoxide 30 days Intra-peritoneal administration	No detectable oocyte loss occurred in rats at the highest dose of 7.4 mmol/kg VCH. In this study, ED50 of VCD and VCH-1,2-epoxide were determined for comparison: ED50 VCD = 0.4 mmol/kg ED50 VCH-1,2-epoxide = 1.4 mmol/kg	VCH did not induce oocyte loss in female rats exposed for 30 days.	Smith *et al*., 1990b
Mouse (B6C3F1) (28 days old) 0, 10, 40, 80 mg/kg (in corn oil) VCD 0, 100, 400, 800 mg/kg (in corn oil) VCH 0, 42.5, 170, 340 mg/kg (in corn oil) VCH-1,2-epoxide or VCH-7,8-epoxide	The dose of VCH which reduced the small oocyte count to 50% that of control (=ED50) was 2.7 mmol/kg In this study, ED50 of VCD, VCH-1,2-epoxide and VCH-7,8-epoxide were determined for comparison: ED50 VCD = 0.2 mmol/kg		Smith *et al*., 1990b

30 days Intra-peritoneal administration	ED50 VCH-1,2-epoxide = 0.5 mmol/kg ED50 VCH-7,8-epoxide = 0.7 mmol/kg		
Rat (Fischer 344) 10/sex/group 0, 50, 100, 200, 400 or 800 mg/kg bw/d (in corn oil by gavage) 13 weeks, 5 days/week	Reduced final body weights in male (≥ 400 mg/kg bw/d) and in female rats (800 mg/kg bw/d) Histopathologic effects: - hyaline droplet degeneration of the proximal convoluted tubules of the kidney in dosed male rats (severity dose related). - Inflammation in the submucosa of the nonglandular portion of the stomach was seen in one male and three females in the 800 mg/kg groups; this acute lesion consisted of focal infiltration of neutrophils and diffuse edema in the gastric submucosa.	This study is considered as valid, although this is a range-finding study, and no ophthalmological examination, no haematology and no clinical biochemistry were performed NOAEL < 50 mg/kg bw/d (male) NOAEL = 200 mg/kg bw/d (female)	NTP, 1986
Mouse (B6C3F1) 10/sex/group 0, 75, 150, 300, 600, or 1200 mg/kg bw/d (in corn oil by gavage) 13 weeks, 5 days/week	Reduced final body weights in female mice receiving 600 mg/kg bw/d Mortality: 9/10 male mice at 1200 mg/kg bw/d and 2/10 and 4/10 female mice at 300 and 1200 mg/kg bw/d, respectively. Histopathological effects: - Reduction (level not specified) in the number of primary follicles and mature graafian follicles in the ovaries of female mice exposed to 1200 mg/kg bw/d (ovaries from the lower dose groups were not similarly examined) - Mild, acute inflammation of the	This study is considered as valid, although this is a range-finding study, and no ophthalmological examination, no haematology and no clinical biochemistry were performed NOAEL = 150 mg/kg bw/d (female) NOAEL = 600 mg/kg bw/d (male)	NTP, 1986

	stomach was seen in the 1200 mg/kg groups in three males that died before the end of the study and in one female that lived to the end of the study.		
Rat (Sprague-Dawley) (male and female) 0, 250, 1000, and 1500 ppm (= 0, 1.11, 4.42, and 6.63 mg/L) by inhalation 13 weeks, 5 days/week, 6 hr/ day	Results: - lethargy (1500 ppm) - decreased body weights (significant) in male rats exposed to 1500 ppm compared to controls - significantly lower body weight gains in male and female rats at 1500 ppm. - increased absolute and/or relative liver weights of male and female rats exposed to 1000 or 1500 ppm - increased relative kidney weight in male rats exposed to 1000 or 1500 ppm - increased accumulation of hyaline droplets in the kidneys of all dosed male rats (although compound- related, the droplets were not accompanied by cytotoxicity) - ovarian atrophy was noted in 2/10 female rats exposed to 1500 ppm VCH. Atrophy in these two rats was morphologically distinct from that seen in mice, in that the primary change was a decrease in the numbers of corpora lutea. Since the decrease on corpora lutea were only observed on 2 of 10 rats and was not observed on previously conducted studies with VCH, the authors considered this effect as spurious and not compound-related. However, in absence of	This study is considered as valid although there is no post-treatment period. NOAEL = 250 ppm In this inhalation study, ovarian atrophy (decrease in the numbers of corpora lutea) was observed in high-dose female rats.	Bevan, 1996

	historical control incidence and of VCH-induced ovarian toxicity in mice, this effect could not be totally disregarded. No effects on: - haematological parameters - clinical chemistry evaluation - urinalysis		
Mouse (B6C3F1) (male/female) 0, 50, 250, or 1000 ppm (= 0, 0.22, 1.11, and 4.42 mg/L) by inhalation 13 weeks, 5 days/week, 6 hr/ day	Results: - lethargy observed in the 1000 ppm VCH-exposed mice. - mortality: all male mice and 5/10 female mice on Test Days 11 or 12, at 1000 ppm (three additional female mice exposed to 1000 ppm VCH died prior to study completion). - ovarian atrophy (level not specified) in females exposed to 1000 ppm (5/10 vs 0/10 in the other groups). The ovarian atrophy was characterized as a severe reduction of all developmental stages of ovarian follicles. - Splenic atrophy in 1/10 females exposed to 50 ppm and 1/10 females exposed to 250 ppm (0/10 in control females) - Testicular atrophy: in 4/10 males exposed to 1000 ppm and 1/10 males exposed to 250 ppm (but the incidence in the control group was 4/10) - Thymic atrophy: in 3/10 males exposed to 1000 ppm and 1/10 males exposed to 250 ppm. This effect was not observed in control and low-dose male groups. This finding	This study is considered as valid although there is no post-treatment period. NOAEL = 250 ppm	Bevan, 1996

	likely represents a secondary, stress-related effect of exposure to a lethal concentration of VCH.		

4.7.1 Non-human information

4.7.1.1 Repeated dose toxicity: oral

In an oral 13-week study in rats and mice, VCH induced decreased final body weights in male and female and hyaline droplet degeneration of the proximal convoluted tubules of the kidney in dosed male rats (severity dose related) (NTP, 1986). Increased incidence of mortality was observed in female and male mice. The number of primary follicles and mature graafian follicles was reduced in the ovaries of high-dose female mice (ovaries from the lower dose groups were not similarly examined). VCH induced inflammation of stomach in rats and mice

4.7.1.2 Repeated dose toxicity: inhalation

After exposure to VCH vapors by inhalation for 13 weeks, ovarian atrophy was observed in female mice (Bevan, 1996). Other effects were lethargy, decreased body weights, and increased incidence of mortality in high-dose mice. Rats were also exposed to VCH vapors. After an exposure period of 13 weeks, lethargy, reduced body weights (in males) and reduced body weight gains (in both sexes), increased liver weight in both male and female rats were observed. Male rats displayed an increased relative kidney weight in high-dose, probably related to an increased accumulation of hyaline droplets in the kidneys seen at all doses. Interestingly, ovarian atrophy was noted in some high-dose female rats. However, atrophy was morphologically distinct from that seen in mice, in that the change was a decrease in the numbers of corpora lutea. Since the decrease on corpora lutea were only observed on 2 of 10 rats and was not observed on previously conducted studies with VCH, the authors considered this effect as spurious and not compound-related. However, in absence of historical control incidence and due to VCH-induced ovarian toxicity in mice, this effect could not be totally disregarded.

4.7.1.3 Repeated dose toxicity: dermal

No dermal data have been reported for VCH.

4.7.1.4 Repeated dose toxicity: other routes

No data

4.7.1.5 Human information

No human data.

4.7.1.6 Other relevant information

Based on information displayed in IR/CSA, Section R.6.2.5.2, the metabolic pathway approach is usually reserved to some toxicological endpoints. Here, data concerning VCD are

presented as supportive evidence. However, the metabolism of VCH is not fast enough to base hazard identification only on studies conducted with that metabolite itself.

