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

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Note on confidential information

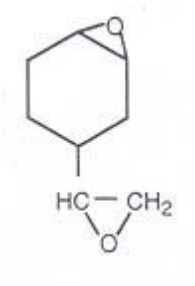
Please be aware that this report is intended to be made publicly available. Therefore it should not contain any confidential information. Such information should be provided in a separate confidential Annex to this report, clearly marked as such.

CONTENTS

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)

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 and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	603-066-00-4	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 H331 H311 H301	GHS08 GHS06 Dgr	H351 H331 H311 H301			
Dossier submitter's proposal	603-066-00-4	7-oxa-3-oxiranylbicyclo[4.1.0] heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	Add Repr. 1B Modify Carc. 1B Acute Tox. 4 Acute Tox. 3 Remove Acute Tox. 3	Add H360F Retain H311 Modify H350 H332 Remove H301	Retain GHS08 GHS06 Dgr	Add H360F Retain H311 Modify H350 H332 Remove H301		dermal: ATE = 680 mg/kg bw inhalation: ATE = 4.656 mg/L	
Resulting entry in Annex VI if adopted by RAC and agreed by Commission	603-066-00-4	7-oxa-3-oxiranylbicyclo[4.1.0] heptane; 1,2-epoxy-4-epoxyethylcyclohexane; 4-vinylcyclohexene diepoxide	203-437-7	106-87-6	Carc. 1B Repr. 1B Acute Tox. 4 Acute Tox. 3	H350 H360F H332 H311	GHS08 GHS06 Dgr	H350 H360F H332 H311		dermal: ATE = 680 mg/kg bw inhalation: ATE = 4.656 mg/L	

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

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	Clear, colourless or pale	ACGIH 2001	

Property	Value	Reference	Comment (e.g. measured or estimated)
kPa	yellow liquid	((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)
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.

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					
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)

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
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 1 000-fold	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 and trials not known, no information on cytotoxicity	Ringo et al., 1982 (Ringo, Brennan et al. 1982)
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)

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
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 of replicates per concentration not known, results shown for only one concentration, no standard deviations reported); No purity.	Turchi et al., 1981 (Turchi, Bonatti et al. 1981)
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,	Positive outcome with and without S9 mix. *Cytotoxicity*: most of	2	NTP 1989 (program 1989)

Method	Cell type	Concentration range*	Results	Klimisch Score**	References
		373 µg/ml	the increases in SCEs occurred in the absence of overt toxicity		
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.

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	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),	NTP 1989 (program 1989); Chhabra et al.,1990 (Chhabra,

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
	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		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.	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 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: 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: 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.	NTP 1989 (program 1989); Chhabra et al., 1990 (Chhabra, 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	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	Tennant et al., 1995 (Tennant, French et al. 1995); Tennant et al., 1996

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
	10/sex (high-dose) - wild type: 5/sex (high dose) Xpo = 24 weeks Xpe = 28 weeks		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, 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: life-table 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)
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	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	Yamamoto et al., 1998 (Yamamoto, Urano et al. 1998)

Species	Design	Exposure levels	Observations and remarks (Klimisch score)*	Reference
	Xpo = 24 weeks Xpe = 26 weeks Statistical analysis tumour incidences: Fisher exact test		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	
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 rats (no further information)	Treated: 10 males and 4 females Control: no data; study duration \;	25 mg/100 g body weight; 2 days/week	Klimisch-score: 3 (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.	Hendry 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 rat), 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.

