Committee for Risk Assessment

RAC

Annex 1

Background document

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

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

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

CLH-O-0000001412-86-301/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

20 September 2019

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

International Chemical Identification:

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

EC Number: 203-437-7

CAS Number: 106-87-6

Index Number: 603-066-00-4

Contact details for dossier submitter:

RIVM, The Netherlands

National Institute for Public Health and the Environment

Centre for Safety of Substances and Products

Bilthoven, The Netherlands

Version number: 0.1 **Date: June 2018**

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

Note on confidential information

Please be aware that this report is intended to be made publicly available. Therefore it should not contain any confidential information. Such information should be provided in a separate confidential Annex to this report, clearly marked as such.

CONTENTS

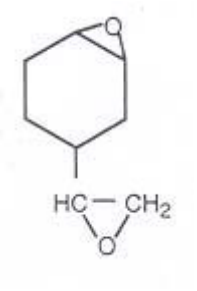
ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	IUPAC names: 3-oxiran-2-yl-7-oxabicyclo[4.1.0]heptane 3-(Epoxyethyl)-7- oxabicyclo[4.1.0]heptane 7-oxa-3-oxiranylbicyclo[4.1.0]heptane
Other names (usual name, trade name, abbreviation)	4-vinylcyclohexene diepoxide; 1,2-Epoxy-4-(epoxyethyl)cyclohexane; 1-(epoxyethyl)-3,4-epoxycyclohexane; 3-(1,2-epoxyethyl)-7-oxabicyclo[4.1.0]heptane; vinylcyclohexene diepoxide; 4-vinyl-1-cyclohexene diepoxide; 4-vinyl-1,2-cyclohexene diepoxide; 4- vinylcyclohexene dioxide; 1-vinyl-3-cyclohexene dioxide; 4-vinyl-1-cyclohexene dioxide
EC number (if available and appropriate)	203-437-7
CAS number (if available)	106-87-6
Molecular formula	$C_8H_{12}O_2$
Structural formula	
Molecular weight or molecular weight range	140.18 g/mol
Degree of purity (%) (if relevant for the entry in Annex VI)	Not specified

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self- classification and labelling (CLP)**
7-oxa-3-oxiranylbicyclo[4.1.0]heptane ; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	unknown	Carc. 2 Acute Tox. 3 * (H301) Acute Tox. 3 * (H311) Acute Tox. 3 * (H331)	Carc. 2 Acute Tox. 3 (H301) Acute Tox. 3 (H311) Acute Tox. 3 (H331)

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**	**The impurity contributes to the classification and labelling**
Unknown				

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 4: Proposed harmonised classification and labelling according to the CLP criteria

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

entry if agreed by RAC and COM		**eptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide**			Repr. 1B Acute Tox. 3 Acute Tox. 4	H350 H360F H311 H332	GHS08 Dgr	H360F H311 H332			

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

Table 5: Reason for not proposing harmonised classification and status under public consultation

Hazard class	**Reason for no classification**	**Within the scope of public consultation**
Explosives	Hazard class not assessed in this dossier	No
Flammable gases (including chemically unstable gases)	Hazard class not assessed in this dossier	No
Oxidising gases	Hazard class not assessed in this dossier	No
Gases under pressure	Hazard class not assessed in this dossier	No
Flammable liquids	Hazard class not assessed in this dossier	No
Flammable solids	Hazard class not assessed in this dossier	No
Self-reactive substances	Hazard class not assessed in this dossier	No
Pyrophoric liquids	Hazard class not assessed in this dossier	No
Pyrophoric solids	Hazard class not assessed in this dossier	No
Self-heating substances	Hazard class not assessed in this dossier	No
Substances which in contact with water emit flammable gases	Hazard class not assessed in this dossier	No
Oxidising liquids	Hazard class not assessed in this dossier	No
Oxidising solids	Hazard class not assessed in this dossier	No
Organic peroxides	Hazard class not assessed in this dossier	No
Corrosive to metals	Hazard class not assessed in this dossier	No
Acute toxicity via oral route		Yes
Acute toxicity via dermal route		Yes
Acute toxicity via inhalation route		Yes
Skin corrosion/irritation	Hazard class not assessed in this dossier	No
Serious eye damage/eye irritation	Hazard class not assessed in this dossier	No
Respiratory sensitisation	Hazard class not assessed in this dossier	No
Skin sensitisation	Hazard class not assessed in this dossier	No
Germ cell mutagenicity		Yes
Carcinogenicity		Yes
Reproductive toxicity		Yes
Specific target organ toxicity-single exposure	Hazard class not assessed in this dossier	/No
Specific target organ toxicity-repeated exposure	Hazard class not assessed in this dossier	No
Aspiration hazard	Hazard class not assessed in this dossier	No
Hazardous to the aquatic environment	Hazard class not assessed in this dossier	No
Hazardous to the ozone layer	Hazard class not assessed in this dossier	No

RAC general comment

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

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

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

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

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

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

Toxicokinetic data in rodents indicate that VCD is absorbed via oral, dermal and inhalation exposure routes (Weil *et al*., 1963 in National Toxicology Program (NTP), 1989). The preliminary results in the NTP indicate that 30 % of the dose applied to the skin is absorbed over a 24-hour period, both in rats and mice (NTP, 1989). By 24 hours, 70 %-80 % of the absorbed dose had been eliminated from the body, virtually all in the urine. The radioactivity remaining in the body was distributed over a number of tissues, with no tissue containing more than 1 % of the applied dose. Toxicokinetic data for the fate after oral administration, however, were not available in the CLH report.

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

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

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

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

4-Vinylcyclohexene diepoxide is classified for carcinogenicity in Annex VI of regulation 5 (EC) No 1272/2008 of the European Parliament as follows: Carc 2 (suspected human carcinogen: H351 suspected of causing cancer), according to the Globally Harmonised System of Classification and Labelling of Chemicals. The classification by the European Commission dates from 1991.

In 1994, IARC concluded that there is inadequate evidence in humans for the carcinogenicity of 4-vinylcyclohexene diepoxide, but that there is sufficient evidence in experimental animals for the carcinogenicity of 4-vinylcyclohexene diepoxide . Therefore, IARC classified 4-vinylcyclohexene diepoxide as possibly carcinogenic to humans (Group 2B) (Cancer. 1994).

In 2008, the Dutch Expert Committee on Occupational Standards, a committee of the Health Council of the Netherlands concluded that 4-vinylcyclohexene diepoxide should be regarded as carcinogenic to humans (comparable to EU category 1B) and that it acts by a stochastic genotoxic mechanism (Netherlands 2008).

This proposal for changing the harmonized classification of 4-vinylcyclohexene diepoxide is based on an update report of the Health Counsil of the Netherlands in 2016, which included an assessment of the requirement for classification on germ cell mutagenicity (Netherlands 2016).

4-Vinylcyclohexene diepoxide is a metabolite of the occupational chemical, 4-vinylcyclohexene for which an advice from RAC is already available (RAC 2012) .

This proposal aims at harmonising the classification applicable to workers in the Netherlands and the harmonised classification in Annex VI of CLP.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

[B.] Justification that action is needed at Community level is required.

Reason for a need for action at Community level:

Change in existing entry due to new interpretation/evaluation of existing data

Further detail on need of action at Community level
The Health Council of the Netherlands published an evaluation of this substance in 2008 and concluded that 4-vinylcyclohexene diepoxide should be regarded as carcinogenic to humans (comparable with CLP category 1B), (Netherlands 2008).

In 2016, the Health Counsil performed a re-evaluation of the mutagenic and carcinogenic properties of 4-vinylcyclohexene diepoxide. The re-evaluation now includes an assessment on the requirement for classification on germ cell mutagenicity. This re-evaluation by the Health Counsil forms the basis for the current proposal for an update of the harmonized classification of 4-vinylcyclohexene diepoxide from Cat. 2 to Cat. 1B for carcinogenicity. Based on the available data, the committee did not recommend a classification as a germ cell mutagen (Netherlands 2016).

In addition, the current proposal assesses the acute toxicty for all three routes as the current harmonised classification is a minimum classification. Furthermore, classification for effects on sexual function and fertility was included based on the identified effects on the ovaries.

5 IDENTIFIED USES

4-vinylcyclohexene diepoxide is used as a chemical intermediate and a diluent for other diepoxides and for epoxy resins derived from bisphenol A and epichlorohydrin (Cancer. 1994, Netherlands 2008). One of the applications is preparation of epoxy resin tissue-embedding agents for electron microscopy (Cancer. 1994, Netherlands 2008). In addion, VCD (often used in combination with Triptolide) has been accepted by the EPA as a rodenticide (EPA Reg Number 91601-1) (www.sensestech.com).

6 DATA SOURCES

This CLH report is based on a recent report of the Health Council of the Netherlands, “4-vinylcyclohexene diepoxide - Evaluation of the carcinogenicity and genotoxicity”, The Hague, February 29th 2016. Starting point of their report were the monographs of the International Agency for Research on Cancer (IARC) and a previous report of the Health Council of the

Netherlands from 2008. In addition a literature research was performed on acute toxicity and reproductive toxicity. VCD is not registered under REACH (January 2018).

7 PHYSICOCHEMICAL PROPERTIES

Table 6: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Clear, colourless or pale yellow liquid	ACGIH 2001 ((ACGIH) 2001)	
Melting/freezing point	-55 °C	ACGIH 2001((ACGIH) 2001)	
Boiling point	227 °C	ACGIH 2001 ((ACGIH) 2001)	
Relative density	-		
Vapour pressure	< 0.13 KPa (20 ^{0}C)	INCHEM 1998	
Surface tension	-		
Water solubility	35.2 g/L, 25 °C	ACGIH 2001 ((ACGIH) 2001)	
Partition coefficient n-octanol/water	0.44 Log Pow		
Flash point	110 °C	ACGIH 2001 ((ACGIH) 2001)	
Flammability	-		
Explosive properties	-		

8 EVALUATION OF PHYSICAL HAZARDS

Not evaluated in this dossier

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

Table 7: Summary table of non-human toxicokinetic studies

Method	Results	Remarks	Reference
Female F344/N rats and B6C3F1 mice Dermal: single application Dose: 0.1 ml and 0.001 ml, respectively, of solutions containing 500 mg/ml (200 pC/ml) [ethylene-^{14}C]4-vinylcyclohexene diepoxide in acetone.	30% of the dose applied to the skin is absorbed over a 24-hour period for both rats and mice; only 1%-3% of the dose remained on the skin at the site of application. By 24 hours, 70%-80% of the absorbed dose had been eliminated from the body, virtually all in the urine. The radioactivity remaining in the body was distributed over a number of tissues, with no tissue containing more than 1% of the applied dose. The liver, muscle, and adipose tissue, however, contained 0.5%-1.6% and 1.2% - 2.9% of the absorbed dose in rat and mouse tissue, respectively. Tissue to blood ratios ranged from 0.3 to 1.5 in rats and from 0.8 to 2.8 in mice (NTP unpublished data in NTP 1998).		The National Toxicology Program (NTP) (program 1989)
Rabbit liver microsomal preparations *In vitro*	4-vinyl-1-cyclohexene diepoxide can be metabolized to monoepoxymonoglycols: 1,2-hydroxy-4-vinylcyclohexane oxide, and 4-(1',2'-dihydroxyethyl)-1-cyclohexane oxide. Formation of these products is catalyzed by epoxide hydrolase.		(Watabe and Sawahata, 1976) (Watabe, Hiratsuka et al. 1980)

Method	Results	Remarks	Reference
Mice Intraperitoneal injection of 4-vinylcyclohexene diepoxide Dose: 500 mg/kg	Conjugation with glutathione is another pathway for metabolism of 4-vinyl-1-cyclo-hexene diepoxide		Giannarini et al. (1981 in NTP 1998) (Giannarini, Citti et al. 1981)

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

4-Vinylcyclohexene diepoxide is absorbed by rodents exposed dermally, orally, or by inhalation (Weil et al., 1963 in NTP) (National Toxicology 1989). The National Toxicology Program (NTP) has studied the fate of a single dermal application of [14C] 4-vinylcyclohexene diepoxide in female F344/N rats and B6C3F1 mice. These studies were conducted to determine if there were differences in disposition which could explain the differences in toxicity observed in rats and mice. Rats and mice received 0.1 ml and 0.001 ml, respectively, of solutions containing 500 mg/ml (200 pC/ml) [ethylene-^{14}C]4-vinylcyclohexene diepoxide in acetone. The preliminary results indicate that 30% of the dose applied to the skin is absorbed over a 24-hour period for both rats and mice; only 1%-3% of the dose remained on the skin at the site of application. By 24 hours, 70%-80% of the absorbed dose had been eliminated from the body, virtually all in the urine. The radioactivity remaining in the body was distributed over a number of tissues, with no tissue containing more than 1% of the applied dose.4 The liver, muscle, and adipose tissue, however, contained 0.5%-1.6% and 1.2%-2.9% of the absorbed dose in rat and mouse tissue, respectively. Tissue to blood ratios ranged from 0.3 to 1.5 in rats and from 0.8 to 2.8 in mice (NTP unpublished data in NTP 1998) (program 1989).

In vitro studies with rabbit liver microsomal preparations showed that 4-vinylcyclohexene diepoxide can be metabolized to monoepoxymono-glycols: 1,2-hydroxy-4-vinylcyclohexane oxide, and 4-(1',2'-dihydroxyethyl)-1-cyclohexane oxide (Watabe and Sawahata, 1976 in NTP 1998) (Watabe, Hiratsuka et al. 1980, program 1989). Formation of these products is catalyzed by epoxide hydrolase. Conjugation with glutathione is another pathway for metabolism of 4-vinylcyclohexene diepoxide, proposed by Giannarini et al. (1981 in NTP 1998) (program 1989), who reported depletion of reduced glutathione in the liver of mice given an intraperitoneal injection of 500 mg/kg 4-vinylcyclohexene diepoxide (program 1989).

10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

Weil et al. (1963) studied 4-vinyl-1-cyclohexene diepoxide for potential acute toxicity evaluating a single-dose oral LD_{50} (2.83 ml/kg) study in rats, a single-dose dermal LD_{50} (0.62 ml/kg) study in rabbits and a skin irritation and corneal injury study in rabbits (Weil, Condra et al. 1963). 4-Vinyl-1-cyclohexene diepoxide was found to be an irritant to skin and eyes (Weil, Condra et al. 1963).

10.1 Acute toxicity - oral route

Table 8: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute oral tox	Rats	4-vinyl-1-cyclohexene diepoxide	Single-dose oral	2.83 ml/kg	(Weil, Condra et al. 1963)
Acute oral tox	Rats	4-vinyl-1-cyclohexene diepoxide	Single oral dose	2130 mg/kg bw	(Dhillon and Von Burg 1996)

Weil et al. investigated acute oral toxicity potential in rats of more than 60 epoxy compounds including 4-Vinyl-1-cyclohexene diepoxide. An LD_{50} of 2.83 ml/kg was established. However, further details on these studies are not given. In a review by Dhillon and Von Burg (Dhillon and Von Burg 1996) in addition to the LD_{50} value determined by Weil et al (1963) also a second LD_{50} value of 2130 mg/kg bw is provided by reference to a secondary source.

10.2.1 Short summary and overall relevance of the provided information on acute oral toxicity

The available data on acute oral toxicity is limited to a study with limited details indicating an LD_{50} of 2.83 ml/kg bw. Using a density of 1.1 g/ml (Lide 1992), this results in a LD_{50} of 3110 mg/kg bw and a secondary reference indicating an LD_{50} value of 2130 mg/kg bw.

10.2.2 Comparison with the CLP criteria

Both available acute oral studies indicate an oral LD_{50} value above 2000 mg/kg bw indicating no requirement for classification. It is unknown on which data the current classification in category 3 is based.

10.2.3 Conclusion on classification and labelling for acute oral toxicity

Based on the available data on the acute oral toxicity of 4-vinyl cyclohexene removal of the classification is proposed.

10.3 Acute toxicity - dermal route

Table 9: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance,**	**Dose levels duration of exposure**	**Value LD_{50}**	**Reference**
Acute dermal tox	Rabbits	4-vinyl-1-cyclohexene diepoxide	Single-dose dermal	0.62 (0.25-1.57) ml/kg bw	(Weil, Condra et al. 1963)

Weil et al. investigated acute dermal toxicity potential in rats of more than 60 epoxy compounds including 4-Vinyl-1-cyclohexene diepoxide. An LD_{50} of 0.62 ml/kg bw was established. However, further details on these studies are not provided.

10.3.1 Short summary and overall relevance of the provided information on acute dermal toxicity

The available data on acute dermal toxicity is limited to a single study with limited details indicating an LD_{50} of 0.62 ml/kg bw. Using a density of 1.1 g/ml (Lide 1992), this results in a LD_{50} of 0.68 g/kg bw.

10.3.2 Comparison with the CLP criteria

According to table 3.1.1 of CLP, substances with a dermal LD_{50} of 0.68 g/kg bw fall within the ranges of category 3 (200 < ATE < 1000 mg/kg bw). This is the same group as the current minimal classification. It is proposed to assign an ATE of 680 mg/kg bw for acute dermal toxicity.

10.3.3 Conclusion on classification and labelling for acute dermal toxicity

Classification in category 3 for acute dermal toxicity is proposed. It is proposed to assign an ATE of 680 mg/kg bw for acute dermal toxicity.

10.4 Acute toxicity - inhalation route

4-Vinyl-1-cyclohexene diepoxide is believed to be mildly toxic by inhalation exposure. A 4-hour LC_{50} was determined to be 800 ppm in rats. Acute effects such as vasodilation and unsteady gait were noted and death occurred during or soon after exposure (Dhillon and Von Burg 1996).

Table 10: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance, form and particle size (MMAD)**	**Dose levels, duration of exposure**	**Value LC_{50}**	**Reference**
Acute inhalation	Rat	4-vinyl-1-cyclohexene diepoxide. Unknown whether tested as vapour or mist	Unknown concentrations for 4 hours.	800 ppm	Dhillon, 1996 (Dhillon and Von Burg 1996)
Acute inhalation	Rat	4-vinyl-1-cyclohexene diepoxide. Vapour	Concentrated vapour for 8 hours	No mortality after 8 hour exposure to the concentrated vapour	Weil et al, 1963 (Weil, Condra et al. 1963)

4-Vinyl-1-cyclohexene diepoxide is believed to be mildly toxic by inhalation exposure. An 4-hour LC_{50} was determined to be 800 ppm in rats. Acute effects such as vasodilation and unsteady gait were noted and death occurred during or soon after exposure (Dhillon and Von Burg 1996). It is unknown whether this inhalation study was performed as a vapour or as a mist. However, based on the saturated vapour pressure of 0.13 hPa, a saturated vapour concentration of 748 mg/m^3 or 128 ppm was calculated. This indicates that the tested concentration of 800 ppm was a mist. The absence of mortality after an 8-hour exposure (Weil, Condra et al. 1963) to the concentrated vapour (assumed to be the saturated vapour concentration of 748 mg/m^3 or 128 ppm) is in line with the LC_{50} as reported by Dhillon et al.

10.4.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

The only available LC_{50} value of 800 ppm (4656 mg/m^3) is based on a secondary reference without any details on the study. The only other information on acute inhalation toxicity indicates that mortality occurs only above the saturated vapour concentration of approximately 748 mg/m^3.

10.4.2 Comparison with the CLP criteria

The only available LC_{50} value of 4656 µg/l would result in clasification in category 4 since for mists and dusts clasification is required between 1 and 5 mg/l. This classification is above the current minimal classification in category 3. It is unknown on which data the current minimal classification is based. It is proposed to assign an ATE of 4.656 mg/l for acute inhalation toxicity.

10.4.3 Conclusion on classification and labelling for acute inhalation toxicity

Based on the available limited data classification in category 4 is proposed. It is proposed to assign an ATE of 4.656 mg/l for acute inhalation toxicity.

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute oral toxicity

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

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

Acute dermal toxicity

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

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

Acute inhalation toxicity

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

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

(0.75 mg/L) or 128 ppm.

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

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

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

Comments received during public consultation

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

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

Additional key elements

Acute oral toxicity

In the NTP technical report (NTP, 1989) on the toxicology and carcinogenicity studies of VCD in F344/N rats and B6C3F1 mice, acute oral and dermal studies are described.

Groups of five rats of each sex were fasted overnight, and then administered a single dose of 187.5, 375, 750, 1 500, or 3 000 mg/kg bw VCD (97 % purity) in corn oil, by gavage. Groups of five mice of each sex were fasted for 4 hours and then administered 375, 750, 1 500, 3 000, or 6 000 mg/kg bw VCD in corn oil by gavage. Animals were observed twice per day for 14 days, and necropsy was performed on all animals.

Survival rates and calculated oral LD_{50} for rats and mice are shown in the tables below.

TABLE H4. SURVIVAL OF RATS IN THE SINGLE-ADMINISTRATION GAVAGE STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE

Dose (mg/kg)	Survival	
	Male	Female (a)
187.5	5/5	5/5
375	5/5	5/5
750	5/5	5/5
1,500	5/5	(b) 4/5
3,000	(c) 0/5	(c) 0/5

(a) LD_{50} by Spearman-Karber procedure: 1,847 mg/kg (95% confidence interval 1,407-2,423 mg/kg)
(b) Day of death: 1
(c) All deaths occurred within 8 hours of dosing.

TABLE H11. SURVIVAL OF MICE IN THE SINGLE-ADMINISTRATION GAVAGE STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE

Dose (mg/kg)	Survival	
	Male (a)	Female (b)
375	5/5	5/5
750	5/5	(c) 4/5
1,500	(d) 3/5	5/5
3,000	(e) 1/5	(f) 2/5
6,000	(g) 0/5	(g) 0/5

(a) LD_{50} by probit analysis: 1,862 mg/kg (95% confidence interval 1,080-3,194 mg/kg)
(b) LD_{50} by probit analysis: 2,358 mg/kg (95% confidence interval 1,327-4,704 mg/kg)
(c) Day of death: 10
(d) Day of death: 2,14
(e) Day of death: 1,1,1,14
(f) Day of death: all 1
(g) All deaths occurred within 8 hours of dosing.

Clinical signs in rats included rapid respiration, staggering gait, burrowing activity, increased eye blinking, and half-closed eyelids; and in mice staggering gait, rough hair coats, and rapid respiration. No lesions were observed at necropsy in either species.

In the Abolaji *et al*. (2016) oral study in female Wistar rats (see section on Reproductive toxicity for further study details), a pilot study was carried out in order to define VCD doses for the main experiment. Female rats were exposed to 100, 250, 500 and 1 000 mg/kg bw of VCD. All animals dosed at 1 000 mg/kg bw died after the first treatment. However, this is a non-guideline study with significant limitations in reporting, and, therefore, considered inadequately reliable for regulatory assessment purposes. For example, number of animals per group is not reported, nor data on survival for other groups.

Acute dermal toxicity

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

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

0.1 mL, to the clipped dorsal interscapular region.

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

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

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

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

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

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

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

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

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

Assessment and comparison with the classification criteria

Acute oral toxicity

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

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

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

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

Category 4:

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

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

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

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

Acute dermal toxicity

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

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

RAC considers that the data for LD_{50} values in rabbits (from Weil *et al*. 1963, and Dhillon and Von Burg 1996) are too limited to support classification as Acute Tox. 3. On the other hand, the NTP studies in rats and mice, which indicate no requirement for classification for acute dermal toxicity, have significant deficiencies in reporting (e.g. it is not known whether it was ensured that VCD is in contact with the skin, and if it was, for how long). Hence, the data are not considered reliable enough to change the classification from Acute Tox. 3 to no classification. It can also be noted that acute toxicity via the dermal route is expected to be lower than via the oral route, where an LD_{50} of 1 847 mg/kg bw was reported.

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

Acute inhalation toxicity

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

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

In RAC's opinion, the uncertainties related to the data from Weil *et al*. (1963) study and a

secondary reference from the review article by Dhillon and Von Burg (1996) are too high to justify changing the classification from Category 3 to Category 4, as proposed by the DS.

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

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

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

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

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

10.5 Skin corrosion/irritation

This hazard class was not assessed.

10.6 Serious eye damage/eye irritation

This hazard class was not assessed.

10.7 Respiratory sensitisation

This hazard class was not assessed.

10.8 Skin sensitisation

This hazard class was not assessed.

10.9 Germ cell mutagenicity

Table 11: Summary of *in vitro* mutagenicity studies

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
Micro-organisms					

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
Reverse mutation	*S. typhimurium* Strains: TA100, TA1535, TA98, TA1537	5 doses: 100, 333, 1000, 3333, 10 000 µg/plate + / - incubation with Liver S9 mix	TA100, TA1535, TA98 positive outcome with and without metabolic activation; TA1537 without metabolic activation equivocal, with activation positive in first trial, equivocal in second trial. *Cytotoxicity*: Nontoxic up to highest concentration tested	2	NTP1989 (program 1989); Mortelmans et al., 1986 (Mortelmans, Haworth et al. 1986)
Reverse mutation	*S. typhimurium* Strains: TA1535, TA98, TA100	0.01, 0.05, 0.1 and 0.5 ml/9 litre desiccator + / - incubation with with Liver S9 mix	TA1535, TA98 and TA100 positive outcome with and without S9 *Cytotoxicity*: no cytotoxicity at the concentrations tested	2	Simmon and Baden 1980 (Simmon and Baden 1980)
Reverse mutation	*S. typhimurium* Strains: TA100 and TA1535	15, 30, 45, 60 µmol	Positive outcome in both TA1535 and TA100 *Cytotoxicity*: 6% and 12% growth inhibition in TA100, 8% and 15% growth inhibition in TA1535 at 48 and 60 µmoles/plate, resp.	2	Frantz and Sinsheimer 1981 (Frantz and Sinsheimer 1981)
Reverse mutation	*S. typhimurium* strains: TA1535, TA100, TA1537, TA98	62.5, 125, 250, 500, 1000, 2000 µg/plate	Positive outcome in TA1535 and TA100 Negative outcome in TA1537 and TA98 *Cytotoxicity*: 2000 µg/plate was toxic	2	El_Tantawy and Hammock 1980 (El-Tantawy and Hammock 1980)
Reverse mutation	*S. typhimurium* TA100	1 and 10 µl/plate (-S9,+S9). 100 µl/plate (no info on S9)	Positive outcome with and without S9. S9 enhanced activity. *Cytotoxicity*: no data	3 (only one strain used, only 3 concentrations used, duplicate plating, no data on cytotoxicity and compound purity)	Murray et al., 1979 (Murray and Cummins 1979)
Reverse mutation	*S. typhimurium* TA100	1 and 10 µmoles/plate	Positive outcome	3 (only one strain used, only 2 concentrations used, not tested with metabolic activation, no data on cytotoxiciy and compound purity, no positive control)	Watabe et al., 1980 (Watabe, Hiratsuka et al. 1980)
Reverse mutation	*S. typhimurium* TA100	Concentratino ranges not specified; 100 µl diluted compound/plate, samples tested over a dilution range of at least	Positive outcome *Cytotoxicity*: no data	3 (limited information on design and results; purity compound unknown; no metabolic activation used, only one strain used, concentrations tested not specified, no data on positive control, number of replicates	Ringo et al., 1982 (Ringo, Brennan et al. 1982)

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
		1 000-fold		and trials not known, no information on cytotoxicity	
Reverse mutation	*S. typhimurium* Strains: TA98, TA100	0.05 and 10 mg + / - incubation with Liver S9 mix	T98 and TA100 positive outcome without and with metabolic activation (NB: numbers of revertants were reported only for tests without S9; authors stated that addition of S9 did not alter the mutagenicity) No cytotoxicity observed	3 (limited information on design and results, no information compound purity and potential solvent used, only two concentrations tested, no information on number of trials, no standard deviations reported for results without S9, numbers of revertants with S9 not reported)	Wade et al., 1979 (Wade, Moyer et al. 1979)
Reverse mutation	*S. typhimurium* Strain: TA100	0.33, 1, 3.3 and 100 mM	Positive outcome. *Cytotoxicity*: about 20% and 60% growth inhibition at 3.3 and 100 mM, resp.	3 (limited information on design and results; purity compound unknown; no metabolic activation used, only one strain used, no information on what has been used as negative control, no data on positive control, number of replicates and trials not known)	Turchi et al., 1981 (Turchi, Bonatti et al. 1981)
Mammalian cells					
Gene mutation	Mouse lymphoma L5178Y tk^+/tk^- cells	0, 25, 50, 100, 200, 400 µg/ml	Positive outcome (Mean mutant frequency (mutants/10E6 clonable cells): at 0 through 200 µg/ml, resp. 48, 157, 273, 895 and 804 (test 1); 96, 175, 274, 590 and 1,595 (test 2).)	2	NTP 1989 (program 1989); McGregor et al., 1988 (McGregor, Brown et al. 1988)
Gene mutation	V79 Chinese hamster cells	4 concentrations up to 10 mM.	Positive outcome *Cytotoxicity*: LD_{50} of 2.3 mM was calculated from survival curve	3 (limited information on design and results; purity compound unknown; no metabolic activation used, no data on positive control, number of replicates per concentration not known, means and standard deviations of mutants not tabulated (results shown only in dose-effect curve), no purity data.	Turchi et al., 1981 (Turchi, Bonatti et al. 1981)
Chromosome aberration	Chinese hamster ovary Cells	- S9 mix: 37.8, 50.3, 62.9 µg/ml + S9 mix: 447, 503, 548 µg/ml	Positive outcome with and without metabolic activation; % of cells with aberrations (* indicates statistical significance): -S9: 3, 43*, 82*, 100* +S9: 5, 33*, 45*, 60* for control through highest concentration, resp.	2	NTP 1989 (program 1989)
Chromosome aberration and micronucleus test	V79 Chinese hamster cells	2 mM (no data on possible other concentrations)	Chromosome aberrations: positive Micronuclei: negative	3 (limited information on design and results; purity compound unknown; no metabolic activation used, no data on positive control, number	Turchi et al., 1981 (Turchi, Bonatti et al. 1981)

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
				of replicates per concentration not known, results shown for only one concentration, no standard deviations reported); No purity.	
Other supporting studies					
Reverse mutation	*Saccharomyces cerevisiae*	25, 50, 75 mM	Positive outcome *Cytotoxicity*: survival 100, 80, 65, 55% at 0 through 75mM, resp.	3 (limited information on design and results, no metabolic activation used, no information on compound purity, no information on potential solvent used, no information on what has been used as negative control, no data on positive control)	Bronzetti et al., 1980 (Bronzetti, Bauer et al. 1980)
Mitotic gene conversion and mitotic cross over	*Saccharomyces cerevisiae*	25, 50, 75 mM	Positive outcome for mitotic gene conversion and mitotic cross over *Cytotoxicity*: survival 100, 80, 65, 55% at 0 through 75mM, resp.	3 (limited information on design and results, no metabolic activation used, no information on compound purity, no information on potential solvent used, no information on what has been used as negative control, no data on positive control)	Bronzetti et al., 1980 (Bronzetti, Bauer et al. 1980)
Sister chromatid exchange	Chinese Hamster Ovary cells	- S9 mix: 1.12, 3.73, 11.2 µg/ml + S9 mix: 37.3, 112, 373 µg/ml	Positive outcome with and without S9 mix. *Cytotoxicity*: most of the increases in SCEs occurred in the absence of overt toxicity	2	NTP 1989 (program 1989)
Comet assay	Human skin biopt	0, 50, 160, 500, 1600 µl/cm^2	Positive outcome *Cytotoxicity*: viability test; not cytotoxic	3 (no validated study)	Rues (Reus, Usta et al. 2012)

* + or - S9, with or without metabolic activation system. (Klimisch, Andreae et al. 1997)

** (Klimisch, Andreae et al. 1997)

4-Vinylcyclohexene diepoxide was found to be mutagenic in various strains of *Salmonella typhimurium*, in the presence and absence of an exogenous metabolic system (Murray and Cummins 1979, Wade, Moyer et al. 1979, El-Tantawy and Hammock 1980, Simmon and Baden 1980, Watabe, Hiratsuka et al. 1980, Frantz and Sinsheimer 1981, Turchi, Bonatti et al. 1981, Ringo, Brennan et al. 1982, Mortelmans, Haworth et al. 1986, program 1989). *Salmonella typhimurium* strain TA100 was used most frequently and consistently showed positive results. Strains TA1535 and TA98, used in four studies, showed positive results in all (TA1535) or three (TA98) studies. Strain TA1537, used in only two studies, was positive with metabolic activation but equivocal or negative without activation. Furthermore, exposure resulted in an increased mutant frequency in L5175Y mouse cells at the heterozygous *tk* locus in the absence of metabolic activation (McGregor, Brown et al. 1988, program 1989). 4-Vinylcyclohexene diepoxide caused an increase in the number of Chinese hamster ovary cells with chromosome aberrations in the presence and absence of metabolic activation (program 1989). Moreover, 4-vinylcyclohexene diepoxide induced sister chromatid exchanges in Chinese hamster ovary cells in the presence and absence of metabolic activation.
The studies with *Saccharomyces cerevisiae* were considered not adequate for genotoxicity assessment because of deficiencies in design and reporting. Two publications of Mabon and Randerath in 1996 on the formation of DNA adducts by 4-vinylcyclohexene diepoxide (not summarized in Table 11) (Mabon and Randerath 1996, Randerath and Mabon 1996) were identified.The authors showed that 4-vinylcyclohexene diepoxide is able to produce DNA-adducts *in vitro* (calf thymus DNA), using the 32P-postlabelling technique (Mabon and Randerath 1996, Randerath and Mabon 1996). The adduct levels were, however, far below those generally found for highly potent carcinogens (such as benzo[a]pyrene) at comparable doses.

