CLH Report

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name: Chloroform

EC Number: 200-663-8

CAS Number: 67-66-3

Submitted by: FRANCE

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CONTENTS

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES 7

1.1 Name and other identifiers of the substance 7

1.2 Composition of the substance 7

1.3 Physico-chemical properties 10

2 MANUFACTURE AND USES 11

2.1 Manufacture 11

2.2 Identified uses 11

2.3 Uses advised against 11

3 CLASSIFICATION AND LABELLING 11

3.1 Classification in Annex I of Directive 67/548/EEC 11

3.2 Self classification(s) 11

4 ENVIRONMENTAL FATE PROPERTIES 12

5 HUMAN HEALTH HAZARD ASSESSMENT 13

5.1 Toxicokinetics (absorption, metabolism, distribution and elimination) 13

5.2 Acute toxicity 13
5.2.1 Acute toxicity: oral 13
5.2.2 Acute toxicity: inhalation 14
5.2.3 Acute toxicity: dermal 15
5.2.4 Acute toxicity: other routes 15
5.2.5 Summary and discussion of acute toxicity 15

5.3 Irritation 15
5.3.1 Skin 15
5.3.2 Eye 16
5.3.3 Respiratory tract 17
5.3.4 Summary and discussion of irritation 18

5.4 Corrosivity 18

5.5 Sensitisation 18
5.5.1 Skin 18
5.5.2 Respiratory system 18
5.5.3 Summary and discussion of sensitisation 18

5.6 Repeated dose toxicity 19
5.6.1 Repeated dose toxicity: oral 19
5.6.2 Repeated dose toxicity: inhalation 20
5.6.3 Repeated dose toxicity: dermal 21
5.6.4 Summary and discussion of repeated dose toxicity: 21

5.7 Mutagenicity 21
5.7.1 In vitro data summary 21

5.7.2 In vivo data summary 23
5.7.3 Detailed description of the key *in vivo* studies 26
5.7.4 Human data 47
5.7.5 Other relevant information 47
5.7.6 Summary and discussion of mutagenicity 47

5.8 Carcinogenicity 51
5.8.1 Carcinogenicity: oral 51
5.8.2 Carcinogenicity: inhalation 53
5.8.3 Carcinogenicity: dermal 54
5.8.4 Carcinogenicity: human data 54
5.8.5 Other relevant information 55
5.8.6 Summary and discussion of carcinogenicity 55

5.9 Toxicity for reproduction 55
5.9.1 Effects on fertility 55
5.9.2 Developmental toxicity 55
5.9.3 Human data 58
5.9.4 Other relevant information 60
5.9.5 Summary and discussion of reproductive toxicity 60