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	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	Kao et al. 1999 (Kao, Sipes et al. 1999)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		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 d (rats).	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 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	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
		single application of 0.1 ml was applied to the 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 micropipette.	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
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 by vaginal cytology), The females were mated with untreated males on	*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 follicles in all control animals regardless of whether they had become pregnant. In contrast, there were essentially no follicles	Haas et al 2007 (Haas, Christian et al. 2007)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		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)).	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)
30 day study with Female cynomolgus macaques (Macaca fascicularis) with a mean age of 9 y	4-vinylcyclohexene diepoxide	<u>Experiment 1</u> At baseline, an ovary was removed to serve as the untreated control and then a poly-L-lacticco-glycolic	<u>Experiment 1</u> 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	Appt 2010 (Appt, Clarkson et al. 2010)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
(range, 6 to 18 y). A poly-L-lacticco-glycolic acid fiber containing 200 mg VCD was placed around the ovary.		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).	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.	
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 × 10^6 sperm/ml.	*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)
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	*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)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		sesame oil (80 mg/kg bw/day) for 15 days, three or four mice per treatment group.		
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 (SD) rats are interperitoneally treated daily with 4-vinylcyclohexene diepoxide VCD).	4-vinylcyclohexene diepoxide	Rats were intraperitoneally (i.p.) injected with a dose of 80 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.	There is a high correlation between premature ovarian failure initiation and ovarian autoimmunity and cardiovascular disorder. Levels of FSH and LH were significantly increased after VCD treated in rats plasma compared to the control groups.	Li 2014 (Li, Fan et al. 2014)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
		Total RNA from ovarian tissue was converted to cDNA and hybridized to mRNA Chip array.		
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. 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.	Mayer 2002 (Mayer, Pearsall et al. 2002)
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	Muhammad 2009 (Muhammad, Goode et al. 2009)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
			likely started with fewer follicles and therefore approached follicle depletion.	
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 neonatal mice	4-vinylcyclohexene diepoxide	Female mice (6–10 animals per treatment 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]).	Results show a mechanism of VCD-induced ovotoxicity involving small preantral follicular destruction and primordial follicle activation.	Sobinoff 2010 (Sobinoff, Pye 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	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	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	Takai 2003 (Takai, Canning et al. 2003)

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
generations), The ASMase- and caspase-2-deficient mouse lines were of a mixed C57BL/6–129/Sv background.		the mean ± sem of the combined results from theanalysis of ovaries collected from three or more mice per genotype per treatment group.	responsible for apoptosis.	
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 individual pairs of treatment-matched males and females were housed for a 15-day breeding cycle.	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 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).	Witmer et al 2017 (Witmer, Raymond-Whish et al. 2017)

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)

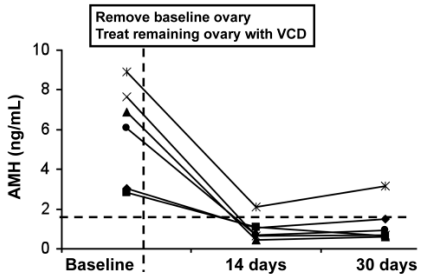
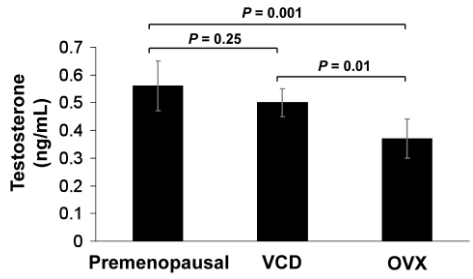


Figure 2: Serum testosterone concentrations (Mean ± SE) of adult cynomolgus monkeys 4 months after sham surgery (premenopausal), placement of VCD fibers next to both ovaries (VCD) or ovariectomy (OVX).



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

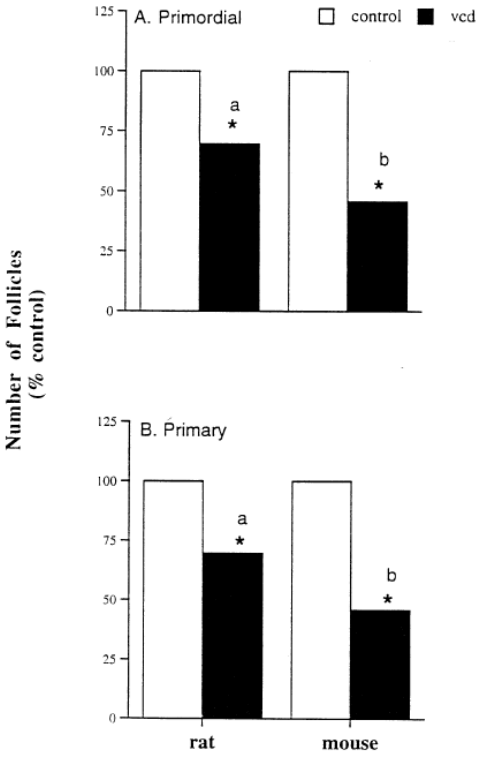


Fig. 3. Comparison on day 12 of the effect of VCD on the number of primordial and primary follicles in rats and mice. Animals were treated daily with VCD (80 mg/kg, i.p.) for 12 d. After the final dosing, ovaries were removed and histologically processed as described in *Materials and Methods*. The number 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).