Overall it is concluded that 4-vinylcyclohexene diepoxide is mutagenic *in vitro* causing gene mutations and chromosomal aberrations.

Table 12: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations if any	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
DNA-adduct formation	4-vinylcyclohexene diepoxide	The assay was applied to skin DNA of mice exposed topically to diepoxides of these dienes	A dose-dependent increase in *in vivo* adduct formation was observed for both compounds.	Mabon 1996 (Mabon and Randerath 1996)
DNA-adduct formation	4-Vinylcyclohexene diepoxide	Assay was performed in female ICR mice	At higher doses (36-225 micro mol/mouse), adduct levels *in vivo* showed a linear dose response, while there was no difference between 14 and 36 micro mol/mouse. The limit of detection was estimated to be 1-3 adducts in 10(8) DNA nucleotides.	Randerath 1996 (Randerath and Mabon 1996)

Mabon and Randerath (1996) also showed that 4-vinylcyclohexene diepoxide is able to produce DNA-adducts in female ICR mice (topical skin application; 17-225 µmol/mouse; once a day for three days), using the 32P-postlabelling technique (Mabon and Randerath 1996, Randerath and Mabon 1996). The adduct levels were, however, far below those generally found for highly potent carcinogens (such as benzo[a]pyrene) at comparable doses. No other *in vivo* mutagenicity studies were retrieved.

Reus et al. 2012 describes the use of *ex vivo* human skin tissue for safety evaluation of chemicals which are direct in contact with the skin. Several chemicals were tested including vinylcyclohexene dioxide. The study showed that human skin obtained from surgery is a promising and robust model for safety evaluation of chemicals that are in direct contact with the skin. Vinylcyclohexene dioxide (VCD) was maximal dosed at 1600 µg/m^2. VCD which has been reported positive for carcinogenicity in rat and mice after dermal exposure, clearly demonstrated a statistically significant increase in %tail DNA at non-cytotoxic dose levels (Reus, Usta et al. 2012).

10.9.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

Below, only data are summarized of a reliable experimental design according to the Klimisch criteria 1 and 2 (Klimisch, Andreae et al. 1997).

Germ cell genotoxicity
As no relevant genotoxicity studies of 4-vinylcyclohexene diepoxide in germ cells were found, it is not possible to make a conclusion whether 4-vinylcyclohexene diepoxide is mutagenic in germ cells.

Somatic cell genotoxicity
Vinylcyclohexene diepoxide was investigated predominantly in *in vitro* genotoxicity tests only for the 3 endpoints of genotoxicity: gene mutations, structural and numerical chromosome aberrations.
In both *in vitro* (calf thymus DNA) and *in vivo* (skin of mice treated topically) studies 4-vinylcyclohexene diepoxide produced DNA-adducts. 4-Vinylcyclohexene diepoxide induced gene mutations in *Salmonella typhimurium* strains in the presence and absence of metabolic activation and in mammalian cells (mouse lymphoma study, tk locus) in the absence of metabolic activation (El-Tantawy and Hammock 1980, Simmon and Baden 1980, Frantz and Sinsheimer 1981, program 1989).

Exposure to vinylcyclohexene diepoxide did also result in an increase in cells with chromosome aberrations with and without metabolic activation. The supporting genotoxicity tests confirmed the positive findings in *in vitro* tests (Table 11). *In vivo*, no other mutagenicity studies were retrieved (Mabon and Randerath 1996, Randerath and Mabon 1996).

10.9.2 Comparison with the CLP criteria

According to the criteria in Annex VI of the European regulation No. 1272/2008, classification as a mutagen in category 1 is warranted when positive evidence for *in vivo* heritable germ cell mutagenicity in humans (1A) or mammals (1B) has been reported. No data have been presented on human germ cell mutagenicity. Overall, due to a lack of data it is concluded that there is no evidence for *in vivo* heritable germ cell mutagenicity of 4-vinylcyclohexene diepoxide. In addition, substances may be categorized in 1B if there are "positive results from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells". The latter may be based on a) "supporting evidence from mutagenicity/ genotoxicity tests in germ cells *in vivo*", or b) "by demonstrating the ability of the substance or its metabolites to interact with the genetic material of germ cells". No evidence has been found for *in vivo* mutagenicity testing in mammals. Regarding the second part of the criterion, there is no evidence that 4-vinylcyclohexene diepoxide is genotoxic in germ cells. Overall, due to lack of data on

germ cell mutagenicity, no evidence exists that 4-vinylcyclohexene diepoxide has the potential to cause mutations to germ cells. If substances do not meet the criteria for classification in category 1, they may be classified in category 2 if there is "positive evidence from experiments in mammals and/or in some cases from *in vitro* experiments obtained from a) somatic cell mutagenicity tests *in vivo*, in mammals" or b) "other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays". However, there is no relevant data from *in vivo* experiments in mammals, only from *in vitro* experiments (El-Tantawy and Hammock 1980, Simmon and Baden 1980, Frantz and Sinsheimer 1981, program 1989). Therefore, we do not recommend a classification as a germ cell mutagen category 2.

10.9.3 Conclusion on classification and labelling for germ cell mutagenicity

Based on the available data, it is not recommend to classify 4-vinylcyclohexene diepoxide as a germ cell mutagen.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

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

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

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

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

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

Comments received during public consultation

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

Assessment and comparison with the classification criteria

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

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

***In vitro* studies**

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

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

Summary results for Klimisch Score 2 studies are presented below:

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

Hammock, 1980	TA98	Without S9 Cytotoxicity: 2 mg/plate	**Negative** in TA1537 and TA98

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

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

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

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

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

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

***Ex vivo* studies**

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

***In vivo* studies**

Although there are no available *in vivo* mutagenicity studies, RAC recognises positive *in vivo* genotoxicity studies, and considers them reliable enough for assessment. The studies showed the formation of DNA adducts by VCD *in vitro* (calf thymus DNA) and *in vivo* in mice, after topical exposure. In the *in vivo* experiment, female ICR mice received, for 3 days, topical applications of acetone (control), VCD or 1,3-butadiene diepoxide (BDE) in acetone at 17, 51 or 153 µmoL/mouse (Mabon and Randerath, 1996), or acetone or VCD at 14.4, 36, 90 or 225 µmoL/mouse (Randerath and Mabon, 1996). Skin DNA was isolated from five mice per group, 5 h after the third treatment, and DNA adducts were measured by the monophosphate ^{32}P-postlabeling assay. The results showed dose-dependent increases in DNA-adduct formation (Table 1 from the Mabon and Randerath (1996) article, presented in *Supplemental information - In depth analyses by RAC in Background document*).

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

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

According to the CLP criteria, "substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2".

Although VCD, as a diepoxide, triggers several alerts for mutagenicity (see reports from ToxTree in *Supplemental information - In depth analyses by RAC*: Figures 1-3), there are no structurally highly similar analogues for VCD in OECD QSAR Toolbox and ToxTree for *in vivo* mutagenicity endpoints. In addition, as pointed out by the DS in the RCOM, Annex VI of the CLP does not include any (di)epoxide with a germ cell mutagenicity classification (regarding

two structural analogues with similar *in vitro* mutagenicity profile that are stated in the NTP Report 1989, please see *Supplemental information - In depth analyses by RAC*). It could therefore be concluded, that VCD currently has no adequate analogues for a reliable read across for *in vivo* mutagenicity prediction. Nevertheless, although VCD-triggered structural alerts cannot fulfil a criterion required by the CLP stated above, they are a supportive evidence for the mutagenicity potential of VCD.

Conclusion

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

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

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

Comparison with the CLP criteria:

- Since there is no data from human population on mutagenicity of VCD, Category 1A is not warranted;
- Also, there is no *in vivo* animal data for heritable germ cell mutagenicity or for somatic cell mutagenicity, therefore Category 1B does not appear justified;
- On the other hand, for VCD there are *in vivo* somatic cell genotoxicity tests (*in vivo* DNA adducts formation in mouse skin), supported by positive results from *in vitro* mutagenicity assays, which could trigger Category 2 classification according to the CLP.

Although available *in vivo* genotoxicity studies on DNA adducts formation (Randerath and Mabon, 1996; Mabon and Randerath, 1996) are open literature studies with some limitations, they are considered reliable enough to serve at least as supportive evidence. Positive findings in these studies are supported by the carcinogenic potential of VCD, regarding both local (skin) and systemic (ovary) tumorigenesis, which is considered to be, at least partially, a consequence of its mutagenicity (Please see the discussion in "*Assessment and comparison with the classification criteria*" in the sections "*RAC evaluation of carcinogenicity*" and "*RAC evaluation of reproductive toxicity*"). Structural alerts for VCD mutagenicity add to this conclusion. Direct ovarian toxicity of VCD indicates that VCD reaches mammalian germ cells.

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

Supplemental information - In depth analyses by RAC

Mabon and Randerath (1996): the formation of DNA adducts by VCD *in vivo* in mice, after topical exposure is presented in the table below.

Table 1
Dose-dependent formation of major DNA adducts in mouse skin treated with BDE or VCD

Dose (μmol/mouse)	Adduct levels (RAL × 10^8 ± S.E.M.)			
	1	2	3	Total
17[a]	5.2 ± 0.2	10.6 ± 0.9	10.8 ± 0.7	26.6 ± 1.8
51[a]	10.6 ± 0.5	23.3 ± 0.4	25.2 ± 1.8	59.1 ± 2.5
153[a]	32.6 ± 1.0	70.2 ± 0.5	82.8 ± 4.9	185.6 ± 5.3
r[b]	>0.99	>0.99	>0.99	>0.99
17[c]	ND[d]	ND	ND	ND
51[c]	19.5 ± 2.3	6.0 ± 1.3	4.4 ± 0.3	29.9 ± 3.4
153[c]	69.9 ± 2.4	28.9 ± 4.8	14.4 ± 1.8	113.2 ± 14.9

[a]Treatment with BDE.
[b]Linear correlation coefficient.
[c]Treatment with VCD.
[d]Adducts not consistently detected.

Results are expressed as relative adduct labelling (RAL × 10^8) values, representing minimum estimates of the number of adducts in 10^8 DNA nucleotides. BDE = 1,3-butadiene diepoxide.

ToxTree reports regarding mutagenicity alerts for VCD

Figure S1. *Alert for DNA binding*

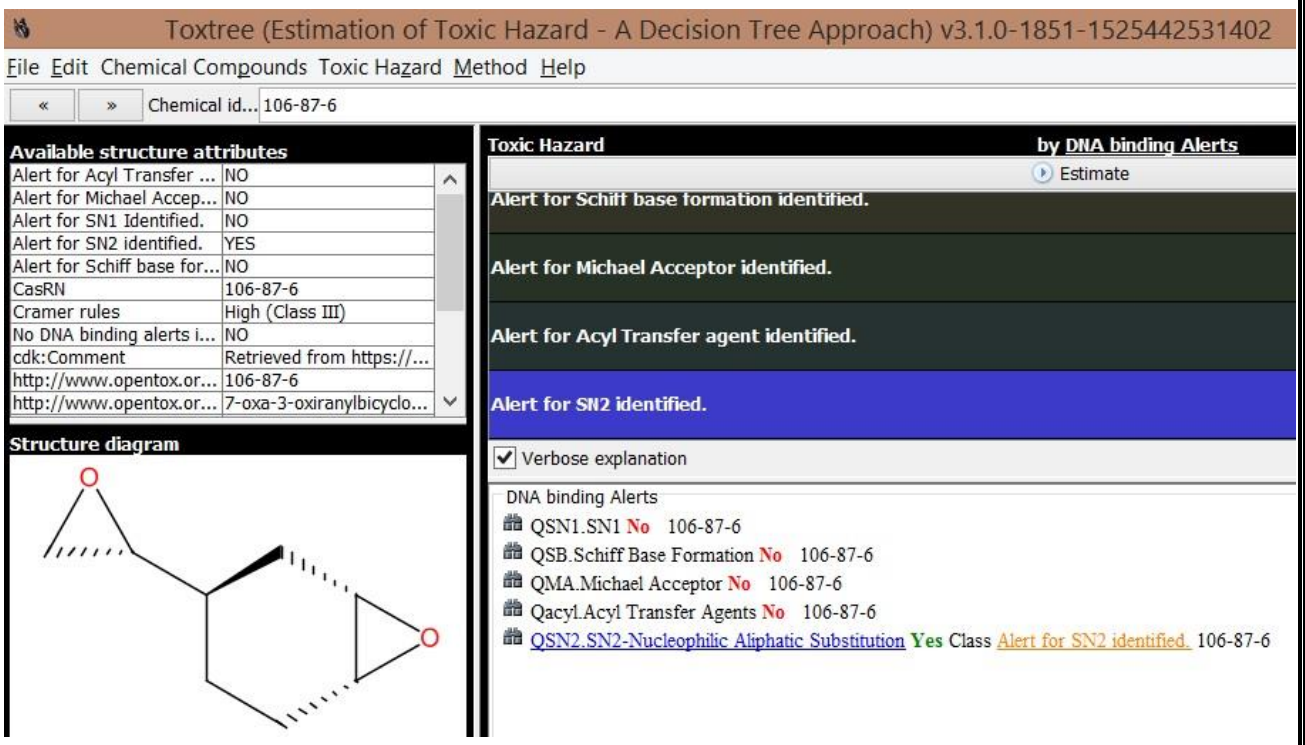


Figure S2. *Alert for in vivo micronucleus assay in rodents*

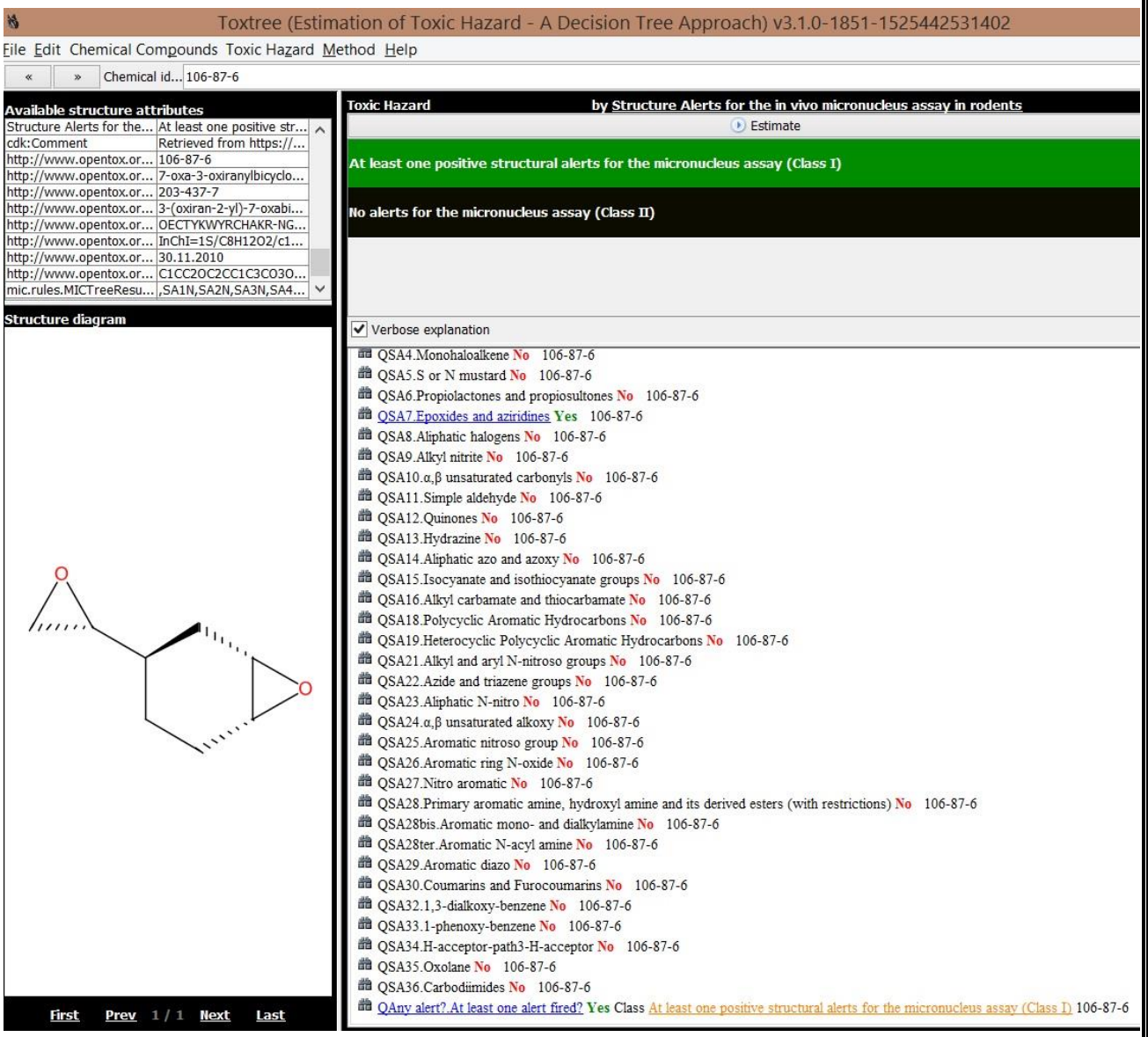
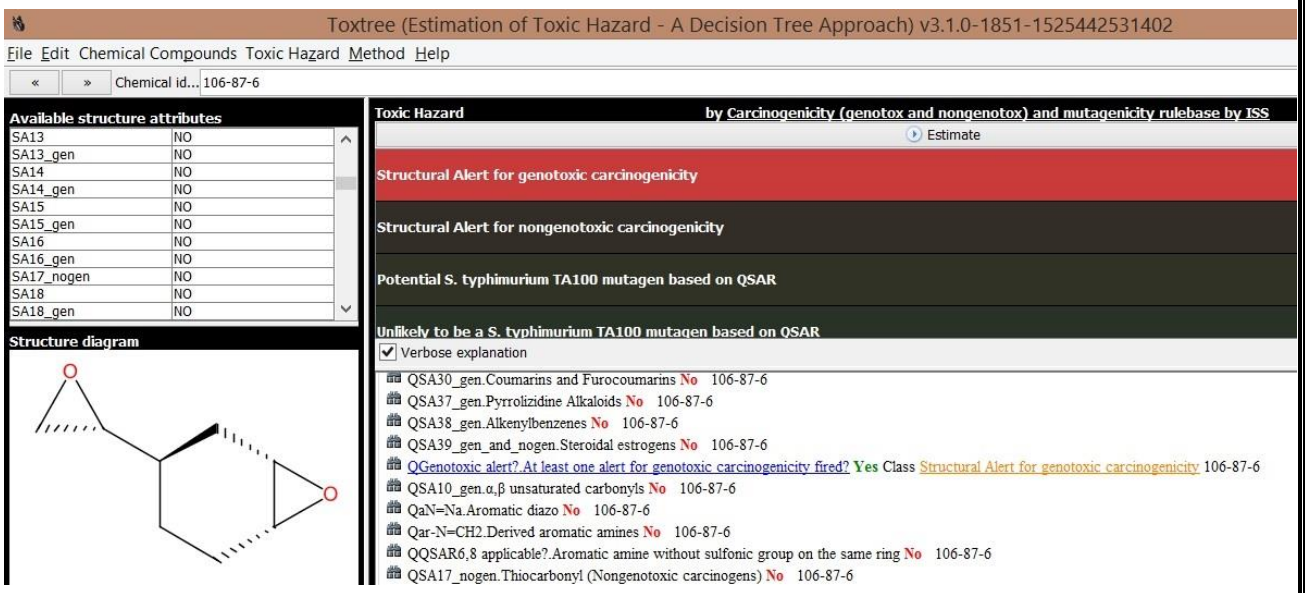


***Figure S3**. Alert for genotoxic carcinogens*



Two structural analogues of VCD, stated in the NTP report (1989)

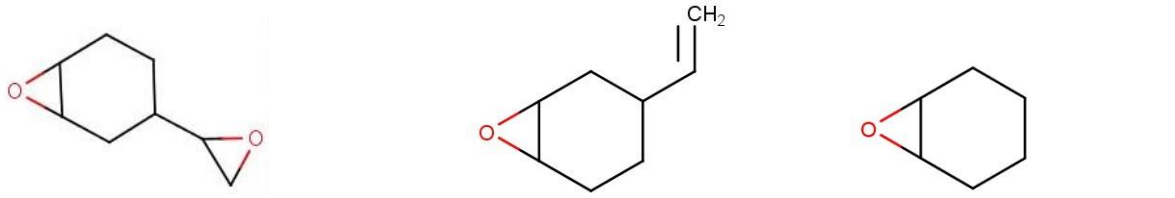
In the NTP report it is stated that the structural analogues of VCD, 3-ethenyl-7-oxabicyc10[4.1.0]heptane (1,2-epoxy-4-vinylcyclohexane, CAS 106-86-5) and 1,2-epoxycyclohexane (CAS 286-20-4), demonstrate similar mutagenic profiles to VCD, since both substances are mutagenic in bacterial assays and in mammalian cells *in vitro*. Nevertheless, these substances have no harmonised classification yet.

According to the classification provided by companies to ECHA in CLP notifications, 1,2-epoxy-4-vinylcyclohexane is toxic to aquatic life with long lasting effects, may cause cancer, is a flammable liquid and vapour, causes serious eye irritation, is harmful if inhaled, is harmful if swallowed, is suspected of causing cancer and causes skin irritation (ECHA Infocard).

According to the classification provided by companies to ECHA in REACH registrations, 1,2-epoxycyclohexane is suspected of causing genetic defects. The REACH registration dossier states that genotoxicity of the test material was determined to be negative (not clastogenic) according to an *in vivo* micronucleus study in male Fischer 344 rats (NTP, 1994), performed according to a similar procedure as in OECD TG 475. However, since several available mutagenicity *in vitro* studies in mammalian cells were positive, the registrant concluded that the overall weight of evidence suggests that the substance is mutagenic, and proposed that the test material should be classified as Muta. 2; H341: Suspected of causing genetic defects.

To conclude, although these two substances are structurally similar to VCD, they do not have harmonised classification and there are no positive data of their mutagenicity *in vivo*. Therefore, they can currently not serve as a basis to classify VCD as Muta. 2, since relevant CLP criteria refers to structure activity relationship to 'known germ cell mutagens' ("substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2").

Figure S4. *Chemical structures of VCD and two analogues, 1,2-epoxy-4-vinylcyclohexane and 1,2-epoxycyclohexane.*



VCD 1,2-epoxy-4-vinylcyclohexane 1,2-epoxycyclohexane

10.10 Carcinogenicity

Data on animal carcinogenicity studies are summarized in Table 13. Regarding tumour development, increased incidences of skin tumours, predominantly squamous cell carcinomas, were observed at the site of application in male and female rats of both doses groups. Details are shown in Table 14.

Table 13: Summary table of animal studies on carcinogenicity

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
Dermal application				
344/N rats	60/sex/dose 5 days/week duration of dosing (Xpo) = 105 weeks duration of experiment (Xpe) = 106-107 weeks 10 animals/sex Study duration 15 months. Statistical analysis tumour incidences: Life table tests, logistic regression tests (with adjustment for intercurrent mortality), Cochran-Armitage trend test, and Fisher exact test	Dermal application, 5 days/week; 0, 15, 30 mg/rat	Klimisch-score: 2 *Neoplastic lesions:* + at 0, 15 and 30 mg, resp. Skin tumours listed below occurred at application site. Skin squamous cell carcinoma 0/50, 33/50 ($p<0.001$), 36/50 ($p<0.001$) in males, 0/50, 16/50 ($p<0.001$), 34/50 ($p<0.001$) in females. Skin squamous cell papilloma 0/50, 3/50, 6/50 ($p<0.05$) in males, 0/50, 0/50, 1/50 in females; animals with this tumour also had a squamous cell carcinoma. Skin basal cell adenoma 0/50, 0/50, 4/50 ($p<0.05$) in males, none in females. Skin basal cell carcinoma 0/50, 1/50, 3/50 in males, 0/50, 3/50, 4/50 ($p<0.05$) in females. Skin sebaceous gland adenoma 0/50, 2/50, 1/50 in males, 1/50, 1/50, 1/50 in females. *General:* survival rates males 7/50, 8/50, 4/50; females 27/50, 23/50, 15/50 at 0, 15 and 30 mg resp. (significantly lower than control between day 637-715 at 15 mg, from day 648 at 30 mg) Body weight about 10% lower than control at 30 mg after week 49 in males and after week 57 in females. *Non-neoplastic lesions:* significantly increased incidence of acanthosis and sebaceous gland hypertrophy at application site at 15 and 30 mg.	NTP 1989 (program 1989); Chhabra et al.,1990 (Chhabra, Huff et al. 1990); Maronpot 1987 (Maronpot 1987)
$B6C3F_1$ mice	60/sex/dose 5 days/week Xpo = 103 weeks Xpe = c. 105 weeks 10 animals/sex	Dermal application: 0, 2.5, 5 and 10 mg/ mouse	Klimisch-score: 2 *Neoplastic lesions*: at 0, 2.5, 5 and 10 mg, resp. Skin squamous cell carcinoma (application site): 0/50, 14/50 ($p<0.001$), 39/50 ($p<0.001$), 42/50 ($p<0.001$) in males, 0/50, 6/50 ($p<0.05$), 37/50 ($p<0.001$), 41/50 ($p<0.001$) in females. Ovary: granulosa cell tumour benign or malignant:	NTP 1989 (program 1989); Chhabra et al., 1990 (Chhabra,

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
	Study duration 15 months. Statistical analysis tumour incidences: Life table tests, logistic regression tests (with adjustment for intercurrent mortality), Cochran-Armitage trend test, and Fisher exact test		0/50, 0/49, 7/49 ($p<0.01$), 12/50 ($p<0.01$). Ovary: benign mixed tumour: 0/50, 0/49, 11/49 ($p<0.001$), 6/50 ($p<0.01$). Lungs: alveolar/bronchiolar adenoma or carcinoma: 4/50, 9/50, 11/50 ($p<0.05$), 7/50 in females. *General*: survivalrates males 38/50, 35/50, 4/50, 0/50, females 30/50, 31/50, 15/50, 10/50 at 0, 2.5, 5 and 10 mg, resp. Body weight lower than control, dose-dependently, at 5 and 10 mg in both sexes (after week 29). Clinical signs: crusts, scales and ulcers at application site. *Non-neoplastic lesions:* (increased incidences of): - Skin: acanthosis, hyper-keratosis and necrotizing inflammation at application site in both sexes at all doses (statistically significant except for inflammation at 2.5 mg); - Ovaries: follicular atrophy and tubular hyperplasia at all doses; - Spleen: hematopoietic cell proliferation, primarily due to hyperplasia of myeloid elements (in response to skin inflammation and neoplasms) in both sexes, most markedly at 5 and 10 mg; - Epididymis: subacute inflammation at 5 and 10 mg.	Huff et al. 1990); Maronpot 1987 (Maronpot 1987)
C57BL/6 mice p53+/-	- p53: 7 male, 8 female - wild-type: 5/sex Treated: - p53: 7 male, 8 female (low-dose) or 10/sex (high-dose) - wild type: 5/sex (high dose) Xpo = 24 weeks Xpe = 28 weeks	Dermal application: 0, 12.5 (p53 only), 25 mg/mouse	Klimisch-score: 2 *Neoplastic lesions*: Skin tumours (squamous cell or basal cell carcinoma or fibrosacroma): p53: 0/7, 2/7, 3/10 in males, 0/8, 0/8, 3/8 in females; none in wild-type mice *General*: Mortality: 2/10 p53 males at 25 mg and 2/8 p53 females at 12.5 mg; no deaths in the other groups. In p53 and wild-type slight, dose-related decrease in weight gain throughout Xpo, reversed after cessation of treatment Skin: Nodular epidermal hyperplasia which appeared to be a continuum with the development of squamous cell carcinomas (no further details).	Tennant et al., 1995 (Tennant, French et al. 1995); Tennant et al., 1996 (Tennant, Spalding et al. 1996)
Swiss-Millerton mice	Treated: 30 males Controls: - vehicle:150 males (3 x 30; 1 x 60) - untreated: 207 males (4 x 27-30; 1 x 60) -benzo(a)pyrene in benzene: 90 (3 x 30) Xpo = Life span Xpe = Life-span Statistical analysis:	Dermal application: ca. 100 mg of solution/application	Klimisch-score: 2 *Neoplastic lesions*: Numbers of mice with tumour (total = papillomas or squamous cell carcinoma [scc]): - treated: total 14, 9 of these scc - vehicle: total 11 (2-5/group), 1 of these scc - untreated: total 13 (0-5/group), 1 of these scc - benzo(a)pyrene: total 49 (10-23/group), 26 of these scc (6-13/group) *General*: Mortality: median survival time: 326 days for treated mice, 262-412 for vehicle controls, 112-345 for untreated controls, 348-370 for positive controls	Van Duuren et al., 1963 (Vanduuren, Nelson et al. 1963)

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
	life-table analysis			
C3H mice (sex not specified)	Treated: 30-40 Control: no Data Xpo= Life-span (max. 21 months) Xpe= Life-span Tumour observations: for papillomas and carcinomas during each painting period. Statistical analysis: no data	Dermal application No quantitative data on doses.	Klimisch score: 3 *Neoplastic lesions*: Skin, application site: - papillomas: in 3 mice - carcinomas: in 1 mouse First tumour appeared at 17 months *General*: Mortality: 18, 6 and 0 survivors at 12, 17 and 24 months, resp.	Weil et al., 1963 (Weil, Condra et al. 1963)
Albino mice (no further information)	Treated: 20 males Control: no Data Xpo= 12 months Xpe= Life-span Method of tumour detection: no data Statistical analysis: no data	Dermal application Ca 16 mg/mouse	Klimisch score: 3 *Neoplastic lesions*: Skin, application site: - Squamous cell carcinoma: 4/9 - Mixed cell sarcoma: 3/9 - Both of above tumours: 2/9 Lung: - adenoma, probably malignant: 1/9 (in mouse with both skin tumours) - adenomata showing no signs of malignancy: 2/9 (in mice with skin carcinomas) *General*: Mortality: Last mouse died at 21 months after initiating treatment. 9 mice died without tumours and 2 died with papillomata that regressed after treatment cessation.	Hendry et al., 1951 (Hendry, Homer et al. 1951)
$CB6F_1$-TgH*ras*2 and wild type $CB6F_1$ mice.	Vehicle control: 10/sex/strain Treated: generally 15/sex/strain/dose Xpo = 24 weeks Xpe = 26 weeks Statistical analysis tumour incidences: Fisher exact test	Dermal application 0, 5, 10 mg/mouse	Klimisch score: 4 (not a representative 2-year study only supportive) *Neoplastic lesions*: Skin papilloma ($p<0.05$ for Tg females dosed with 10 mg), forestomach papilloma, thymic lymphoma, lung adenoma: increased incidences in treated Tg and non-Tg mice compared to vehicle controls (incidences in Tg mice were higher than in non-Tg mice). Skin squamous cell carcinomas and spleen hemangiosarcomas in treated Tg mice (not in treated non-Tg mice). *General*: No data	Yamamoto et al., 1998 (Yamamoto, Urano et al. 1998)
Intraperitoneal injection				
Female Sprague-Dawley rats,	Young (1month old): • treated: 12 and 21 rats at low- and high-dose, resp. • vehicle control: 17 Rats (interim kill after 15 doses: 10 high-dose rats, 7 controls); Mature (3 months old): • treated: 7 and 12 rats at low- and high-dose, resp. • vehicle control: 17 rats	Intraperitoneally 25 doses between post-natal days (PND) 35-68 (young rats) or PND 94-119 (mature rats) Dose: 80 (low) and 160 (high) mg/kg body weight/day	Klimisch score: 3 (no individual animal data reported, low number of animals used, route of exposure not relevant) *Neoplastic lesions:* Young rats: dose-related acceleration of onset and increase of incidence of fibroadenoma at low- and high-dose (from 38% to 84%); • Mature rats: tumour onset and incidence (0% in all groups) not affected.	Wright et al., 2011 (Wright, Frye et al. 2011)
Albino	Treated: 10 males and	25 mg/100 g body	Klimisch-score: 3	Hendry

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
rats (no further information)	4 females Control: no data; study duration \;	weight; 2 days/week	(Not adequate for carcinogenicity assessment. Deficiencies: contaminated test material of unknown purity used, very limited information on study design and results, insufficient number of animals used, no controls, short exposure period, route of exposure not relevant, limited information on non-cancer effects.) *Neoplastic lesions:* Mixed-cell sarcoma tissue in peritoneal cavity and large area of lung infiltrated with tumour tissue: in one male at 7 months.	et al., 1951 (Hendry, Homer et al. 1951)
C57 Black mice	Treated: 20 (sex not specified) Control: no data	Exposure route, frequency and duration, vehicle, purity test material, observation period, method of tumour detection: no data Concentration: 0.5 mM	Klimisch score: 3 (Not adequate for carcinogenicity assessment. Deficiencies: very limited information on study design and results, sex animals not specified, low number of animals used, no data on purity of test material and exposure conditions, no data on noncancer effects.) *Tumours* (in survivors): Skin tumours: 1/16 Malignant lymphomas: 4/16 First tumour (type not specified) appeared at 14 months	Kotin and Falk 1963 (Kotin and Falk 1963)
33 day study with Female rats	Young SD rats were administered 25 intraperitoneal (i.p.) doses of VCD between post-natal days (PND) 35–68	80 mg/kg or 160 mg/kg; n = 12 and 21, respectively, or vehicle (1.25 µL/g/d DMSO;n = 17). They were monitored for 22 months for persistent estrus and tumor development.	Final mammary tumor incidence in both low and high dose VCD animals significantly exceeded that of controls (83–85% vs. 38%;). Additionally, VCD had a dose-dependent effect on tumor burden, as the average number of tumors per affected rat was double and triple that of controls with 80 and 160 mg/kg VCD, respectively.	Wright 2011 (Wright, Frye et al. 2011)

The carcinogenicity studies in experimental animals are summarized in Table 13. The summarized studies comprise seven dermal studies (six in mice and one in rats), two studies in intraperitoneally exposed rats and one study in mice using an unspecified administration route. No long-term oral and inhalation studies were identified.