5.10 Other effects 60

5.11 Derivation of DNEL(s) or other quantitative or qualitative measure for dose response 60

6 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES 61

7 ENVIRONMENTAL HAZARD ASSESSMENT 62

TABLES

Table 1: Summary of physico- chemical properties 10
Table 3 Summary of acute oral toxicity....................13
Table 4 Summary of acute inhalation toxicity....................14
Table 5 Summary of acute dermal toxicity....................15
Table 6 Summary of skin irritation....................15
Table 7 Summary of eye irritation....................16
Table 8 Summary of respiratory tract irritation....................17
Table 9 Summary of oral RDT....................19
Table 10 Summary of inhalation RDT....................20
Table 11 Summary of in vitro mutagenicity....................21
Table 12 Summary of in vivo mutagenicity....................23
Table 13: Frequency of micronucleated kidney cells in rats treated with chloroform. 27
Table 14: Results of the micronucleus test on mouse bone marrow. 27
Table 15: Frequencies of micronucleated polychromatic erythrocytes. 28
Table 16: 29
Table 17: Number of micronuclei/500 PCE for a single mouse for each compound....................29
Table 18: Mean group body weight evolution over the dosing period of the assay from day 1 to day 6 per sex compared to concurrent vehicle controls....................30
Table 19: Number of micronuclei/500 PCE for a single mouse for each compound....................31
Table 20....................33
Table 21: Relationship between dose and trialomethanes (THM)-induced CA 12h after i.p. injection 34
Table 22: Variation over time of THM-induced CA 35
Table 23: Relationship between dose and THM-induced CA 35
Table 24: Variation of THM-induced CA 36
Table 25....................36
Table 26: LacI mutant frequencies in Chloroform-treated Mice. 39
Table 27: Studies on possible covalent binding of ^{14}C from [14C]$CHCl_3$ to mouse liver DNA or RNA. 40
Table 28: DNA strand break induction by THMs....................41
Table 29: Induction of UDS by chemicals in the in vivo – in vitro hepatocyte DNA repair assay. 42
Table 30: Summary of key studies. 44
Table 31 Dose-Response Relationships for the Incidences of Renal Tumors Induced by Chloroform Exposures in the Male Rat Study (Nagano et al., 2006)....................52
Table 32 Summary of oral carcinogenicity....................52
Table 33 Summary of inhalation carcinogenicity....................54
Table 34 Summary of effects on fertility....................55
Table 35 Main maternal parameters following exposure to chloroform by inhalation....................56
Table 36 Main fetal parameters following exposure to chloroform by inhalation....................56
Table 37 Summary for developmental toxicity....................57
Table 38 Semen analysis after 1 year exposure (Chang et al.,2001) 58

Figure 1 37
Figure 2: DNA repair in the liver of mice treated with dimethylnitrosamine (DMN) or chloroform ($CHCl_3$) relative to control group 41
Figure 3: The two pathways of chloroform bioactivation. 48
Figure 4: Hypothesis for micronucleus formation and chromosomal aberration after exposure to chloroform. 50

PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

Substance Name: Chloroform (Methane, trichloro-)

EC Number: 200-663-8

CAS number: **67-66-3**

Registration number (s): -

Purity: > 99%

Impurities: 1,1-dichloroethylene; chloromethane; bromochloromethane; carbon tertrachloride

Chloroform was on the 2nd priority list of the Existing Substances Regulation and its classification was reviewed in the context of the Risk Assessment procedure as it was a requirement to harmonise classification for all endpoints.

The need to revise the current harmonised classification was identified including the need to revise the specific concentration limits applied (i.e. Xn; R22 (>5%) and Xn; R48/20/22 (>5%)).

Revision of the health classification of chloroform was discussed at ECB by the TC C&L in September 2007 (see annex 1).

The TC C&L agreed on addition of classifications Xn; R20, Xi; R36 and Repr. Cat. 3; R63 based on the FR proposal. They also agreed not to revise existing classifications Xn; R22, Xi; R38 and Carc. Cat. 3; R40 and not to classify chloroform with Xi; R37 (initially proposed by France) as the nasal effects reported were rather covered by Xn; R48/20. Further, the TC C&L agreed that R48/22 could be deleted as effects were only seen at high doses. The narcotic effects, covered by Xn; R20 under Directive 67/548, trigger classification with STOT Single 3 under the CLP Regulation. The follow-up of the discussion having taken place in the TC C&L regarding R20 and R22 (see annex 1) do not mention any discussion regarding specific concentration limits. It is supposed that the agreement on the corresponding classifications R22 and R48/20 imply agreement on the withdrawal of these specific concentration limits.

No agreement could be reach by the TC C&L on mutagenicity and this report is submitted to ECHA in order to state on the classification for this endpoint.

Environmental classification of chloroform was discussed and no classification was agreed by the TC C&L in January 2007 (see annex 2). Environmental classification is therefore not presented in this dossier. Further information can be found in the transitional dossier.