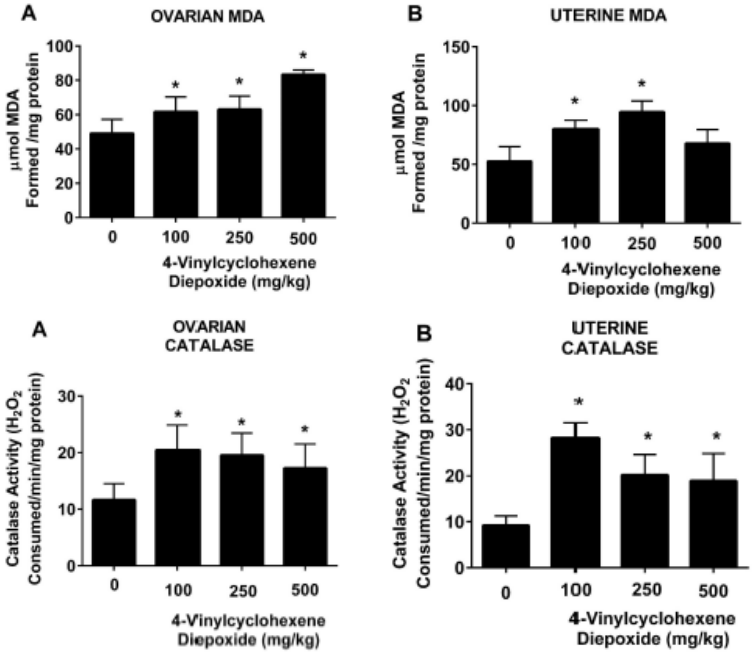
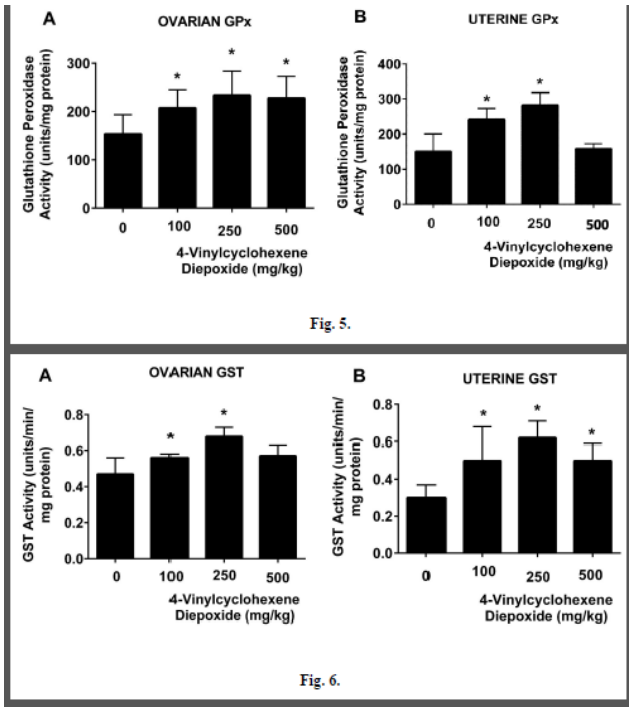


Figure 5. Ovarian and uterine glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities in rats (Abolaji, Adedara et al. 2016).



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).

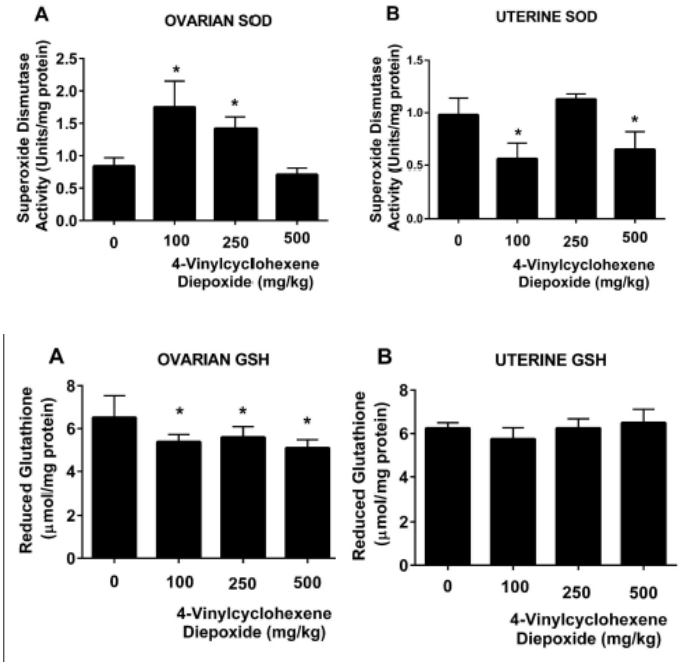
Hormones (IU/L)	Control	VCD (mg/kg Body Weight) 100	250	500
LH	17.50 ± 2.52	17.50 ± 0.71	16.00 ± 1.00	25.67 ± 2.52*
FSH	13.00 ± 1.41	13.00 ± 1.41	11.50 ± 0.71	21.67 ± 1.53*
PRL	24.00 ± 3.61	15.33 ± 1.15*	17.67 ± 1.53*	25.33 ± 2.52
PRG	5.26 ± 0.64	5.31 ± 0.32	5.15 ± 0.07	3.33 ± 0.49*
ESTR	35.67 ± 5.51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