Table 12: Summary table of relevant repeated dose toxicity studies for VCD

Method	Results	Remarks	Reference
Rat (Fischer 344) (28 days old) 10/sex/group 0, 10, 40, 80 mg/kg (in corn oil) VCD 0, 100, 400, 800 mg/kg (in corn oil) VCH 0, 42.5, 170, 340 mg/kg (in corn oil) VCH-1,2-epoxide or VCH-7,8-epoxide 30 days Intra-peritoneal administration	The dose of VCD which reduced the small oocyte count to 50% that of control (=ED50) was 0.4 mmol/kg. In this study, ED50 of VCH and VCH-1,2-epoxide were determined for comparison: ED50 VCH > 7.4 mmol/kg ED50 VCH-1,2-epoxide = 1.4 mmol/kg	VCD is the most potent ovotoxicant in rats after an i.p. administration, compared to VCH and VCH monoepoxides.	Smith *et al*., 1990b
Mouse (B6C3F1) (28 days old) 0, 10, 40, 80 mg/kg (in corn oil) VCD 0, 100, 400, 800 mg/kg (in corn oil) VCH 0, 42.5, 170, 340 mg/kg (in corn oil) VCH-1,2-epoxide or VCH-7,8-epoxide 30 days Intra-peritoneal administration	The dose of VCD which reduced the small oocyte count to 50% that of control (=ED50) was 0.2 mmol/kg In this study, ED50 of VCH, VCH-1,2-epoxide and VCH-7,8-epoxide were determined for comparison: ED50 VCH = 2.7 mmol/kg ED50 VCH-1,2-epoxide = 0.5 mmol/kg ED50 VCH-7,8-epoxide = 0.7 mmol/kg	VCD is the most potent ovotoxicant in mice after an i.p. administration, compared to VCH and VCH monoepoxides. VCD has a similar ovotoxic potency both in rats and mice.	Smith *et al*., 1990b
Rat (Fischer 344) 0, 3.75, 7.5, 15, 30, 60 mg/rat (in acetone) 13 weeks, 5 days/week Dermal exposure	No mortality Reduced final body weights in the high-dose groups. Clinical signs at high dose: redness, scabs, and ulceration at the application site and burrowing behavior after dermal application. Histopathologic effects: - Hyperplasia of the sebaceous glands and acanthosis (hyperplasia) and hyperkeratosis of the squamous epithelium were seen at the site of	$NOAEL_{syst}$ = 30 mg/rat $NOAEL_{local}$ < 15 mg/rat	NTP, 1989

	application in all treated groups. The severity of the lesions was greatest at 60 mg/rat.		
Mouse (B6C3F1) 10/sex/group 0, 0.625, 1.25, 2.5, 5, 10 mg/mouse (in acetone) 13 weeks, 5 days/week Dermal exposure	No effect on mortality and on body weights. Increased relative liver (in all treated males and in the two highest dose group females) and kidney weights (from 1.25 and 2.5 in males and females, respectively). Histopathological effects: - Compound-related lesions of the skin included sebaceous gland hyperplasia and acanthosis (hyperplasia) (8/10 males and 2/10 females that received 10 mg/mouse and 1/10 males that received 5 mg/mouse) and hyperkeratosis of the stratified squamous epithelium at the site of application (8/10 males and 8/10 females that received 10 mg/mouse and 5/10 males and 6/10 females that received 5 mg/ mouse). - Diffuse ovarian atrophy observed in all females that received 10 mg/mouse and in 4/10 females that received 5 mg/mouse. - Uterine atrophy in 2/10 females that received 10 mg/mouse	$NOAEL_{syst}$ < 0.625 mg/ mouse (M) $NOAEL_{syst}$ = 1.25 mg/ mouse (F) $NOAEL_{local}$ = 2.5 mg/ mouse	NTP, 1989

4.7.1.7 Summary and discussion of repeated dose toxicity

Repeated dose toxicity data are presented for information as they may provide relevant data for assessment of carcinogenicity and no classification is discussed and proposed for this endpoint.

VCH produced a similarity of effects whatever the route of exposure (by oral gavage or by inhalation). Indeed, inflammation of stomach, ovarian atrophy in female mice and hyaline droplet degeneration in male rats were observed via both routes of exposure. Other toxic effects were mortality in mice, and reduced body weight. Inflammation of stomach observed in rats and mice

exposed orally to VCH is probably due to the route of exposure (gavage) since these effects were not observed in the 13-week inhalation study.
It should be noted that ovaries were target organs in both rodent species in the 13-week inhalation study, but not in the 13-week oral toxicity (mice only) and in the oral carcinogenicity (mice only) studies. Histopathological findings were different since in mice severe reduction of all developmental stages of ovarian follicles was observed, whereas only the number of corpora lutea was decreased in female rats.
In the 13-week oral NTP study and the 13-week inhalation study, ovarian toxicity was observed in high-dose female mice only, concomitantly to increased mortality. Since no histopathological examination of ovaries from lower dose groups in the NTP study was performed (since this study was aimed to determine dose range for the carcinogenicity study), no information on VCH-induced ovarian toxicity at non lethal doses is available. Although examination of all treated animals was carried out in the 13-week inhalation, the spacing between mid- and high-dose groups could be too large to observe any effect on ovary. However, in a reproductive study (Grizzle *et al*., 1994), the number of primordial oocytes/follicles in F1 high-dose female mice was decreased by ca. 33%. No mortality occurred in these animals, but they displayed some toxicity effects: decreased body weight from PND 77-117 (8-9%), increased relative liver weight (ca. 8%) and increased feed consumption were observed in these animals (see section 4.12). But, once again, in this study, only F1 control and high-dose groups were examined.

It should be noted that VCD, the ultimate metabolite of VCH, is a very potent ovotoxicant both in rats and mice after an i.p. administration. It is assumed that the ovotoxicity of VCH is attributed to that of VCD and that the epoxidation of VCH in VCD is required to affect ovary. 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 when i.p. administered. Interestingly, VCD is not able to produce ovotoxicity in rats after a 13-week dermal exposure. This could be due to differences in distribution of VCD to the target tissue (i.e. ovary) or absorption/excretion rates compared to an i.p. administration.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

Not evaluated in this dossier.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 13: Summary table of relevant in vitro and in vivo mutagenicity studies

Method	Results	Remarks	Reference
~ OECD guideline 471 S. typhimurium TA 1535, TA 1537, TA 98 and TA 100 with and without metabolic activation (rat or hamster S9 fractions) Preincubation protocol 0, 3.3, 10, 33, 100, 333, 1,000 µg/plate in DMSO	VCH did not produce increase in revertants in strains TA100, TA1535, TA1537, and TA98, with or without metabolic activation (rat or hamster S9), when tested according to the pre-incubation protocol. Doses up to 1000 µg per plate were used for each experiment, except without metabolic activation where the highest dose studied was 333 µg per plate (no explanation given in the NTP study report). Cytotoxicity was observed in TA100 strain incubated with 1000 µg per plate, with rat S9.	This study is considered as valid. VCH was not mutagenic in this bacterial reverse mutation assay.	NTP, 1986
Mouse lymphoma assay (without S9: 0, 20, 30, 40, 50, 60, 80, and 120 µg/mL in ethanol; with S9: 0, 30, 40, 50, 60, 80, 100 and 120 µg/mL in ethanol) Positive control: MMS (without S9) and 3-MC (with S9)	Without S9 (duplicate): Negative Cytotoxicity : 120 µg/mL With S9: 1st and 2nd experiment: equivocal; 3rd experiment: positive	Only tabulated results available on the NTP website	NTP, undated
~ OECD guideline 474 Crl:CD BR (Sprague-Dawley) rats Male and female 5/sex/group Inhalation study (vapors) 2-day study: 0, 500, 1000, 2000 ppm VCH; 13-week-study: 0, 250, 1000, 1500 ppm VCH 2-day study: 6 h/ day on two consecutive days 13-week study: 6 h/day, 5 days/week	VCH did not induce micronuclei in rats after a 2-day or a 13-week period exposure. Toxicity: 2-day study: Clinical signs of toxicity were noted in rats and included decreased responsiveness to sound stimulus, inactivity, and narcosis/sleep induction during both exposures in each VCH treatment group. Animal arousal occurred within approximatively 10 minutes after cessation of exposure. No clinical signs of toxicity were noted in rats prior to each exposure or during the recovery period. In rats, body weights for the 2000 ppm VCH-exposed group were significantly lowered at both the 24- and 48-h post-exposure time points compared to the controls (102 and 70%, respectively) .	Only 1000 PCE per animal were scored (the actual OECD 474 TG recommends to score a minimum of 2000 immature erythrocytes per animal for the incidence of micronucleated immature erythrocytes) No individual data available No historical negative/positive control data	Bevan, 2001