Proposed classification based on Directive 67/548/EEC criteria:

- Xn; R20/22
- Xn; R48/20
- Xi ; R36/38
- [Muta cat. 3; R68]
- Carc. Cat. 3; R40

- Repr. Cat. 3; R63

- Not classified for the environment

Proposed classification based on CLP criteria:

- Acute Tox. 3 – H331
- Acute Tox. 4 – H 302
- STOT Rep. 1 – H 372
- STOT Single 3 – H336
- Eye Irrit. 2 – H319
- Skin Irrit. 2 – H315
- [Muta. 2 – H341]
- Carc. 2 – H351
- Repr. 2 – H361d

- Not classified for the environment

Proposed labelling:

Xn

R:20/22-36/38-40-48/20-63-68

S: 2-36/37

Proposed specific concentration limits (if any): none

Proposed notes (if any): none

JUSTIFICATION

1 IDENTITY OF THE SUBSTANCE AND PHYSICAL AND CHEMICAL PROPERTIES

1.1 Name and other identifiers of the substance

Chemical Name: Chloroform (Methane, trichloro-)

EC Number: 200-663-8

CAS Number: 67-66-3

IUPAC Name: Chloroform

1.2 Composition of the substance

Consituents

Chemical Name: Chloroform

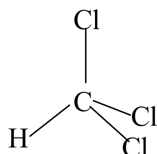
EC Number: 200-663-8

CAS Number: 67-66-3

IUPAC Name: Chloroform

Molecular Formula: $CHCl_3$

Structural Formula:



Molecular Weight: 119.5 g/mol

Typical concentration (% w/w): ≥ 99% w/w

Concentration range (% w/w):

Impurities

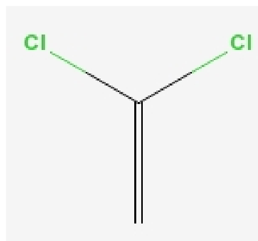
Chemical Name: 1,1-dichloroethylene

EC Number: 200-864-0

CAS Number: 75-35-4

IUPAC Name: 1,1-dichloroethene

Molecular Formula: $C_2H_2Cl_2$

Structural Formula: 

Molecular Weight: 96.9 g/mol

Typical concentration (% w/w): < 0.002 % w/w

Concentration range (% w/w): -

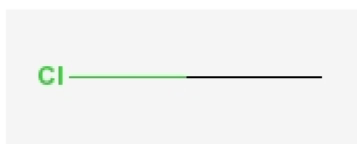
Chemical Name: chloromethane

EC Number: 200-817-4

CAS Number: 74-87-3

IUPAC Name: chloromethane

Molecular Formula: CH_3Cl

Structural Formula: 

Molecular Weight: 50.4 g/mol

Typical concentration (% w/w): < 0.005 % w/w

Concentration range (% w/w): -

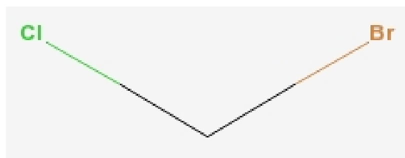
Chemical Name: bromochloromethane

EC Number: 200-826-3

CAS Number: 74-97-5

IUPAC Name: bromo(chloro)methane

Molecular Formula: CH_2BrCl

Structural Formula: 

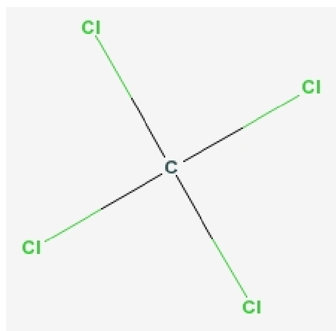
Molecular Weight: 129.3 g/mol

Typical concentration (% w/w): unknown

Concentration range (% w/w): -

Chemical Name: carbon tetrachloride

EC Number: 200-262-8

CAS Number:	56-23-5
IUPAC Name:	tetrachloromethane
Molecular Formula:	CCl_4
Structural Formula:	
Molecular Weight:	153.8 g/mol
Typical concentration (% w/w):	unknown
Concentration range (% w/w):	-

Additives

Chemical Name:	unknown
EC Number:	
CAS Number:	
IUPAC Name:	
Molecular Formula:	
Structural Formula:	
Molecular Weight:	
Typical concentration (% w/w):	< 1% w/w
Concentration range (% w/w):	-