The National Toxicology Program (NTP) performed carcinogenicity studies in rats and mice (Chhabra, Huff et al. 1990, program 2011). Groups of 60 male and 60 female F344/N rats and B6C3F1 mice received 4-vinylcyclohexene diepoxide by topical application at doses of 0 (vehicle), 15 or 30 mg/animal (rats) five days per week for 105 weeks, and 0 (vehicle), 2.5, 5 or 10 mg/animal (mice), five days per week up to 103 weeks. At month 15, ten animals from each group were sacrificed for interim histopathological examination. (Table 14)

Table 14: Survival and skin tumour incidences in F344 rats, which were given 4-vinylcyclohexene diepoxide by dermal application for 2 years (program 1989).

	Overall rates			Terminal rates[a]		
Dose (mg/rat)	0	15	30	0	15	30
Survival						
males	7/50	8/50	4/50			
females	27/50	23/50	15/50			
Tumour incidences						
males						
skin: basal cell adenoma or carcinoma	0/50	1/50	6/50	0/7	0/8	1/4
skin: squamous cell papilloma	0/50	3/50	6/50*	0/7	1/8	0/4
skin: squamous cell carcinoma	0/50	33/50**	36/50**	0/7	8/8	4/4
females						
skin: basal cell carcinoma	0/50	3/50	4/50*	0/27	2/23	2/15
skin: squamous cell carcinoma	0/50	16/50**	34/50**	0/27	14/23	15/15

[a] Terminal rates are tumour incidence rates in animals, which were still alive at 105 weeks.
* $p<0.05$ versus vehicle control; ** $p<0.01$ versus vehicle control.

Table 15: Survival and tumour incidences in mice, which were given 4-vinylcyclohexene diepoxide by dermal application for 2 years (program 1989).

	Overall rates				Terminal rates[a]			
Dose (mg/mouse)	0	2.5	5	10	0	2.5	5	10
Survival								
males	38/50	35/50	4/50	0/50				
females	30/50	31/50	15/50	12/50§				
Tumour incidences								
males								
skin: squamous cell carcinoma	0/50	15/50**	39/50**	42/50**	0/38	10/35	4/4	0/0
females								
skin: squamous cell carcinoma	0/50	6/50*	37/50**	41/50**	0/30	3/31	15/15	0/0
ovary: luteoma, granulosa cell tumour, benign mixed tumour, or malignant cell tumour	1/50	0/49	17/49*	18/50*	1/30	0/31	7/14	0/0
lung: alveolar/bronchiolar adenoma or carcinoma	4/50	9/50	11/50*	7/50	3/30	7/31	4/15	0/0

[a] Terminal rates are tumour incidence rates in animals, which were still alive at 105 weeks.
§ Number of animals alive at week 85. * $p<0.05$ versus vehicle control; ** $p<0.01$ versus vehicle control.

Table 16: Survival of rats in the two-year dermal studies of 4-vinylcyclohexene diepoxide (program 1989).

	Vehicle Control	15 mg/Rat	30 mg/Rat
MALE (a)			
Animals initially in study	50	50	50
Natural deaths	12	5	16
Moribund kills	31	37	29
Animals surviving until study termination	7	8	4
Killed accidentally	0	0	1
Survival P values (b)	0.524	0.487	0.590
FEMALE (a)			
Animals initially in study	50	50	50
Natural deaths	8	13	21
Moribund kills	15	14	14
Animals surviving until study termination	27	23	15
Survival P values (b)	0.007	0.262	0.005

(a) First day of termination period: 743
(b) The result of the life table trend test is in the vehicle control column, and the results of the life table pairwise comparisons with the vehicle controls are in the dosed columns.

Survival in male rats was very low for all groups, controls included, but showed no significant differences between dosed males and controls (Table 16). Survival of high-dosed females was significantly lower compared to controls after day 648 and survival of low-dosed females was significantly lower between days 637 and 715. In the second year of the study, male and female rats of the high-dose group had slightly lower body weights than the controls.
Regarding tumour development, increased incidences of skin tumours at the site of application were observed in male and female rats in all dose groups (Table 14). No other treatment related tumours were observed. At the site of application, the treated animals had also significantly increased nonneoplastic skin lesions, such as acanthosis and sebaceous gland hypertrophy.

As in rats, 4-vinylcyclohexene diepoxide induced squamous cell carcinomas at the site of application in male and female mice, as shown in Table 15. No other treatment related skin tumours were observed. However, nonneoplastic skin lesions, such as acanthosis, hyperkeratosis and necrotizing inflammation (mid and high dose groups), were found to be significantly increased in both male and female mice at all dose groups. Furthermore, in treated female mice a significant increase of number of animals with ovarian tumours were observed compared to vehicle controls (see table 15). Also in female mice, an increased incidence of lung tumours in the mid-dose group was found, but not in the highdose group. No other treatment related tumours were found in any of the exposed groups.

Tennant et al. (1995, 1996) used 4-vinylcyclohexene diepoxide as model compound to examine the potential of transgenic mouse models to identify carcinogens and non-carcinogens (Tennant, French et al. 1995, Tennant, Spalding et al. 1996). He used p53-deficient C57BL/6 mice which are susceptible to tumour development due to reduced expression of the p53 tumour suppressor gene. After dermal application of 4-vinylcyclohexene diepoxide at 12.5 or 25 mg/animal, two times per week for 24 weeks, treated transgenic mice developed the same type of squamous cell tumours at the application site as did normal mice in the two-year dermal carcinogenicity study of the NTP2.

Yamamoto et al. used 4-vinylcyclohexene diepoxide as model carcinogen to validate a transgenic mouse bioassay, using *ras*H2 (CB6F1) mice carrying the human prototype c-Ha-*ras* gene, for rapid carcinogenicity testing (Yamamoto, Urano et al. 1998). In various human and animal tumours *ras* genes are activated by point mutations. Therefore, this transgenic mouse line should be vulnerable to developing tumours. 4-Vinylcyclohexene diepoxide was applied to the dorsal skin of the transgenic (Tg) and non-transgenic mice (non-Tg mice) at 5 or 10 mg/kg bw/day, five times per week for 24 weeks. 4-Vinylcyclohexene diepoxide induced skin papillomas around the site of application 26 weeks after initiation of treatment; the incidence of skin papillomas was statistically significantly increased in high-dose female Tg mice compared with vehicle control Tg mice. At the high-dose the incidence of skin papillomas was significantly higher in Tg mice (both sexes) than in non-Tg mice. Furthermore, forestomach papilloma, thymic lymphoma and lung adenoma were induced in treated Tg mice and, to a lesser extent, in treated non-Tg mice. Additionally, skin squamous cell carcinomas and spleen hemangiosarcomas were observed in Tg mice but not in non-Tg mice. The review of Yamamoto et al. does not present further details on study design and results.

The studies of the NTP were well performed and reported and, therefore, considered suitable for assessing the carcinogenic potential of 4-vinylcyclohexene diepoxide. In the NTP studies 4-vinylcyclohexene diepoxide was carcinogenic for F344/N rats and B6C3F1 mice of both sexes, causing skin (application site) squamous cell neoplasms (predominantly carcinomas) and basal cell

neoplasms (adenomas and carcinomas) in rats and skin squamous cell carcinomas in mice. In addition, 4-vinylcyclohexene diepoxide induced ovarian neoplasms (benign or malignant granulosa cell tumours, benign mixed tumours) and possibly lung neoplasms (alveolar/bronchiolar adenomas or carcinomas) in female mice. Two dermal studies in transgenic mice provided supportive evidence for the carcinogenicity of 4-vinylcyclohexene diepoxide in mice. P53-deficient C57BL/6 mice developed the same type of skin squamous cell tumours at the application site as did normal mice in the two-year mouse study by the NTP. In rasH2 (CB6F1) mice 4-vinylcyclohexene diepoxide induced skin papillomas around the site of application, forestomach papilloma, thymic lymphoma, lung adenoma, squamous cell carcinoma and spleen hemangiosarcoma. Most of these tumours was also induced in the treated non-transgenic CB6F1 included in this study.

Although the design of the above studies in transgenic mice differs considerably from that of a conventional two-year rodent carcinogenicity bioassay, these studies provide supportive evidence for the carcinogenicity of 4-vinylcyclohexene diepoxide in mice.The NTP studies showed that mice were more susceptible to 4-vinylcyclohexene diepoxide-induced ovarian toxicity and carcinogenicity than rats. A plausible explanation for this observation is a difference in detoxification capacity. Hoyer and Sipes (1996) referred to a study which showed that the mouse, as compared with the rat, has a reduced capacity to convert 4-vinylcyclohexene diepoxide to its inactive tetrol derivate (Hoyer and Sipes 1996).

Human carcinogenicity

There is no literature available regarding human exposure to 4-vinylcyclohexene diepoxide leading to carcinogenicity.

Other relevant information

No transformation studies on the potential carcinogenicity of 4-vinylcyclohexene diepoxide were available.

10.10.1 Short summary and overall relevance of the provided information on carcinogenicity

No data on the carcinogenicity of 4-vinylcyclohexene diepoxide in humans were available.

The 2-year bioassays conducted by NTP showed that skin application of 4-vinylcyclohexene diepoxide produced squamous cell neoplasms (predominantly carcinomas) and basal cell neoplasms (adenomas and carcinomas) in male and female rats and skin squamous cell carcinomas in male and female mice. In female mice 4-vinylcyclohexene diepoxide also induced ovarian neoplasms (benign or malignant granulosa cell tumours, benign mixed tumours) and possibly lung neoplasms (alveolar/bronchiolar adenomas or carcinomas). The tumours in the skin and ovaries are considered to be relevant for humans. An increase in the incidence of lung tumours in a mouse carcinogenicity study is generally considered to have little relevance to man. Moreover, in the mouse study with 4-vinylcyclohexene diepoxide the incidence of lung tumours was increased in only one sex and this finding was not unequivocally related to treatment.

The carcinogenic mechanism through which 4-vinylcyclohexene diepoxide exerts its effect on ovarian follicles is not completely understood. The results of the genotoxicity studies in the previous section provide evidence for a stochastic mechanism. Further it has been proposed that elevated levels of gonadotropins in response to oocyte depletion (due to the loss of negative feed-back on the hypothalamic-pituitary axis) act as promotors of ovarian tumour development. However, this hypothesis is not uniformly supported by experimental results (Hoyer and Sipes 1996).

Table 17: Compilation of factors to be taken into consideration in the hazard assessment

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
Consistent increase in tumours was observed in both rats and mice	The observed increase in dermal tumours at the site of exposure normally has a low to zero incidence in control animals	The increase in tumours is limited to the site of exposure in rats but is extended to at least the ovaries in mice.	The skin tumours in both rats and mice progress to malignancy. The ovary tumours in female mice were partly malignant.	Tumour latency was significantly reduced as no such tumours were observed in the control animals	Skin tumours were observed in both male and female rats and mice	Local skin toxicity due to the irritating properties may have contributed to the formation of tumours. However, especially in rats the local effects were limited to acanthosis and sebaceous gland hypertrophy but no necroses or inflammation, indicating no excessive toxicity. In mice necrotizing inflammation was observed at relevant concentrations indicating that excessive toxicity may have contributed.	The dermal route is considered relevant to humans.	The available information indicates that both local irritation and probable local mutagencity have contributed to the increase in dermal tumours. Both mechanisms are considered relevant to humans.

The table with the factors (table 17) to be taken into consideration when assesing the overall level of concern in general support classification in category 1B as the observed tumours were consistent between species and sexe (dermal tumours), and have a low incidence in control animals and progress to malignancy. The only concern is whether the dermal tumours could be secondary to the local skin toxicity. However, in rats the local toxicity is limited to acanthosis and sebaceous gland hypertrophy but no necroses or inflammation. In addition, based on the available data the substance is expected to be genotoxic and mutagenic in the skin. Therefore, it is likely that the contribution of the local toxicity to the tumour formation in rats is limited. In mice, local necrotic inflamation is also observed and could have a higher contribution to the formation of skin tumours. However, local toxicity can be expected from high exposure to mutagenic substances and some mutations will result in cell death. Overall, it is considered unlikely that the formation of tumours is limited to concentrations only inducing excessive toxicity.

10.10.2 Comparison with the CLP criteria

No data on the carcinogenicity of 4-vinylcyclohexene diepoxide in humans were available. Adequate studies on carcinogenicity in experimental animals were available for the dermal route. In these studies 4-vinylcyclohexene diepoxide was carcinogenic in rats and mice of both sexes, causing skin (application site) squamous cell neoplasms (predominantly carcinomas) and basal cell neoplasms (adenomas and carcinomas) in rats and skin squamous cell carcinomas in mice. In addition, 4-vinylcyclohexene diepoxide induced ovarian neoplasms (benign and malignant granulosa cell tumours, benign mixed tumours) and posibly lung neoplasms (alveolar/bronchiolar adenomas or carcinomas) in female mice. The contribution of excessive local toxixcity is considered to be limited.

According to the CLP criteria, 4-vinylcyclohexene diepoxide should, therefore, be classified as “presumed to be as carcinogenic to humans”, which corresponds to classification in catergory 1B. Supporting evidence is that the substances shows genotoxic properties in bacterial and mammalian cells *in vitro*, genotoxicity ex-vivo in the skin and DNA adducts *in vitro* and *in vivo*.

10.10.3 Conclusion on classification and labelling for carcinogenicity

4-vinylcyclohexene diepoxide is "presumed to be carcinogenic to man", and classifying the substance in category 1B is proposed. As no information on the carcinogencity by other routes is available but also systemic tumours are observed in mice, a limitation to a single route is not warranted. The available information on potency do not justify an SCL.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

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

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

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

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

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

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

- consistent substance-related increase in skin tumours was observed in both rats and mice of both genders, and tumours progressed to malignancy;

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

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

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

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

Comments received during public consultation

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

Assessment and comparison with the classification criteria

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

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

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

NTP study in rats

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

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

General toxicity

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

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

Neoplastic changes

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

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

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

dose. Hyperkeratosis was observed only at the high dose. The latent period for development of skin neoplasms was shorter at the high dose compared to the low dose (e.g. the day of first observation of squamous cell carcinoma in the skin was day 596 at low dose and day 543 at high dose in males; Table A3 and B3 in NTP Report, 1989).

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

Non-neoplastic changes

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

NTP study in mice

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

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

General toxicity

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

Neoplastic changes

In both genders, the incidence of skin squamous cell carcinomas at the application site was

significantly higher than in controls and in non-application skin areas in exposed animals. In exposed groups, they were above the HCD range (NTP HCD ranges from relevant time period). Increased incidence occurred already after 15-month exposure, and was dose-dependent. In many animals, carcinomas metastasised to lymph nodes or visceral organs. Squamous cell carcinomas occurred at an incidence slightly above the HCD range also in skin areas away from the site of VCD application. The study authors considered that this could be due to inadvertent spread of the study material away from the application site, but also due to metastasising process (via subcutaneous lymph nodes) from skin carcinoma at the application site. The latent period for development of skin neoplasms was shorter at higher doses compared to lower doses. For example, the day of first observation of squamous cell carcinoma in the skin was day 525, 411 and 376 at low, mid and high dose, respectively, in males, and day 642, 402 and 376 at low, mid and high dose, respectively, in females (Table C3 and D3 in NTP, 1989).

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

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

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

<u>Non-neoplastic changes</u>

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

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

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

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

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

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

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

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

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

Conclusions

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

In both rodent species, a dose-related increase in incidence of benign and malignant skin

tumours was observed, especially of squamous cell carcinoma. The increase was observed in both genders, in the absence of marked general toxicity. In mice, dermal exposure to VCD was also related to increased incidence of ovarian tumours, already in the absence of pronounced general toxicity.

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

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

Due to the fact that:

- there are no data on carcinogenetic effects of VCD in humans, which would warrant classification in Category 1A, while
- there are animal experiments showing sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen):
    - dose-dependent, significant increase in the incidence of benign and malignant skin tumours was observed in two rodent species (rats and mice), and in both genders,
    - tumours in extra-dermal sites, away from the site of exposure, were also observed (ovarian tumours in mice),
    - latent period for tumour development followed inverse dose-response relationship,
    - tumours were observed already at dose levels which did not induce significant general or skin toxicity,

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

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

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

Supplemental information - In depth analyses by RAC

2-year NTP carcinogenicity study in rats (1989)

Table S1. NTP 2-year dermal carcinogenicity study in rats (1989)

Dose level (mg VCD/rat/day)	**Males**			**HCD**[‡]	**Females**			**HCD**[‡]
	0	**15**	**30**	(range)	**0**	**15**	**30**	(range)
At 15 months								
Number of rats/group	10	10	10		10	10	10	
Survival (N)	10	10	10		10	10	10	
Body weight (% of Control)	(ref)	97	90		(ref)	98	93	
Organ relative weights	n.e.	n.e.	n.e.		n.e.	n.e.	n.e.	
Neoplastic changes								
Skin, squamous cell carcinoma (application site)	0	0	2	0/50 -2/49	0	0	0	
Forestomach, squamous cell carcinoma	0	0	0		0	0	1	NA
After 2 years								
Number of rats/group	50	50	50		50	50	50	
Survival [N (%)]	7 (14)	8 (16)	4 (8)		27 (54)	23 (46)	15 (30)*	
Body weight (% of Control)[§]	(ref)	102	89		(ref)	103	86	
Neoplastic skin changes – overall rates, application site								
Basal cell adenoma or carcinoma	0	1	6	0/50 – 4/50	0	3[†]	4[†]*	0/50 – 1/50
Squamous cell papilloma	0	3	6*	0/50 -2/49	0	0	0	0/50 -1/50
Squamous cell carcinoma	0	33*	36*	0/50 -2/49	0	16*	34*	0/50 -1/49
Neoplastic skin changes – overall rates, non-application site								
Basal cell adenoma	1	0	0	0/50 – 3/50	1	1	0	0/50 – 1/50
Basal cell carcinoma	1	1	0	0/50 – 2/50	0	0	0	0/50 – 1/50
Trichoepithelioma	0	0	1		0	0	0	
Neoplastic skin changes –other organs								
Urinary bladder papilloma	0	0	0		0	2	0	0/50 - 1/49

Results are presented as number of animals, if not stated otherwise.
*P < 0.05; statistically significant compared to controls
†Basal cell carcinoma
‡NTP historical control data base (Haseman *et al.*, 1984, 1985) for studies of at least 104 weeks, including 1 596 male and 1 643 female rats
§In animals that survived until the end of experiment
n.e. – no effect
NA – not available
Organ relative weights for necropsies performed after 15 months were not available.

TABLE 6. NUMBERS OF RATS WITH SELECTED SKIN LESIONS IN THE FIFTEEN-MONTH DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE (a)

Lesion	Male (mg/rat)			Female (mg/rat)		
	0	15	30	0	15	30
Acanthosis	0	**7	**9	0	*5	**10
Hyperkeratosis	0	0	*5	0	0	**10
Sebaceous gland hyperplasia	0	**7	**8	0	0	**10
Squamous cell carcinoma	0	0	2	0	0	0

(a) Ten animals were examined in each group.
*P<0.05 vs. controls
**P<0.01 vs. controls

TABLE 9. NUMBERS OF RATS WITH SELECTED SKIN LESIONS IN THE TWO-YEAR DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE (a)

Site/Lesion	Male (mg/rat)			Female (mg/rat)		
	0	15	30	0	15	30
Skin, application site (b)						
Skin, scapula						
Acanthosis	0	**39	**40	4	**33	**42
Sebaceous gland hypertrophy	0	**28	**39	1	**20	**43
Basal cell adenoma	0	0	*4	0	0	0
Basal cell carcinoma	0	1	2	0	3	*4
Basal cell carcinoma (multiple)	0	0	1	0	0	0
Sebaceous gland adenoma	0	1	1	0	1	1
Squamous papilloma	0	3	*6	0	0	1
Squamous cell carcinoma	0	**10	**12	0	**8	**12
Squamous cell carcinoma (multiple)	0	**22	**24	0	**8	**22
Skin, back						
Acanthosis	0	*6	**8	1	4	**11
Sebaceous gland hypertrophy	0	4	**8	0	0	**15
Sebaceous gland adenoma	0	1	0	1	0	0
Squamous cell carcinoma	0	2	1	0	0	1
Skin, nonapplication site (c)						
Acanthosis	0	2	1	0	0	0
Sebaceous gland hypertrophy	0	1	1	0	0	0
Basal cell adenoma	1	0	0	1	1	0
Basal cell carcinoma	1	1	0	0	0	0
Trichoepithelioma	0	0	1	0	0	0

(a) Most dosed animals had more than one lesion; 50 animals were examined in each group.
(b) Skin, application site, includes skin from the interscapular region where chemical was applied (skin, scapula) and skin adjacent to site of application (skin, back).
(c) Skin, nonapplication site, is skin from areas distant from application site.
*P<0.05 vs. the vehicle controls
**P<0.01 vs. the vehicle controls

2-year NTP carcinogenicity study in mice

Table S2. NTP 2-year dermal carcinogenicity study in mice (1989)

	Males				HCD	Females				HCD
Dose level (mg VCD/mouse/day)	**0**	**2.5**	**5**	**10**	(range)	**0**	**2.5**	**5**	**10**	(range)
At 15 months										
Number of rats/group	10	10	10	10		10	10	10	10	
Survival (N)	10	10	10	10		10	10	10	10	
Body weight (% of Control)	(ref)	97	99	87		(ref)	103	106	101	
Uterine relative weight (% of Control)	-	-	-	-				31	42	
Neoplastic changes										
Sebaceous gland adenoma	0	0	0	0		0	1	1	1/10	
Keratoacanthoma	0	0	0	1		0	0	0	0/10	
Benign basosquamous skin tumous	0	0	0	1		0	0	0	0/10	
Squamous skin papilloma	0	0	1	2		0	0	1	1/10	
Squamous skin cell carcinoma	0	0	2	8*		0	0	2	5/10*	
Ovarian granulosa cell tumour	-	-	-	-					2/9	
Ovarian papillary cystadenoma	-	-	-	-					1/9	
Non-neoplastic changes										
Sebaceous gland hyperthrophy/hyperplasia	0	4	10*	7*		0	5*	9*	10*	
Ovarian atrophy	-	-	-	-		1	10	10	10	
Tubular hyperplasia	-	-	-	-				8	9	
After 2 years										
Number of rats/group	50	50	50	50		50	50	50	50	
Survival [N (%)]	38 (76)	35 (70)	4 (8)*	0*		30 (60)	31 (62)	15 (30)*	12 (24)§*	
Body weight (% of Control)Dossier Submitter†	(ref)	104	88	NA		(ref)	102	88	NA‡	
Neoplastic skin changes – overall rates, application site										
Malignant basosquamous tumour	0	2	0	3		0	0	1	1	
Basal cell carcinoma	0	0	1	0		0	0	0	0	
Squamous cell carcinoma	0	15*	39*	42*	0/50-1/49	0	6*	37*	41*	0/50-1/48
Neoplastic skin changes – overall rates, non-application site										
Squamous cell carcinoma	0	1	2	3	0/50-1/49	0	0	3	2	0/50-1/48
Neoplastic skin changes –other organs										
Ovarian tumours¶	-	-	-	-		1	0	17*	18*	0/50-3/47
Lung tumours#	10	11	9	3		4	9	11*	7	0/50-8/50
Non-neoplastic changes										
Spleen, haematopoietic cell proliferation	2	7	31	39		3	7	28	30	
Bilateral ovarian atrophy	-	-	-	-		3	32	29	39	
Bilateral tubular hyperplasia, ovary	-	-	-	-		4	20	21	21	
Epididymis, subacute inflammation	0	0	6	13		-	-	-	-	

Results are presented as number of animals, if not stated otherwise
*P < 0.05; statistically significant compared to controls
†In animals that survived until the end of study
‡At 84 weeks surviving animals were killed

[§]The number of mice alive at week 85 when all survivors of this group were killed
[¶]Luteoma, granulosa cell tumour, benign mixed tumour, or malignant granulosa cell tumour
[#]Alveolar/bronchiolar adenoma or carcinoma.
(ref) = reference value
NA = not applicable
HCD = NTP historical control data base (Haseman *et al*., 1984, 1985) for studies of at least 104 weeks, including > 1 600 male and > 1 600 female mice

TABLE 16. NUMBERS OF MICE WITH SELECTED SKIN LESIONS IN THE FIFTEEN-MONTH DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE (a)

Lesion	Male (mg/mouse)				Female (mg/mouse)			
	0	2.5	5	10	0	2.5	5	10
Acanthosis	0	2	**10	**8	0	2	**9	**10
Hyperkeratosis	0	0	0	2	0	1	0	4
Sebaceous gland hyperplasia/hypertrophy	0	4	**10	**7	0	*5	**9	**10
Sebaceous gland adenoma	0	0	0	0	0	1	1	1
Keratoacanthoma	0	0	0	1	0	0	0	0
Benign basosquamous tumor	0	0	0	1	0	0	0	0
Squamous papilloma	0	0	1	2	0	0	1	1
Squamous cell carcinoma	0	0	2	**8	0	0	2	*5

(a) Ten animals were examined in each group.
*P<0.05 vs. controls
**P<0.01 vs. controls

TABLE 19. NUMBERS OF MICE WITH SELECTED LESIONS OF THE SKIN IN THE TWO-YEAR DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE (a)

Site/Lesion	Male (mg/mouse)				Female (mg/mouse)			
	0	2.5	5	10	0	2.5	5	10 (b)
Skin, application site (c)								
Skin, scapula								
Acanthosis	1	**35	**38	**35	4	**31	**41	**36
Hyperkeratosis	1	**12	**14	**21	1	**27	**29	**20
Necrotizing inflammation	1	4	**12	**15	2	5	**15	**16
Malignant basosquamous tumor	0	2	0	3	0	0	1	1
Basal cell carcinoma	0	0	1	0	0	0	0	0
Squamous cell carcinoma	0	**10	**27	**37	0	*5	**14	**31
Squamous cell carcinoma (multiple)	0	2	**12	*5	0	1	**23	**10
Skin, back								
Acanthosis	0	*6	*6	2	1	0	5	4
Hyperkeratosis	0	1	4	4	0	1	4	2
Necrotizing inflammation	0	0	0	1	0	0	*5	1
Squamous cell carcinoma	0	4	0	0	0	1	3	0
Squamous cell carcinoma (multiple)	0	0	0	0	0	0	1	0
Skin, nonapplication site (d)								
Acanthosis	0	*5	*6	*5	4	0	5	4
Hyperkeratosis	0	1	2	0	1	0	5	2
Necrotizing inflammation	0	0	*6	1	1	0	6	6
Squamous cell carcinoma	0	1	2	3	0	0	3	2
Squamous cell carcinoma (multiple)	0	0	1	0	0	0	0	1

(a) Fifty animals were examined in each group.
(b) Survivors were killed during week 85.
(c) Skin, application site, includes skin from the interscapular region where chemical was applied (skin, scapula) and skin adjacent to site of application (skin, back).
(d) Skin, nonapplication site, is skin from areas distant from application site.
*P<0.05 vs. vehicle controls
**P<0.01 vs. vehicle controls

NTP historical control data (Haseman *et al*., 1984, 1985) included in NTP report (1989) for those tumours in rats and mice that showed compound-related effects.

HCD for rats

TABLE A4a. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM BASAL CELL TUMORS IN MALE F344/N RATS (a)

	Incidence in Controls		
	Benign	Malignant	Benign or Malignant
No 2-year dermal studies using acetone as a vehicle are included in the historical data base.			
Overall Historical Incidence for Untreated Controls			
TOTAL	(b) 20/1,596 (1.3%)	(c) 10/1,596 (0.6%)	(d) 30/1,596 (1.9%)
SD (e)	1.82%	1.07%	2.16%
Range (f)			
High	3/50	2/50	4/50
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Includes 4 trichoepitheliomas, 1 adnexal adenoma, 4 sebaceous gland adenomas, and 11 basal cell tumors
(c) Basal cell carcinomas; one adenocarcinoma, NOS, was also observed.
(d) Includes 4 trichoepitheliomas, 1 adnexal adenoma, 4 sebaceous gland adenomas, 1 adenocarcinoma, 11 basal cell tumors, and 9 basal cell carcinomas
(e) Standard deviation
(f) Range and SD are presented for groups of 35 or more animals.

TABLE A4b. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM SQUAMOUS CELL TUMORS IN MALE F344/N RATS (a)

	Incidence in Controls		
	Papilloma	Carcinoma	Papilloma or Carcinoma
No 2-year dermal studies using acetone as a vehicle are included in the historical data base.			
Overall Historical Incidence for Untreated Controls			
TOTAL	(b) 21/1,596 (1.3%)	10/1,596 (0.6%)	(b) 31/1,596 (1.9%)
SD (c)	1.50%	1.08%	1.81%
Range (d)			
High	2/49	2/49	3/49
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Includes one papilloma, NOS
(c) Standard deviation
(d) Range and SD are presented for groups of 35 or more animals.

TABLE B4a. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM BASAL CELL TUMORS IN FEMALE F344/N RATS (a)

	Incidence in Controls		
	Benign	Malignant	Benign or Malignant
No 2-year dermal studies using acetone as a vehicle are included in the historical data base.			
Overall Historical Incidence for Untreated Controls			
TOTAL	(b) 3/1,643 (0.2%)	(c) 4/1,643 (0.2%)	(d) 7/1,643 (0.4%)
SD (e)	0.58%	0.66%	0.83%
Range (f)			
High	1/50	1/50	1/50
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Includes one trichoepithelioma
(c) All basal cell carcinomas
(d) Includes one trichoepithelioma, two basal cell tumors, and four basal cell carcinomas
(e) Standard deviation
(f) Range and SD are presented for groups of 35 or more animals.