The data are expressed as mean ± SD, n=7. 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.

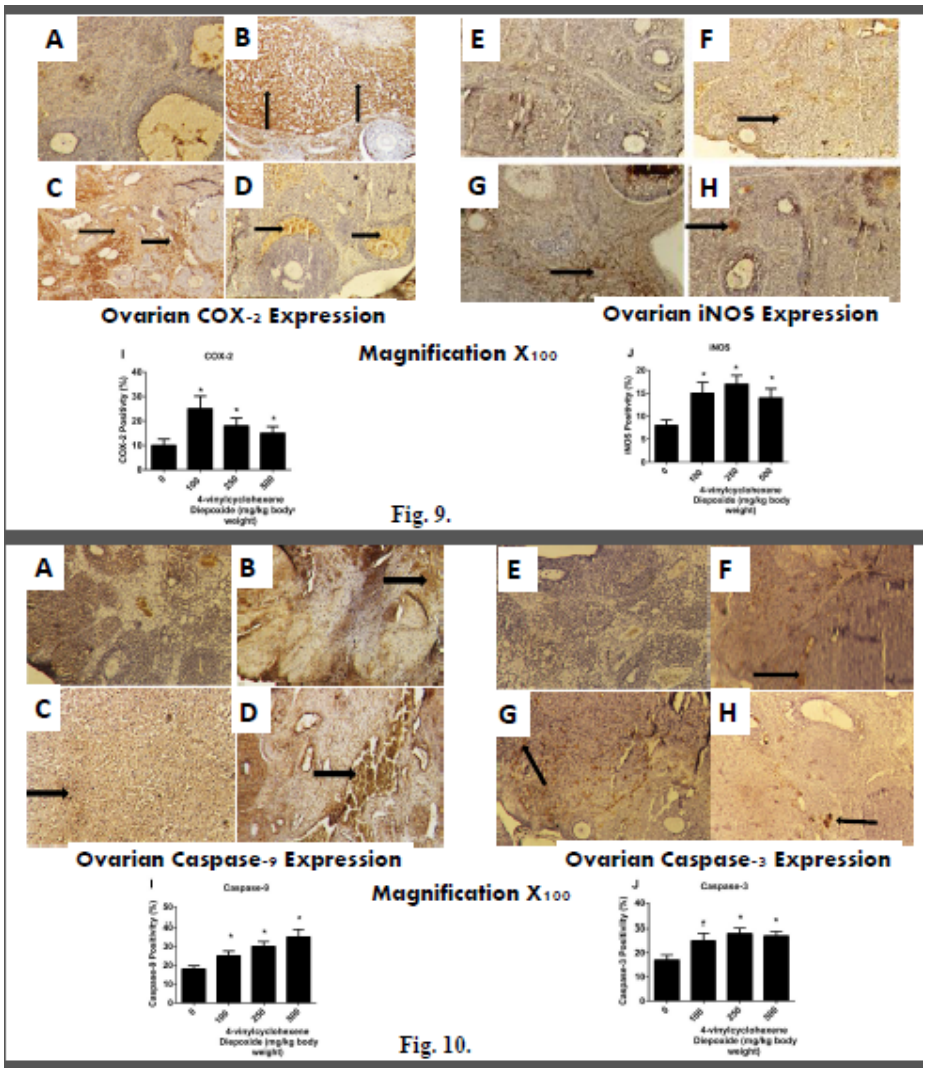


Figure 7. Immunohistochemical expressions of ovarian cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), Caspase-9 and Caspase-3 (Abolaji, Adedara et al. 2016).