	13-week study: There was no compound-related mortality in rats exposed to VCH. Clinical signs of toxicity were evident in male and female rats in all VCH-exposure groups. The most prevalent signs were lethargy, clear discharge from the mouth, and stained fur. Body weight gain for male rats exposed to 1000 ppm and 1500 ppm VCH were significantly lower from controls (12-15% reduction).		
~ OECD guideline 474 B6C3F1/CrBR mice Male and female 5/sex/group Inhalation study (vapors) 2-day study: 0, 250, 500, 1000 ppm VCH; 13-week-study: 0, 50, 250, 1000 ppm VCH 2-day study: 6 h/ day on two consecutive days 13-week study: 6 h/day, 5 days/week	VCH did not induce micronuclei in mice after a 2-day or a 13-week period exposure. However, the mean MN/PCE/1000 PCE for mice exposed to 500 ppm for 2 days was twice as much the control value at 24-h post-exposure sampling time. Toxicity: 2-day study: Clinical signs of toxicity were not observed in mice. Body weight gain for the 1000 ppm VCH-exposed male mice were significantly less than controls at the 24-h post-exposure timepoint. I 13-week study: There was significant compound-related mortality in mice exposed to VCH. All of the male mice (10/10) and 5/10 female mice exposed to 1000 ppm VCH died on test days 11 or 12. Three additional high-dose females died prior to study completion. No clinical signs of toxicity were observed in the 250 ppm VCH-exposed mice; however, tremors and lethargy were observed in one male and two female 50 ppm VCH-exposed mice. Body weight gain for female mice exposed to 250 ppm VCH were significantly lower from controls (82% reduction)	No toxicity observed in female mice in the 2-d study. Due to the high mortality and the high reduction of body weight gain in the high-dose female mice, 250 ppm is considered as the MTD in the 13-week study. 1,3-butadiene (1000 ppm) was used as a concurrent positive control substance (no historical data) Only 1000 PCE per animal were scored (the actual OECD 474 TG recommends to score a minimum of 2000 immature erythrocytes per animal for the incidence of micronucleated immature erythrocytes) No individual data available No historical negative/positive control data	Bevan, 2001

4.9.1 Non-human information

4.9.1.1 In vitro data

In a bacterial reverse mutation assay (Ames test), did not produce increase in revertants in strains TA100, TA1535, TA1537, and TA98, with or without metabolic activation (rat or hamster S9), when tested according to the pre-incubation protocol. Doses up to 1000 µg per plate were used for each experiment, except without metabolic activation where the highest dose studied was 333 µg per plate (no explanation given in the NTP study report. Cytotoxicity was observed in TA100 strain incubated with 1000 µg per plate, with rat S9 (NTP, 1986). It should be noted that *in vitro* systems may be inappropriate to test VCH since rat S9 may fail to metabolise VCH into the ultimate metabolite VCD. Interestingly, a mouse lymphoma assay was found positive in a NTP study (not published but results available on the NTP website (http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=mouselymphoma.studyDetails&study_no=971117&cas_no=100-40-3&endpointlist=ML,ML-N) (NTP, undated). However, although the only *in vitro* assay using cells from the species sensitive to VCH ovotoxicity is positive, the validity of this assay is not ensured since only the tabulated results are available on the NTP website

4.9.1.2 In vivo data

Micronuclei were assessed in rats and mice exposed by inhalation to VCH vapors for two consecutive days or for 13 weeks (Bevan *et al*., 2001). VCH concentrations were: 0, 250 (mice only), 500, 1000, 2000 (rats only) ppm VCH in the 2-day study; and 0, 50 (mice only), 250, 1000, 1500 (rats only) ppm VCH in the 13-week-study. VCH did not induce micronuclei in rats and mice. Signs of toxicity and decreased body weight gain were observed in treated rats. Body weight gain was decreased in high-dose male mice in the 2-day study and increased mortality was observed in the high-dose groups in the 13-week study. However, validity of the results is questioned since only 1000 PCE per animal were scored (the actual OECD 474 TG recommends to score a minimum of 2000 immature erythrocytes per animal for the incidence of micronucleated immature erythrocytes), no individual data are available, and no historical negative/positive control data are available (especially for 1,3-butadiene used as a positive control in the mouse micronucleus assay)). This information is of importance given that the mean MN/PCE/1000 PCE for mice exposed to 500 ppm for 2 days was twice as much the control value at 24-h post-exposure sampling time.

4.9.2 Human information

No human data.

4.9.3 Other relevant information

Table 14 Summary table of relevant mutagenicity studies for VCD

Method	Results	Remarks	Reference
Reverse mutation test *Salmonella typhimurium* TA100, TA1535, TA1537, TA98 strains	TA100, TA1535 and TA98: positive with/without S9 (from 50, 170 and 500 µg/mL, respectively) TA1537: equivocal without S9 and positive with S9, from 1700 µg/mL	/	NTP, 1989
Gene conversion *Saccharomyces cerevisiae*	Positive without S9 from 3500 µg/mL	Not tested with S9	IARC, 1994
Mitotic crossing-over *Saccharomyces cerevisiae*	Positive without S9 from 3500 µg/mL	Not tested with S9	IARC, 1994
Reverse mutation *Saccharomyces cerevisiae*	Positive without S9 from 3500 µg/mL	Not tested with S9	IARC, 1994
Micronucleus formation *Allium cepa*	Positive without S9 from 700 µg/mL	Not tested with S9	IARC, 1994
Micronucleus formation *Vicia faba*	Positive without S9 from 1400 µg/mL	Not tested with S9	IARC, 1994
Gene mutation Chinese hamster V79 lung cells, *hprt* locus	Positive without S9 from 140 µg/mL	Not tested with S9	IARC, 1994
Gene mutation Chinese hamster V79 lung cells, *hprt* locus	Positive without S9 from 700 µg/mL	Not tested with S9	IARC, 1994
Mouse L5178Y lymphoma cells	Positive from 25 µg/mL	Not tested with S9	NTP, 1989
Sister chromatid exchanges Chinese hamster ovary cells	Positive from 3.73 µg/mL without S9 and from 37.3 µg/mL with S9		NTP, 1989
Micronucleus formation Chinese hamster V79 lung cells	Negative without S9	Not tested with S9	IARC, 1994
Chromosome aberration assay Chinese hamster ovary cells	Positive from 37.8 µg/mL without S9 and from 447 µg/mL with S9	/	NTP, 1989

4.9.4 Summary and discussion of mutagenicity

The mutagenicity database for VCH is limited. VCH was not mutagenic to four strains of *Salmonella typhimurium* with or without metabolic activation. The S9 used comes from hamster or rats. Metabolism data have shown that rat is not a potent species for transforming VCH into VCD. Therefore, the added value of this test regarding VCH mutagenicity *in vitro* with metabolic activation is questioned. Interestingly, a mouse lymphoma assay performed by the NTP was found

positive with metabolic activation (NTP, undated). However, although this is the only *in vitro* assay using cells from the species sensitive to VCH ovotoxicity and it is positive, the validity of this assay is not ensured since only the tabulated results are available on the NTP website. VCH did not increase micronucleus frequency in rats and mice *in vivo*. But, rats are probably less sensitive than mice due to differences in VCH metabolism, and the validity of the assay using mice is questioned (see section 4.9.1.2). However, the diepoxide metabolite of VCH, VCD, was mutagenic to *Salmonella typhimurium* and to *Saccharomyces cerevisiae* (NTP, 1989; IARC, 1994). It also caused gene conversion and mitotic crossing-over in *S. cerevisiae*. Micronuclei were induced by the compound in cells of two plant species, *Allium cepa* and *Vìcia faba*, but not in Chinese hamster V79 lung cells (without S9).VCD induced mutations at both the hprt and tk loci in cultured mammalian cells. In rodent cell lines, it induced sister chromatid exchange and chromosomal aberrations. No *in vivo* assay is available, although it is mutagenic in several *in vitro* tests and carcinogenic in rats and mice. However, it was found to form DNA adducts in mice dermally exposed to VCH (Randerath and Mabon, 1996). Overall, we are of the opinion that the genotoxic potential of VCH and VCD has not been sufficiently investigated to conclude on classification for VCH.

4.9.5 Comparison with criteria

4.9.6 Conclusions on classification and labelling

Information regarding mutagenicity are displayed as supporting evidence for the carcinogenicity endpoint. No classification is discussed and proposed for this endpoint for VCH but this endpoint would deserve to be further investigated by an *in vivo* study.