TABLE B4b. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM SQUAMOUS CELL TUMORS IN FEMALE F344/N RATS (a)

	Incidence in Controls		
	Papilloma	Carcinoma	Papilloma or Carcinoma
No 2-year dermal studies using acetone as a vehicle are included in the historical data base.			
Overall Historical Incidence for Untreated Controls			
TOTAL	(b) 4/1,643 (0.2%)	3/1,643 (0.2%)	(b) 7/1,643 (0.4%)
SD (c)	0.66%	0.59%	0.83%
Range (d)			
High	1/50	1/49	1/49
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Includes two papillomas, NOS
(c) Standard deviation
(d) Range and SD are presented for groups of 35 or more animals.

HCD for mice

TABLE C4. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM SQUAMOUS CELL TUMORS IN MALE B6C3F$_1$ MICE (a)

Study	Incidence in Controls		
	Papilloma	Carcinoma	Papilloma or Carcinoma
Historical Incidence in Dermal Studies Using Acetone as a Vehicle (b)			
JP-5 navy fuel	0/50	0/50	0/50
Marine diesel fuel	1/50	0/50	1/50
TOTAL	1/100 (1.0%)	0/100	1/100 (1.0%)
Overall Historical Incidence for Untreated Controls			
TOTAL	(c) 4/1,692 (0.2%)	5/1,692 (0.3%)	(c) 9/1,692 (0.5%)
SD (d)	0.82%	0.72%	1.02%
Range (e)			
High	2/50	1/49	2/50
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Studies conducted at Litton Bionetics, Inc.
(c) Includes one papilloma, NOS
(d) Standard deviation
(e) Range and SD are presented for groups of 35 or more animals.

TABLE D4a. HISTORICAL INCIDENCE OF INTEGUMENTARY SYSTEM SQUAMOUS CELL TUMORS IN FEMALE B6C3F$_1$ MICE (a)

Study	Incidence in Controls		
	Papilloma	Carcinoma	Papilloma or Carcinoma
Historical Incidence in Dermal Studies Using Acetone as a Vehicle (b)			
JP-5 navy fuel	0/48	0/48	0/48
Marine diesel fuel	0/50	0/50	0/50
TOTAL	0/98 (0.0%)	0/98 (0.0%)	0/98 (0.0%)
Overall Historical Incidence for Untreated Controls			
TOTAL	2/1,689 (0.1%)	2/1,689 (0.1%)	4/1,689 (0.2%)
SD (c)	0.49%	0.49%	0.84%
Range (d)			
High	1/48	1/48	2/48
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Studies conducted at Litton Bionetics, Inc.
(c) Standard deviation
(d) Range and SD are presented for groups of 35 or more animals.

TABLE D4b. HISTORICAL INCIDENCE OF OVARIAN TUMORS IN FEMALE B6C3F$_1$ MICE (a)

Study	Incidence of Granulosa Cell Tumors in Controls
Historical Incidence in Dermal Studies Using Acetone as a Vehicle (b)	
JP-5 navy fuel	(c) 1/47
Marine diesel fuel	0/50
TOTAL	1/97 (1.0%)
Overall Historical Incidence for Untreated Controls	
TOTAL	(d) 16/1,577 (1.0%)
SD (e)	1.71%
Range (f)	
High	3/47
Low	0/49

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Studies conducted at Litton Bionetics, Inc.
(c) Luteoma
(d) Includes four luteomas, two benign mixed tumors, and one granulosa cell carcinoma
(e) Standard deviation
(f) Range and SD are presented for groups of 35 or more animals.

TABLE D4c. HISTORICAL INCIDENCE OF ALVEOLAR/BRONCHIOLAR TUMORS IN FEMALE B6C3F$_1$ MICE (a)

Study	Incidence in Controls		
	Adenoma	Carcinoma	Adenoma or Carcinoma
Historical Incidence in Dermal Studies Using Acetone as a Vehicle (b)			
JP-5 navy fuel	0/48	3/48	3/48
Marine diesel fuel	0/50	0/50	0/50
TOTAL	0/98	3/98 (3.1%)	3/98 (3.1%)
Overall Historical Incidence for Untreated Controls			
TOTAL	73/1,676 (4.4%)	35/1,676 (2.1%)	107/1,676 (6.4%)
SD (c)	3.35%	1.68%	3.76%
Range (d)			
High	6/49	3/50	8/50
Low	0/50	0/50	0/50

(a) Data as of May 12, 1988, for studies of at least 104 weeks
(b) Studies conducted at Litton Bionetics, Inc.
(c) Standard deviation
(d) Range and SD are presented for groups of 35 or more animals.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

10.11 Reproductive toxicity

10.11.1 Adverse effects on sexual function and fertility

Table 18: Summary table of animal studies on adverse effects on sexual function and fertility

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Controlled periclinical trial with nonhuman primates: 4 adult female cynomolgus monkeys of 8, 9, 12 and 13 years old.	4-vinylcyclohexene diepoxide	Once-daily intramuscular injections for 15 days of vehicle control sesame oil (n=1) or 250 (n=1), 160 (n=1) or 80 (n=1) mg/kg bw/day. Archived ovaries from two premenopausal monkeys (8 and 13 years old) were used as additional untreated comparators. Ovaries were removed 27 days after treatment and pathological determinations were made at necropsy (after 240 days).	At 250 mg/kg bw/day nearly complete elimination of primordial, intermediate, primary and secondary follicles was achieved, at 160 mg/kg bw/day a 50% elimination and at 80 mg/kg bw/day no elimination was achieved. No gross or histological lesions in the organs studied were found at postmortum evaluations after 9 months.	Appt et al. 2006 (Appt, Kaplan et al. 2006)
Female F344 rats, C57BL/6 mice, or AhR-deficient (_/_,AhRKO) mice.	4-Vinylcyclo-hexene diepoxide	Immature F344 rats, or C57BL/6 or AhR_/_ mice (d28) were weighed and dosed daily with one or more of the following treatments: vehicle sesame oil, VCD (80 mg/kg bw/day i.p.) and/or a AhR antagonist alpha-alphanaphthoflavone 20 or 80 mg/kg bw/day.	Compared with controls, VCD caused a 60% reduction ($P < 0.05$) in primordial and primary follicles in mice and rats.	Thompson et al. 2005 (Thompson, Bourguet et al. 2005)
Reproductive function study with: male and female Sprague-Dawley rats. Male rats were only used for mating purposes.	4-Vinylcyclo-hexene diepoxide	Intraperitoneally injection of doses; 0, 5, 20, 80 mg/kg bw/day from 2 weeks prior to mating to Day 7 of gestation (n=10 in each group/sex).	At necropsy, number of implanted embryos, rate of implantation decreased and the rate of preimplantation loss showed an increasing tendency in the 80 mg/kg group. The pre-implantation loss was considered to be a consequence of the decrease in small follicles. No changes observed in animals given 5 or 20 mg/kg bw/day. Histopathologically, the ovaries showed a decrease in number of small follicles at 80 mg/kg bw/day. Decreased absolute and relative ovary weights (left) in 80 mg/kg bw/day group.	Kodama et al. 2008 (Kodama, Yoshida et al. 2009)
Study with: Female Fischer 344 rats and B6C3F1 mice (age Day 28)	4-Vinyl-cyclo-hexene diepoxide	Daily dosed (vehicle or 80 mg/kg bw/day VCD, intraperitoneally) The same dose of VCD was used in both rats (n =3–6) and mice (n= 3), and dosing was for 6, 8, 10, or 12 d (mice and rats), or 15	Significant loss of primordial and primary follicles ($P < 0.05$) was measured on day 12 in both rats and mice. However, when compared with controls, follicle loss on that day was greater ($P < 0.05$) in mice (64.2 ± 4.5%) than in rats (34.7 ±4.9%). VCD-dependent increase ($P < 0.05$) in percent atretic *primordial* follicles was first	Kao et al. 1999 (Kao, Sipes et al. 1999)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		d (rats).	observed 4 h after the final dose in mice on Day 8 (VCD-treated, 44.4 ± 3.1% vs. control, 26.9 ± 5.4%). Conversely, in rats, this significant increase was not seen until Day 10 (VCD-treated, 44.3 ± 1.3% vs. control, 23.1 ± 4.0%). A VCD-dependent increase in percent atretic *primary* follicles was not observed in either species before Day 12. There was no significant effect on growing or preantral follicles on any day in either species.	
Two- or four week repeated dose study with: female Sprague-Dawley rats	4-Vinyl-cyclo-hexene diepoxide	Intraperitoneally dosed at 0, 5, 10 and 80 mg/kg bw/day VCD once a day for 2 or 4 weeks. (n=10 in each group)	In the 4-week study a decrease in small follicles was observed in the ovaries at 20 and 80 mg/kg bw/day. In the 2-week study , the same change was observed at 80 mg/kg bw/day.	Ito et al. 2009 (Ito, Mafune et al. 2009)
13-week dermal study with male and female B6C3F1 mice	4-vinylcyclohexene diepoxide	Ten mice of each sex per dose group were administered 0, 0.625, 1.25, 2.5, 5, or 10 mg VCD in acetone by dermal application to the clipped dorsal interscapular region, 5 days per week for 13 weeks. The interscapular region was clipped once per week. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. A single application of 0.1 ml was applied to the in-terscapular region of mice. The dose mixture was applied uniformly at the site of application.	There was no mortality in either study. No compound-related deaths occurred (doses up to 10 mg/mouse). Diffuse ovarian atrophy was observed in all females that received 10 mg/mouse and in 4/10 females that received 5 mg/mouse. Uterine atrophy was seen in 2/10 females that received 10 mg/mouse.	NTP 1989 (program 1989);
2-year study with $B6C3F_1$ mice	4-vinylcyclohexene diepoxide	Two-year studies were conducted by administering VCD in acetone by dermal application to the clipped dorsal interscapular re-gion for 103 weeks to groups of 60 mice of each sex at 0, 2.5, 5, or 10 mg per animal. The interscapular region was clipped once per week. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. For mice, a single application of 0.1 ml was applied to the	Ovary: Follicular atrophy and tubular hyperplasia were observed at increased ($P<0.001$) incidences in exposed mice (atrophy: vehicle control, 12/50;low dose, 43/49; mid dose, 42/49; high dose, 47/50; tubular hyperplasia: 5/50; 35/49; 38/49; 34/50). Ovarian atrophy was characterized by a complete absence of follicles and corpora lutea, whereas tubular hyperplasia consisted of multiple epithelial lined tubular structures extending from the surface epithelium into the interior of the ovary.	NTP 1989 (program 1989); Chhabra et al., 1990 (Chhabra, Huff et al. 1990);

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		interscapular region. The dose mixture was applied uniformly at the site of application.		
15 months dermal study with B6C3F$_1$ mice	4-vinylcyclohexene diepoxide	VCD in acetone was administered by dermal application to the clipped dorsal interscapular region for 15 months to groups of 60 mice of each sex at 0, 2.5, 5, or 10 mg per animal. The interscapular region was clipped once per week. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. For mice, a single application of 0.1 ml was applied to the interscapular region. The dose mixture was applied uniformly at the site of application.	*General*: survivalrates males 38/50, 35/50, 4/50, 0/50, females 30/50, 31/50, 15/50, 10/50 at 0, 2.5, 5 and 10 mg, resp. Body weight lower than control, dose-dependently, at 5 and 10 mg in both sexes (after week 29). *Non-neoplastic lesions:* Clinical signs: crusts, scales and ulcers at application site. - Ovaries: follicular atrophy and tubular hyperplasia at all doses; - Epididymis: subacute inflammation at 5 and 10 mg.	NTP 1989 (program 1989); Chhabra et al., 1990 (Chhabra, Huff et al. 1990); Maronpot 1987 (Maronpot 1987)
13 week oral study with male and female B6C3F1 mice	4-vinylcyclohexene diepoxide	Studies were conducted by administering VCD in corn oil by gavage, for 5 days per week, for 13 weeks to groups of 10 mice of each sex at 0, 62.5, 125, 250, 500, 1000 mg/kg bw/day.	Compound-related lesions were seen in the forestomach, testis, ovary, and uterus. Ovary: Diffuse ovarian atrophy was seen in 5/10 females receiving 250 mg/kg bw/day, 6/10 receiving 500 mg/kg bw/day, and 10/10 receiving 1000 mg/kg bw/day. Uterine atrophy was present in 7/10 mice receiving 1000 mg/kg bw/day. Multifocal to diffuse testicular degeneration was present in 8/10 males receiving 250 mg/kg bw/day, 8/10 receiving 500 mg/kg bw/day, and 9/10 receiving 1000 mg/kg bw/day.	NTP 1989 (program 1989);
2 year dermal study with male and female F344/N rats	4-vinylcyclohexene diepoxide	Two-year studies were conducted by administering VCD in acetone by dermal application to the clipped dorsal interscapular region for 5 days per week for 105 weeks to groups of 60 rats of each sex at 0, 15, or 30 mg per animal. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. Three 0.l ml consecutive applications were administered to rats with a 100 µl	No effects on ovaria. Acanthosis and sebaceous gland hypertrophy of skin from the scapula or back were observed at substantially increased incidences in ex- posed male and female rats. Squamous cell papillomas in male rats and squamous cell carcinomas in male and female rats were observed only in exposed rats.	NTP (National Toxicology 1989)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		micropipette.		
13 week dermal study with male and female F344/N rats	4-vinylcyclohexene diepoxide	Ten rats of each sex were administered 0, 3.75, 7.5, 15, 30, or 60 mg VCD in acetone by dermal application to the clipped dorsal interscapular region, 5 days per week for 13 weeks. The interscapular region was clipped once per week. The volume and concentration of the dose mixtures were not adjust-ed with changes in body weight. Three 0.1-ml consecutive applications were administered to rats with a 100 µ1 micropipette. The dose mixture was applied uniformly at the site of application.	No chemical related effects on survival or body weights. Compound-related clinical signs observed from week 7 or 11 included redness, scabbiness, and ulceration on the back at the application site and burrowing behavior after dermal application in the 60 mg/rat groups. Thymus weight to body weight ratios for males receiving 30 or 60 mg/rat were significantly lower than that for vehicle controls	NTP (National Toxicology 1989)
15 month dermal study with male and female F344/N rats	4-vinylcyclohexene diepoxide	15 month studies were conducted by administering VCD in acetone by dermal application to the clipped dorsal interscapular region, 5 days per week for 15 months to groups of 60 rats of each sex at 0, 15, or 30 mg per animal. The volume and concentration of the dose mixtures were not adjusted with changes in body weight. Three 0.l-ml consecutive applications were administered to rats with a 100 µ1 micropipette.	Organ weight to body weight ratios were not affected by dermal administration of 4-vinyl-l- cyclohexene diepoxide (Two of 10 male rats that received 30 mg had a squamous cell carcinoma of the skin at or adjacent to the site of application (Acanthosis was seen in exposed rats (mild severity at 30 mg/rat and minimal severity at 15 mg/rat); hyperkeratosis was observed for rats in the 30 mg/rat groups. One female receiving 30 mg/rat had a squamous cell carcinoma of the forestomach.	NTP (National Toxicology 1989)
17 day study with female C57BL mice	4-vinylcyclohexene diepoxide	Randomly selected (n=7-8 per group) female mice (28 d of age) were administered intraperitoneally VCD dissolved in sesame oil (160 mg/kg bw/day) or with sesame oil only (vehicle control) for 15, 17, 20, or 22 d. For fertility evaluation, more mature animals(age 91 d) were used to ensure that reproductive cyclicityhad become established (as determined	*General*: Maternal body weight was significantly reduced by 9% compared to controls. *Ovary:* A significant reduction in pregancies and pregnancies per copulatory plug was observed. The difference between group 1 and 2 was attributed to the presence of unaffected secondary and antral follicles in group 1 animals. No live fetuses were recorded in group 2. Histologic evaluation of the ovaries collected from control and VCDtreated animals in both groups revealed numerous primordial, primary, secondary, and antral	Haas et al 2007 (Haas, Christian et al. 2007)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		by vaginal cytology), The females were mated with untreated males on either the second proestrus after exposure (group 1; control (n = 5) and VCD-treated (n = 14)) or the first proestrus after day 20 after exposure (group 2; (control, n = 6; VCD-treated, n = 8)).	follicles in all control animals regardless of whether they had become pregnant. In contrast, there were essentially no follicles of any size in ovaries of VCD-treated animals regardless of whether they had become pregnant. The only detectable follicle population in those animals was an occasional antral follicle in animals that had become pregnant. *Fertility* There was a greater number of resorbed fetuses in group 1 VCD treated (1.9± 0.4) mice compared with controls (0.6± 0.2; $P < 0.05$). This count was lower than the 5 resorbed fetuses counted in the 1 group 2 VCD-treated mouse that became pregnant.	
28 day study with adult female Wistar rats	4-vinylcyclohexene diepoxide	The rats (10 rats/dose group) were orally administered with VCD (100, 250 and 500 mg/kg bw/day) for twenty-eight days. Control rats only received orally corn oil.	*General:* Compared to control (P< 0.05) VCD increased ovarian and uterine malondialdehyde (MDA) level (ovarian:100-500 mg/kg bw/day, uterine: 100 and 250 mg/kg bw/day), and catalase (ovarian+uterine:100-500 mg/kg bw/day), glutathione peroxidase (GPx) (ovarian:100-500 mg/kg bw/day, uterine: 100 and 250 mg/kg bw/day), and glutathione S-transferase (GST) activities (ovarian:100-250 mg/kg, uterine: 100-500 mg/kg bw/day in rats ($p < 0.05$). VCD exposure decreased the levels of progesterone (500 mg/kg), prolactin (100-250 mg/kw bw/day) and estrogen(500 mg/kg), but increased the levels of luteinizing hormone and follicle stimulating hormone (500 mg/kg bw/day). Also, VCD (100-250 mg/kg bw/day) increased ovarian superoxide dismutase (SOD) activity, and depleted uterine SOD activity (100 and 500 mg/kg bw/day) and ovarian glutathione (GSH) level in rats ($p < 0.05$) (100-500 mg/kg bw/day). *Ovary:* The histopathology of the ovary revealed large cystic follicles and scanty number of follicles following VCD administration. This is suggestive of ovotoxicity and corroborates existing literature that VCD depletes follicular number in rats. The observation that uterine histopathology revealed a gradual loss of the endometrial cells may be due to VCD-induced oxidative stress as shown above, thus representing another target of VCD toxicity not previously reported in the literature. VCD induced reproductive dysfunctions in rats via ovarian and uterine oxidative damage, hormonal imbalance, as well as inflammation and apoptosis in the ovary of rats.	Abolaji 2016 (Abolaji, Adedara et al. 2016)

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
30 day study with Female cynomolgus macaques (Macaca fascicularis) with a mean age of 9 y (range, 6 to 18 y). A poly-L-lacticco-glycolic acid fiber containing 200 mg VCD was placed around the ovary.	4-vinylcyclohexene diepoxide	Experiment 1 At baseline, an ovary was removed to serve as the untreated control and then a poly-L-lacticco-glycolic acid fiber containing 200 mg VCD was placed around the remaining ovaryfor 30 days in 8 monkeys. Experiment 2 Female monkeys in the VCD treatment group (n = 29) underwent a single laparotomy (as described for experiment 1) to place VCD fibers around both ovaries (200 mg VCD per ovary). These fibers were left in place, and the ovaries were not removed. Both ovaries were removed in an additional 20 monkeys so that they could serve as OVX comparators, and 20 monkeys were anesthetized but did not undergo laparotomy (premenopausal controls).	Experiment 1 At day 30 after surgical placement of a biodegradable fiber containing approximately 200 mg (VCD) next to one ovary in each of 8 monkeys, primordial follicles were reduced by approximately 70%, with a corresponding decrease (83%) in antimüllerian hormone (AMH, a serum marker of ovarian follicle numbers). At 4 mo after VCD-treatment of both ovaries in 29 monkeys (approximately 200 mg VCD per ovary), AMH was reduced 56% from baseline, testosterone was unchanged, and follicular phase estradiol was slightly increased. Data indicate that VCD treatment markedly reduced primordial follicles while preserving larger estradiol- and testosterone-producing follicles and ovarian stroma, a condition that mimics ROR in women. Experiment 2 Marked reductions in AMH were present after VCD fibers were placed adjacent to both ovaries (baseline, 14.1±2.0 ng/mL; after VCD treatment, 6.24±1.1 ng/mL; P = 0.001), with 14 of 29 monkeys having concentrations less than 3 ng/mL.	Appt 2010 (Appt, Clarkson et al. 2010)
2 week oral study with female rats (Simonson albino rats (a Sprague-Dawley derived strain))	4-vinylcyclohexene diepoxide	Female rats received 0.02 or 0.04% VCD in drinking water for 2 weeks prior to oocyte recovery; control females received plain drinking water. The estimated exposure of 20 and 40 mg/kg b.w. / day (estimated from an average water consumption of 10 ml / 100 g b.w.) would be expected to have a small effect on primordial and primary oocyte numbers and is one-fourth and one-half the levels that reduced primordial and primary oocyte numbers by two-thirds and reduced estrous cyclicity. Three replicates with each treatment in each replicate consisting of 3–6 females were evaluated. Oocytes were inseminated with sperm preincubated at 0.5	*General:* No effect on final weight or weight gain. *Ovary:* The fragility of oocytes may be slightly affected (51, 35, and 36% of the oocytes remained after removal of the zona pellucida for control, 0.02% VCD-exposed, and 0.04% VCD-exposed females, S.E.M. = 7; $P < 0.10$ for the comparisons with oocytes from control females). There was no effect of VCD at these exposure levels on the fertilizability of oocytes.	Berger 2003 (Berger and Horner 2003)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		× 10^6 sperm/ml.		
15 day study with female wild-type C57 mice (4 weeks old) using intraperitoneal injections	4-vinylcyclohexene diepoxide	Female mice were administered daily intraperitoneal injections of either sesame oil (2.5 ml/kg, vehicle control) or VCD (Sigma) dissolved in sesame oil (80 mg/kg bw/day) for 15 days, three or four mice per treatment group.	*Ovary*: 15 days of VCD treatment reduced the number of primordial follicles by 45% and primary follicles by 55% that of control mice, respectively, with a weak toxic effect on follicles of higher grade.	Chen, 2015 (Chen, Kang et al. 2015)
11-20 months study testing VCD on reproductive function in 1 and 3 month old female Sprague-Dawley rats.	4-vinylcyclohexene diepoxide	Vehicle (DMSO; *n* = 38), VCD at 80 mg/kg bw/day (low-dose VCD; *n* = 11), and VCD at 160 mg/kg bw/day (high-dose VCD; *n* = 40) was administered intraperitoneally (total volume, 0.25 to 0.35 mL) for a total of 25 doses, either on consecutive days or 5 times each week to female rats.	*General:* Adult rats treated with either dose of VCD weighed less (*P*< 0.001) than did vehicle-treated controls throughout the dosing period, with a final weight loss at end of dosing of less than 20% in high-dose VCD rats. Adult animals surviving high-dose VCD lost weight relative to baseline weights (*P* < 0.01), as compared with continued weight increases in vehicle-treated and low-dose VCD rats during the same period. Moreover, a significant (*P* < 0.001) number of high-dose VCD adult rats (*n* = 10) died or were euthanized (*n* = 1) during treatment (76% survivorship) as compared with no deaths in the vehicle or low-dose VCD groups. *Ovary:* Twenty-five daily doses of VCD (80 or 160 mg/kg daily compared with vehicle alone) depleted ovarian follicles in a dose-dependent fashion in rats of both ages, accelerated the onset of acyclicity, and caused dose-dependent increases in follicle-stimulating hormone that exceeded those naturally occurring with age in control rats but left serum levels of 17β−estradiol unchanged, with continued ovarian production of androstenedione. High-dose VCD caused considerable nonovarian toxicities in 3-mo-old Sprague–Dawley rats, making this an unsuitable model. In contrast, 1-mo-old rats had more robust dosedependent increases in follicle-stimulating hormone without evidence of systemic toxicity in response to either VCD dose.	Frye 2012 (Frye, Lukefahr et al. 2012)
15 week study with Fischer 344 rats.	4-vinylcyclohexene diepoxide	Rats were dosed daily for 15 days with vehicle control or VCD (80 mg/kg bw/day i.p.).	*Ovary*: Dosing of female rats with 4-vinylcyclohexene diepoxide (VCD), for 30 days destroyed the majority of ovarian primordial follicles.	Hoyer 2001 (Hoyer, Devine et al. 2001)
6 weeks study where female Sprague Dawley	4-vinylcyclohexene diepoxide	Rats were intraperitoneally (i.p.) injected with a dose of 80	There is a high correlation between premature ovarian failure initiation and ovarian autoimmunity and cardiovascular	Li 2014 (Li, Fan et al. 2014)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
(SD) rats are interperitoneally treated daily with 4-vinylcyclohexene diepoxide VCD).		mg/kg bw/day VCD (n=6) dissolved in 0.8 mL sesame oil , or the equal volumes of sesame oil in vehicle (n=6). Six weeks later after the final injection of VCD, all rats were scarified for tissue harvesting. Total RNA from ovarian tissue was converted to cDNA and hybridized to mRNA Chip array.	disorder. Levels of FSH and LH were significantly increased after VCD treated in rats plasma compared to the control groups.	
10 or 20 day study where female B6C3F1 mice are dosed with VCD at a concentration of 160 mg/kg/day.		Female mice (28 days old, n=8), were interperitoneally dosed daily for 10 or 20 days with VCD (160 mg/kg bw/d) or sesame oil. Animals were evaluated for reproductive function on day 10, 20, 35 after the onset of dosing, and on the day of follicle depletion.	VCD reduced the number of primordial (by 93.2%) and primary (by 85.1%) follicles after 10 days of dosing. All primordial and primary follicles were lost after 20 days of dosing. Relative to controls, in 20-day-dosed mice, there was a reduction of ($P<0.05$) in the number of all classes of ovarian follicles in VCD-treated animals. Follicle numbers in those animals were primordial (102.4±22.2 control, 0.3 ± 0.2 VCD; follicles counted per ovary), small primary (27.6 ± 4.7 control, 0.2±0.2 VCD; follicles counted per ovary), large primary (7.8±1.9 control, 0±0 VCD; follicles counted per ovary), secondary (59.4±4.7 control, 15.5±3.5 VCD;follicles counted per ovary and antral follicles (28.0 ± 3.1 control, 20.5± 1.8 VCD; follicles counted per ovary).	Lohff 2006 (Lohff, Christian et al. 2006)
30 day study with female Fisher 344 rats using intraperitoneal injections	4-vinylcyclohexene diepoxide	Female 28-day rats (n=5 per group) were dosed (30days) with VCD (80 mg/kg bw per day, i.p.) or vehicle, and animals were evaluated for reproductive function at subsequent time points for up to 360 days.	*General:* On day 360, total body weights were not different between VCD-treated and control groups. *Ovary*: VCD caused a loss in the number of preantral follicles at day 30 (137.2 ± 43.12, control; 49 ± 16.14, VCD follicles counted per ovary; $P < 0.05$). Treatment with VCD reduced ($P < 0.05$) the number of primordial (31 ± 5.4% of control), and primary (48.6 ± 9.5% of control) follicles on day 30. Following cessation of dosing, relative to control, primordial, primary, and secondary follicles were progressively lost ($P <0.05$) with time. The number of antral follicles in VCD-treated animals was reduced ($P < 0.05$) relative to control beginning on day 120. Concomitant with loss of follicles was a decrease in corpora Lutea. In summary, short-term dosing of rats (30 days) caused premature ovarian failure by 360 days after dosing.	Mayer 2002 (Mayer, Pearsall et al. 2002)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			VCD-induced follicle loss resulted in ovarian atrophy, disrupted cyclicity, increased plasma levels of FSH, and variable levels of circulating 17-estradiol at that time.	
30 day interperitoneally study with female Sprague–Dawley rats	4-vinylcyclohexene diepoxide	Adult and peripubertal rats were injected intraperitoneally daily for 30 d with vehicle or VCD at 40 or 80 mg/kg bw/day. Each treatment group consisted of 10 adult and 10 peripubertal rats,	*General*: Treatment with VCD did not affect body weight, but food intake was reduced in both adult and peripubertal rats treated with 80 mg/kg bw/day VCD. *Ovary*: At 80 mg/kg bw/day, VCD destroyed primordial and primary follicles to a similar extent in both adult and peripubertal animals, although adult rats likely started with fewer follicles and therefore approached follicle depletion.	Muhammad 2009 (Muhammad, Goode et al. 2009)
10 day interperitoneally study with young female Syrian hamsters (Mesocricetus auratus)	4-vinylcyclohexene diepoxide	Eight-week-old female hamsters were treated with 400 mg/kg bw/day VCD once daily for 10 days by i.p. injection. Control females were given daily vehicle (1:1 mixture of 0.9% saline and DMSO) injections. Injections were administered under isoflurane anesthesia (3%)during the light phase of the light–dark cycle (between 08:00 and10:00 EST).	*General:* Uterine mass did not differ between the two groups. Mean body masses at the start of treatment and at the time of behavioral testing were not significantly different. *Ovary*: VCD-treated females had significantly fewer primordial, primary, and secondary follicles than vehicle-treated controls. The number of primordial and secondary ovarian follicles in VCD-treated females did not differ from those counted in the ovaries of untreated 15-month-old females. VCD-treated females had fewer primary follicles than 15-month-old females. The number of ovarian follicles was not correlated with the mate preference behaviors of VCD-treated or control females.	Roosa 2015A (Roosa and Place 2015)
10 day study with female Siberian hamsters	4-vinylcyclohexene diepoxide	Siberian hamsterswere treated with VCD prepared in a 1:1 mixture of 0.9% saline and DMSO. (240 mg/kg bw/day i.p. for 10 days) during short days, and outcomes were compared with reproductively active females that were maintained and treated in long days. One week following the final injection, animals were either euthanized to harvest ovaries for the follicle counts (Experiment 1, n=10 per group) or remained in the study to evaluate their fertility (Experiment 2; n = 18–20/group).	Primordial follicle numbers were significantly reduced by VCD under both day lengths, and reproductive quiescence in shortdays did not appear to render the ovaries less susceptible to VCD-induced follicle depletion. Independentof day length and reproductive state, VCD-treated hamsters weaned substantially fewer offspring than controls. These results suggest that time of year may not be an important consideration for optimizinguse of VCD in the field when the target pest species is a seasonally breeding rodent.	Roosa 2015 B (Roosa, Mukai et al. 2015)
7 day study with female swiss	4-vinylcyclohexene	Female mice (6–10 animals per treatment	Results show a mechanism of VCD-induced ovotoxicity involving small	Sobinoff 2010 (Sobinoff, Pye

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
neonatal mice	diepoxide	group) were treated intraperitoneally with VCD 40, 80 mg/kg/day or sesame oil containing vehicle control (< 0.5 ml/kg/day dimethyl sulfoxide [DMSO]).	preantral follicular destruction and primordial follicle activation.	et al. 2010)
15 day study with female mice; The Ahr-, Bax-, and caspase-3-deficient mouse lines were C57BL/6 congenic (*i.e.* more than nine generations), The ASMase- and caspase-2-deficient mouse lines were of a mixed C57BL/6–129/Sv background.	4-vinylcyclohexene diepoxide	Female mice were given once-daily i.p. injections of either vehicle (sesame oil) or VCD (80 mg/kg bw/day; for 15 d. The data shown represent the mean ± sem of the combined results from theanalysis of ovaries collected from three or more mice per genotype per treatment group.	In addition to confirming that Bax, caspase-2, and caspase-3 are functionally important mediators of VCD-induced ovotoxicity, the present study supports the concept that the specific pathway used by follicles to die is determined by both the developmental status of the oocyte and the stimulus responsible for apoptosis.	Takai 2003 (Takai, Canning et al. 2003)
10 day study with female KM mice	4-vinylcyclohexene diepoxide	Forty-five KM mice weighing 18-22 g were divided into three groups, one group of mice were treated with intraperitoneal injection of VCD mixed with sesame oil at 160 mg/kg bw/day for consecutive 10 days, one group of mice were treated with sesame oil at 10 ml/kg as the vehicle control group, the last group of mice were treated with sterile water as the control group. All the mice were weighed weekly and sacrificed at day 60 after the termination of treatment	Number of ovarian follicles and corpus luteum moderately decreased in VCD group. The reduction of antral follicles would lead to no mature oocyte for fertilization and induce infertility. Uterine pathological examination indicated fat cells mildly infiltrated into uterine mucosa and mild edema presented in uterine mucosa, which may induce abortion without a good development environment. There was no obvious pathological change in ovary and uterus for seasame oil and the control group. 4-vinylcyclohexene diepoxide also hindered the mice growing ($p < 0.05$) with damaged ovary and uterus; the body weight of mice feed by 35% galactose food pellet increased slowly ($p < 0.05$) with dramatically higher serum concentration level of galactose, albumin, and total protein ($p < 0.001$) and injured ovary.	Zhang 2016 (Zhang, Yan et al. 2016)
15 day study with female and male SD rats	4-vinylcyclohexene diepoxide	Females and males were randomly separated into two groups (n=8/group) to be exposed daily to liquid emulsion (control bait) or liquid emulsion (active bait) VCD (0.109%) by voluntary oral consumption through a Dyet's feeding tube (35 or 50 ml). Following 21 days of daily exposure, bait was discontinued and	Testes weights in treated males were lower ($P< 0.05$). Testis volume was also lower ($P < 0.05$) than controls in treated males. Likewise, epididymis weights were lower ($P < 0.05$) in treated males, compared with controls. Circulating testosterone levels in males at the time of tissue collection were not different ($P < 0.05$) between groups (0.83 ± 0.15 ng/ml control; 0.99 ± 0.19 ng/ml treated). The tubule from the treated rat shows disorganized placement of germ cells, loss of integrity in the germ cell epithelium, and a greatly reduced number	Witmer et al 2017 (Witmer, Raymond-Whish et al. 2017)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		individual pairs of treatment-matched males and females were housed for a 15-day breeding cycle.	of spermatozoa in the adluminal space. Ovarian weights were lower ($P < 0.05$) in treated females than in controls. Relative to control females, in treated females there was a reduction ($P < 0.05$) of 53% in primordial, 35% in primary, and 48% in secondary follicles. There were no differences ($P< 0.05$) in numbers of antral follicles between groups (control, 3.4 ± 0.73 follicles counted; treatment, 3.3 ± 0.06 follicles counted).	