1.3 Physico-chemical properties

Table 1: Summary of physico- chemical properties

REACH ref Annex, §	Property	IUCLID section	Value
VII, 7.1	Physical state at 20°C and 101.3 KPa	3.1	Liquid
VII, 7.2	Melting/freezing point	3.2	-63.5ºC
VII, 7.3	Boiling point	3.3	61.3ºC
VII, 7.4	Relative density	3.4 density	
VII, 7.5	Vapour pressure	3.6	209 hPa at 20ºC
VII, 7.6	Surface tension	3.10	
VII, 7.7	Water solubility	3.8	8700 mg/L at 23ºC
VII, 7.8	Partition coefficient n-octanol/water (log value)	3.7 partition coefficient	Log Kow 1.97
VII, 7.9	Flash point	3.11	
VII, 7.10	Flammability	3.13	
VII, 7.11	Explosive properties	3.14	
VII, 7.12	Self-ignition temperature		
VII, 7.13	Oxidising properties	3.15	
VII, 7.14	Granulometry	3.5	none
XI, 7.15	Stability in organic solvents and identity of relevant degradation products	3.17	
XI, 7.16	Dissociation constant	3.21	
XI, 7.17,	Viscosity	3.22	
	Auto flammability	3.12	
	Reactivity towards container material	3.18	
	Thermal stability	3.19	

2 MANUFACTURE AND USES

2.1 Manufacture

2.2 Identified uses

2.3 Uses advised against

3 CLASSIFICATION AND LABELLING

3.1 Classification in Annex I of Directive 67/548/EEC

According to Annex VI of CLP, chloroform is currently classified as follows (19th ATP):

Index number: 602-006-00-4

Xn; R22-48/20/22
Xi; R38
Carc. Cat. 3; R40

Specific concentration limits apply for Xn; R22 (>5%) and Xn; R48/20/22 (>5%).

3.2 Self classification(s)

4 ENVIRONMENTAL FATE PROPERTIES

This section is not covered in this dossier. Further information can be found in the transitional dossier.

5 HUMAN HEALTH HAZARD ASSESSMENT

5.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

Chloroform is well absorbed, metabolized and eliminated by mammals after oral, inhalation or dermal exposure. Chloroform is hence widely distributed all around the organism, via blood circulation and, due to its liposolubility, preferentially in fatty tissues and in the brain. Uptake and storage of chloroform in adipose tissue can be substantial, with daily exposures potentially leading to accumulation, particularly in obese persons.

Chloroform is mainly metabolised in liver and both oxidative and reductive pathways of chloroform have been identified, although data *in vivo* are limited. The major metabolite is carbon dioxide, generated by oxidative pathway *in vivo*; this main pathway generates also reactive metabolites, including phosgene. The reductive pathway generates the dichloromethylcarbene free radical. Both pathways proceed through a cytochrome P450-dependent enzymatic activation step ant their balance depends on species, tissue, dose and oxygen tension. Phosgene is produced by oxidative dechlorination of chloroform to trichloromethanol, which spontaneously dehydrochlorinates.

The electrophilic metabolic phosgene binds covalently to nucleophilic components of tissue proteins and also interacts with other cellular nucleophiles and, to some extent, to the polar heads of phospholipids. Phosgene can also react with water to release carbon dioxide and hydrochloric acid.

Available literature data show that chloroform toxicity is due to its metabolites: phosgene is supposed to be responsible for irreversible bindings to liver components. Chloroform can cross the placenta and it is expected to appear in human colostrum and mature breast milk.

5.2 Acute toxicity

5.2.1 Acute toxicity: oral

Acute toxicity varies depending upon the strain, sex and vehicle. In mice the oral LD50 values range from 36 to 1366 mg chloroform/kg body weight, whereas for rats, they range from 450 to 2000 mg chloroform/kg body weight. In general, chloroform elicits the same symptoms of toxicity in humans as in animals. The mean lethal oral dose for an adult is estimated to be about 45 g (640 mg/kg bw), but large interindividual differences in susceptibility occur.