4.10 Carcinogenicity

Table 15: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
Rat (Fischer 344) (7 weeks old) 50 /sex/group 103 weeks, 5 days per week 0, 200 or 400 mg/kg bw/d (gavage, in corn oil)	Results: Mortality: - male: control 17/50, low 37/50*, high 45/50* - female: control 10/50; low 22/50; high 36/50* (P<0.001 for all groups except low dose female rats, for which P = 0.022) The survivals of the high- and low-dose male rats were significantly lower than that of the vehicle controls after week 5 (43 high-dose rats vs 49 control rats) and week 88 (26 low-dose rats vs 36 control rats), respectively. In female rats, the survivals of the high- and low-dose groups were significantly reduced after week 3 (42 high-dose rats vs 50 control rats) and week 102 (31 low-dose rats vs 41 control rats), respectively. Body weights: no effects, except for high dose males late in the study Neoplasic and non-neoplasic effects: - slightly increased incidence of epithelial hyperplasia of the forestomach in males (1/50, 3/50, 5/47 [1]) • slightly increased incidence squamous-cell papillomas or carcinomas (combined) of the skin in high-dose males (terminal rates[2]: control, 0/33; low-dose,	Only two concentrations used Purity: 98%; impurities in two lots of test chemical included 0.01 % butylated hydroxytoluene in one and 0.005% tert-butylcatechol in the other, which had been added as inhibitors of peroxide formation Due to the high incidence of mortality in low and high-dose-rats, it is not possible to relate the increased incidence of squamous-cell papillomas or carcinomas (combined) of the skin to exposure to VCH. Nevertheless, the increased incidence of adenomas or squamous-cell carcinomas (combined) of the clitoral gland in low-dose female rats can be taken into account since the survival rate of this group is similar to control until week 102.	NTP, 1986 Collins *et al*., 1987

[2] Terminal rate: rate at the end of the study taking into account animals sacrificed at 104 weeks only

	0/13; high-dose 1/5 (20%); and overall rates[3]: control, 0/50; low-dose, 1/50 (2%); high-dose, 4/50* (8%)) (NTP historical incidence: 1.9% (range: 0-10%)) - marginally increased incidence of adenomas or squamous-cell carcinomas (combined) of the clitoral gland in low dose female rats (survival more similar to control) (terminal rates: control, 1/40 (3%); low-dose, 5/28* (18%); high-dose 0/13; and overall rates: control, 1/50 (2%); low-dose, 5/50* (10%); high-dose, 0/49) (NTP historical incidence: 2.1% (range: 0-8%))		
Mouse (B6C3F1) (7 weeks old) 50 /sex/group 103 weeks, 5 days per week 0, 200 or 400 mg/kg (gavage, in corn oil)	Results: Decreased body weights and mortality in both males (control, 13/50; low-dose, 11/50; high-dose, 43/50*) and females (10/50, 11/50, 33/50*, respectively) in the high-dose groups. Neither gross observations nor histopathologic evaluations revealed a specific cause of death in any of the dosed mouse groups. The survivals of the high dose male and female mice were significantly lower than that of the vehicle controls after week 29 and after week 32, respectively. Non-neoplasic effects: - mild, acute inflammatory lesions and epithelial hyperplasia of the forestomach, especially in males (0/47, 7/50, 7/46) - increased incidence of a number of other non-neoplasic lesions: lung congestion, (high dose	Only two concentrations used Purity: 98%; impurities in two lots of test chemical included 0.01 % butylated hydroxytoluene in one and 0.005% tert-butylcatechol in the other, which had been added as inhibitors of peroxide formation) Due to the extensive and early incidence of mortality in male mice, it was not possible to evaluate the carcinogenic potential of VCH. Nevertheless, survival is similar in control and treated female mice. Based on the significantly increased incidence of granulosa-cell tumours of the ovary and of mixed	NTP, 1986 Collins *et al*., 1987

[3] Overall rate: animals which died during the study and animals sacrificed at the end of the study are counted

	male and female) splenic red pulp atrophy in high dose males, congestion of the adrenal gland in high dose females, cytologic alteration of the adrenal cortex in low and high dose females Neoplasic effects: Females: - significant treatment-related increase in the incidence of: • granulosa-cell tumours or carcinomas of the ovary (terminal rates: control, 1/39 (3%); low-dose, 9/38* (24%); high-dose 7/16* (44%); and overall rates: control, 1/49 (2%); low-dose, 10/48* (21%); high-dose, 13/47* (28%)) [2] • and of mixed benign tumours composed of epithelial and granulosa cells of the ovary (terminal rates: control, 0/39, low-dose, 24/38* (63%), high-dose, 4/16* (25%); and overall rates: control, 0/49, low-dose, 25/48* (52%), and high-dose: 11/47* (23%)). [2] (uncommon ovarian neoplasms: incidence in historical corn oil control female B6C3F1 mice: 1.2% = 12/1028 animals; historical control for granulosa cell carcinoma : 0.1% (=1/1028x100); and for granulosa cell tumors: 0.2% (=2/1028x100)) - slight increase of adrenal gland adenoma in high-dose group (terminal rates: control, 0/40; low-dose; 3/39 (8%); high-dose: 2/17 (12%); overall rates: control, 0/50; low-dose, 3/49 (6%); high-dose; 4/48* (8%)) (NTP historical incidence: 0.7%; range: 0-4.3%)) The incidences of granulosa-cell	tumours composed of epithelial and granulosa cells of the ovary, VCH is considered as carcinogenic to female mice.	

	hyperplasia and tubular-cell hyperplasia of the ovary were also increased in treated females. Males : - increased incidences of malignant lymphomas and alveolar/bronchiolar adenomas or carcinomas (combined) of the lung seen in the males surviving to the end of the study (malignant lymphomas: terminal rates: 3/37 (8%); 5/39 (13%); 4/7 (57%); overall rates: control, 4/50; low-dose, 7/50; high-dose, 5/50; p = 0.01; incidental tumour trend test; p = 0.001 incidental pair-wise test for high dose versus control; alveolo-bronchiolar adenomas or carcinomas [combined]: terminal rates: 3/37 (8%); 9/39* (23%); 3/7* (43%); overall rates: control, 4/49; low-dose, 11/50; high-dose, 4/50; p = 0.047; incidental tumour trend test) were slightly increased in treated males (NTP historical incidence: 14.3%)) (the extensive mortality seen in the high-dose male mice confounded the interpretation of these incidences).		

* Statistically significant
1 The forestomachs from 47 high-dose male rats were examined.
2 With regard to terminal rates, they should normally be based on a total of 40 control, 39 low-dose, and 17 high-dose animals. However, ovary tissues from one female rat per group were not examined.

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

Carcinogenic studies were performed with mice and rats (NTP, 1986; Collins, 1987). It should be emphasized that increased incidence of some tumors have been observed in male mice and male and female rats, but, due to poor survival in these groups, the interpretation of the data is difficult. This early excessive mortality may have masked increased incidences of different types of tumors. However, the results observed in female mice are considered as valid. The most pronounced effect is the significant treatment-related increased incidence of granulosa-cell tumours of the ovary (overall rates: control, 2.0%; low-dose, 21%*; high-dose 28%*) and of mixed tumours composed of epithelial and granulosa cells of the ovary (0%, 52%*, 23%*, respectively) (uncommon ovarian neoplasms; incidence in NTP historical corn oil control female B6C3F1 mice: 1.2% = 12/1028 animals) in female mice. Despite significant mortality in high-dose female mice, ovary tumors

could not be confounded with an excessive toxicity since the incidence is statistically significant even at the low dose at which mortality was similar to control.

4.10.1.2 Carcinogenicity: inhalation

No data available.

4.10.1.3 Carcinogenicity: dermal

No data available.

4.10.2 Human information

No human data.