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)

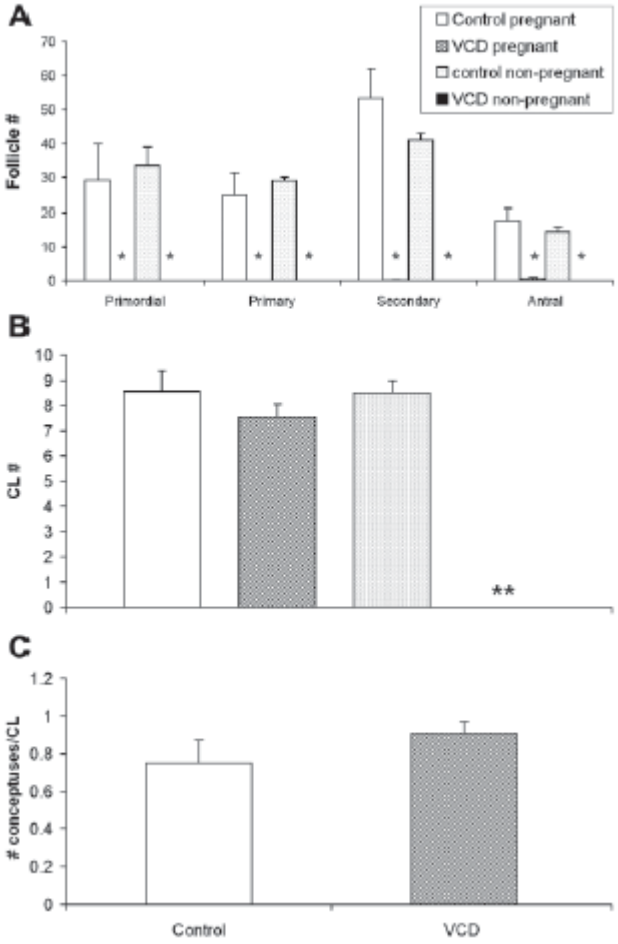
	Control (n = 13)	VCD: group 1[a] (n = 14)	VCD: group 2[b] (n = 8)
No. of plugs/no. of times with males	89.7 ± 7.9	71.3 ± 10.1	83.3 ± 12.6
No. of pregnancies/no. of times with males	64.1 ± 12.1	60.0 ± 11.1	12.5 ± 12.5[c]
No. of pregnancies/no. of copulatory plugs	57.7 ± 12.5	60.0 ± 12.1	12.5 ± 12.5[c]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

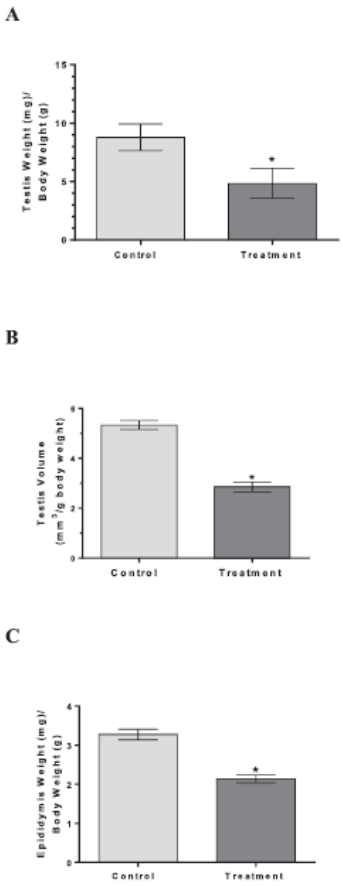
Studies with male animals (testes)

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).