Introduction

Many studies on the ovarian toxicity of VCD are available in the public literature, including studies using VCD as a model to study menopausal effects and to determine the protection by other substances for the ovotoxicity of VCD. Most of these studies were performed using ip exposure. As ip exposure allows direct contact of VCD with the ovaries these studies may not be representative of studies using normal routes of exposure. In addition, no studies are available in which VCD was applied using a relevant route of exposure and determining the effects on fertility. Therefore, the provided summaries will mainly be limited to studies on the effects of VCD using normal routes of exposure and on studies which also determine the effects of ovotoxicity on fertility. However, to provide some information on the other studies with VCD and to provide an historical overview of the research on VCD relevant parts of the review by Kappeler and Hoyer (2012) on VCD as a model for ovotoxicity are provided below.

Animal studies

The National Toxicology Program (NTP) conducted two long term studies with 4-vinylcyclohexene (VCH) and VCD with the purpose of screening for carcinogenic potential in mice and rats (National Toxicology 1989). Animals were exposed to the chemicals over a two year period. Although the studies concluded that there was some carcinogenic potential with VCH and VCD following this lengthy exposure, an interesting observation was the appearance of ovarian and uterine atrophy in exposed mice within 13 weeks of exposure. Additionally, no visible ovarian follicles or corpora lutea were observed at the end of exposure. No similar effects were observed in rats. These findings suggested that these ovarian effects might result from direct damage to ovarian follicles. Because the apparent infertility was seen to be irreversible, this suggested further that one effect of VCH and VCD could be the result of destruction of the primordial follicle pool (Hoyer and Sipes 2007). These reports prompted initiation of an ongoing investigation into possible ovarian effects of VCH and VCD in mice and rats.

Initial studies were designed to investigate why mice developed ovarian damage from these chemicals, but rats appeared to be resistant. In a 30-day dosing experiment comparing VCH and VCD in mice and rats, VCH produced a dose-responsive loss of small ovarian follicles in mice, whereas, it was ineffective in rats (Smith, Carter et al. 1990). However, both mice and rats were susceptible to the monoepoxide and diepoxide metabolites of VCH. Further, VCD caused follicle destruction at 2.5–3 times lower doses than did the monoepoxide in both species. This study suggested that VCD is the ovotoxic form and VCH represents the parent form of the compound. A further structure–activity study supported that conclusion (Doerr, Hooser et al. 1995). Subsequently, studies addressing the metabolism of VCH and VCD led to the hypothesis that in mice VCH is more readily bioactivated to VCD, and VCD is less readily detoxified, as compared with rats (Hoyer and Sipes 2007). Further studies have determined that the mouse and rat ovary possess the enzymatic capabilities to bioactivate and detoxify VCH and VCD, respectively (Cannady, Dyer et al. 2003, Rajapaksa, Cannady et al. 2007, Keating, Rajapaksa et al. 2008, Keating, Sipes et al. 2008, Keating, Sen et al. 2010). Therefore, the ovary itself may directly contribute to the degree of follicle damage produced by exposure to xenobiotic agents.

An early question in these studies was whether the observed ovotoxicity with VCH/VCD was due to direct ovarian targeting, or resulted from disruptions in hypothalamic-pituitary signaling. In the hypothalamic-pituitary-ovarian axis of regulation, gonadotropin releasing hormone (GnRH) released by the hypothalamus signals the pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Hoyer and devine 2002). Consequently, ovarian hormones (17β-estradiol, progesterone, inhibin) provide a negative feedback on GnRH and LH/FSH. Thus, it was reasoned that if VCH/VCD targets the hypothalamus/pituitary, LH/FSH levels would drop and this would precede the observed loss of small pre-antral follicles. Conversely, if the chemicals target the ovary, follicle loss would precede an increase in LH/FSH (resulting from loss of negative feedback). In a longterm study following 30 days of dosing mice with VCH, substantial small pre-antral follicle loss was observed at the end of dosing, whereas, circulating FSH levels did not rise until 240 days after the onset of dosing (Hooser, Douds et al. 1994). Therefore, it was concluded that VCH/VCD directly targets the ovary, and mechanistic investigations into VCD effects in the ovary were undertaken.

Dosing studies to that point had only investigated ovarian effects following 30 days of repeated dosing with VCH/VCD. Under those conditions, follicle populations in all stages of development (primordial, primary, secondary, antral) had been seen to be targeted. Thus, it was unknown whether VCH/VCD directly targets all sizes of ovarian follicles, or is selective for a specific population. Two time course studies identified that VCD directly targets primordial and primary follicles (Springer, McAsey et al. 1996, Kao, Sipes et al. 1999). Additionally, it was concluded that VCD-induced ovotoxicity requires repeated daily dosing (Springer, McAsey et al. 1996, Borman, VanDePol et al. 1999). An investigation into the nature of VCD's effects on ovarian follicles was made. Two types of death, necrosis and apoptosis, can be a mechanism by which cells are destroyed. Cell death by necrosis usually occurs in response to injury and elicits an inflammatory response in surrounding tissue. Conversely, apoptosis is a physiological process of programmed cell death (Wyllie, Kerr et al. 1980). Thus, it was of interest to determine whether VCD causes necrosis (toxic response) or apoptosis (programmed cell death) in ovarian follicles. A morphological investigation determined that VCD causes accelerated atresia (apoptotic cell death) rather than necrosis (Springer, McAsey et al. 1996). In reaching that conclusion, it was postulated that due to the specificity for primordial and primary follicles, and because cell death is via a natural process (atresia), women who might be exposed to similar chemicals would only experience early menopause without first experiencing disruptions in menstrual cyclicity. Therefore, VCD appeared to be an ideal model chemical to study selective effects of xenobiotics on primordial and primary follicles." (as summarised by Kappeler and Hoyer, 2012 (Kappeler and Hoyer 2012))

Monkeys

Appt et al. 2006 studied the effect of 4-vinylcyclohexene diepoxide in nonhuman primates that received once-daily intramuscular injections for 15 days of 250, 160 or 80 mg/kg bw/day (Appt, Kaplan et al. 2006). Four female cynomolgus monkeys of similar age (8-12 years) were used for this study . At 250 mg/kg bw/day nearly complete elimination of primordial, intermediate, primary and secondary follicles was achieved, at 160 mg/kg bw/day a 50% elimination and at 80 mg/kg bw/day no elimination was achieved. No gross of histological lesions in the organs studied were found at postmortum evaluations after 9 months (Appt, Kaplan et al. 2006). These findings show that the monkey ovary is susceptible to VCD and that as in rodents, primordial and primary follicles are targeted selectively. Follicle counts for two control ovaries from permenopausal monkeys and for monkeys treated with vehicle only and with VCD (80, 160, 250 mg/kg bw/day) are listed in table 19.

Table 19: Ovarian follicle counts (Appt, Kaplan et al. 2006).

Ovarian follicle counts.

Follicle counts by treatment	Age (y)	Primordial follicles	Intermediate follicles	Primary follicles	Secondary follicles	Antral follicles
Archived control ovary	8	5,036	2,720	257	103	10
Archived control ovary	13	3,132	1,603	167	45	7
Vehicle control	13	3,791	3,910	689	473	22
Mean of control ovaries	11.3	3,986	2,744	371	207	13
VCD (250 mg/kg)	12	168	377	82	59	8
VCD (160 mg/kg)	9	1,961	3,127	167	25	4
VCD (80 mg/kg)	8	7,837	5,173	236	110	9

Appt. Ovarian follicle destruction in monkeys. Fertil Steril 2006.

Appt et al 2010 studied the effect of 4-vinylcyclohexene diepoxide in nonhuman primates got a surgical placement of a bio degradable fiber containing approximately 200 mg VCD next to one ovary of 8 monkeys (Appt, Clarkson et al. 2010). At day 30 primordial follicles were reduced by approximately 70%, with a corresponding decrease (83%) in antimüllerian hormone (AMH, a serum marker of ovarian follicle numbers) (Figure 1). At 4 mo after VCD-treatment of both ovaries in 29 monkeys (approximately 200 mg VCD per ovary), AMH was reduced 56% from baseline, testosterone was unchanged, and follicular phase estradiol was slightly increased (Figure 2). Data indicate that VCD treatment markedly reduced primordial follicles while preserving larger estradiol- and testosterone-producing follicles and ovarian stroma, a condition that mimics ROR in women.

Figure 1: Serum concentrations of antimüllerian hormone (AMH) measured before the removal of one ovary (baseline) and 14 and 30 days after treatment of the remaining ovary with approximately 200 mg VCD. Data are shown as AMH concentration (ng/mL) per monkey (n=6)

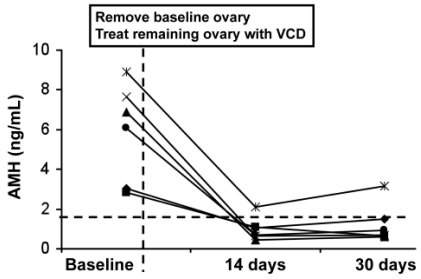
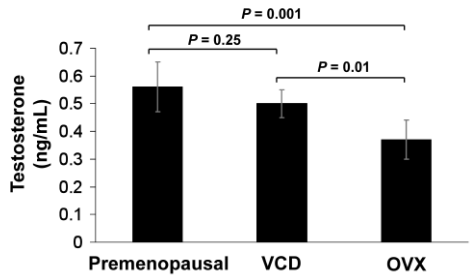


Figure 2: Serum testosterone concentrations (Mean ± SE) of adult cynomolgus monkeys 4 months after sham surgery (premenopausal), placement of VCD fibers next to both ovaries (VCD) or ovariectomy (OVX).



Rats

Kodama et al. conducted a study to evaluate female reproductive function in female rats (Kodama, Yoshida et al. 2009). VCD was intraperitoneally administered to female rats SD, n=10) at 0, 5, 20, 80 mg/kg bw/day from 2 weeks prior to mating to Day 7 of gestation (for at least 3 weeks). No mortality and no test compound-related changes of the estrous cycle were observed at any dose level. Maternal body weight was reduced on day 14 of pregnancy (table 20). At the necropsy of pregnant females, the number of implanted embryos and rate of implantation tended to decrease and the rate of preimplantation loss tended to increase in the 80

mg/kg bw/day dosed group. However, these changes were not statistically significant. The pre-implantation loss was considered to be a consequence of the decrease in small follicles since histopathologically the ovaries showed a decrease in number of small follicles at 80 mg/kg bw/day. A decrease of the small follicles of the ovaries was observed at the highest dose compared to controls (table 21). No test compound-related changes were observed on histopathological examination of the vagina, uterus and pituitary gland at any dose level (Kodama, Yoshida et al. 2009). Based on the results of Kodama et al. the NOAEL for reproductive toxicity in female rats was considered to be 20 mg/kg bw/day. and the NOAEL for development of the next generation in females was considered to be more than 80 mg/kg bw/day.

Table 20: Summary of results of female fertility study (Kodama, 2009).

Dose (mg/kg/day)	0 (Control)	5	20	80
No. of animals (Females)	10	10	10	10
Clinical observations	-	-	-	-
Body weight (g)				
Day 14 of treatment	255.3	251.3	256.6	244.3
Day 14 of pregnancy	334.9	335.9	334.6	313.1 *
Mean estrous cycle (days)	4.4	4.1	4.1	4.2
Irregular estrous cycle 1)	2 / 10	0 / 10	1 / 10	0 / 10
No. of animals mated	10	10	10	10
No. of animals copulated	10	10	10	10
No. of pregnant females	10	10	9	10
Necropsy findings	-	-	-	-
Mean No. of corpora lutea	18.7	19.1	19.8	20.7
Mean No. of implantations	18.0	17.9	18.8	16.6
Mean % implantations	94.7	96.5	94.9	81.5
Mean % preimplantation loss	7.4	9.2	8.1	18.5
Mean No. of live embryos	11.1	8.8	9.8	9.2
Mean No. of dead embryos	6.9	9.1	9.0	7.4
Mean % postimplantation loss	35.0	49.0	46.8	44.1

1) Irregular cycle means the interval between two estrous cycles was longer than 5 days, or estrous was recorded on more than 2 consecutive days.
-: No noteworthy findings, *: $p < 0.05$

Table 21: Histopathological changes (Kodama, 2009).

Dose (mg/kg)	0			5			20			80		
No. of ovaries	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10
Ovary: Decrease in small follicles	0	3	1	2	1	1	1	1	2	9	8	10
Slight	-	3	1	1	1	1	1	-	1	6	1	4
Moderate	-	-	-	1	-	-	-	-	1	2	4	5
Marked	-	-	-	-	-	-	-	1	-	1	3	1

R: right ovary, L: left ovary, W: whole body, -: no noteworthy findings

Kao et al. 1999 studied ovotoxicity effects of VCD in femals rats and mice which were daily dosed with a vehicle or 80 mg/kg bw/day intraperitoneally for 6,8,10 or 12 days. Significant loss of primordial and primary follicles ($P < 0.05$) was measured on day 12 in both rats and mice (figure 3). A significant increase in % atretic primary follicles (apoptosis) was found after 4 hours after the final dose in mice on day 8 in mice. In rats this significant increase was not seen until day 10 (Kao, Sipes et al. 1999).

Figure 3

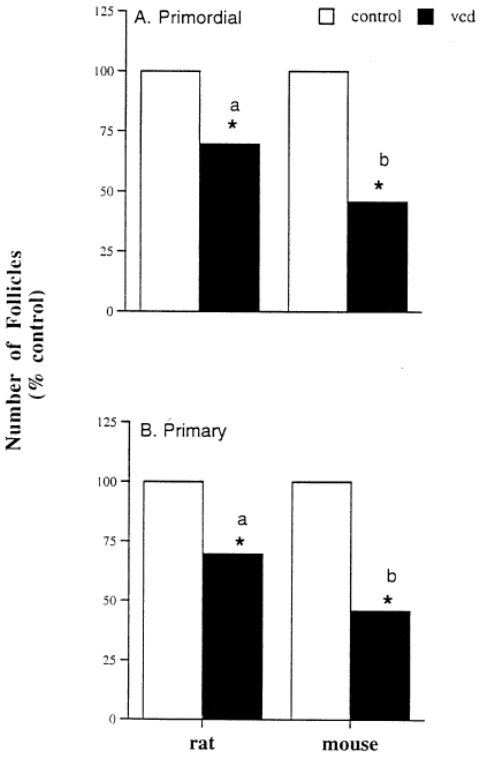


Fig. 3. Comparison on day 12 of the effect of VCD on the number of primordial and primary follicles in rats and mice. Animals were treated daily with VCD (80 mg/kg, i.p.) for 12 d. After the final dosing, ovaries were removed and histologically processed as described in *Materials and Methods*. The number of oocytes contained in primordial and primary follicles were counted in every 20th section (mice) or 40th section (rats) as described in *Materials and Methods*. A) primordial, and B) primary follicles in rats and mice (values are expressed as percent control ±SE; rat, $n = 6$; mouse, $n = 3$; $*P < 0.05$ different from control; a, b = different between groups.

Ito et al. 2009 evaluated ovarian toxicity in a two- or four-week repeated dose study of 4-vinylcyclohexene diepoxide in female rats. Rats were intraperitoneally dosed at 0, 5, 10 and 80 mg/kg bw/day once a day for 2 or 4 weeks. In the 4-week study a decrease in small follicles was observed in the ovaries at 20 and 80 mg/kg bw/day (Table 22). In the 2-week study , the same change was observed at 80 mg/kg bw/day (Ito, Mafune et al. 2009).

Table 22: Histopathological findings of ovaries in 2- or 4-week study of 4-vinylcyclohexene diepoxide (Ito, Mafune et al. 2009)

2-week study												
Dose (mg/kg)		0			5			20			80	
No. of animals		10			10			10			10	
No. examined	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10
Decrease in small follicles	0	0	0	0	0	0	0	0	0	2	2	3
Slight	–	–	–	–	–	–	–	–	–	1	0	1
Moderate	–	–	–	–	–	–	–	–	–	0	1	2
Severe	–	–	–	–	–	–	–	–	–	1	1	0
4-week study												
Dose (mg/kg)		0			5			20			80	
No. of animals		10			10			10			10	
No. examined	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10	R:10	L:10	W:10
Decrease in small follicles	0	0	0	0	0	0	2	2	2	7	8	9
Slight	–	–	–	–	–	–	2	2	1	3	3	2
Moderate	–	–	–	–	–	–	0	0	1	1	4	4
Severe	–	–	–	–	–	–	0	0	0	3	1	3

R: right ovary, L: left ovary, W: whole body, -: no noteworthy findings

4-Vinylcyclohexene diepoxide (VCD) induces ovotoxicity in rodents and therefore can be seen as an industrial occupational health hazard chemical. Chemicals that destroy primordial follicles are of concern to women because exposure can result in premature ovarian failure (early menopause).

NTP studies

13 week study

In the 13-week dermal studies, all rats survived to the end of the studies (doses up to 60 mg/rat) (Annex 1 table 1) (National Toxicology 1989). The final mean body weights of the 60 mg/rat groups were 9%-14% lower than those of the vehicle controls. Compound-related clinical signs in the 60 mg/rat groups observed during the second half of the studies included redness, scabs, and ulceration at the application site and burrowing behavior after dermal application. Hyperplasia of the sebaceous glands and acanthosis (hyperplasia) and hyperkeratosis of the squamous epithelium were seen at the site of application (annex 1 table 2). The severity of the lesions was greatest at 60 mg/rat. Ulcers of the skin were seen in 3/10 males that received 60 mg/rat. Acute to chronic inflammation of the epidermis from the application site was observed for rats administered 60 mg/rat.

15-month study

Organ weight to body weight ratios were not affected by dermal administration of 4-vinyl-l-cyclohexene diepoxide (annex 1 table 3) (National Toxicology 1989). Two of 10 male rats that received 30 mg had a squamous cell carcinoma of the skin at or adjacent to the site of application (annex 1 table 4). Acanthosis was seen in exposed rats (mild severity at 30 mg/rat and minimal severity at 15 mg/rat); hyperkeratosis was observed for rats in the 30 mg/rat groups. One female receiving 30 mg/rat had a squamous cell carcinoma of the forestomach.

2 year study

Two-year studies were conducted by administering 4-vinyl-1-cyclohexene diepoxide in acetone by dermal application, 5 days per week for 105 weeks to groups of 60 rats of each sex at 0, 15, or 30 mg/animal (National Toxicology 1989). In general, the body weights and survival were lower in mid and high dose groups than in vehicle controls (annex 1 Table 5).

The survival was lower in exposed groups (annex 1 Table 6), primarily because of neoplasms (survival at week 105--male rats: vehicle control, 7/50; low dose, 8/50; high dose, 4/50; female rats: 27/50; 23/50; 15/50. No significant differences in survival were observed between any groups of male rats; however, survival at the end of the study was very low for all groups, including vehicle controls. Acanthosis and sebaceous gland hypertrophy of skin from the scapula or back were observed at substantially increased incidences in ex- posed male and female rats. Squamous cell papillomas in male rats and squamous cell carcinomas in male and female rats were observed only in exposed rats (annex 1 Table 7).

Abolaji et al. 2016 investigated VCD-induced reproductive dysfunction in female Wistar rats (Abolaji, Adedara et al. 2016). The rats were orally administered with VCD (100, 250 and 500 mg/kg bw/day) for twenty-eight days. Thereafter, we evaluated selected biomarkers of oxidative damage, inflammation, endocrine disruption, and apoptosis. It was observed that VCD increased ovarian and uterine malondialdehyde (MDA) level, and catalase (see figure 4), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in rats ($p < 0.05$). (see figure 5),

Figure 4. Ovarian and uterine malondialdehyde (MDA) and catalase levels in rats (Abolaji, Adedara et al. 2016).

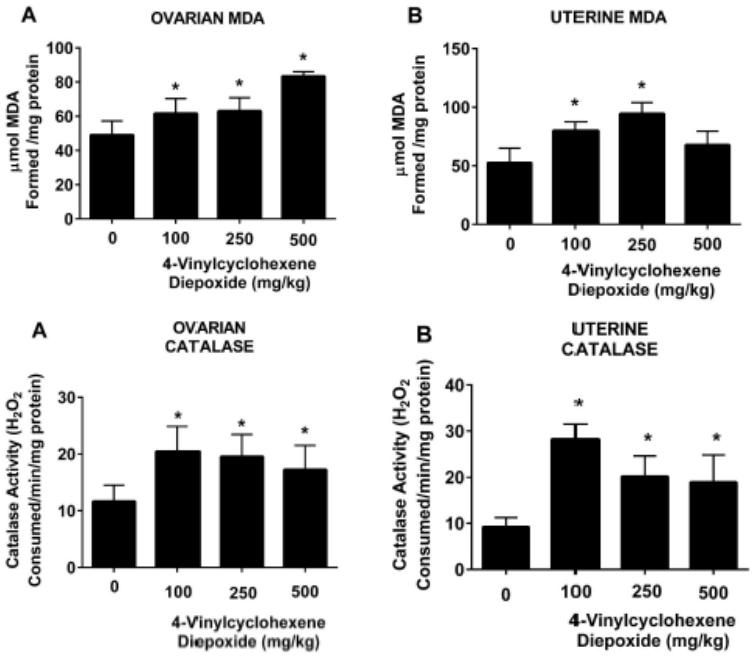


Figure 5. Ovarian and uterine glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in rats (Abolaji, Adedara et al. 2016).

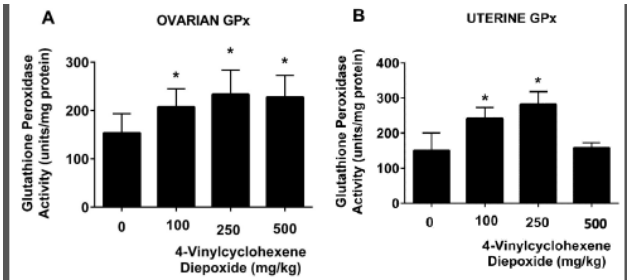


Fig. 5.

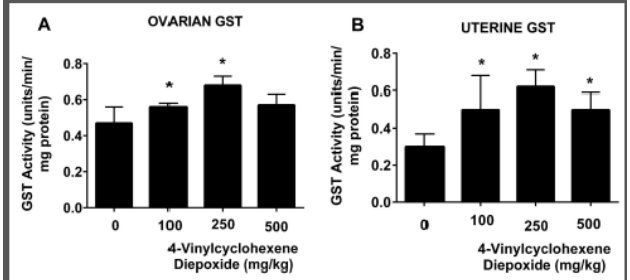


Fig. 6.

VCD exposure decreased the levels of progesterone, prolactin and estrogen, but increased the levels of luteinizing hormone and follicle stimulating hormone (Table 23).

Table 23: Effects of VCD on serum reproductive hormones of female rats (Abolaji, Adedara et al. 2016).

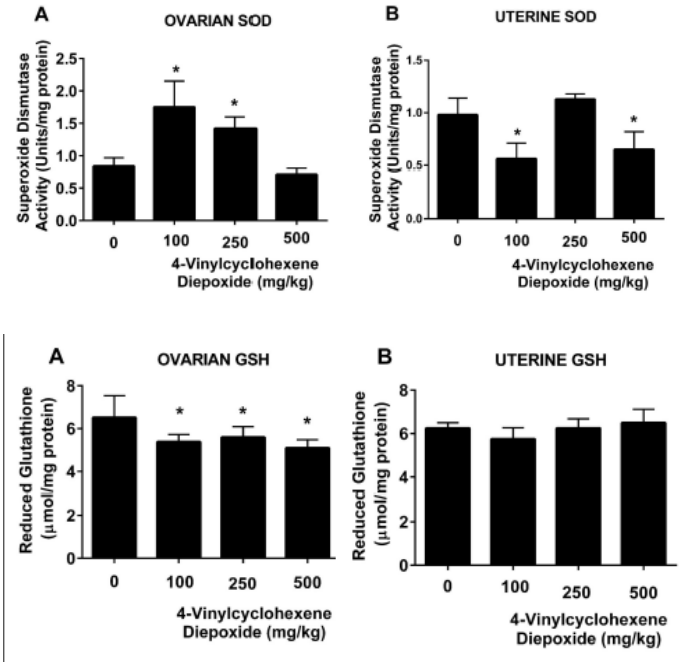
		VCD (mg/kg Body Weight)		
Hormones (IU/L)	Control	100	250	500
LH	17.50 ± 2.52	17.50 ± 0.71	16.00 ± 1.00	25.67 ± 2.52*
FSH	13.00 ± 1.41	13.00 ± 1.41	11.50 ± 0.71	21.67 ± 1.53*
PRL	24.00 ± 3.61	15.33 ± 1.15*	17.67 ± 1.53*	25.33 ± 2.52
PRG	5.26 ± 0.64	5.31 ± 0.32	5.15 ± 0.07	3.33 ± 0.49*
ESTR	35.67 ± 5.51	32.67 ± 2.08	39.33 ± 2.52	25.33 ± 2.52*

The data are expressed as mean ± SD, n=7. Values differ significantly from control ($p < 0.05$).

LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; PRL, Prolactin; PRG, progesterone; ESTR, Estrogen.

Also, VCD increased ovarian superoxide dismutase (SOD) activity, and depleted uterine SOD activity and ovarian glutathione (GSH) level in rats ($p < 0.05$) (See figure 6).

Figure 6. Ovarian and uterine superoxide dismutase (SOD) activity, and glutathione (GSH) levels in rats (Abolaji, Adedara et al. 2016)



Lastly, VCD markedly increase immunohistochemical expressions of ovarian cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), Caspase-9 and Caspase-3 (see figure 7). Overall, VCD induced reproductive dysfunctions in rats via ovarian and uterine oxidative damage, hormonal imbalance, as well as inflammation and apoptosis in the ovary of rats.

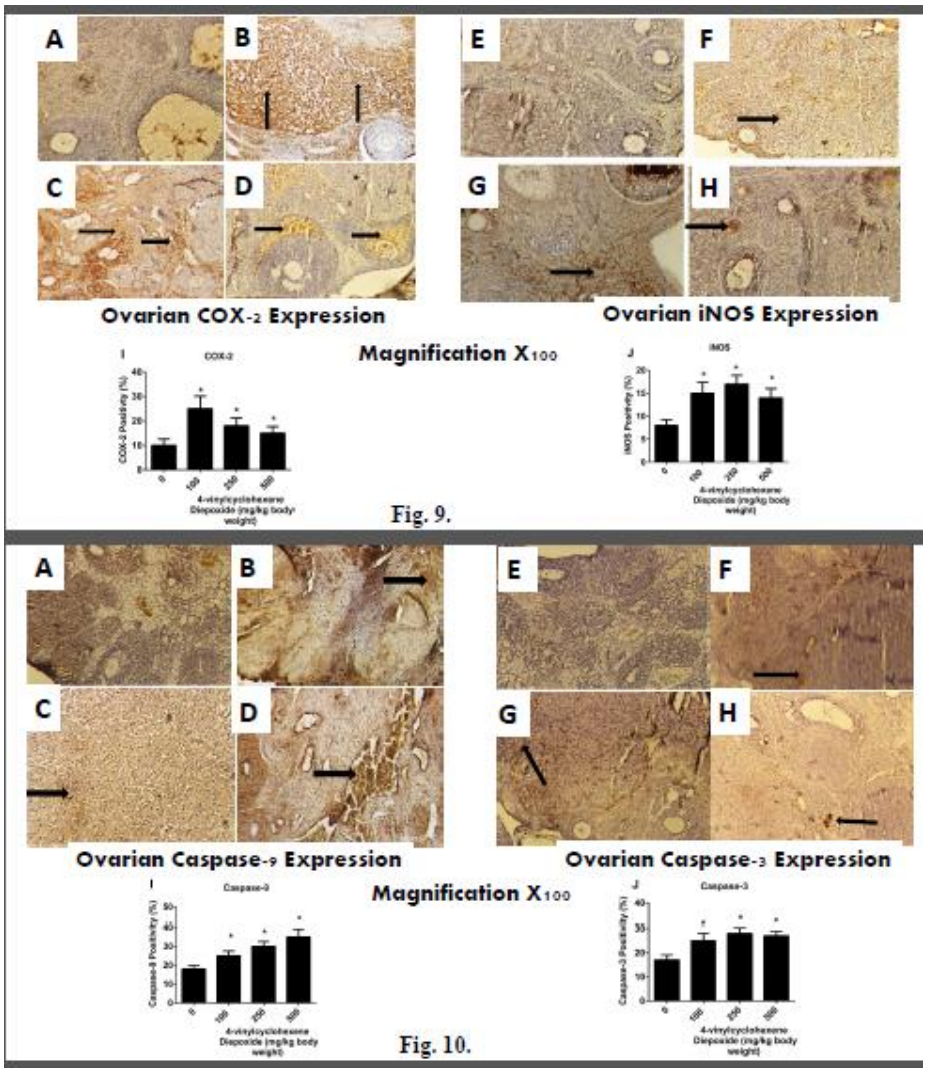


Figure 7. Immunohistochemical expressions of ovarian cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), Caspase-9 and Caspase-3 (Abolaji, Adedara et al. 2016).

The histopathology of the ovary revealed large cystic follicles and scanty number of follicles following VCD administration. This is suggestive of ovotoxicity and corroborates existing literature that VCD depletes follicular number in rats. The observation that uterine histopathology revealed a gradual loss of the endometrial cells may be due to VCD-induced oxidative stress as shown above, thus representing another target of VCD toxicity not previously reported in the literature. Data suggest that the VCD-induced reproductive toxicity in female rats occurs via the combined impact of oxidative damage, inflammation, apoptosis and hormonal disruption, thus contributing to the available information in the literature on the toxicity of VCD in rats.

Berger et al 2003 did a study with female rats which received 0.02 or 0.05% VCD in drinking water for 2 weeks prior to oocyte recovery (Berger and Horner 2003). The 4-vinylcyclohexene diepoxide had no effect on final weight or weight gain (Table 24). The fragility of oocytes may be slightly affected (51, 35, and 36% of the oocytes remained after removal of the zona pellucida for control, 0.02% 4-vinylcyclohexene diepoxide-exposed, and 0.04% 4-vinylcyclohexene diepoxide-exposed females, S.E.M. = 7; $P < 0.10$ for the comparisons with oocytes from control females). There was no effect of vinylcyclohexene diepoxide at these exposure levels on the fertilizability of oocytes.

Table 24: Weight and reproductive parameters in female rats after exposure to 4-vinylcyclohexene diepoxide (Berger and Horner 2003).

	Control[a]	0.02% 4-vinylcyclohexene diepoxide[a]	0.04% 4-vinylcyclohexene diepoxide[a]	S.E.M.
Weight gain (g)	68.2	68.2	70.0	2.6
Final weight (g)	143.3	146.4	150.1	4.7
Percentage of females ovulating	100	92	100	6
Number of oocytes recovered per ovulating female	25	30	20	3
Percentage of oocytes remaining after removal of the zona pellucida	51	35[b]	36[b]	7
Percentage of oocytes fertilized	74	72	72	2
Penetrated sperm/oocyte	1.02	1.38	1.34	0.23

[a] Values are least square means; $n = 3$.
[b] Value differs from mean for control females, $P < 0.10$.