4.10.3 Other relevant information

Table 16: Summary table of relevant carcinogenicity studies for VCD

Method	Results	Remarks	Reference
Rat (Fischer 344) (7-8 weeks old) 60 /sex/group 105 weeks, 5 days per week 0, 15, 30 mg/rat (in acetone) Dermal application	Mortality: - male: control 43/50, low 42/50, high 46/50 - female: control 23/50; low 27/50; high 35/50 Neoplasic and non-neoplasic effects: - increased incidences of acanthosis and sebaceous gland hypertrophy of skin from the scapula or back (M + F) - squamous cell papillomas (M) and squamous cell carcinomas (M + F) (squamous cell carcinomas: male: vehicle control, 0/50; low dose, 33/50; high dose, 36/50; female: 0/50; 16/50; 34/50). - increased incidences of basal cell adenomas or carcinomas (combined) (male: 0/50; 1/50; 6/50; female: 0/50; 3/50; 4/50).	Only two concentrations used	NTP, 1989
Mouse (B6C3F1) (8-9 weeks old) 60 /sex/group	Mortality: - male: control 12/50, low 15/50, mid 46/50*, high		NTP, 1989

103 weeks, 5 days per week 0, 2.5, 5, 10 mg/mouse (in acetone) Dermal application	50/50* (wk 85) - female: control 20/50; low 19/50, mid 35/50, high 50/50* (wk 83) Neoplasic and non-neoplasic effects: - acanthosis, hyperkeratosis, and necrotizing inflammation of the skin over the scapula or back. - squamous cell carcinomas (male: vehicle control, 0/50; low dose, 14/50; mid dose, 39/50; high dose, 42/50; female: 0/50; 6/50; 37/50; 41/50). - increased follicular atrophy and tubular hyperplasia of the ovary (atrophy: 12/50; 43/49; 42/49; 47/50; tubular hyperplasia: 5/50; 35/49; 38/49; 34/50). - benign or malignant granulosa cell tumors (0/50; 0/49; 7/49; 12/50) and benign mixed tumors (0/50; 0/49; 11/49; 6/50) in mid- and high-dose females. Increased combined incidences of luteomas, granulosa cell tumors, benign mixed tumors, or malignant granulosa cell tumors in mid- and high-dose female mice [overall rate: 1/50 (2%); 0/49 (0%); 17/49 (35%); 18/50 (36%); terminal rate: 1/30 (3%); 0/31 (0%); 7/14 (50%); 0/0]. - marginally increased incidences of alveolar bronchiolar adenomas or carcinomas (combined) in exposed female mice (4/50; 9/50; 11/50; 7/50).		

4.10.4 Summary and discussion of carcinogenicity

An oral carcinogenic study was performed with mice and rats (NTP, 1986; Collins *et al*., 1987). A significant increase in mortality was observed in treated rats and mice, except in the low-dose female mice. Different types of tumors were observed in these animals displaying poor survival

(squamous-cell papillomas or carcinomas (combined) of the skin in high-dose male rats, adrenal gland adenoma in high-dose female mice, malignant lymphomas and alveolar/bronchiolar adenomas or carcinomas (combined) of the lung seen in the male mice surviving to the end of the study). However, these findings could not be interpreted with certainty to evaluate the carcinogenic potential of VCH. This early excessive mortality may have masked increased incidences of different types of tumors. However, the results observed in female mice are considered as valid. Nevertheless, a significant increased incidence of ovary tumors in low-dose (for which mortality was similar to controls) and high-dose female mice was considered relevant taking into account the very low historical control incidence of these types of tumors (terminal rates: control, 1/39 (3%); low-dose, 9/38* (24%); high-dose 7/16* (44%); and overall rates: control, 1/49 (2%); low-dose, 10/48* (21%); high-dose, 13/47* (28%); incidence in historical corn oil control female B6C3F1 mice: 1.2% = 12/1028 animals; historical control for granulosa cell carcinoma : 0.1%; and for granulosa cell tumors: 0.2%). In addition, the incidence of squamous-cell carcinomas (combined) of the clitoral gland in low dose female rats is slightly increased in low-dose female rats for which the survival was significantly different from that of control only after week 102. No increased incidence was noted in the high-dose female rats. But it could be explained by the severe mortality observed in this group (terminal rates: control, 1/40 (3%); low-dose, 5/28* (18%); high-dose 0/13; and overall rates: control, 1/50 (2%); low-dose, 5/50* (10%); high-dose, 0/49; NTP historical incidence: 2.1% (range: 0-8%))

It should be noted that VCH-diepoxide (VCD), the ultimate metabolite of VCH, is also able to produce ovarian tumors in mice and to cause ovotoxicity in mice (but also in rats, contrary to VCH). Indeed, VCD is classified by IARC as possibly carcinogenic to humans (Group 2B). It induced ovary tumors after dermal exposure in mice, and skin tumors in rats and mice at the site of application. It is genotoxic in many *in vitro* assays: it was mutagenic in *Salmonella typhimurium* (Ames test), in *Saccharomyces cerevisiae* (gene conversion and mitotic crossing-over), cultured mammalian cells (at both the hprt and tk loci), in rodent cell lines (sister chromatid exchange and chromosomal aberrations), and in two plant species, *Allium cepa* and *Vìcia faba* (micronucleus test). VCD has a greater ovotoxicity potency in rodents than VCH after an i.p. administration and is able to destroy small oocytes both in rats and mice when administered by i.p. Interestingly, VCD is not able to produce ovotoxicity in rats after a 13-week or a 105-week dermal exposure. This could be due to differences in distribution of VCD to the target tissue (i.e. ovary) or absorption/excretion rates compared to an i.p. administration. Based on the fact that VCD is ovotoxic in rats by i.p. and that it is a critical step in ovarian tumor induction, VCD could be expected to cause ovary tumors in rats when orally or intraperitoneally administered. However, since no oral or i.p. carcinogenesis study in rats exposed to VCD has been found in the literature, it is not possible to prove this assumption. Moreover, VCD induced skin tumors in rats at the site of application. Since VCH was orally (and not dermally) administered to rats, it is not possible to know if VCH could produce local tumors like VCD.

It is remarkable that VCH and VCD are both ovotoxic (in mice, and in both mice and rats, respectively) and ovarian carcinogens in mice. In rodents, it was demonstrated that VCH is primarily submitted to epoxidation catalyzed by hepatic CYP to produce monoepoxides: the major metabolite being VCH-1,2-epoxide. Then VCH-1,2-epoxide undergoes an epoxidation to produce VCD. It has been demonstrated that VCH-induced ovarian tumors are dependent on the metabolism of VCH to the diepoxide metabolite, VCD, which is responsible for the destruction of oocytes, which is a critical step in the induction of ovary carcinogenesis. Detoxication of VCH epoxides can be mediated by glutathione conjugation and/or hydrolysis by epoxide hydrolase. However, mice produced the epoxide metabolites at a higher rate of formation, and they detoxified less efficiently the epoxides by e.g. epoxide hydrolases compared to rats. This could explain the difference in susceptibility to VCH-mediated ovotoxicity in mice and rats. In the oral carcinogenesis study, rats

may be resistant to ovarian tumor induction by VCH because of the amount of VCH converted to epoxides is insufficient to produce oocyte destruction, or perhaps due to the poor survival in these animals, which do not allow to draw a firm conclusion for the carcinogenic effects of VCH in rats.

Based on the review of ovarian toxicity and carcinogenicity in eight national toxicology program studies, Maronpot demonstrated a relationship between antecedent ovarian hypoplasia, atrophy, and hyperplasia, and subsequent ovarian neoplasia (Maronpot, 1987). In addition, he observed that pathologic changes in other tissues such as the adrenal glands and uterus were associated with the treatment-related ovarian changes (it should be reminded that congestion of the adrenal gland and slight increase of adrenal gland adenoma in high-dose female mice were observed in the oral carcinogenicity study with VCH). The findings were interpreted as indicating a relationship between previous ovarian toxicity and subsequent ovarian neoplasia. Therefore, ovarian atrophy observed in female mice exposed to VCH is considered as an early event in VCH-induced ovarian carcinogenesis in mice.

More recently, a mode of action for VCH-induced ovotoxicity and ovary carcinogenesis in mice has been proposed (Hoyer and Sipes (review; 2007); Bevan (communication; 2009)). After epoxidation of VCH into mono-epoxides and diepoxide (VCD), mainly in liver, VCD enters the systemic circulation and is distributed throughout the body. Upon reaching the ovary, VCD selectively destroys the primordial and primary follicles. Indeed, in the NTP oral 13-week study, a reduction in the number of primary follicles and mature graafian follicles in the ovaries was observed in female mice (but not in female rats) exposed to VCH (NTP; 1986). In an inhalation 13-week study, female mice exhibited also ovarian atrophy, characterized as a severe reduction of all developmental stages of ovarian follicles (Bevan *et al*., 1996). VCD is assumed to produce ovarian atresia through a mechanism involving programmed cell death or apoptosis. Repeated exposures to VCH ultimately result in premature ovarian failure (premature menopause), characterized by no estrous cyclicity, due to complete follicular loss. In mice given daily intraperitoneal injections of 800 mg/kg VCH for 30 days, there was >90% loss of the small pre-antral follicles at the end of the dosing period. At 240 days of the study (210 days following VCH treatment), there were few widely scattered oocytes in small and growing follicles; however, at 360 days, no oocytes at any stage were observed in the VCH-treated mice. The complete loss of oocytes at 360 days coincided with the loss of estrous cyclicity, indicating ovarian failure. 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 the promotion of ovarian tumors. At the time of ovarian failure, VCH-treated mice showed lesions in the ovary that appear similar to preneoplastic lesions reported in a genetically susceptible strain of mice for granulosa cell tumors.