Table 2 Summary of acute oral toxicity

Animal species & strain	Number of animals per dose level	Doses, route of administration, vehicle	LD_{50} (mg/kg bw)	Reference year
Mouse C3H/Tif	Not reported	Oral, sesame oil vehicle	36 for males (kidney damage) 353 for females	Pericin & Thomann 1979 in IPCS 1994
Rat Sprague-Dawley (14 days old)	Not reported	Oral, undiluted	450 for male and female	Kimura et al., 1971

Conclusion

Kidney damage induced in male mice are related to very sensitive strain (C3H/Tif), thus it is not considered relevant for acute toxicity classification. Due to oral 200 < DL50 ≤ 2000mg/kg for rats, female mice (C3H/Tif) or mice of other strains, the application of R22 is indicated.

There is no need to maintain the specific concentration levels of the 19th ATP.

Classification R22 (CLP Acute Tox 4 – H302) was agreed at TC C&L in September 2007.

5.2.2 Acute toxicity: inhalation

Chloroform LC50 values of 6.2 g/m^3 and 9.2 g/m^3 have been reported for 6 h inhalation exposure in mice and rats respectively. Mice are more susceptible than rats to acute chloroform toxicity for both exposure routes. A LOAEL of 2.5 mg/l is based on effects on the kidneys and liver of mice and rats.

Table 3 Summary of acute inhalation toxicity

Animal species & strain	**Number of animals per dose level**	**Doses, route of administration, vehicle**	**LC_{50} (mg/l)**	**Reference year**
Mouse, OF1	Not reported	Inhalation, 6h	LC_{50}= 6.2 mg/l	Gradiski et al., 1978 in CICAD, 2004
Mouse, BDF1	10/sex/dose	Inhalation vapor, 6h/d, 5d/week, 2 weeks 500, 1000, 2000, 4000, 8000 ppm (2.5, 5, 10, 20, 40 mg/l)	LOAEC 2.5 mg/l necrosis and cytoplasmic basophilia of the kidney proximal tubules in males and centrilobular necrosis of the liver in females mortality rates 9/10 for 2.5 and 5 mg/l ; 10/10 over 5 mg/l Atrophy and metaplasia of olfactory epithelium	Kasai et al., 2002
Rat	Not reported	Inhalation, 6h	LC_{50}= 9.2 mg/l	Bonnet et al., 1980 in CICAD, 2004
Rat, F344	10/sex/dose	Inhalation vapor, 6h/d, 5d/week, 2 weeks 500, 1000, 2000, 4000, 8000 ppm (2.5, 5, 10, 20, 40 mg/l)	LOAEC 2.5 mg/l for vacuolic changes in proximal tubules of the kidneys and in the central area of the liver mortality rates for male and female 0/10 for 2.5 and 5 mg/l ; 10/10 over 5 mg/l Atrophy and disarrangement of olfactory epithelium, oedema of the lamina propria of the nasal cavity (all doses)	Kasai et al., 2002

Conclusion

Based on inhalation 2 < DL50 ≤ 20 mg/l for mice and rats the application of R20 is indicated.

Classification R20 (CLP Acute Tox – H331) was agreed at TC C&L in September 2007.

5.2.3 Acute toxicity: dermal

Only one dermal study was available for rabbits, a single application of chloroform (1.0, 2.0, or 3.98 g/kg) for 24h did not result in any deaths. However, extensive necrosis of the skin and considerable weight loss occurred at all levels. Animals were sacrificed for study 2 weeks after exposure. All treated rabbits exhibited degenerative changes in the kidney tubules graded in intensity with dosage levels. The livers were not grossly affected (Torkelson et al., 1976).

Table 4 Summary of acute dermal toxicity

Animal species & strain	**Number of animals per dose level**	**Doses, route of administration, vehicle**	**Result**	**Reference year**
Rabbit	2	Dermal 1.0, 2.0, 3.98 g/kg 24h occlusive	LOAEL= 1.0 g/kg necrosis of the skin, weight loss degenerative changes in the kidney tubules (dose relationship)	Torkelson et al., 1976

No classification is required for dermal acute toxicity

5.2.4 Acute toxicity: other routes

No data

5.2.5 Summary and discussion of acute toxicity

Classification R22 and R20 (CLP Acute Tox 4 – H302 and Acute Tox 3 –H331) were agreed at TC C&L in September 2007. No classification is required for dermal acute toxicity.