Mice

Mature female mice (91 days old) were exposed daily to 160 mg/kg bw/day VCD via ip exposure for 17 days (Haas, Christian et al. 2007). The females were mated with untreated males on either the second proestrus after exposure (group 1) or the first proestrus after day 20 after exposure (group 2). Maternal body weight was significantly reduced by 9% compared to controls. A significant reduction in pregancies and pregnancies per copulatory plug was observed (table 25). The difference between group 1 and 2 was attributed to the presence of unaffected secondary and antral follicles in group 1 animals. No live fetuses were recorded in group 2.

Table 25: Mating effiency (%; mean ± standard deviation) (Haas, Christian et al. 2007)

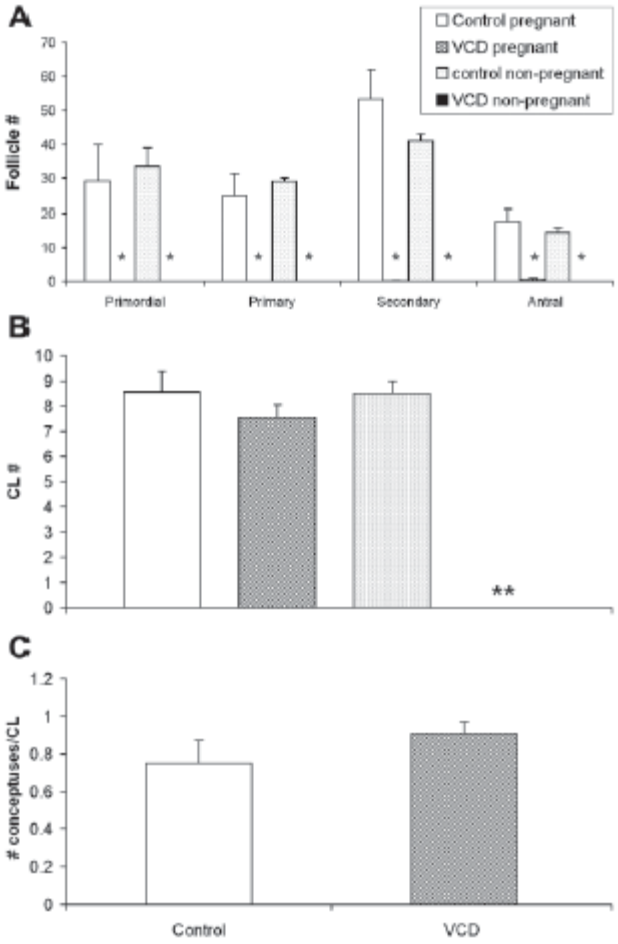
	Control (n = 13)	VCD: group 1[a] (n = 14)	VCD: group 2[b] (n = 8)
No. of plugs/no. of times with males	89.7 ± 7.9	71.3 ± 10.1	83.3 ± 12.6
No. of pregnancies/no. of times with males	64.1 ± 12.1	60.0 ± 11.1	12.5 ± 12.5[c]
No. of pregnancies/no. of copulatory plugs	57.7 ± 12.5	60.0 ± 12.1	12.5 ± 12.5[c]
Pregnancy rate	76.9 ± 12.2	73.3 ± 11.8	12.5 ± 12.5[c]

[a]Group 1 mice were mated on the second proestrus after the final dose of VCD.
[b]Group 2 mice were mated on the first proestrus at least 20 d after the final dose of VCD.
[c]$P < 0.05$ compared with value for control.

In group 1, all control mice and 10 of the 14 VCD-treated mice became pregnant. Of the 5 VCD-treated mice that established pregnancies, 5 became pregnant after their first mating attempt, and the other 5 became pregnant after the second mating attempt. The overall delay in conception in group 1 animals is representative of subfertility in aging women, in whom the time required to become pregnant increases. Although group 2 VCD-treated mice had regular cycles and displayed copulatory plugs after mating, only 1 established a pregnancy, and it did not result in any viable fetuses. Therefore, fertility clearly was impaired even though evidence of ovarian function was still present (proestrus and copulatory plugs).

Histologic evaluation of the ovaries collected from control and VCD treated animals in both groups (Figure 8 A) revealed numerous primordial, primary, secondary, and antral follicles in all control animals regardless of whether they had become pregnant. In contrast, there were essentially no follicles of any size in ovaries of VCD-treated animals regardless of whether they had become pregnant. The only detectable follicle population in those animals was an occasional antral follicle in animals that had become pregnant. CL were counted in ovaries from all animals (Figure 8 B). There were no differences in numbers of CL between control animals (pregnant or nonpregnant) and VCD-treated animals that became pregnant. However, the ovaries of VCD-treated animals that did not become pregnant contained no detectable CL, suggesting that no ovulation had occurred. The number of concepti (live fetuses plus resorptions) was calculated and compared with the number of CL for each animal (Figure 8 C). The ratio of numbers of concepti to CL did not differ between the control and VCD-treated pregnant animals.

Figure 8: Ovarian morphology in pregnant versus nonpregnant mice (Haas, Christian et al. 2007).



As expected, all control animals had numerous follicles of all sizes, whereas only a few antral follicles were seen in the VCD-treated animals that had become pregnant. No antral follicles were observed in VCD-treated mice that did not become pregnant. These findings confirm that ovarian failure was approaching rapidly in VCD-treated animals. In support of that conclusion, ovarian weights were lower in VCD-treated pregnant mice than in control pregnant mice, and ovarian weights from VCD-treated nonpregnant mice were lower than in VCD-treated mice that were pregnant. Therefore, ovarian atrophy had already occurred to some extent in those VCD-treated mice that became pregnant and to an even greater extent in VCD-treated mice that did not become pregnant.

It is unlikely that exposure to VCD directly caused the observed effect on fertility, because the more severe problems were seen in the second group that were mated longer after VCD dosing had been stopped. The group mated earlier still showed evidence of fertility, although slightly impaired, whereas the later group demonstrated almost complete infertility. In addition, VCD dosing did not affect weights of tissues other than ovaries, uteri, and adrenals effects presumably resulting from loss of ovarian function.
In conclusion, VCD-treated C57BL/6J mice displayed proestrus and copulatory plugs, yet as the period of impending ovarian failure progressed, the mice demonstrated evidence of subfertility (group 1) and eventually infertility (group 2).

NTP

13 weeks study

In mice, no compound-related deaths occurred after applications of up to 10 mg/mouse in 13-week dermal studies, and final mean body weights of exposed and vehicle control mice were similar (Annex 1 Table 8) (National Toxicology 1989). Relative liver and kidney weights increased with dose. Compound-related lesions of the skin included sebaceous gland hyperplasia and acanthosis (hyperplasia) and hyperkeratosis of the stratified squamous epithelium at the site of application (annex 1 Table 9). In the 13-week dermal study with mice diffuse ovarian atrophy was observed in all females that received 10 mg/mouse and in 4/10 females that received 5 mg/mouse. Ovarian atrophy was also considered to be compound related. In the 13-week oral studies, the major target organ of toxicity in rats and mice was the forestomach, as indicated by hyperkeratosis and hyperplasia of the stratified squamous

epithelium. In female mice, ovarian atrophy was seen in 4-vinyl-1-cyclohexene diepoxide-dosed groups. Compound-related inflammation of the stomach mucosal layer in the oral studies and of skin in the dermal studies in rats and mice suggests that 4-vinyl-1-cyclohex-ene diepoxide is a direct irritant at the site of contact.

15 months study

In the 15-month study with mice, benign and malignant neoplasms of the ovary occurred in mid and high dose female mice. tubular hyperplasia of the ovarian surface epithelium was seen in most of the animals at 5 or 10 mg/mouse but not at 2.5 mg/mouse. Two of nine animals in the high dose group and one animal in the mid dose group had granulosa cell tumors of the ovary. At the end of the study, the incidences of these neoplasms were similar in mid and high dose groups, and no ovarian neoplasms were seen in the 2.5 mg/ mouse group. In the ovaries, follicular atrophy and tubular hyperplasia was observed at all doses.

Compound-related nonneoplastic skin lesions in mice included acanthosis, hyperkeratosis, and sebaceous gland hyperplasidhypertrophy (annex 1 table 10). Squamous cell papillomas and carcinomas were seen in mice that received 5 or 10 mg/mouse; none was seen in vehicle control or low dose groups (papillomas-- male: mid dose, 1/10; high dose, 2/10; female: 1/10; 1/10; carcinomas--male: 2/10; 8/10; female: 2/10; 5/10). One vehicle control and all exposed female mice had atrophy of the ovary. Hyperplasia of the ovarian surface epithelium was seen in 8/10 females receiving 5 mg/mouse and 9/9 females receiving 10 mg/mouse. Two of nine females receiving 10 mg/mouse had granulosa cell tumors of the ovary, and 1/9 females receiving 10 mg/mouse had an ovarian papillary cystadenoma.

2 year study

Groups of 60 mice of each sex were administered 0, 2.5, 5, or 10 mg/animal on the same schedule for 103 weeks.

In general, the body weights and survival were lower in mid and high dose groups than in vehicle controls male mice: vehicle control, 38/50; low dose, 35/50; mid dose, 4/50; high dose, 0/50; female mice: 30/50; 31/50; 15/50; 0/50). All high dose male mice died by week 83; the 10 surviving high dose female mice were killed during week 85.(Annex 1 Table 11)

For exposed mice, acanthosis, hyperkeratosis, and necrotizing inflammation of the skin were observed over the scapula or back. Squamous cell carcinomas were found only in exposed mice (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).(annex 1 table 12) The in cidences of squamous cell carcinomas in exposed mice were significantly greater than those in vehicle controls (annex 1 Table 13).

Follicular atrophy and tubular hyperplasia of the ovary in female mice were significantly increased (atrophy: 12/50; 43/49; 42/49; 47/50; tubular hyperplasia: 5/50; 35/49; 38/49; 34/50) (annex 1 Table 14). Mid and high dose females had benign or malignant granulosa cell tumors (0/50; 0149; 7/49; 12/50) and benign mixed tumors (0/50;0/49; 11/49; 6/50). The combined incidences of luteomas, granulosa cell tumors, benign mixed tumors, or malignant granulosa cell tumors in mid and high dose female mice were increased (1/50; 0/49; 17/49; 18/50).

Subacute inflammation was observed at increased incidences in mid (P>0.05) and high (P 0.01) dose male mice (vehicle control, 0/50; low dose 0/50; mid dose 6/50; high dose 13/49).

Studies with male animals (testes)

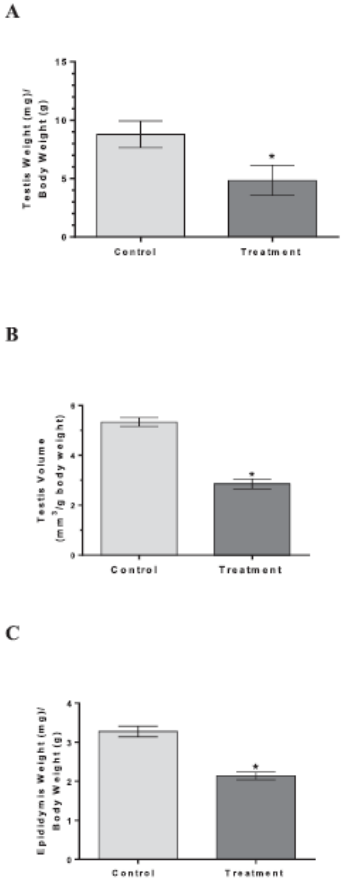
There are also some studies about effects of VCD on male animals. Effectiviness of orally delivered VCD on the fertility of male rats and mice has been examined (Hooser, DeMerell et al. 1995, Schmuki 2009, Burd 2014). Males intraperitoneally injected with VCD (40-320 mg/kg/day; 5-30 days) had reduced testicular weights and testicular damage. Cessation of treatment resulted in recovery of the testicular tissue (Hooser, DeMerell et al. 1995) suggesting that if fertility effects had occurred, such effects were likely reversible. Fifteen days of oral VCD gavage (500 mg/kg/day) caused SD rat testicular and epididymal weights to increase (day 47 post-treatment). No effects on reproductive function of treated males were reported (Schmuki 2009). These findings in male rodents are not surprising considering the continual nature of the spermatogenesis cycle (Clermont 1972).

Adedarra et al 2016 investigated the influence of VCD on testicular and epidydimal functions following oral exposure of Wistar rata to VCD at 0,100,250,500 mg/kg bw/day for 28 days. They found that exposure to VCD induces testicular and epidydimal dysfunctions via endocrine suppression, disruption of antioxidant enzymes activities, increase in biomarkers of oxidative stress, inflammation and apoptosis in rats (Adedara, Abolaji et al. 2016). Administration of VCD significantly decreased the body weight gain and organo-somatic indices of the testes and epididymis. When compared with the control, VCD significantly decreased superoxide dismutase and catalase activities in the testes whereas it significantly decreased superoxide dismutase activity but increased catalase activity in the epididymis. Moreover, while glutathione peroxidase activity and glutathione level remain unaffected, exposure of rats to VCD significantly increased glutathione S-transferase activity as well as hydrogen peroxide and malondialdehyde levels in testes and epididymis of the treated rats. The spermiogram of VCD-treated rats showed significant decrease in epididymal sperm count, sperm progressive motility, testicular sperm number and daily sperm production when compared with the control. Administration of VCD significantly decreased circulatory concentrations of follicle-stimulating

hormone, luteinizing hormone and testosterone along with testicular and epididymal degeneration in the treated rats. Immunohistochemical analysis showed significantly increased cyclooxygenase-2, inducible nitric oxide synthase, caspase-9 and caspase-3 protein expressions in the testes of VCD-treated rats.

In another study by Witmer et al 2017, male rats were given daily acces to active liquid bait (VCD, 0.109%;treated) for 15 days (Witmer, Raymond-Whish et al. 2017). Testes weights in treated males were lower ($P < 0.05$; Fig. 9A). Testis volume was also lower ($P < 0.05$) than controls in treated males (Fig. 9B). Likewise, epididymis weights were lower ($P < 0.05$) in treated males, compared with controls (Fig. 9C). Circulating testosterone levels in males at the time of tissue collection were not different ($P < 0.05$) between groups (0.83 ± 0.15 ng/ml control; 0.99 ± 0.19 ng/ml treated). The tubule from the treated rat shows disorganized placement of germ cells, loss of integrity in the germ cell epithelium, and a greatly reduced number of spermatozoa in the adluminal space. This provided morphological evidence of reduced fertility in treated males.

Figure 9: Testes and epididymis weights in males consumingn active bait. Wild-caught Normal male rats were allowed to consume control (light bars) or active (dark bars) bait.



Mechanistic information

To confirm that VCD-induced ovotoxicity is via accelerated atresia (apoptosis), more mechanistic investigations were required. Because VCD selectively targets primordial and primary follicles, a method to isolate those small follicles from the greater untargeted amount of ovarian tissue was needed. Thus, a method for isolation of small pre-antral follicles from the ovaries of animals that had been dosed with VCD was developed (Flaws, Salyers et al. 1994). By that method, ovaries are collected from rats that have been dosed daily with VCD. Following gentle dissociation of the ovaries with collagenase, intact follicles of all sizes are recovered in suspension. The dissociate is passed through a 250 μm pore filter to exclude larger antral follicles (non-targets). Pre-calibrated Pasteur pipettes are then used to hand sort follicles in the filtrate into two populations, fraction 1 (primordial and primary, targeted by VCD) and fraction 2 (secondary, non-targets). The result is a fraction highly enriched in the target population of follicles, as well as a fraction containing non-targeted follicles (used to distinguish effects that are specifically due to VCD). Having the ability to prepare a cellular fraction containing primordial and primary follicles, biochemical analyses of the effects of VCD on apoptotic signaling pathways were conducted. In some cell types, an apoptotic signal for apoptosis occurs at an intracellular checkpoint involving the

Bcl-2 family of proto-oncogenes (Reed 1997). Several members of this family can heterodimerize to modulate cellular apoptosis versus survival. Pro-apoptotic Bax and Bad can direct a cell death signal, whereas, Bcl-2 and its homolog Bcl-xl function in an anti-apoptotic manner. Using fraction 1 follicles isolated from rats dosed (15 days) with vehicle control or VCD (80 mg/kg), the following experiments determined that, compared with controls, the Bad/Bax response was increased by VCD (Hu, Christian et al. 2001), and this culminated in activation of caspase-3 activity (pro-apoptotic executioner protease) (Hu, Christian et al. 2001). Another intracellular pathway associated with apoptosis is the mitogen activated protein kinase family (MAPK) (Marshall 1995). The pro-apoptotic branch of this family included c-Jun-N-terminal kinase (JNK). Using the isolated follicle approach this pathway was also investigated (Hu, Flaws et al. 2002). As with Bcl-2, the pro-apoptotic branch of MAPK involving JNK was also seen to be activated by VCD (Liu, Wang et al. 2015) . In both pathways, the VCD response was selective for fraction 1 follicles (target population) as compared with fraction 2 follicles which were insensitive. This helped confirm that the observed effects were those specifically elicited by VCD.

In Vitro Culture Studies

An *in vitro* culture system has been established to assess the precise mechanisms underlying the accelerated follicle loss induced by VCD (Devine, Sipes et al. 2002). The culture system consists of ovaries from postnatal day (PND) 4 rats placed on a membrane floating in 0.5 ml culture medium. This allows the ovary sufficient access to both oxygen and nutrients from the medium. Environmental toxins, like VCD, can then be added to the medium and the cellular mechanism of follicular loss can be evaluated. Since the PND4 rat ovary is highly enriched in primordial and primary follicles and VCD selectively targets these early follicular stages, the neonatal rat ovarian culture system is especially useful for evaluating ovotoxicity by VCD. *In vivo* studies must also contend with metabolic contributions from other tissues, such as the liver, to clearance of VCD, which adds complexity to those studies and is avoided in the culture system. The *in vitro* studies have demonstrated that the ovarian organ cultures mimic the physiological response *in vivo*, making it a valuable tool for mechanistic studies (Devine, Sipes et al. 2002). A time course of VCD exposure has also shown that a depletion of both primordial and primary follicles occurs following 6 days in culture (Keating, C et al. 2009).

A diverse group of growth factors has been recognized as important for follicular survival and development. Investigations into the ability of some of these growth factors to override VCD-induced ovotoxicity have been made. Those growth factors include: granulosa cellassociated factors, kit ligand (KITLG), leukemia inhibitory factor (LIF), growth and differentiation factor 9 (GDF9), and bone morphogenic factor 4(BMP4); oocyte-associated factors, glial cell line-derived neurotrophic factor (GDNF), platelet-derived growth factor isoform B (PDGFB), fibroblast growth factor 2 (FGF2), and an ovarian thecal cell factor, fibroblast growth factor 7 (FGF7) (Fernandez, Keating et al. 2008, Mark-Kappeler, Sen et al. 2011).Amongst all of the growth factors tested, only KITLG demonstrated an ability to attenuate VCD-induced ovotoxicity . The growth factor KITLG binds to its oocyte-associated receptor, KIT, which plays an important role in follicular survival, and is able to act as an anti-apoptotic factor in oocytes of primordial follicles (Parrott and Skinner 1999, Jin, Han et al. 2005). Because only endogenous KITLG was shown to protect against VCD-induced ovotoxicity, this suggested that the KIT/KITLG signaling pathway is involved in the ability of VCD to target primordial and primary follicles.

KITLG and KIT interaction plays an important role in the communication between the oocyte and surrounding granulosa cells by activating downstream pathway members. This plays a vital role in oocyte survival (Liu, Rajareddy et al. 2006). Therefore, investigations into the effect of VCD on KIT/KITLG and members of its cellular signaling cascade were undertaken. Relative to controls, on day 4 of VCD exposure there was a decrease in mRNA encoding Kit and on day 6 an increase in mRNA encoding Kitlg (Fernandez, Keating et al. 2008). VCD also decreased levels of KIT protein on the oocyte pericytoplasmic membrane following 4 days of exposure (Keating, Fernandez et al. 2011). The binding of KITLG to KIT has been shown to activate the PI3K signaling pathway (Reddy, Shen et al. 2005). AKT functions as an important downstream molecule in the PI3K signaling pathway. Once phosphorylated, activated ovarian AKT translocates to the nucleus, and plays a role in primordial to primary follicle activation and recruitment (Reddy, Shen et al. 2005, Liu, Rajareddy et al. 2006). In assessing VCD exposure, a decrease in oocyte nuclear pAKT protein was observed on day 2 of culture supporting a decrease in its activity. This demonstrated an early downstream response to VCD interaction with KIT receptor signalling (Keating, Fernandez et al. 2011).

Autophosphorylation of KIT activates its signaling cascade. Therefore, the effect of VCD on phospho-KIT (pKIT) was also investigated. A decrease in pKIT protein was observed with VCD exposure after 2 days of culture. This observation supported that VCD-induced ovotoxicity is initiated by direct interaction with KIT. Additional experiments were conducted to further analyze a possible interaction between VCD and KIT using an antimouse KIT2 (ACK2) antibody. ACK2 has been shown to bind to KIT and block its signaling activity. This results in inhibited oocyte growth, and increased follicular atresia (Packer, Hsu et al. 1994, Carlsson, Laitinen et al. 2006) . There was no effect of VCD or ACK2 on total KIT protein following 2 days in culture, however, ACK2±VCD caused a decrease in pKIT protein (Mark-Kappeler, Sen et al. 2011) . This suggests a similar mechanism of interaction with KIT between ACK2 and VCD. Additionally, the effects of an anti-mouse KIT4 (ACK4) antibody on the ovary were evaluated and compared with those of ACK2. ACK4 recognizes the KIT receptor, but it is directed against a different epitope than ACK2. While ACK2 is an antagonist of KIT, ACK4 binds to the receptor but does not block its function in hemopoietic progenitor cells (Ogawa, Matsuzaki et al. 1991). On day 2 of *in vitro* incubation of ovaries with ACK4 and VCD there was a partial attenuation of the decreased phosphorylation of KIT protein caused by VCD (Mark-Kappeler, Sen et al. 2011). This suggests that ACK4, by binding to KIT, can protect it from interacting with VCD. Alternatively, ACK4 binding to KIT may change the conformation of KIT and interfere with its ability to be targeted by VCD. Overall, these results provided further evidence for a direct interaction between VCD and KIT.

In summary, the collective findings reveal that VCD interacts directly with membranebound KIT and its downstream signaling pathway in the oocyte to cause follicular destruction. Initially, there is a decrease in phosphorylation of KIT and AKT on day 2 of VCD exposure, relative to controls (post-translational signaling effects). Subsequently, on day 4 of VCD exposure there is a decrease in expression of KIT (mRNA and protein), as well as, a decrease in mRNA encoding AKT (transcriptional effects). Lastly, there is an increase in KITLG mRNA on day 6 of VCD exposure, relative to controls. All of these effects on the KIT/KITLG signaling pathway show that post-translational signaling effects of VCD precede transcriptional effects and the result is small follicle loss on day 6 of VCD exposure . (as summarised by Kappeler and Hoyer, 2012)

Tuck et al 2015, characterised mRNA expression of *c-kit* and *KITL* isoforms and the localisation of c-kit and KITL proteins in adult human premenopausal ovaries (Tuck, Robker et al. 2015). The c-kit/kit ligand (KITL) signalling axis is an essential component of ovarian folliculogenesis in mammals, but little is known about expression and localisation of its key components in the ovaries of reproductive age women. Both c-kit mRNA isoforms, known as GNNK+ and GNNK-, were detected in human ovarian cortex, while KITL protein isoforms (KITL1 and KITL2) were present in ovarian cortex and human granulosa cells. Immunohistochemistry showed expression of KITL and c-kit protein in multiple cell types within follicles throughout development, from primordial follicles to large antral follicles, in addition to atretic follicles. Oocytes of all follicle stages expressed c-kit protein exclusively. Interestingly, unlike animal models, expression of both proteins displayed a less cell-type specific distribution with immunostaining present in granulosa, theca and stromal cells, suggesting that autocrine signalling occurs within the human ovary. The presence of c-kit protein in adult granulosa cells is supported by other studies, which demonstrated the presence of *c-kit* mRNA and protein in pregranulosa cells and granulosa cells of primordial follicles in human fetal ovaries (Hoyer, Byskov et al. 2005, Carlsson, Laitinen et al. 2006). This finding suggests that the roles of KITL previously established in animal models may also be present in the human ovary, and furthermore, that these functions are perhaps regulated in a differential manner (e.g., autocrine versus paracrine) (Tuck, Robker et al. 2015). c-kit and KITL protein was also found to be co-expressed in the theca layer of all antral follicles. This suggests that the roles of KITL in formation and function of the theca layer, as shown in the bovine ovary (Parrott and Skinner 1997, Parrott and Skinner 1998), may remain conserved in the human ovary (Tuck, Robker et al. 2015). The presence of increased levels of KITL2 in preantral granulosa cells may suggest that KITL plays a greater or more prolonged role during early human folliculogenesis.

Results of Chen et al 2015, indicated that maternal Rictor is not required for preimplantation embryonic development (Chen, Kang et al. 2015). However, disruption of Rictor in oocytes causes early depletion of functional ovarian follicles, aberrant gonadal hormone secretion, and secondary subfertility in cKO mice, reminiscent of POF phenotypes. It was concluded that Rictor/mTORC2 plays a critical role in folliculogenesis, follicle survival, and female fertility and that its inactivation in oocytes causes POF.

10.11.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Adequate studies on reproductive toxicity in experimental animals were available for the intraperitoneal and intramuscular route. In these studies 4-vinylcyclohexene diepoxide was ovotoxic in female rats and mice inducing a significant loss of primordial and primary follicles rats and mice. Ovotoxicity was also induced in non human primates after intramuscular injection where primordial and primary follicles were targeted selectively. However, these routes of exposure are less relevant for human exposure. Especially ip treatment could result in direct contact of VCD with the ovaria. However, also in dermal studies with mice, comparable effects on the ovaries were observed. The general toxicity in these studies were limited to local effects on the skin. Seen the limited general toxicity and the identified mechanism for the induction of ovotoxicity in rats and mice, it is considered that the observed ovotoxicity is a direct effect of VCD and not secondary to the general toxicity. In addition, some studies (Haas, Christian et al. 2007, Kodama, Yoshida et al. 2009)) but not all show that VCD also induces a reduction in the number of offspring. As ovotoxicity is observed in 4 species (rat, mouse, hamster and nonhuman primates) it is considered likely that this effect is also relevant to humans. In addition Tuck et al 2015 have demonstrated the presence of KITL and c-kit in the adult human ovary throughout follicle development, in addition to showing the presence of each isoform. This suggests that the KITL/c-kit system is involved in human folliculogenesis (Tuck, Robker et al. 2015). Exposure to VCD has also an effect on male animals. Exposure of rats to VCD induces testicular and epididymal dysfunctions via endocrine suppression, disruption of antioxidant enzymes activities, increase in biomarkers of oxidative stress, inflammation and apoptosis in rats. Males intraperitoneally injected with VCD had reduced testicular weights and testicular damage. Cessation of treatment resulted in recovery of the testicular tissue suggesting that if fertility effects had occurred, such effects were likely reversible. No effects on reproductive function of treated males were reported. These findings in male rodents are not surprising considering the continual nature of the spermatogenesis cycle.

Seen the limited general toxicity and the identified mechanism for the induction of ovotoxicity in rats and mice, it is considered that the observed ovotoxicity is a direct effect of VCD and not secondary to the general toxicity.

10.11.3 Comparison with the CLP criteria

As there are no human data on the effects on sexual function and fertility by VCD, classification in category 1A (known human reproductive toxicant) is not warranted.
As the effect in female animals is specifically targetted to the ovaries, after dermal, intramuscular and intraperitoneal exposure, it is considered an effect on sexual function and fertility. Although there are no data on the effect of the ovotoxicity on the resulting fertility via relevant routes of exposure, the observed ovotoxicity is considered to result in a reduction of the number of offspring. The intraperitoneal and intramuscular routes of exposure are less relevant for human exposure. Especially ip treatment could result in direct contact of VCD with the ovaria. However, also in dermal and oral studies with mice and oral studies with rats, comparable effects on the ovaries were observed. The general toxicity in these studies were limited to local effects on the skin. Seen the limited general toxicity and the identified mechanism for the induction of ovotoxicity in rats and mice, it is considered that the observed ovotoxicity is a direct effect of VCD and not secondary to the general toxicity. As ovotoxicity is observed in 4 species (rat, mouse, hamster and nonhuman primates) it is considered likely that this effect is also relevant to humans. In addition the presence of KITL and c-kit in the adult human ovary has been demonstrated throughout follicle development, in addition to showing the presence of each isoform. This suggests that the KITL/c-kit system which is a target for VCD in animals is also involved in human folliculogenesis and supports that the ovotoxicity effect is also relevant to humans. Further, there is clear evience for effects on the testis but no information regarding effects on male fertility. Therefore, altogether classification in category 1B is warranted and not category 2.

10.11.4 Adverse effects on development

No data on the developmental toxicity of 4-vinylcyclohexene diepoxide in humans are available and only limited data in animals.

Some data in the fertility study on VCD shows an increase in resorptions (Haas, Christian et al. 2007). Fertility was evaluated on gestational day 16 after ip exposure before mating. In group 1 (mated on the second proestrus after exposure), cycle length, pregnancy rate, and number of live fetuses did not differ between VCD-treated animals and controls, but VCD-treated mice required more matings to become pregnant and had more resorptions. In group 1, the number of resorbed fetuses was significantly increased (1.9 ± 0.4 versus 0.6 ± 0.2 in controls) (figure 10). This is considered an effect on development and not on fertility. Numbers of live fetuses did not differ between group 1 VCD treated and control mice, but there was a greater number of resorptions in the VCD-treated group. Therefore, the conception rate was similar between groups mated soon after primordial follicle loss. However, as there was no exposure during the in utero development, the value of this study for developmental clasification is difficult to assess.

Kodama et al 2009 did a fertility study with female mice which were intraperitoneally injected with doses of 0, 5, 20, 80 mg/kg bw/day VCD from 2 weeks prior to mating to Day 7 of gestation. No effects on reproductive potential at any dose level were observed. At the necropsy of pregnant females, the number of implanted embryos and rate of implantation tended to decrease in the 80 mg/kg bw/day group. No changes were observed in animals given 5 or 20 mg/kg bw/day (Kodama, Yoshida et al. 2009).

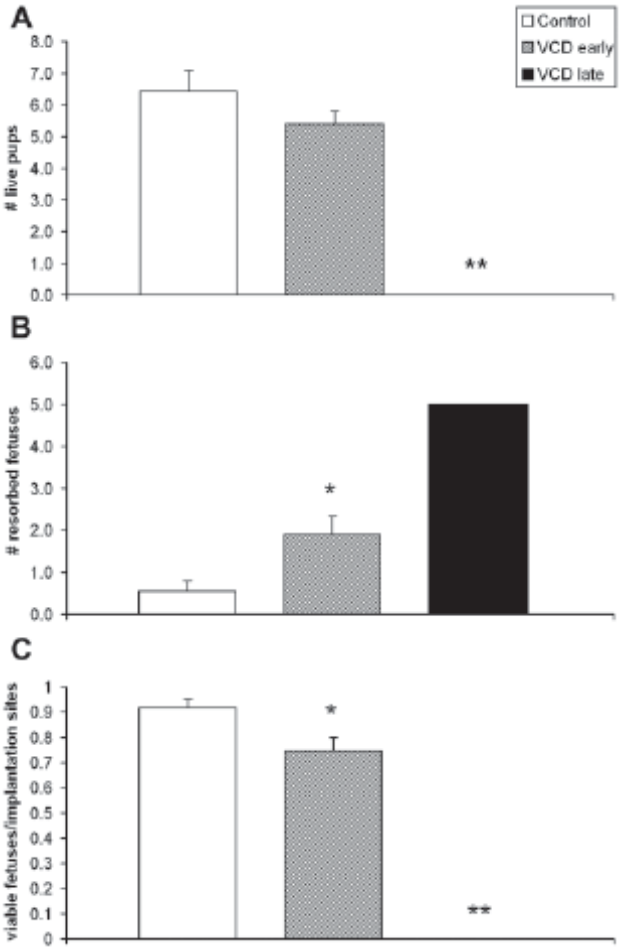


Figure 10. Effect of impeding ovarian failure on fertility outcome. Mature female C7BL/6 mice were dosed daily with vehicle control or VCD (160 mg/kg bw/day intraperitoneally) for 17 d. Control and VCD-treated mice were mated with adult males. Fertility was evaluated on gestational day 16. Measurements in control (open bars n=9), group 1 VCD-treated (crosshatched bars n=10), and Group 2 VCD treated (closed bars n=1) mice were made of A) number of viable foetuses and B) number of resorptions. The ratio of C) viable foetuses versus implantation sites was calculated. Values are represented as mean ± standard error, *P< 0.05 compared with value for controls; **P<0.003 versus value for controls.