Based on the well-described mechanism by which ovarian tumors are produced, it can be concluded that VCH can lead to ovarian tumors in mice. This mechanism might occur in women. Indeed, the Sapphire Group reports that VCD has been shown to selectively deplete primordial and primary follicles in the ovaries of non human primates (Macaca fascicularis) (TCEQ, 2011). The physiology and anatomy of non human primates are more similar to humans than rodents. The finding that VCD depletes primordial and primary follicles in non human primates is strong evidence that the MOA for VCH-induced ovarian cancer is plausible in humans. Humans and non human primates possess the same ability to metabolize VCH as rodents, specifically CYP2A, 2B and 2E1 and epoxide hydrolase, as well as glutathione transferase in organs, such as the liver, lung and ovaries. This group also reports that the incidence of ovarian cancer in women climbs dramatically around the age at which most women reach menopause. The onset of menopause, which happens at approximately 51 years of age, involves changes in gonadotropin levels as a result of cessation of ovarian function and menstrual cycle. The complete cessation of ovarian function results in the loss

of negative feedback of ovarian steroids (i.e., -estradiol) on gonadotropins. In 2 to 3 years after menopause, gonadotropin levels are particularly high, such that the concentrations of FSH and LH reach a peak of 10-20 times and 3-4 times the values recorded during the proliferative phase of the menstrual cycle, respectively.

Finally, the question is whether the VCH-induced ovary carcinogenesis is relevant to human.

Since it was demonstrated that human hepatic microsomes are able to catalyse *in vitro* the epoxidation of VCH in monoepoxides (Smith and Sipes, 1991), and that epoxidation of VCH into monoepoxides and epoxidation of these latter in VCD can occur in isolated human CYPs, it cannot be ruled out that this reaction could occur in human exposed to VCH. Humans were expected to be less efficient in the conversion of VCH into VCD based on the results of the study of Smith and Sipes (1991) which determined the rate of formation of VCH-1,2-epoxide in human liver microsomes to be 13- and 2-fold slower than in mouse and rat hepatocyte microsomes (results from a previous study (Smith *et al.,* 1990a)), respectively. The dossier submitter is of the opinion that direct comparison of these rates is difficult to interpret, especially given the variability in the rate of formation of VCH monoepoxides mentioned in the literature. Indeed, it appears that the difference in the rate of formation of VCH monoepoxides (i.e. the critical step thought to account for the higher mouse sensitivity towards VCH-induced ovotoxicity) in rat liver microsomes compared to mouse liver microsomes varies from study to study [rate of formation of VCH-1,2-epoxide in mice / rate of formation of VCH-1,2-epoxide in rats = 1.9 to 55.5; and rate of formation of VCH-7,8-epoxide in mice / rate of formation of VCH-7,8-epoxide in rats = 1.6 to 13, depending on the publication (Fontaine *et al*., 2001a; Keller *et al*., 1997 respectively)]. Taking into account the results from the study of Fontaine *et al*. (2001a), the rate of formation of VCH-1,2-epoxide in human liver microsomes is only 1.3-fold lower than in mice and even 1.4-fold higher than in rats (if the highest rate determined in women instead of the mean rate of formation of VCH-1,2-epoxide formation, it is even 1.4 and 2.7 higher than in rodents in mice and rats, respectively). Consequently, the assumption that metabolism of VCH by human is less efficient than by rats and mice is uncertain.

Nevertheless, although a MOA by which VCH-induced ovary carcinogenesis could occur via oocyte depletion leading to ovary tumors is plausible, there is uncertainty that a genotoxic component does not play a role in the VCH carcinogenicity. Indeed, no firm conclusion can be drawn about the genotoxic potential of VCH or VCD (see section 4.9) and this MOA does not account for the increased incidence of adenomas or squamous-cell carcinomas (combined) of the clitoral gland in low-dose female rats for which the survival is similar to control until week 102. Other tumors occurred in other tissues from male mice and male and female rats, but, due to the poor conditions of the animals, these findings could be misleading.

4.10.5 Comparison with criteria

The dossier submitter has argued for classification as Carc. 1B. Their rationale is as follows:

The CLP criteria for classification in Carc. 1 are as follows:

"Known or presumed human carcinogens
A substance is classified in Category 1 for carcinogenicity on the basis of epidemiological and/or animal data. A substance may be further distinguished as:

Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or
Category 1B: Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence. The classification in Category 1A and 1B is based on strength of evidence together with additional considerations (see section 3.6.2.2). Such evidence may be derived from:

- *human studies that establish a causal relationship between human exposure to a substance and the development of cancer (known human carcinogen); or*
- *animal experiments for which there is sufficient (1) evidence to demonstrate animal carcinogenicity (presumed human carcinogen). In addition, on a case-by-case basis, scientific judgement may warrant a decision of presumed human carcinogenicity derived from studies showing limited evidence of carcinogenicity in humans together with limited evidence of carcinogenicity in experimental animals."*

In the CLP, sufficient evidence of carcinogenicity is defined as when *"a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. 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 regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;"*
Limited evidence of carcinogenicity is defined as when *"the data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies; (c) the agent increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential; or (d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs."*

According to these criteria, a classification in Carc. Cat. 1A is not warranted because of the lack of epidemiological studies.

However, based on experimental studies in rodents, a causal relationship between the oral exposure to VCH and the increased incidence of ovary tumors has been demonstrated in female mice. The incidences of mixed tumours composed of epithelial and granulosa cells is 0%, 52%, and 23%, in control, low- and high-dose female mice, respectively. It should be noted that these types of tumors are uncommon ovarian neoplasms, with an incidence in NTP historical corn oil control female B6C3F1 mice of 1.2%. The mechanism by which VCH induces ovary tumors is well understood. VCH is epoxided in the ultimate metabolite VCD in rodents (with a greater extent in mice compared to rats). Then VCD exerts its ovotoxicity in destroying small oocytes via apoptosis, which is a critical step in the VCH-induced ovarian tumors. Consequently, 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 the promotion of ovarian tumors

According to IR/CSA (Section R.6.2.5.2), "data from hazard identification studies conducted with […] primary metabolite itself can be used to identify hazards for the parent compound." . Therefore, ovotoxicity and carcinogenicity data of VCD can be used to support the classification of VCH.

VCD is a more potent ovotoxic in mice, compared to VCH, and is ovotoxic in both mice and rats, unlike VCH which is not ovotoxic in rats in a 13-week toxicity study, probably because the amount of VCD formed in rats is not enough to exert ovarian toxicity. Moreover, a dermal carcinogenicity demonstrated that its ovotoxicity leads to ovary carcinogenesis in female mice like VCH. This type of tumors was not observed in rats (only local skin tumors were produced at the site of application). Ovotoxicity was demonstrated as an increase of cysts. However, since VCH is able to destroy small oocytes in female rats by i.p. administration and not after a dermal exposure, it could be expected that VCD produces ovotoxicity (and ovarian carcinogenicity) after i.p. administration but not after dermal exposure, probably because of differences in absorption/excretion/distribution of VCD via these routes. No oral or i.p. carcinogenesis study in rats exposed to VCH is available to us in order to confirm this assumption.

It should also be emphasised that, although a MOA by which VCH-induced ovary carcinogenesis could occur via oocyte depletion leading to ovary tumors is plausible, there is uncertainty that a genotoxic component does not play a role in the VCH carcinogenicity. Indeed, no firm conclusion can be drawn about the genotoxic potential of VCH or VCD (see section 4.9) and this MOA does not account for the increased incidence of adenomas or squamous-cell carcinomas (combined) of the clitoral gland in low-dose female rats for which the survival is similar to control until week 102. Other tumors occurred in other tissues from male mice and male and female rats, but, due to the poor conditions of the animals, these findings could be misleading.