5.3 Irritation

5.3.1 Skin

Table 5 Summary of skin irritation

Animal species & strain	**Number of animals**	**Doses**	**Result**	**Reference**
Rabbit	Not reported	Liquid chloroform 24h, occlusive 10 applications for ears	ear: hyperemia and exfoliation after 1 to 4 applications belly: slight hyperemia with moderate necrosis and	Torkelson et al., 1976 in CICAD 2004

		2 applications for bellies	eschar formation delayed healing of the skin	

Conclusion

Based on the rabbit study and on the previous classification, R38 irritating to skin is indicated.

Classification R38 (CLP Skin Irrit 2 – H315) was agreed at TC C&L in September 2007.

5.3.2 Eye

Two animal studies were available: in the first one, chloroform produced severe eye irritation to NZW rabbits. The effects had disappeared 2-3 weeks after application, except for one rabbit that still showed corneal opacity after 3 weeks (Duprat et al., 1976); in the second, chloroform caused slight irritation of the conjunctiva which was barely detectable 1 week after treatment and slight but definite corneal injury evidenced by staining with fluorescein (Torkelson et al., 1976).

In man, exposure to concentrated chloroform vapours causes a stinging sensation in the eye. Splashing of the liquid into the eye evokes burning, pain and redness of the conjunctival tissue. Occasional injury of the corneal epithelium will recover fully within a few days (IPCS, 1994).

Table 6 Summary of eye irritation

Animal species & strain	Number of animals	Doses	Result	Reference
Rabbit, NZW	6	Undiluted chloroform, doses not specified	6/6 severe eye irritation, with pupils dilation (mydriasis) and corneal inflammation (keratitis) 4/6 translucent zones in the cornea	Duprat et al., 1976
Rabbit	3	Undiluted chloroform, doses not specified 1 eye rinsed after 30s	Slight irritation of the conjunctiva Slight but definite corneal injury	Torkelson et al., 1976

Conclusion

Based on the rabbit studies reporting corneal injury and human data showing reversible corneal effects, the application of R36 irritating to eyes, is indicated.

Classification R36 (CLP Eye Irrit 2 –H319) was agreed at TC C&L in September 2007.

5.3.3 Respiratory tract

Kasai et al., (2002), conducted two experiments in mice and rats with inhalation doses from 12 to 8000 ppm during 2 or 13 weeks. Significant increases of nasal lesions were reported as degeneration of the olfactory epithelium in male mice exposed to 25 ppm, thickening of the bone in nasal septum and eosinophilic changes of olfactory and respiratory epithelia in female mice at 12 ppm, as well as mineralization and atrophy of the olfactory epithelium observed for rats at 25 ppm.

Mery et al. (1994) exposed F344 rats and B6C3F1 mice to chloroform for 6h/day during 7 days to exposure concentrations ranging from 1 to 300 ppm. Examination of the nasal passages revealed that chloroform caused a complex set of responses in the ethmoid turbinates, predominantly in rats. These lesions were most severe peripherally and generally spared the tissue adjacent to the medial airways. The changes were characterized by atrophy of Bowman's glands, new bone formation (LOAEL= 10 ppm), and increased labelling index in S phase periosteal cells (LOAEL= 10 ppm). At 30 and 100 ppm, new osseous spicules were present at the beginning of the first endoturbinate, while at 271 ppm, the width of the new bone was almost doubled compared to controls. The only change noted in the mouse was increased cell proliferation without osseous hyperplasia. The authors proposed that the osseous changes induced by chloroform exposure may be secondary to primary degeneration of adjacent Bowman's glands. The NOAEL values for these responses ranged from 3-100 ppm, with histological and induced cell proliferation being the most sensitive indices of effect.