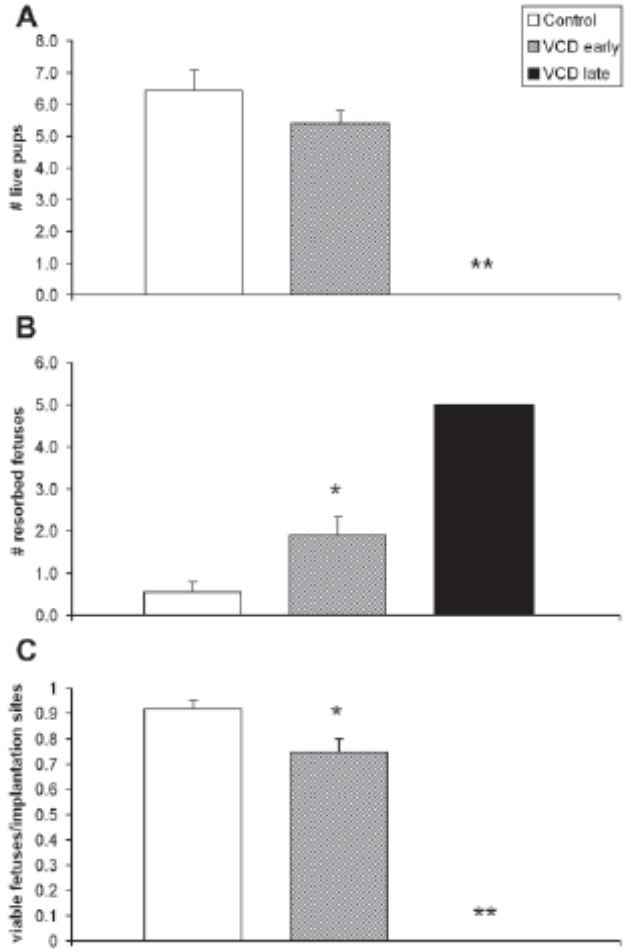


Figure 10. Effect of impeding ovarian failure on fertility outcome. Mature female C7BL/6 mice were dosed daily with vehicle control or VCD (160 mg/kg bw/day intraperitoneally) for 17 d. Control and VCD-treated mice were mated with adult males. Fertility was evaluated on gestational day 16. Measurements 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.

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

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15 ANNEXES

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

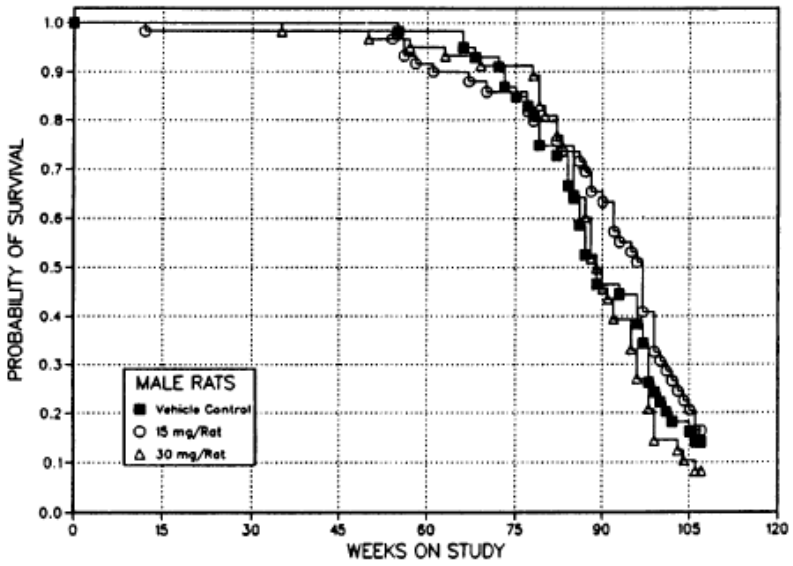
Table5: Survival of rats in the 2 year dermal studies of VCD

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.



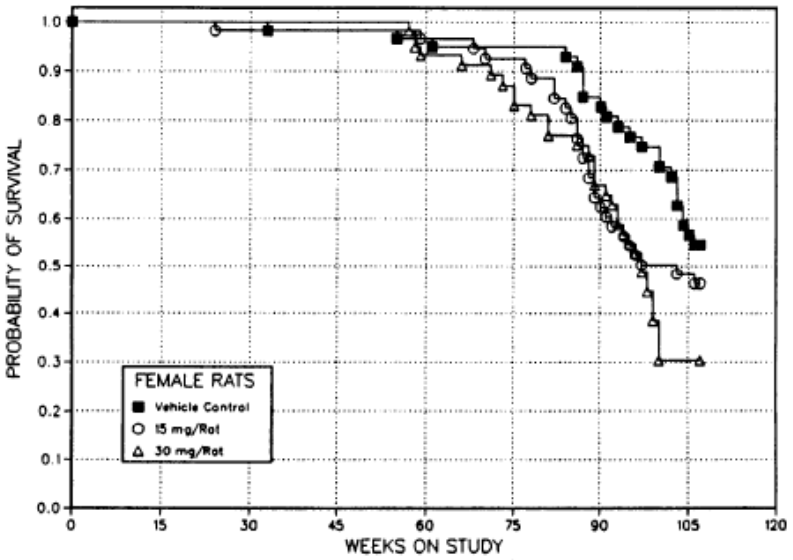


Table7: Skin tumors in rats in the 2 year dermal studies of VCD.

	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%)