It is concluded that the data provided in the report provide **sufficient evidence** of carcinogenic effects of VCH. Moreover, human hepatocytes are able to epoxide VCH into monoepoxides *in vitro*, and isolated human CYPs are able to epoxide VCH into monoepoxides and VCH monoepoxides into the diepoxide metabolite VCD. Therefore, there is no mechanistic evidence that could lead to think that these effects are not relevant for humans.

According to the dossier submitter, classification **Carc. 1B –H350** is therefore warranted (Carc. Cat. 2; R45 according to Directive 67/548/EEC). As no data are available by inhalation or dermal route, it is proposed not to specify route of exposure in the hazard statement.

Classification in Carc. 1A is not appropriate as it should be based on human data and no human data specific of VCH are available.

According to the dossier submitter, classification as Carc. 2 is not appropriate as the mechanism of action for inducing ovary tumors is well defined (metabolisation of VCH in VCD, which destroys small oocytes, which is a critical step in the outcome of ovary tumor). Moreover it occurred at a high incidence whereas the historical control incidence is low (1.2%).

4.10.6 Conclusions on classification and labelling

The dossier submitter argued that based on the increased incidence of ovarian tumors in female mice exposed to VCH for two years by gavage, a classification as **Carc. 1B – H350: May cause cancer** (Carc. Cat. 2; R45 according to Directive 67/548/EEC) is proposed for VCH, with no specific route of exposure added. However, the RAC disagreed with the dossier submitter's proposal and recommended that VCH be classified as Carc. 2 – H351: suspected of causing cancer in humans (Carc. Cat. 3; R40 according to Directive 67/548/EEC).

4.11 Toxicity for reproduction

The data are reported to support ovotoxicity of VCH in mice. Due to the lack of OECD guideline studies, the classification for this endpoint is not discussed in the proposal. Additional information should be required while evaluating the substance.

Although results obtained in the 90-day toxicity study on mice show in females a reduction in the number of primary follicles and mature graafian follicles in the ovaries of female mice exposed to 1200 mg/kg bw/d (ovaries from the lower dose groups were not similarly examined) and that one of the metabolites (VCD) is a known ovotoxic, only one reprotoxicity study (Reproductive assessment by continuous breeding (RACB) protocol) is available in the literature. The RACB protocol is summarized to allow comparison with current OECD test guideline 416 (Figure). For the F0 cohabitation and lactation phases, 100 male and 100 female Swiss (CD-1) mice, 11 weeks of age, were assigned to four dose groups as follows: control group, 40 males and 40 females, and each treatment group, 20 males and 20 females. The doses of VCH were 100, 250, and 500 mg/kg bw/day in corn oil, administered once daily per os. Body weights were taken once weekly for determining dose rate. Feed and water consumption were monitored during treatment weeks 1, 2, 5, 9, 13, and 18 (the last week for females only).

During Week 1 of exposure to VCH, animals were housed two per cage by sex by dose group. During Weeks 2 through 15 of exposure, animals were housed in breeding pairs within dose groups, and newborn litters were euthanized immediately after evaluation. Starting at Week 16 of exposure, the breeding pairs were separated, and F_0 females were allowed to deliver and rear the final litter until PND 21. Because no deleterious reproductive effects were observed during the 14-week cohabitation period, the F0 crossover mating was not conducted, and a limited F1 generation fertility assessment was conducted using only control and high-dose F1 animals. All F0 males were singly housed beginning at Week 16 and then euthanized without a necropsy during Week 17, and all F0 females were euthanized without a necropsy shortly after their litters reached PND 21. Only pups from the control and high-dose groups were weaned. Treatments were administered to all F0 animals until euthanization. Data collected during the F0 cohabitation were date of delivery of each litter, number, sex and weight of pups per litter, number of litters per breeding pairs, PND 0 dam body weight, and feed, water, and body weight data during Weeks 1, 2, 5, 9, 12, and 18 (females). On PND 0, 4, 7, 14, and 21, surviving pups were counted, sexed and weighed for all dams delivering a litter after Week 16.

During the F1 fertility assessment, the F1 generation from the control and high-dose groups were housed two per cage by sex within dose beginning at weaning (PND 21). Oral dosing of VCH in corn oil was initiated on PND 22 (prior to direct oral dosing, possible indirect exposure to VCH may have occurred through the gametes, *in utero*, or during lactation). Dose rate was based upon weekly body weights. At 74 ± 10 days of age, 20 males and 20 females per dose group were housed as nonsibling breeding pairs for 7 days or until a vaginal copulatory plug was observed, whichever was sooner. Litter data resulting from the F1 cohabiation were collected as described for the F0 cohabitation. After delivery of the litters, vaginal smears were collected from F1 females for 12 days. All F1 parents were euthanized and necropsied. Feed and water consumption were measured during Weeks 1 (breeding), 2, 3, and 4 of the F1 fertility assessment.

At the necropsy, the body, paired kidney with attached adrenal, and liver weights were collected for both sexes immediately following CO_2 asphyxiation. For males, rights testis, left testis with

attached epididymis, right epididymis, prostate, and seminal vesicles with coagulating glands (glandular secretions not removed) were weighed at necropsy. Paired ovaries (with attached oviducts) and uterus (with upper half of vagina) were weighed in females. Evaluation of sperm parameters included right cauda epididymis sperm motility, concentration, and morphology, and homogenization-resistant right testis spermatid concentration. All tissues, except ovaries with attached oviducts, were fixed in 10% neutral buffer formalin. The left testis and epididymis were embedded in glycol methacrylate, sectioned at 2.5-µm thickness, and stained with the hematoxylin/PAS. Paired ovaries with attached oviducts were fixed in Bouin/s fixative for 24 h and then transferred to 70% ethanol to await embedding into paraffin. Ovaries were serially sectioned at 6-µm thickness and every 20^{th} section was mounted, stained with hematoxylin and eosin, and evaluated for the number of primary, growing, and antral follicles.