10.11.5 Short summary and overall relevance of the provided information on adverse effects on development

No data on the developmental toxicity of 4-vinylcyclohexene diepoxide in humans are available and only limited data in animals.

10.11.6 Comparison with the CLP criteria

No data on the reproductive toxicity of 4-vinylcyclohexene diepoxide in humans are available and only limited data in animals. Therefore, classification is not applicable due to absence of data.

10.11.7 Adverse effects on or via lactation

There are no studies in which animals were exposed to VCD during postnatal development via the mother.

10.11.8 Short summary and overall relevance of the provided information on effects on or via lactation

There are no studies in which animals were exposed to VCD during postnatal development via the mother.

10.11.9 Comparison with the CLP criteria

As there are no studies in which animals were exposed to VCD during postnatal development via the mother, no classification is warranted based on absence of data.

10.11.10 Conclusion on classification and labelling for reproductive toxicity

According to the CLP criteria, 4-vinylcyclohexene diepoxide should be classified as "presumed to be as a reproductive toxicant to humans", which corresponds to classification in catergory 1B.

The hazard statement H360F: "*May damage fertility"* is applicable.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

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

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

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

The DS concluded that since:

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

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

Developmental toxicity and Effects on or via lactation

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

Comments received during public consultation

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

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

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

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

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

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

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

Other studies are non-guideline studies reported in peer-reviewed journals, and in a majority of them, non-standard routes of exposure (for regulatory purposes) were applied (e.g.

intraperitoneal or intramuscular route). Additionally, in many of these studies general toxicity was not reported. Nevertheless, they could serve as supporting evidence, as well as to provide information on mechanisms of ovarian toxicity of VCD. Ovarian toxicity of VCD has been extensively studied, including experiments in which VCD was used as a model to study menopausal effects, or as a substance for pest control.

Oral Studies

16-day oral NTP studies in rats and mice

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

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

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

13-week oral NTP studies in rats and mice

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

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

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

In *female mice*, a dose-dependent increase in the incidence of diffuse ovarian atrophy was observed at 250, 500 and 1 000 mg/kg bw/d. Increased incidence of uterine atrophy was present at the top dose (1 000 mg/kg bw/d). No compound-related deaths occurred in females, and the final mean body weights of mice that received 500 or 1 000 mg/kg bw/d were only 3 % or 6 % lower, respectively, compared to controls. An accident that happened

during the experiment, when 9 females (one at 62.5 mg/kg bw/d, two at 125 mg/kg bw/d, 4 at 250 mg/kg bw/d, and 2 at 1 000 mg/kg bw/d) delivered litters during week 4 of the study, after they escaped from their cages, is not expected to invalidate the study results.

Non-NTP oral studies

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

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

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

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

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

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

Although in the study of Witmer *et al*. (2017), ovarian changes typical for VCD treatment were observed (reduced number of primordial, primary and secondary follicles in treated animals compared to unexposed control), this study was not considered by RAC as appropriate for

reproductive toxicity assessment, and was not further evaluated. Namely, the animals (inbred SD rats and wild-caught Norway rats) were concomitantly exposed (in the same liquid bait) to VCD and another reproductive toxic substance, triptolide. Although triptolide was present as a minor component in the mixture (0.001 %), and it is considered to affect mainly larger ovarian preantral and antral follicles (while VCD is selective for small preantral follicles), triptolide has also been shown to affect primordial follicles in mice (Zeng *et al*., 2016), cause menstrual cycle disruptions in women, affect oestrous cycles in female rats, cause infertility in male rats (Witmer *et al*., 2017), and induce mitochondrial damage in mouse Sertoli cells (Cheng *et al*., 2018). Therefore, possible interference with the VCD reproductive toxic effects cannot be excluded.

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

Dermal Studies

13-week dermal NTP studies in rats and mice

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

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

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

2-year dermal NTP studies in rats and mice

Male and female F344/N rats and male and female B6C3F1 mice, 60 animals per group and gender, were administered VCD in acetone by dermal application to the clipped dorsal

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

In rats, general and dermal toxicity was not marked, as described in the section on carcinogenicity. The only adverse effect in reproductive organs, potentially related to VCD exposure, was an increased incidence of ovarian cyst (8 % in controls, 10 % at 15 mg/rat/d, and 18 % at 30 mg/rat/d).

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

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

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

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

<u>Studies with intraperitoneal, intramuscular or adjacent-to-ovary exposure</u>

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

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

primates; primordial and primary follicles in rats and mice, and primordial, intermediate, primary and secondary follicles in Cynomolgus macaques monkeys.

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

Adverse effects on development of the offspring

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

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

Conclusions

Adverse effects on sexual function and fertility

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

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

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

- 13-week oral NTP study in rats: smaller uterine horns (at 500 mg/kg bw/d);
- 13-week oral NTP study in mice: dose-dependent increase in the incidence of ovarian

atrophy (at 250, 500 and 1 000 mg/kg bw/d), increased incidence of uterine atrophy (at 1 000 mg/kg bw/d);

- 13-week dermal NTP studies in mice: high incidence of ovarian atrophy (at 5 and 10 mg/mouse/d);
- 2-year dermal NTP studies in mice: increased incidence of ovarian atrophy (at 2.5, 5 and 10 mg/mouse/d at 15-month evaluation; at 2.5 mg/mouse/d at 2-year evaluation) and lower uterine weight (at 5 and 10 mg/mouse/d at 15-month evaluation).

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

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

Although in these studies, functional fertility parameters were not directly evaluated, evidence of toxic effects of VCD on ovaries, uteri and testes in rodents was clear, especially regarding ovarian toxicity (follicle depletion) in mice. These findings, according to the CLP Regulation[2] and the CLP Guidance[3], justify classification for reproductive fertility. As supporting evidence, intraperitoneal study in mice showed decreased fertility index at dose level without marked general toxicity (Haas *et al*., 2007).

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

Since:

- there are no human data on the effects on sexual function and fertility by VCD, which would warrant classification in Category 1A,
- adverse effects on reproductive organs (ovaries, uteri and/or testes) were observed in two rodent species (mice and rats) after both oral and dermal exposure,
- these effects were observed at VCD dose levels without or with only limited general toxicity,
- even when these effects were observed at VCD doses with more pronounced general toxicity, they were not considered to be a secondary consequence of systemic toxicity,

[2] CLP Regulation, paragraph 3.7.1.3. Adverse effects on sexual function and fertility: "Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, … premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems".

[3] ECHA CLP Guidance 2017: "Use of data from standard repeat dose tests, Fertility effects: Toxicological effects, including marked effects, observed in a standard repeat dose study could be considered valid for the pre-mating phase for adult females and the pre- and post-mating phase for adult males".

taking into account that they were continuation of reproductive toxic effects observed at lower VCD doses, and considering mechanism of VCD ovarian toxicity (direct toxic effects on oocytes), and

- there is no indication that mechanisms of VCD-related ovarian and uterine toxicity are not relevant for humans as well,

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

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

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

Adverse effects on development of the offspring

Since there are no available guideline studies in which developmental toxicity was assessed, and the available non-guideline studies from open literature used non-standard exposure route and study protocols which greatly differed from those recommended by OECD TGs, **RAC agrees with the DS that no classification for developmental toxicity of VCD is warranted due to lack of data**.

Effects on or via lactation

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

Supplemental information - In depth analyses by RAC

Adverse effects on sexual function and fertility

NTP oral studies

Table. Adverse effects on fertility in oral NTP studies in mice and rats

Table S3. Adverse effects on fertility in oral NTP studies in mice and rats

	Males						Females					
16-day oral study in mice												
VCD dose (mg/kg bw/day):	**0**	**125**	**250**	**500**	**1000**	**2000**	**0**	**125**	**250**	**500**	**1000**	**2000**
General toxicity												
VCD-related mortality (a)	0/5	1/5	0/5	0/5	2/5	5/5	0/5	0/5	0/5	0/5	0/5	5/5
Final body weight (% of Control)	(ref)	100	107	103	98	NA	(ref)	98	99	98	99	NA
Clinical signs	-	-	-	-	-	+ (b)	-	-	-	-	-	+ (b)
Reproductive toxicity												
Testicular degeneration					4/5	NA						
13-week oral study in mice												
VCD dose (mg/kg bw/day):	**0**	**62.5**	**125**	**250**	**500**	**1000**	**0**	**62.5**	**125**	**250**	**500**	**1000**
General toxicity												
VCD-related mortality (a)	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Final body weight (% of Control)	(ref)	102	99	99	87	85	(ref)	97	103	105	97	94
Reproductive toxicity												
Ovarian atrophy							0/10	0/10	0/10	5/10	6/10	10/10
Uterine atrophy							0/10	0/10	1/10	0/10	0/10	7/10
Testicular degeneration	1/10	0/10	0/10	8/10	8/10	9/10						
13-week oral study in rats												
VCD dose (mg/kg bw/day):	**0**	**62.5**	**125**	**250**	**500**	**1000**	**0**	**62.5**	**125**	**250**	**500**	**1000**
General toxicity												
VCD-related mortality (a)	0/10	0/10	0/10	0/10	0/10	3/10	0/10	0/10	0/10	0/10	0/10	6/10
Final body weight (% of Control)	(ref)	105	102	99	94	77	(ref)	97	95	95	93	80
Clinical signs				(d)	(c)(d)	(c)(d)					(c)(d)	(c)(d)
Reproductive toxicity												
Smaller uterine horns											+	+
Smaller than normal testes					+	+						
Testicular degeneration						1/10						

(ref) = referent value; NA = not applicable; (a) Mortality due to gavage error is not reported in the table; (b) Hyperpnoea, burrowing behaviour and half-closed eyelids; (c) Burrowing behaviour and closed eyes; (d) Excessive salivation

NTP dermal studies

Table. Adverse effects on fertility in dermal NTP study in mice

Table S4. Adverse effects on fertility in dermal NTP study in mice

	Males						Females					
13-week dermal study												
VCD dose (mg/mouse/day):	**0**	**0.625**	**1.25**	**2.5**	**5**	**10**	**0**	**0.625**	**1.25**	**2.5**	**5**	**10**
General and skin toxicity												
VCD-related mortality (a)	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Final body weight (% of Control)	(ref)	99	103	98	98	97	(ref)	98	101	97	100	96
Necrotising skin inflammation	0/10	NE	NE	0/10	0/10	1/10	0/10	NE	NE	0/10	0/10	0/10
Reproductive toxicity												
Ovarian atrophy							NR				4/10	10/10
Uterine atrophy							NR					2/10
2-year dermal study												
VCD dose (mg/mouse/day):	**0**			**2.5**	**5**	**10**	**0**			**2.5**	**5**	**10**
• **At 15 months**												
General toxicity												
VCD-related mortality (a)	0/10			0/10	0/10	0/10	0/10			0/10	0/10	0/10
Final body weight (% of Control)	(ref)			97	99	87	(ref)			103	106	101
Reproductive toxicity												
Ovarian atrophy							1/10			10/10	10/10	10/10
Uterine relative weight (% of Con)											31	42
• **At 2 years**												
General toxicity												
VCD-related mortality (b)	9/50			15/50	44/50	50/50	17/50			18/50	36/50	38/50
Final body weight (% of Control)	(ref)			104	88	NA	(ref)			102	88	NA
Necrotising skin inflammation	1/50			4/50	12/50	15/50	2/50			5/50	15/50	16/50
Reproductive toxicity												
Ovarian atrophy							3/50			32/50	29/50	39/50
Epididymis, subacute inflammation	0/50			0/50	6/50	13/50						

(ref) = referent value; NA = not applicable; NE = not evaluated; NR = not reported, but in 13-week oral NTP study in mice ovarian and uterine atrophy was not found, and in chronic dermal NTP study in mice ovarian atrophy was found in 1/10 females at 15-month evaluation and in 3/50 females during the remaining period of 2-year study; (a) Mortality due to gavage error is not reported in the table; (b) Natural deaths and moribund kills.

Non-NTP oral studies

Abolaji *et al*. (2016) – Serum reproductive hormones in female rats

Table 1. Effects of VCD on serum reproductive hormones of female rats

Hormones (IU/L)	Control	VCD (mg/kg Body Weight) 100	250	500
LH	17.50 ± 2.52	17.50 ± 0.71	16.00 ± 1.00	25.67 ± 2.52*
FSH	13.00 ± 1.41	13.00 ± 1.41	11.50 ± 0.71	21.67 ± 1.53*
PRL	24.00 ± 3.61	15.33 ± 1.15*	17.67 ± 1.53*	25.33 ± 2.52
PRG	5.26 ± 0.64	5.31 ± 0.32	5.15 ± 0.07	3.33 ± 0.49*
ESTR	35.67 ± 5.51	32.67 ± 2.08	39.33 ± 2.52	25.33 ± 2.52*

The data are expressed as mean ± SD, n=7
*Significantly different from control (P < 0.05).
LH = luteinizing hormone
FSH = follicle stimulating hormone
PRL = prolactin
PRG = progesterone
ESTR = oestrogen

Berger and Horner (2003) – Exposure to VCD

Table 4
Weight and reproductive parameters in female rats after exposure to 4-vinylcyclohexene diepoxide

	Control[a]	0.02% 4-vinylcyclohexene diepoxide[a]	0.04% 4-vinylcyclohexene diepoxide[a]	S.E.M.
Weight gain (g)	68.2	68.2	70.0	2.6
Final weight (g)	143.3	146.4	150.1	4.7
Percentage of females ovulating	100	92	100	6
Number of oocytes recovered per ovulating female	25	30	20	3
Percentage of oocytes remaining after removal of the zona pellucida	51	35[b]	36[b]	7
Percentage of oocytes fertilized	74	72	72	2
Penetrated sperm/oocyte	1.02	1.38	1.34	0.23

[a] Values are least square means; $n = 3$.
[b] Value differs from mean for control females, $P < 0.10$.

The fragility of oocytes was calculated as the percentage of oocytes remaining after acidic treatment to remove the Zona pellucida (Tyrode's treatment, pH 2.5) of the total number of oocytes collected from oviducts and ovaries treated with hyaluronidase.

10.12 Specific target organ toxicity-single exposure

Not evaluated in this dossier.

10.13 Specific target organ toxicity-repeated exposure

Not evaluated in this dossier.

10.14 Aspiration hazard

Not evaluated in this dossier.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

11.1 Rapid degradability of organic substances

Not evaluated in this dossier.

11.2 Environmental transformation of metals or inorganic metals compounds

Not evaluated in this dossier.

11.3 Environmental fate and other relevant information

Not evaluated in this dossier.

11.4 Bioaccumulation

Not evaluated in this dossier.

11.5 Acute aquatic hazard

Not evaluated in this dossier.

11.6 Long-term aquatic hazard

Not evaluated in this dossier.

11.7 Comparison with the CLP criteria

Not evaluated in this dossier.

11.8 CONCLUSION ON CLASSIFICATION AND LABELLING FOR ENVIRONMENTAL HAZARDS

Not evaluated in this dossier.

12 EVALUATION OF ADDITIONAL HAZARDS

Not evaluated in this dossier.

13 ADDITIONAL LABELLING

14 REFERENCES

(ACGIH), A. C. o. G. I. H. (2001). Vinyl cyclohexene dioxide. Documentation of the threshold limit values and biological exposure indices. Cincinnati: ACGIH;.

Abolaji, A. O., I. A. Adedara, A. O. Abajingin, O. J. Fatunmibi, E. O. Ladipo and E. O. Farombi (2016). "Evidence of oxidative damage and reproductive dysfunction accompanying 4-vinylcyclohexene diepoxide exposure in female Wistar rats." Reprod Toxicol.

Adedara, I. A., A. O. Abolaji, E. O. Ladipo, O. J. Fatunmibi, A. O. Abajingin and E. O. Farombi (2016). "4-Vinylcyclohexene diepoxide disrupts sperm characteristics, endocrine balance and redox status in testes and epididymis of rats." Redox Report: 1-11.

Appt, S. E., T. B. Clarkson, P. B. Hoyer, N. D. Kock, A. K. Goode, M. C. May, J. T. Persyn, N. K. Vail, K. F. Ethun, H. Chen, N. Sen and J. R. Kaplan (2010). "Experimental induction of reduced ovarian reserve in a nonhuman primate model (Macaca fascicularis)." Comp Med **60**(5): 380-388.

Appt, S. E., J. R. Kaplan, T. B. Clarkson, J. M. Cline, P. J. Christian and P. B. Hoyer (2006). "Destruction of primordial ovarian follicles in adult cynomolgus macaques after exposure to 4-vinylcyclohexene diepoxide: a nonhuman primate model of the menopausal transition." Fertil Steril **86**(4 Suppl): 1210-1216.

Berger, T. and C. M. Horner (2003). "In vivo exposure of female rats to toxicants may affect oocyte quality." Reprod Toxicol **17**(3): 273-281.

Borman, S. M., B. J. VanDePol, S. Kao, K. E. Thompson, I. G. Sipes and P. B. Hoyer (1999). "A single dose of the ovotoxicant 4-vinylcyclohexene diepoxide is protective in rat primary ovarian follicles." Toxicol Appl Pharmacol **158**(3): 244-252.

Bronzetti, G., C. Bauer, C. Corsi, C. Leporini and R. Nieri (1980). "Genetic effects of vinylcyclohexene diepoxide in yeast." Boll Soc Ital Biol Sper **56**(18): 1803-1806.

Burd, A. M. (2014). "In vivo and in vitro studies of 4-vinylcyclohexene diepoxide in wild-caught female brushtail possums (Trichosurus vulpecula) and Norway rats (Rattus norvegicus) and its potential as a fertility control agent."

Cancer., I. A. f. R. o. (1994). 4-vinylcyclohexene diepoxide. IARC Monographs on evaluating carcinogenic risk of chemicals in humans. Lyon, France. **60:** 361-372.

Cannady, E. A., C. A. Dyer, P. J. Christian, I. G. Sipes and P. B. Hoyer (2003). "Expression and activity of cytochromes P450 2E1, 2A, and 2B in the mouse ovary: the effect of 4-vinylcyclohexene and its diepoxide metabolite." Toxicol Sci **73**(2): 423-430.

Carlsson, I. B., M. P. Laitinen, J. E. Scott, H. Louhio, L. Velentzis, T. Tuuri, J. Aaltonen, O. Ritvos, R. M. Winston and O. Hovatta (2006). "Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture." Reproduction **131**(4): 641-649.

Chen, Z., X. Kang, L. Wang, H. Dong, C. Wang, Z. Xiong, W. Zhao, C. Jia, J. Lin, W. Zhang, W. Yuan, M. Zhong, H. Du and X. Bai (2015). "Rictor/mTORC2 pathway in oocytes regulates folliculogenesis, and its inactivation causes premature ovarian failure." J Biol Chem **290**(10): 6387-6396.

Chhabra, R. S., J. Huff, J. Haseman, M. P. Jokinen and M. Hetjmancik (1990). "Dermal toxicity and carcinogenicity of 4-vinyl-1-cyclohexene diepoxide in Fischer rats and B6C3F1 mice." Fundam Appl Toxicol **14**(4): 752-763.

Clermont, Y. (1972). "Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal." Physiological Reviews **52**: 198-236.

Devine, P. J., I. G. Sipes, M. K. Skinner and P. B. Hoyer (2002). "Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide." Toxicol Appl Pharmacol **184**(2): 107-115.

Dhillon, S. and R. Von Burg (1996). "Vinylcyclohexene dioxide." J Appl Toxicol **16**(5): 465-468.

Doerr, J. K., S. B. Hooser, B. J. Smith and I. G. Sipes (1995). "Ovarian toxicity of 4-vinylcyclohexene and related olefins in B6C3F1 mice: role of diepoxides." Chem Res Toxicol **8**(7): 963-969.

El-Tantawy, M. A. and B. D. Hammock (1980). "The effect of hepatic microsomal and cytosolic subcellular fractions on the mutagenic activity of epoxide-containing compounds in the Salmonella assay." Mutat Res **79**(1): 59-71.

Fernandez, S. M., A. F. Keating, P. J. Christian, N. Sen, J. B. Hoying, H. L. Brooks and P. B. Hoyer (2008). "Involvement of the KIT/KITL signaling pathway in 4-vinylcyclohexene diepoxide-induced ovarian follicle loss in rats." Biol Reprod **79**(2): 318-327.

Flaws, J. A., K. L. Salyers, I. G. Sipes and P. B. Hoyer (1994). "Reduced ability of rat preantral ovarian follicles to metabolize 4-vinyl-1-cyclohexene diepoxide in vitro." Toxicol Appl Pharmacol **126**(2): 286-294.

Frantz, S. W. and J. E. Sinsheimer (1981). "Bacterial mutagenicity and toxicity of cycloaliphatic epoxides." Mutat Res **90**(1): 67-78.

Frye, J. B., A. L. Lukefahr, L. E. Wright, S. L. Marion, P. B. Hoyer and J. L. Funk (2012). "Modeling perimenopause in Sprague-Dawley rats by chemical manipulation of the transition to ovarian failure." Comp Med **62**(3): 193-202.
Giannarini, C., L. Citti, P. G. Gervasi and G. Turchi (1981). "Effects of 4-vinylcyclohexene and its main oxirane metabolite on mouse hepatic microsomal enzymes and glutathione levels." Toxicol Lett **8**(1-2): 115-121.
Haas, J. R., P. J. Christian and P. B. Hoyer (2007). "Effects of impending ovarian failure induced by 4-vinylcyclohexene diepoxide on fertility in C57BL/6 female mice." Comp Med **57**(5): 443-449.
Hendry, J. A., R. F. Homer, F. L. Rose and A. L. Walpole (1951). "Cytotoxic agents: II. Bis-epoxides and related compounds." Br J Pharmacol Chemother **6**(2): 235-255.
Hooser, S. B., D. G. DeMerell, D. A. Douds, P. Hoyer and I. G. Sipes (1995). "Testicular germ cell toxicity caused by vinylcyclohexene diepoxide in mice." Reprod Toxicol **9**(4): 359-367.
Hooser, S. B., D. P. Douds, D. G. DeMerell, P. B. Hoyer and I. G. Sipes (1994). "Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene." Reprod Toxicol **8**(4): 315-323.
Hoyer, P. B. and P. J. devine (2002). "The female reproductive system. ." Handbook of toxicology: 573-595.
Hoyer, P. B., P. J. Devine, X. Hu, K. E. Thompson and I. G. Sipes (2001). "Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model." Toxicol Pathol **29**(1): 91-99.
Hoyer, P. B. and I. G. Sipes (1996). "Assessment of follicle destruction in chemical-induced ovarian toxicity." Annu Rev Pharmacol Toxicol **36**: 307-331.
Hoyer, P. B. and I. G. Sipes (2007). "Development of an animal model for ovotoxicity using 4-vinylcyclohexene: a case study." Birth Defects Res B Dev Reprod Toxicol **80**(2): 113-125.
Hoyer, P. E., A. G. Byskov and K. Mollgard (2005). "Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries." Mol Cell Endocrinol **234**(1-2): 1-10.
Hu, X., P. Christian, I. G. Sipes and P. B. Hoyer (2001). "Expression and redistribution of cellular Bad, Bax, and Bcl-X(L) protein is associated with VCD-induced ovotoxicity in rats." Biol Reprod **65**(5): 1489-1495.
Hu, X., P. J. Christian, K. E. Thompson, I. G. Sipes and P. B. Hoyer (2001). "Apoptosis induced in rats by 4-vinylcyclohexene diepoxide is associated with activation of the caspase cascades." Biol Reprod **65**(1): 87-93.
Hu, X., J. A. Flaws, I. G. Sipes and P. B. Hoyer (2002). "Activation of mitogen-activated protein kinases and AP-1 transcription factor in ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats." Biol Reprod **67**(3): 718-724.
Ito, A., N. Mafune and T. Kimura (2009). "Collaborative work on evaluation of ovarian toxicity. 4) Two- or four-week repeated dose study of 4-vinylcyclohexene diepoxide in female rats." J Toxicol Sci **34 Suppl 1**: SP53-58.
Jin, X., C. S. Han, F. Q. Yu, P. Wei, Z. Y. Hu and Y. X. Liu (2005). "Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction." Mol Reprod Dev **70**(1): 82-90.
Kao, S. W., I. G. Sipes and P. B. Hoyer (1999). "Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice." Reprod Toxicol **13**(1): 67-75.
Kappeler, C. J. and P. B. Hoyer (2012). "4-vinylcyclohexene diepoxide: a model chemical for ovotoxicity." Syst Biol Reprod Med **58**(1): 57-62.
Keating, A. F., J. M. C, N. Sen, I. G. Sipes and P. B. Hoyer (2009). "Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7, 12-dimethylbenz[a]anthracene in neonatal rat ovaries." Toxicol Appl Pharmacol **241**(2): 127-134.
Keating, A. F., S. M. Fernandez, C. J. Mark-Kappeler, N. Sen, I. G. Sipes and P. B. Hoyer (2011). "Inhibition of PIK3 signaling pathway members by the ovotoxicant 4-vinylcyclohexene diepoxide in rats." Biol Reprod **84**(4): 743-751.
Keating, A. F., K. S. Rajapaksa, I. G. Sipes and P. B. Hoyer (2008). "Effect of CYP2E1 gene deletion in mice on expression of microsomal epoxide hydrolase in response to VCD exposure." Toxicol Sci **105**(2): 351-359.
Keating, A. F., N. Sen, I. G. Sipes and P. B. Hoyer (2010). "Dual protective role for glutathione S-transferase class pi against VCD-induced ovotoxicity in the rat ovary." Toxicol Appl Pharmacol **247**(2): 71-75.
Keating, A. F., I. G. Sipes and P. B. Hoyer (2008). "Expression of ovarian microsomal epoxide hydrolase and glutathione S-transferase during onset of VCD-induced ovotoxicity in B6C3F(1) mice." Toxicol Appl Pharmacol **230**(1): 109-116.

Klimisch, H., M. Andreae and U. Tillmann (1997). "A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data." Regul Toxicol Pharmacol **25**(1): 1-5.
Kodama, T., J. Yoshida, T. Miwa, D. Hasegawa and T. Masuyama (2009). "Collaborative work on evaluation of ovarian toxicity. 4) Effects of fertility study of 4-vinylcyclohexene diepoxide in female rats." J Toxicol Sci **34 Suppl 1**: SP59-63.
Kotin, P. and H. L. Falk (1963). "Organic peroxides, hydrogen peroxide, epoxides, and neoplasia." Radiat Res **Suppl 3**: 193-211.
Li, J., S. Fan, D. Han, J. Xie, H. Kuang and P. Ge (2014). "Microarray gene expression profiling and bioinformatics analysis of premature ovarian failure in a rat model." Exp Mol Pathol **97**(3): 535-541.
Lide, D. R. (1992). CRC Handbook of Chemistry and Physiscs
Liu, K., S. Rajareddy, L. Liu, K. Jagarlamudi, K. Boman, G. Selstam and P. Reddy (2006). "Control of mammalian oocyte growth and early follicular development by the oocyte PI3 kinase pathway: new roles for an old timer." Dev Biol **299**(1): 1-11.
Liu, W., L. Y. Wang, X. X. Xing and G. W. Fan (2015). "Conditions and possible mechanisms of VCD-induced ovarian failure." Altern Lab Anim **43**(6): 385-392.
Lohff, J. C., P. J. Christian, S. L. Marion and P. B. Hoyer (2006). "Effect of duration of dosing on onset of ovarian failure in a chemical-induced mouse model of perimenopause." Menopause **13**(3): 482-488.
Mabon, N. and K. Randerath (1996). "32P-postlabeling of 1,3-butadiene and 4-vinyl-1-cyclohexene metabolite-DNA adducts: in vitro and in vivo applications." Toxicology **113**(1-3): 341-344.
Mark-Kappeler, C. J., N. Sen, A. Lukefahr, L. McKee, I. G. Sipes, J. Konhilas and P. B. Hoyer (2011). "Inhibition of ovarian KIT phosphorylation by the ovotoxicant 4-vinylcyclohexene diepoxide in rats." Biol Reprod **85**(4): 755-762.
Maronpot, R. R. (1987). "Ovarian toxicity and carcinogenicity in eight recent National Toxicology Program studies." Environ Health Perspect **73**: 125-130.
Marshall, C. J. (1995). "Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation." Cell **80**(2): 179-185.
Mayer, L. P., N. A. Pearsall, P. J. Christian, P. J. Devine, C. M. Payne, M. K. McCuskey, S. L. Marion, I. G. Sipes and P. B. Hoyer (2002). "Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide." Reprod Toxicol **16**(6): 775-781.
McGregor, D. B., A. Brown, P. Cattanach, I. Edwards, D. McBride, C. Riach and W. J. Caspary (1988). "Responses of the L5178Y tk+/tk- mouse lymphoma cell forward mutation assay: III. 72 coded chemicals." Environ Mol Mutagen **12**(1): 85-154.
Mortelmans, K., S. Haworth, T. Lawlor, W. Speck, B. Tainer and E. Zeiger (1986). "Salmonella mutagenicity tests: II. Results from the testing of 270 chemicals." Environ Mutagen **8 Suppl 7**: 1-119.
Muhammad, F. S., A. K. Goode, N. D. Kock, E. A. Arifin, J. M. Cline, M. R. Adams, P. B. Hoyer, P. J. Christian, S. Isom, J. R. Kaplan and S. E. Appt (2009). "Effects of 4-vinylcyclohexene diepoxide on peripubertal and adult Sprague-Dawley rats: ovarian, clinical, and pathologic outcomes." Comp Med **59**(1): 46-59.
Murray, M. P. and J. E. Cummins (1979). "Mutagenic activity of epoxy embedding reagents employed in electron microscopy." Environ Mutagen **1**(4): 307-313.
National Toxicology, P. (1989). "NTP Toxicology and Carcinogenesis Studies of 4-Vinyl-cyclohexene-diepoxide(CAS No. 106-87-6) in F344/N Rats and B6C3F1 Mice (Dermal Studies)." Natl Toxicol Program Tech Rep Ser **362**.
Netherlands, H. C. o. t. (2008). 4-Vinylcyclohexene diepoxide; Evaluation of the carcinogenicity and genotoxicity. H. C. o. t. Netherlands. The Hague
Netherlands, H. C. o. t. (2016). 4-Vinylcyclohexene diepoxide. Evaluation of the carcinogenicity and genotoxicity. . H. C. o. t. Netherlands. The Hague.
Ogawa, M., Y. Matsuzaki, S. Nishikawa, S. Hayashi, T. Kunisada, T. Sudo, T. Kina, H. Nakauchi and S. Nishikawa (1991). "Expression and function of c-kit in hemopoietic progenitor cells." J Exp Med **174**(1): 63-71.
Packer, A. I., Y. C. Hsu, P. Besmer and R. F. Bachvarova (1994). "The ligand of the c-kit receptor promotes oocyte growth." Dev Biol **161**(1): 194-205.