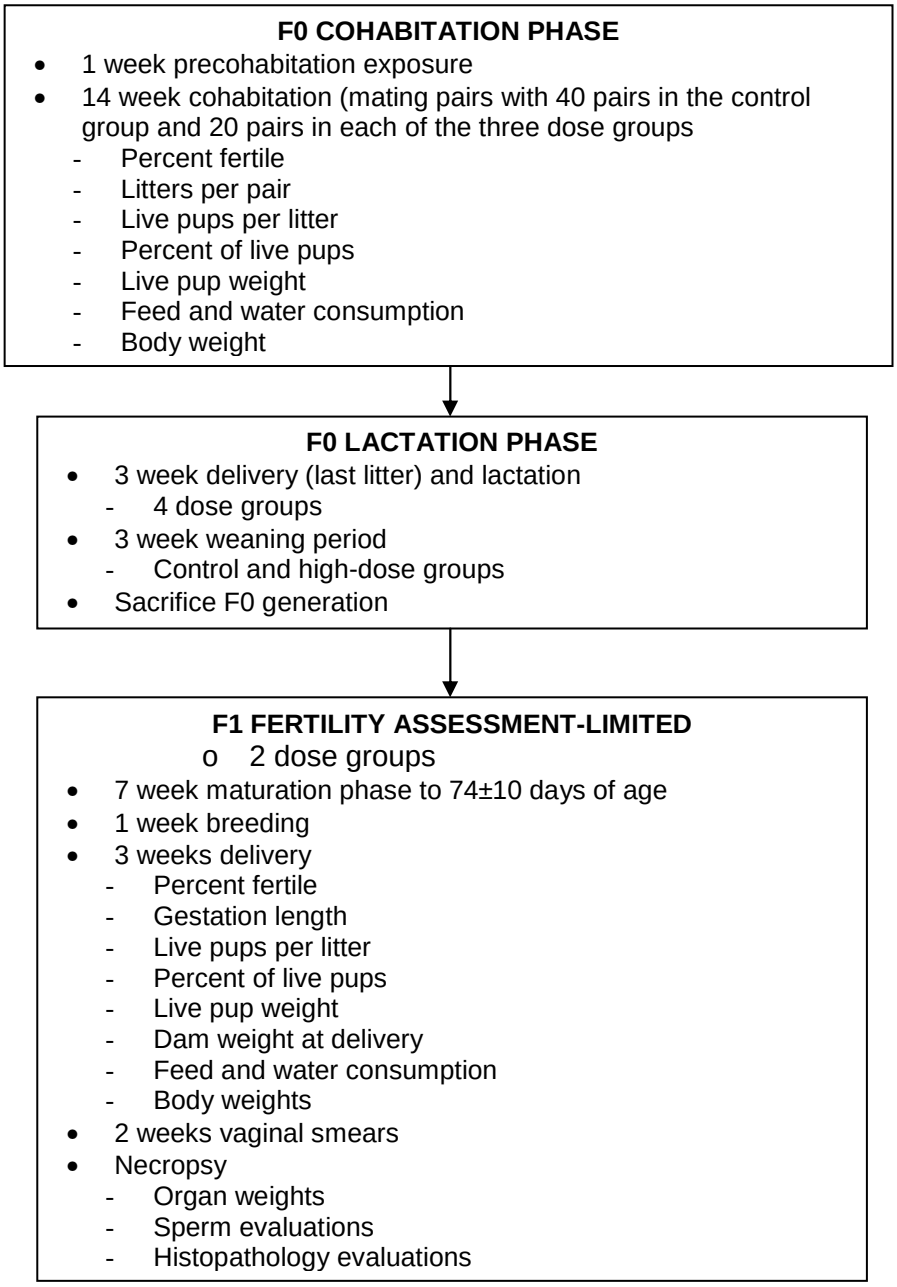


Figure 2 – VCH reproductive assessment by continuous breeding flow diagram (Grizzle *et al.*, 1994)

This study is only reported to support ovotoxicity of VCH in mice. Classification regarding reprotoxic effects is not proposed here. Nevertheless, we consider that more data should be gathered before evaluating in depth VCH reprotoxicity. Indeed, the screening study available confirms VCH effect on testicular sperm concentration and oocytes/follicles without apparently impacting fertility. A mechanism of action for the VCH-induced ovotoxicity in mice has been proposed [Hoyer and Sipes (review; 2007); Bevan (communication; 2009)]. After epoxidation of VCH into mono-epoxides and diepoxide (VCD), mainly in liver, VCD enters the systemic circulation and is distributed throughout the body. Upon reaching the ovary, VCD selectively destroys the primordial

and primary follicles. Indeed, in the NTP oral 13-week study, a reduction in the number of primary follicles and mature graafian follicles in the ovaries was observed in female mice (but not in female rats) exposed to VCH (NTP; 1986). In an inhalation 13-week study, female mice exhibited also ovarian atrophy, characterized as a severe reduction of all developmental stages of ovarian follicles (Bevan *et al*., 1996). VCD is assumed to produce ovarian atresia through a mechanism involving programmed cell death or apoptosis. Repeated exposures to VCH ultimately result in premature ovarian failure (premature menopause), characterized by no estrous cyclicity, due to complete follicular loss. In mice given daily intraperitoneal injections of 800 mg/kg VCH for 30 days, there was >90% loss of the small pre-antral follicles at the end of the dosing period. At 240 days of the study (210 days following VCH treatment), there were few widely scattered oocytes in small and growing follicles; however, at 360 days, no oocytes at any stage were observed in the VCH-treated mice. The complete loss of oocytes at 360 days coincided with the loss of estrous cyclicity, indicating ovarian failure. 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 the promotion of ovarian tumors. At the time of ovarian failure, VCH-treated mice showed lesions in the ovary that appear similar to preneoplastic lesions reported in a genetically susceptible strain of mice for granulosa cell tumors.

This mechanism might occur in women. Indeed, the Sapphire Group reports that VCD has been shown to selectively deplete primordial and primary follicles in the ovaries of non human primates (Macaca fascicularis) (TCEQ, 2011). The physiology and anatomy of non human primates are more similar to humans than rodents. The finding that VCD depletes primordial and primary follicles in non human primates is strong evidence that the MOA for VCH-induced ovarian cancer is plausible in humans. Humans and non human primates possess the same ability to metabolize VCH as rodents, specifically CYP2A, 2B and 2E1 and epoxide hydrolase, as well as glutathione transferase in organs, such as the liver, lung and ovaries. This group also reports that the incidence of ovarian cancer in women climbs dramatically around the age at which most women reach menopause. The onset of menopause, which happens at approximately 51 years of age, involves changes in gonadotropin levels as a result of cessation of ovarian function and menstrual cycle. The complete cessation of ovarian function results in the loss of negative feedback of ovarian steroids (i.e., -estradiol) on gonadotropins. In 2 to 3 years after menopause, gonadotropin levels are particularly high, such that the concentrations of FSH and LH reach a peak of 10-20 times and 3-4 times the values recorded during the proliferative phase of the menstrual cycle, respectively.

Table 17: Summary table of relevant reproductive toxicity studies

Method	Results	Remarks	Reference
Mouse (CD-1 (ICR) BR outbred Swiss albino) (11 weeks old) 20 /sex/treated group 40/sex/control group 0, 100, 250 or 500 mg/kg (gavage, in corn oil) Exposure period: F0 males: 14 weeks (from 11	Results: F0: 0, 100, 250 or 500 mg/kg: No effect on mortality, feed/water consumption and clinical signs. 500 mg/kg - Slightly decreased	Reproductive effects of VCH in mice were assessed via the continuous breeding (RACB) protocol.	Grizzle *et al*., 1994

weeks of age) F0 females: 20 weeks (from 11 weeks of age) F1 males: 14 weeks (from 22 days of age) F1 females: 16 weeks (from 22 days of age)	postpartum dam weight (F) F1 : 0, 100, 250 or 500 mg/kg: No effect on mortality, clinical signs and water consumption. 500 mg/kg - Decreased mean body weight from PND77-117 (males: PND 77, 31.51 g vs 34.07 g in controls, and PND 117, 32.79 g vs 35.24 g in controls; females: PND 77, 26.20 g vs 28.40 g in controls, and PND 117, 28.00 g vs 30.60 g in controls) Increased relative liver weight (males: 60.46 vs 55.59 mg/g bw in controls; females: 62.08 vs 57.52 mg/g bw in controls) - Increased feed consumption (M + F) - Increased epididymal sperm motility (but within the historical control range) (85.5% vs 68.9% in control group) - Decreased testicular sperm concentration ($11.3x10^4$/mg testis tissue vs $13.6x10^4$/mg testis tissue in control group) - 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) - No effect on oestrus - No effect on mating index, fertility index, number of live pups per litter, number of F2 pups born alive, sex ratio		

4.11.1 Effects on fertility

4.11.1.1 Non-human information

Reproductive effects of VCH were assessed in Swiss mice via the continuous breeding (RACB) protocol (Grizzle *et al*., 1994). Mice were exposed to 0, 100, 250 or 500 mg/kg VCH in corn oil by gavage. The treatment did not induce changes in mortality, feed/water consumption and clinical signs in treated parental generation, but a slightly decreased postpartum weight was observed in dam treated with 500 mg/kg bw/d VCH. In F1 mice, VCH did not affect mortality, clinical signs and 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), 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)] were decreased, although reproductive capacity was not altered in F0 and in F1.

4.11.1.2 Human information

No data

4.11.2 Developmental toxicity

4.11.2.1 Non-human information

No data

4.11.2.2 Human information

No data

4.11.3 Other relevant information

No data

4.12 Other effects

Not evaluated in this dossier.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated in this dossier.

6 OTHER INFORMATION

Information considered in this report was collected by a literature search performed on PubMed up to July 2010. References regarding toxicity and metabolism of VCH that were not evaluated by IARC for their classification in 1994 are mentioned in the references by an exponent 2 in the list below.

The dossier submitter noted that "as we have seen that VCH was planned for registration on November 30th, 2010, a consultation was performed by emailing concerned registrants in order to require the existing data they would like to be considered. No response was collected."

Apparently, VCH was not registered by August 15th, 2011 but various notifications for its classification were available:

Notification	Flam. Liq.	Asp. Tox. 1	Acute Tox. 4	Skin Irrit. 2	Eye Dam. 1	Eye Irrit. 2	Carc. 2	Repr. 2	Aquatic Chronic
1		x		x			x	x	3
2	2	x		x			x	x	3
3	2								
4	2								
5	1		x						
6									
7	2	x		x	x				3
8	2			x	x		x	x	2
9	2		x	x					
10	2		x	x		x	x		2
11	2			x		x			
12	2			x		x			
13									
14	2		x	x		x	x		
15									
16	2	x		x			x		

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8 ANNEXES