Parrott, J. A. and M. K. Skinner (1997). "Direct actions of kit-ligand on theca cell growth and differentiation during follicle development." Endocrinology **138**(9): 3819-3827.
Parrott, J. A. and M. K. Skinner (1998). "Thecal cell-granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and Kit ligand during ovarian follicular development." Endocrinology **139**(5): 2240-2245.
Parrott, J. A. and M. K. Skinner (1999). "Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis." Endocrinology **140**(9): 4262-4271.
program, N. T. (1989). Toxicology and Carcinogenesis Studies of 4-Vinyl-1-cyclohexene Diepoxide (CAS No. 106-87-6) in F344/N Rats and B6C3F1 Mice (Dermal Studies). N. I. o. Health. Research Triangle Park, NC,.
program, N. T. (2011). 4-Vinyl-1-cyclohexene Diepoxide. Report on Carcinogens. N. I. o. Health. Research Triangle Park, NC.
RAC, C. f. R. A. (2012). Opinion proposing harmonised classification and labelling at EU level of 4 vinylcyclohexene (VCH). ECHA/RAC/CLH-O-0000002966-62-01/F. Helsinki, Finland.
Rajapaksa, K. S., E. A. Cannady, I. G. Sipes and P. B. Hoyer (2007). "Involvement of CYP 2E1 enzyme in ovotoxicity caused by 4-vinylcyclohexene and its metabolites." Toxicol Appl Pharmacol **221**(2): 215-221.
Randerath, K. and N. Mabon (1996). "In vitro and in vivo (32)P-postlabeling analysis of 4-vinyl-1-cyclohexene (butadiene dimer) diepoxide-DNA adducts." Cancer Lett **101**(1): 67-72.
Reddy, P., L. Shen, C. Ren, K. Boman, E. Lundin, U. Ottander, P. Lindgren, Y. X. Liu, Q. Y. Sun and K. Liu (2005). "Activation of Akt (PKB) and suppression of FKHRL1 in mouse and rat oocytes by stem cell factor during follicular activation and development." Dev Biol **281**(2): 160-170.
Reed, J. C. (1997). "Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies." Semin Hematol **34**(4 Suppl 5): 9-19.
Reus, A. A., M. Usta and C. A. Krul (2012). "The use of ex vivo human skin tissue for genotoxicity testing." Toxicol Appl Pharmacol **261**(2): 154-163.
Ringo, D. L., E. F. Brennan and E. H. Cota-Robles (1982). "Epoxy resins are mutagenic: implications for electron microscopists." J Ultrastruct Res **80**(3): 280-287.
Roosa, K. A., M. Mukai and N. J. Place (2015). "4-Vinylcyclohexene diepoxide reduces fertility in female Siberian hamsters when treated during their reproductively active and quiescent states." Reprod Toxicol **51**: 40-46.
Roosa, K. A. and N. J. Place (2015). "Mate preference for dominant vs. subordinate males in young female Syrian hamsters (Mesocricetus auratus) following chemically-accelerated ovarian follicle depletion." Physiol Behav **152**(Pt A): 41-46.
Schmuki, S. (2009). "Effects of oral 4-vinylcyclohexene diepoxide (VCD) on exposure on spermatorgenesis and steriodogenesis in Sprague Dawley rats." Biological Sciences **Northern Arizona University, Flagstaff, Arizona**: 71.
Simmon, V. F. and J. M. Baden (1980). "Mutagenic activity of vinyl compounds and derived epoxides." Mutat Res **78**(3): 227-231.
Smith, B. J., D. E. Carter and I. G. Sipes (1990). "Comparison of the disposition and in vitro metabolism of 4-vinylcyclohexene in the female mouse and rat." Toxicol Appl Pharmacol **105**(3): 364-371.
Sobinoff, A. P., V. Pye, B. Nixon, S. D. Roman and E. A. McLaughlin (2010). "Adding insult to injury: effects of xenobiotic-induced preantral ovotoxicity on ovarian development and oocyte fusibility." Toxicol Sci **118**(2): 653-666.
Springer, L. N., M. E. McAsey, J. A. Flaws, J. L. Tilly, I. G. Sipes and P. B. Hoyer (1996). "Involvement of apoptosis in 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats." Toxicol Appl Pharmacol **139**(2): 394-401.
Takai, Y., J. Canning, G. I. Perez, J. K. Pru, J. J. Schlezinger, D. H. Sherr, R. N. Kolesnick, J. Yuan, R. A. Flavell, S. J. Korsmeyer and J. L. Tilly (2003). "Bax, caspase-2, and caspase-3 are required for ovarian follicle loss caused by 4-vinylcyclohexene diepoxide exposure of female mice in vivo." Endocrinology **144**(1): 69-74.
Tennant, R. W., J. E. French and J. W. Spalding (1995). "Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models." Environ Health Perspect **103**(10): 942-950.

Tennant, R. W., J. Spalding and J. E. French (1996). "Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens." Mutat Res **365**(1-3): 119-127.
Thompson, K. E., S. M. Bourguet, P. J. Christian, J. C. Benedict, I. G. Sipes, J. A. Flaws and P. B. Hoyer (2005). "Differences between rats and mice in the involvement of the aryl hydrocarbon receptor in 4-vinylcyclohexene diepoxide-induced ovarian follicle loss." Toxicol Appl Pharmacol **203**(2): 114-123.
Tuck, A. R., R. L. Robker, R. J. Norman, W. D. Tilley and T. E. Hickey (2015). "Expression and localisation of c-kit and KITL in the adult human ovary." J Ovarian Res **8**: 31.
Turchi, G., S. Bonatti, L. Citti, P. G. Gervasi and A. Abbondandolo (1981). "Alkylating properties and genetic activity of 4-vinylcyclohexene metabolites and structurally related epoxides." Mutat Res **83**(3): 419-430.
Vanduuren, B. L., N. Nelson, L. Orris, E. D. Palmes and F. L. Schmitt (1963). "Carcinogenicity of Epoxides, Lactones, and Peroxy Compounds." J Natl Cancer Inst **31**: 41-55.
Wade, M. J., J. W. Moyer and C. H. Hine (1979). "Mutagenic action of a series of epoxides." Mutat Res **66**(4): 367-371.
Watabe, T., A. Hiratsuka, M. Isobe and N. Ozawa (1980). "Metabolism of d-limonene by hepatic microsomes to non-mutagenic epoxides toward Salmonella typhimurium." Biochem Pharmacol **29**(7): 1068-1071.
Weil, C. S., N. Condra, C. Haun and J. A. Striegel (1963). "Experimental Carcinogenicity and Acute Toxicity of Representative Epoxides." Am Ind Hyg Assoc J **24**: 305-325.
Witmer, G. W., S. Raymond-Whish, R. S. Moulton, B. R. Pyzyna, E. M. Calloway, C. A. Dyer, L. P. Mayer and P. B. Hoyer (2017). "Compromised Fertility in Free Feeding of Wild-Caught Norway Rats (Rattus Norvegicus) with a Liquid Bait Containing 4-Vinylcyclohexene Diepoxide and Triptolide." J Zoo Wildl Med **48**(1): 80-90.
Wright, L. E., J. B. Frye, A. L. Lukefahr, S. L. Marion, P. B. Hoyer, D. G. Besselsen and J. L. Funk (2011). "4-Vinylcyclohexene diepoxide (VCD) inhibits mammary epithelial differentiation and induces fibroadenoma formation in female Sprague Dawley rats." Reprod Toxicol **32**(1): 26-32.
Wyllie, A. H., J. F. Kerr and A. R. Currie (1980). "Cell death: the significance of apoptosis." Int Rev Cytol **68**: 251-306.
Yamamoto, S., K. Urano, H. Koizumi, S. Wakana, K. Hioki, K. Mitsumori, Y. Kurokawa, Y. Hayashi and T. Nomura (1998). "Validation of transgenic mice carrying the human prototype c-Ha-ras gene as a bioassay model for rapid carcinogenicity testing." Environ Health Perspect **106 Suppl 1**: 57-69.
Zhang, T., D. Yan, Y. Yang, A. Ma, L. Li, Z. Wang, Q. Pan and Z. Sun (2016). "The comparison of animal models for premature ovarian failure established by several different source of inducers." Regul Toxicol Pharmacol **81**: 223-232.

Additional references

Cheng Y, Chen G, Wang L, Kong J, Pan J, Xi Y, Shen F, Huang Z (2018) Triptolide-induced mitochondrial damage dysregulates fatty acid metabolism in mouse sertoli cells. Toxicol Lett 292:136-50.

EC Guidelines for setting specific concentration limits for carcinogens in Annex I of Directive 67/548/EEC, 1999

Endocrine disruption: a guidance document for histologic evaluation of endocrine and reproductive tests. Part 3. Female reproductive system. OECD, 2008

Goggin M, Sangaraju D, Walker VE, Wickliffe J, Swenberg JA, Tretyakova N. Persistence and repair of bifunctional DNA adducts in tissues of laboratory animals exposed to 1,3-butadiene by inhalation. Chem Res Toxicol 2011;24:809-17.

International Agency for Research on Cancer (IARC) (1976) 1-Epoxyethyl-3,4-epoxycyclohexane. Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man, Vol. 11. Cadmium, Nickel, Some Epoxides, Miscellaneous Industrial Chemicals and General Considerations on Volatile Anaesthetics. Lyon, France: IARC, pp. 141-45.

Jamieson S, Fuller PJ (2012) Molecular pathogenesis of granulosa cell tumors of the ovary. Endocr Rev ;33:109-44.

Malgorzata D, Malgorzata G, Malgorzata K-S, Tabarowski Z (2016) The primordial to primary follicle transition — a reliable marker of ovarian function. In: Insights from Animal Reproduction, Chapter 6. InTech, http://dx.doi.org/10.5772/62138

Schurdak ME, Randerath K, Randerath K (1989) Effects of route of administration on tissue distribution of DNA adducts in mice - comparison of 7h-dibenzo(c,g)carbazole, benzo(a)pyrene, and 2-acetylaminofluorene. Cancer Res 49: 2633-38

Shell Chemical Company, Unpublished Report, 2401 Crow Canyon Rd., San Romon, CA 94583; cited in RTECS, Registry of Toxic Effects of Chemical Substances [database on-line]. NIOSH, Bethesda, MD 1995

Zeng Y, Sun H, Li Y, Shao M, Han P, Yu X, He L, Xu Y, Li S (2017) Exposure to triptolide affects follicle development in NIH mice: Role of endoplasmic reticulum stress in granulosa cell apoptosis. Hum Exp Toxicol 36:82-92.

15 ANNEXES

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON 7-OXA-3-OXIRANYLBICYCLO[4.1.0]HEPTANE; 1,2-EPOXY-4-EPOXYETHYLCYCLOHEXANE; 4-VINYLCYCLOHEXENE DIEPOXIDE

Annex I - NTP studies (National Toxicology 1989)

Table1: Survival and mean body weights of rats in the 13-week dermal studies of VCD

Dose (mg/rat)	Survival (a)	Mean Body Weights (grams) Initial (b)	Final	Change (c)	Final Weight Relative to Vehicle Controls (percent)
MALE					
0	10/10	160 ± 3	337 ± 6	+177 ± 6	
3.75	10/10	159 ± 4	346 ± 7	+187 ± 4	102.7
7.5	10/10	159 ± 4	333 ± 9	+174 ± 7	98.8
15	10/10	155 ± 4	343 ± 2	+188 ± 4	101.8
30	10/10	157 ± 4	338 ± 6	+181 ± 7	100.3
60	10/10	158 ± 5	291 ± 8	+133 ± 5	86.4
FEMALE					
0	10/10	132 ± 2	200 ± 4	+68 ± 3	
3.75	10/10	128 ± 4	205 ± 3	+77 ± 4	102.5
7.5	10/10	128 ± 3	203 ± 2	+75 ± 3	101.5
15	10/10	121 ± 5	194 ± 3	+73 ± 4	97.0
30	10/10	123 ± 4	197 ± 3	+74 ± 4	98.5
60	10/10	129 ± 3	182 ± 2	+53 ± 2	91.0

(a) Number surviving/number initially in the group
(b) Initial group mean body weight ± standard error of the mean
(c) Mean body weight change of the group ± standard error of the mean

Table2: Number of rats with selected skin lesions at the application site in the 13 week dermal studies of VCD.

TABLE 5. NUMBERS OF RATS WITH SELECTED SKIN LESIONS AT THE APPLICATION SITE IN THE THIRTEEN-WEEK DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE (a)

Lesion	Male (mg/rat) 0	15	30	60	Female (mg/rat) 0	15	30	60
Acute to chronic inflammation	0	0	0	3	0	3	2	2
Hyperkeratosis	0	2	**9	**10	0	**10	**9	**10
Parakeratosis	0	0	2	**10	0	0	0	**8
Acanthosis	0	1	**9	**10	0	**9	*4	**10
Necrotizing inflammation	0	0	0	3	0	0	0	**6
Ulcers 0	0	0	3	0	0	0	0	
Sebaceous gland hyperplasia	0	0	**6	**10	0	0	2	**10

(a) Ten animals were examined in each group.
*P<0.05 vs. controls
**P<0.01 vs. controls

Table3: Organ weight to body weight ratios for rats in the 15 month dermal studies of VCD

Organ	Vehicle Control	15 mg/Rat	30 mg/Rat
MALE			
Body weight (b)	486 ± 4.5	480 ± 6.8	**442 ± 5.0
Brain	4.2 ± 0.06	4.3 ± 0.06	**4.6 ± 0.06
Kidney	4.2 ± 0.11	4.0 ± 0.04	4.2 ± 0.15
Liver	40.5 ± 1.13	37.1 ± 1.22	38.2 ± 1.25
Right testis	3.7 ± 0.34	3.4 ± 0.14	3.6 ± 0.47
FEMALE			
Body weight (b)	297 ± 4.0	292 ± 7.5	281 ± 7.5
Brain	6.3 ± 0.08	6.4 ± 0.14	6.7 ± 0.15
Uterus	2.3 ± 0.22	2.1 ± 0.14	2.3 ± 0.43
Ovary	0.5 ± 0.05	0.4 ± 0.01	0.5 ± 0.03
Kidney	3.9 ± 0.10	4.0 ± 0.07	4.0 ± 0.10
Liver	37.0 ± 1.86	35.7 ± 0.54	35.2 ± 1.01

(a) Mean ± standard error for groups of 10 animals in milligrams per gram unless otherwise specified; P values are vs. the vehicle controls by Dunnett's test (Dunnett, 1980) or Williams' test (Williams, 1971, 1972).
(b) Absolute body weight in grams
**P<0.01

Table4: numbers of rats with selected skin lesions in the 15 month dermal studies of VCD.

Lesion	Male (mg/rat)			Female (mg/rat)		
	0	15	30	0	15	30
Acanthosis	0	**7	**9	0	*5	**10
Hyperkeratosis	0	0	*5	0	0	**10
Sebaceous gland hyperplasia	0	**7	**8	0	0	**10
Squamous cell carcinoma	0	0	2	0	0	0

(a) Ten animals were examined in each group.
*P<0.05 vs. controls
**P<0.01 vs. controls

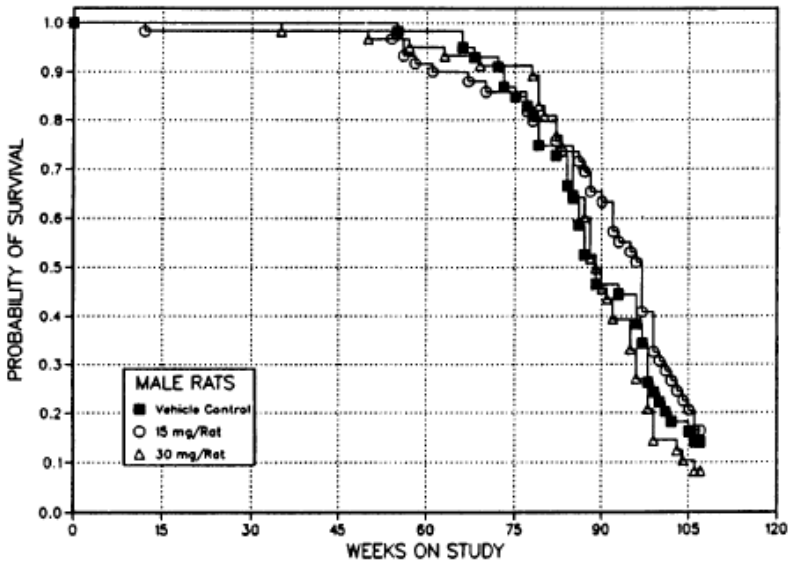
Table5: Survival of rats in the 2 year dermal studies of VCD

TABLE 8. SURVIVAL OF RATS IN THE TWO-YEAR DERMAL STUDIES OF 4-VINYL-1-CYCLOHEXENE DIEPOXIDE

	Vehicle Control	15 mg/Rat	30 mg/Rat
MALE (a)			
Animals initially in study	50	50	50
Natural deaths	12	5	16
Moribund kills	31	37	29
Animals surviving until study termination	7	8	4
Killed accidentally	0	0	1
Survival P values (b)	0.524	0.487	0.590
FEMALE (a)			
Animals initially in study	50	50	50
Natural deaths	8	13	21
Moribund kills	15	14	14
Animals surviving until study termination	27	23	15
Survival P values (b)	0.007	0.262	0.005

(a) First day of termination period: 743
(b) The result of the life table trend test is in the vehicle control column, and the results of the life table pairwise comparisons with the vehicle controls are in the dosed columns.

Table6: Kaplan-Meier survival curves for rats administered VCD in acetone by dermal application for 2 years.



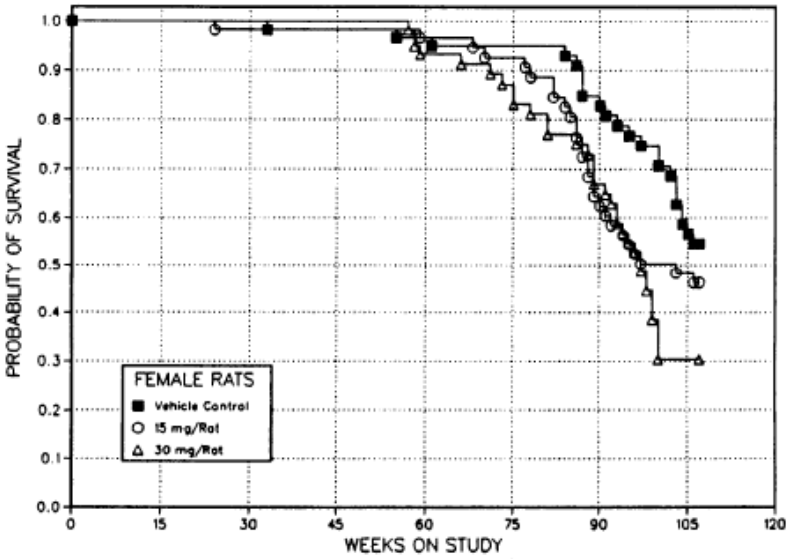


Table7: Skin tumors in rats in the 2 year dermal studies of VCD.

	Vehicle Control	15 mg/Rat	30 mg/Rat
MALE			
Application Site (Scapula or Back)			
Sebaceous Gland Adenoma			
Overall Rates	0/50 (0%)	2/50 (4%)	1/50 (2%)
Basal Cell Adenoma			
Overall Rates	0/50 (0%)	0/50 (0%)	4/50 (8%)
Terminal Rates	0/7 (0%)	0/8 (0%)	1/4 (25%)
Day of First Observation			668
Logistic Regression Tests	P=0.008	(b)	P=0.040
Basal Cell Carcinoma			
Overall Rates	0/50 (0%)	1/50 (2%)	3/50 (6%)
Terminal Rates	0/7 (0%)	0/8 (0%)	0/4 (0%)
Day of First Observation		642	595
Logistic Regression Tests	P=0.055	P=0.502	P=0.110
Basal Cell Adenoma or Basal Cell Carcinoma			
Overall Rates	0/50 (0%)	1/50 (2%)	6/50 (12%)
Terminal Rates	0/7 (0%)	0/8 (0%)	1/4 (25%)
Day of First Observation		642	595
Logistic Regression Tests	P=0.003	P=0.502	P=0.011
Squamous Cell Papilloma (c)			
Overall Rates	0/50 (0%)	3/50 (6%)	6/50 (12%)
Terminal Rates	0/7 (0%)	1/8 (13%)	0/4 (0%)
Day of First Observation		688	595
Logistic Regression Tests	P=0.006	P=0.159	P=0.015
Squamous Cell Carcinoma (d)			
Overall Rates	0/50 (0%)	33/50 (66%)	36/50 (72%)
Terminal Rates	0/7 (0%)	8/8 (100%)	4/4 (100%)
Day of First Observation		596	543
Logistic Regression Tests	P<0.001	P<0.001	P<0.001
Nonapplication Site			
Basal Cell Adenoma			
Overall Rates	1/50 (2%)	0/50 (0%)	0/50 (0%)
Basal Cell Carcinoma			
Overall Rates	1/50 (2%)	1/50 (2%)	0/50 (0%)
Trichoepithelioma			
Overall Rates	0/50 (0%)	0/50 (0%)	1/50 (2%)
Trichoepithelioma, Basal Cell Adenoma, or Basal Cell Carcinoma (e)			
Overall Rates	2/50 (4%)	1/50 (2%)	1/50 (2%)

Table7: Skin tumors in rats in the 2 year dermal studies of VCD. (continued)

	Vehicle Control	15 mg/Rat	30 mg/Rat
FEMALE			
Application Site			
Basal Cell Carcinoma			
Overall Rates	0/50 (0%)	3/50 (6%)	4/50 (8%)
Terminal Rates	0/27 (0%)	2/23 (9%)	2/15 (13%)
Day of First Observation		739	654
Logistic Regression Tests	P=0.015	P=0.081	P=0.032
Sebaceous Gland Adenoma			
Overall Rates	1/50 (2%)	1/50 (2%)	1/50 (2%)
Squamous Cell Carcinoma (f)			
Overall Rates	0/50 (0%)	16/50 (32%)	(g) 34/50 (68%)
Terminal Rates	0/27 (0%)	14/23 (61%)	15/15 (100%)
Day of First Observation		625	601
Logistic Regression Tests	P<0.001	P<0.001	P<0.001
Nonapplication Site			
Basal Cell Adenoma			
Overall Rates	1/50 (2%)	1/50 (2%)	0/50 (0%)
Sebaceous Gland Adenoma, Basal Cell Adenoma, or Basal Cell Carcinoma (h)			
Overall Rates	1/50 (2%)	1/50 (2%)	0/50 (0%)

(a) For a complete explanation of the entries in this table, see Table A3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) No P value is reported because no tumors were observed in the 15 mg/rat and vehicle control groups.
(c) All squamous cell papillomas were observed in animals also bearing a squamous cell carcinoma.
(d) Historical incidence of squamous cell papillomas or carcinomas (combined) in untreated controls (mean ± SD): 31/1,596 (2% ± 2%)
(e) Historical incidence in untreated controls (mean ± SD): 30/1,596 (2% ± 2%)
(f) Historical incidence of squamous cell papillomas or carcinomas (combined) in untreated controls (mean ± SD): 7/1,643 (0.4% ± 0.8%)
(g) A squamous cell papilloma was observed in an animal also bearing a squamous cell carcinoma.
(h) Historical incidence in untreated controls (mean ± SD): 7/1,643 (0.4% ± 0.8%)

Table8: Survival and mean body weights of mice in the 13 week dermal studies of VCD

Dose (mg/mouse)	Survival (a)	Mean Body Weights (grams) Initial (b)	Final	Change (c)	Final Weight Relative to Vehicle Controls (percent)
MALE					
0	10/10	21.8 ± 0.9	28.3 ± 0.5	+6.5 ± 1.0	
0.625	9/10	21.0 ± 0.8	28.0 ± 0.6	+6.9 ± 0.7	98.9
1.25	10/10	23.9 ± 0.6	29.2 ± 0.4	+5.3 ± 0.7	103.2
2.5	10/10	23.3 ± 0.7	27.6 ± 0.5	+4.3 ± 0.4	97.5
5	9/10	21.9 ± 0.8	27.8 ± 0.6	+5.4 ± 0.8	98.2
10	10/10	20.8 ± 0.6	27.4 ± 0.4	+6.6 ± 0.5	96.8
FEMALE					
0	10/10	17.6 ± 0.5	24.2 ± 0.5	+6.6 ± 0.4	
0.625	10/10	17.9 ± 0.4	23.6 ± 0.5	+5.7 ± 0.5	97.5
1.25	10/10	16.5 ± 0.3	24.5 ± 0.5	+8.0 ± 0.5	101.2
2.5	10/10	18.0 ± 0.4	23.4 ± 0.6	+5.4 ± 0.5	96.7
5	10/10	17.7 ± 0.6	24.2 ± 0.6	+6.5 ± 0.5	100.0
10	10/10	17.6 ± 0.4	23.3 ± 0.2	+5.7 ± 0.4	96.3

(a) Number surviving/number initially in the group; all deaths were judged to be accidental.
(b) Initial group mean body weight ± standard error of the mean. Subsequent calculations are based on animals surviving to the end of the study.
(c) Mean body weight change of the survivors ± standard error of the mean

Table9: Numbers of mice with selected skin lesions at the application site in the 13 week dermal studies of VCD

Lesion	Male (mg/rat) 0	2.5	5	10	Female (mg/rat) 0	2.5	5	10
Acute to chronic inflammation	0	0	1	1	0	0	0	0
Hyperkeratosis	0	0	*5	**8	0	0	**6	**8
Parakeratosis	0	0	0	3	0	0	0	1
Acanthosis	0	0	1	**8	0	0	0	2
Necrotizing inflammation	0	0	0	1	0	0	0	0

(a) Ten animals were examined in each group.
*P < 0.05 vs. controls
**P < 0.01 vs. controls

Table10: Numbers of mice with selected skin lesions in the 15 month dermal studies of VCD

Lesion	Male (mg/mouse)				Female (mg/mouse)			
	0	2.5	5	10	0	2.5	5	10
Acanthosis	0	2	**10	**8	0	2	**9	**10
Hyperkeratosis	0	0	0	2	0	1	0	4
Sebaceous gland hyperplasia/hypertrophy	0	4	**10	**7	0	*5	**9	**10
Sebaceous gland adenoma	0	0	0	0	0	1	1	1
Keratoacanthoma	0	0	0	1	0	0	0	0
Benign basosquamous tumor	0	0	0	1	0	0	0	0
Squamous papilloma	0	0	1	2	0	0	1	1
Squamous cell carcinoma	0	0	2	**8	0	0	2	*5

(a) Ten animals were examined in each group.
*P<0.05 vs. controls
**P<0.01 vs. controls

Table11: Survival of mice in the 2 year dermal studies of VCD

	Vehicle Control	2.5 mg/Mouse	5 mg/Mouse	10 mg/Mouse
MALE (a)				
Animals initially in study	50	50	50	50
Natural deaths	6	11	17	20
Moribund kills	3	4	27	30
Animals surviving until study termination	38	35	4	0
Killed accidentally	3	0	2	0
Survival P values (b)	<0.001	0.306	<0.001	<0.001
FEMALE (a)				
Animals initially in study	50	50	50	50
Natural deaths	7	8	13	9
Moribund kills	10	10	(c) 23	29
Animals surviving until study termination	30	31	15	(d) 12
Killed accidentally	3	1	0	0
Survival P values (b)	<0.001	0.990	0.001	<0.001

(a) First day of termination period: 729
(b) The result of the life table trend test is in the vehicle control column, and the results of the life table pairwise comparisons with the vehicle controls are in the dosed columns.
(c) One moribund animal was killed during the termination period and was combined, for statistical purposes, with those killed at termination.
(d) The number of mice alive at week 85 when all survivors of this group were killed

Table 12: Numbers of mice with selected lesions of the skin in the 2 year dermal studies of VCD

Site/Lesion	Male (mg/mouse) 0	2.5	5	10	Female (mg/mouse) 0	2.5	5	10 (b)
Skin, application site (c)								
Skin, scapula								
Acanthosis	1	**35	**38	**35	4	**31	**41	**36
Hyperkeratosis	1	**12	**14	**21	1	**27	**29	**20
Necrotizing inflammation	1	4	**12	**15	2	5	**15	**16
Malignant basosquamous tumor	0	2	0	3	0	0	1	1
Basal cell carcinoma	0	0	1	0	0	0	0	0
Squamous cell carcinoma	0	**10	**27	**37	0	*5	**14	**31
Squamous cell carcinoma (multiple)	0	2	**12	*5	0	1	**23	**10
Skin, back								
Acanthosis	0	*6	*6	2	1	0	5	4
Hyperkeratosis	0	1	4	4	0	1	4	2
Necrotizing inflammation	0	0	0	1	0	0	*5	1
Squamous cell carcinoma	0	4	0	0	0	1	3	0
Squamous cell carcinoma (multiple)	0	0	0	0	0	0	1	0
Skin, nonapplication site (d)								
Acanthosis	0	*5	*6	*5	4	0	5	4
Hyperkeratosis	0	1	2	0	1	0	5	2
Necrotizing inflammation	0	0	*6	1	1	0	6	6
Squamous cell carcinoma	0	1	2	3	0	0	3	2
Squamous cell carcinoma (multiple)	0	0	1	0	0	0	0	1

(a) Fifty animals were examined in each group.
(b) Survivors were killed during week 85.
(c) Skin, application site, includes skin from the interscapular region where chemical was applied (skin, scapula) and skin adjacent to site of application (skin, back).
(d) Skin, nonapplication site, is skin from areas distant from application site.
*P<0.05 vs. vehicle controls
**P<0.01 vs. vehicle controls

Table 13: Skin tumors in mice in the 2 year dermal studies of VCD

	Vehicle Control	2.5 mg/Mouse	5 mg/Mouse	10 mg/Mouse
MALE				
Application Site (Scapula or Back)				
Squamous Cell Carcinoma (b)				
Overall Rates	0/50 (0%)	14/50 (28%)	39/50 (78%)	42/50 (84%)
Terminal Rates	0/38 (0%)	10/35 (29%)	4/4 (100%)	0/0
Day of First Observation		525	411	376
Logistic Regression Tests	P<0.001	P<0.001	P<0.001	P<0.001
FEMALE				
Application Site (Scapula or Back)				
Squamous Cell Carcinoma (c)				
Overall Rates	0/50 (0%)	6/50 (12%)	37/50 (74%)	41/50 (82%)
Terminal Rates	0/30 (0%)	3/31 (10%)	15/15 (100%)	0/0
Day of First Observation		642	402	376
Logistic Regression Tests	P<0.001	P=0.016	P<0.001	P<0.001

(a) For a complete explanation of the entries in this table, see Table C3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Historical incidence of papillomas or carcinomas (combined) in dermal studies using acetone as a vehicle: 1/100 (1%); historical incidence in untreated controls (mean ± SD): 9/1,692 (0.5% ± 1%)
(c) Historical incidence of papillomas or carcinomas (combined) in dermal studies using acetone as a vehicle: 0/98; historical incidence in untreated controls (mean ± SD): 4/1,689 (0.2% ± 0.8%)

Table 14: Ovarian tumors in female mice in the 2 year dermal study of VCD

	Vehicle Control	2.5 mg/Mouse	5 mg/Mouse	10 mg/Mouse (b)
Luteoma				
Overall Rates	1/50 (2%)	0/49 (0%)	0/49 (0%)	0/50 (0%)
Benign Mixed Tumor				
Overall Rates	0/50 (0%)	0/49 (0%)	11/49 (22%)	6/50 (12%)
Terminal Rates	0/30 (0%)	0/31 (0%)	5/14 (36%)	0/0
Day of First Observation			497	474
Logistic Regression Tests	P<0.001	(c)	P<0.001	P=0.024
Granulosa Cell Tumor				
Overall Rates	0/50 (0%)	0/49 (0%)	5/49 (10%)	10/50 (20%)
Terminal Rates	0/30 (0%)	0/31 (0%)	2/14 (14%)	0/0
Day of First Observation			679	388
Logistic Regression Tests	P<0.001	(c)	P=0.013	P=0.006
Malignant Granulosa Cell Tumor				
Overall Rates	0/50 (0%)	0/49 (0%)	2/49 (4%)	2/50 (4%)
Granulosa Cell Tumor or Malignant Granulosa Cell Tumor				
Overall Rates	0/50 (0%)	0/49 (0%)	7/49 (14%)	12/50 (24%)
Terminal Rates	0/30 (0%)	0/31 (0%)	2/14 (14%)	0/0
Day of First Observation			579	388
Logistic Regression Tests	P<0.001	(c)	P=0.004	P=0.001
Luteoma, Granulosa Cell Tumor, or Benign Mixed Tumor				
Overall Rates	1/50 (2%)	0/49 (0%)	15/49 (31%)	16/50 (32%)
Terminal Rates	1/30 (3%)	0/31 (0%)	7/14 (50%)	0/0
Day of First Observation	729		497	388
Logistic Regression Tests	P<0.001	P=0.493N	P<0.001	P<0.001
Luteoma, Granulosa Cell Tumor, Benign Mixed Tumor, or Malignant Granulosa Cell Tumor (d)				
Overall Rates	1/50 (2%)	0/49 (0%)	17/49 (35%)	18/50 (36%)
Terminal Rates	1/30 (3%)	0/31 (0%)	7/14 (50%)	0/0
Day of First Observation	729		497	388
Logistic Regression Tests	P<0.001	P=0.493N	P<0.001	P<0.001

(a) For a complete explanation of the entries in this table, see Table D3 (footnotes); the statistical analyses used are discussed in Section II (Statistical Methods).
(b) Survivors were killed during week 85.
(c) No P value is reported because no tumors were observed in the 2.5 mg/mouse and vehicle control groups.
(d) Historical incidence in dermal studies using acetone as a vehicle (mean): 1/97 (1%); historical incidence in untreated controls (mean ± SD): 16/1,577 (1% ± 2%)