Table 7 Summary of respiratory tract irritation

Animal species & strain	**Number of animals**	**Doses**	**Result**	**Reference**
Rat, F344	Not reported	0, 1, 3, 10, 30, 100, or 271 ppm 6 hr/day for 7 days	NOAEL= 3 ppm atrophy of Bowman's glands, new bone formation, and increased labeling index in S phase periosteal cells	Mery et al., 1994
Rat, F344 Inhalation	10/sex/dose	vapour, 6h/d, 5d/week, 2 weeks 500, 1000, 2000, 4000, 8000 ppm	Desquamation, atrophy and disarrangement of the olfactory epithelium but also oedema of the lamina propria of the nasal cavity in both sexes 100% mortality ≥ 2000 ppm	Kasai et al., 2002
Mouse, BDF1 Inhalation	10/sex/dose	vapour, 6h/d, 5d/week, 2 weeks 500, 1000, 2000, 4000, 8000 ppm	Atrophy and metaplasia in the olfactory epithelium in males Degeneration, necrosis and disarrangement of olfactory and respiratory epithelia in females 100% mortality ≥ 2000 ppm	Kasai et al., 2002

Conclusion

Considering the results of inhalation studies and the nasal lesion observed, chloroform is irritating to respiratory system. This effect is already covered by the classification R48/20 proposed in section 5.6.2.

5.3.4 Summary and discussion of irritation

Classification R38 and R36 (CLP Skin Irrit 2 –H315 and Eye Irrit 2 –H319) were agreed at TC C&L in September 2007.

5.4 Corrosivity

5.5 Sensitisation

A sensitisation test on chloroform was reported (Chiaki et al., 2002). This study was designed to evaluate the skin sensitizing potency of chloroform, and it was performed to further evaluate the differences between Guinea Pig Maximization Test (GPMT) and Local Lymph Node Assay (LLNA, RI Method). No positive reaction was observed in any method for sensitization.

5.5.1 Skin

5.5.2 Respiratory system

5.5.3 Summary and discussion of sensitisation

No classification is required for sensitisation.

5.6 Repeated dose toxicity

5.6.1 Repeated dose toxicity: oral

Table 8 Summary of oral RDT

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference
Mouse, B6C3F1	10/sex/dose	Corn oil or Emulphor 60, 130 and 270 mg/kg bw/d, 90 days	females LOAEL 60 mg/kg : increased liver weight, vacuolation, lipid accumulation in the liver	Bull et al., 1986
Mouse, CD-1	7-12 /sex/dose	Drinking water 0, 50, 125, 250 mg/kg bw/d, 90 days	LOAEL 50 mg/kg: increased liver weight and increased hepatic microsomal activity in females Small intertubular collections of chronic inflammatory cells in kidneys, generalized hydropic degeneration of hepatocytes and small focal collections of lymphocytes in liver	Munson et al., 1982
Rat	Not reported	263 mg/kg bw 90 days Stomach tube	LOAEL 263 mg/kg: Fatty changes and increased liver weight	US EPA, 1980
Beagle Dog	7-15 male & female	15, 30 mg/kg bw/d 7.5 years Toothpaste	LOAEL 15 mg/kg: increase alanine aminotransferase (ALAT) levels 15 mg/kg: fatty cysts in the liver. Incidences: control – 15mg – 30mg 1/15 – 6/7 – 6/7 for males 0/12 – 3/8 – 7/8 for females	Heywood et al., 1979

Conclusion

Repeated exposure to chloroform induced hepatic effects in rats and mice but the effects are not sufficiently severe at the dose of 50 mg/kg to justify a classification for oral RDT.

No classification R48/22 was agreed at the TC C&L in September 2007.

5.6.2 Repeated dose toxicity: inhalation

Table 9 Summary of inhalation RDT

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference
Mouse, BDF1	10/sex/dose	Vapor, 6h/day, 5d/w, 13weeks 12, 25, 50, 100 or 200 ppm (60, 124, 248, 496, 992 mg/m^3)	LOAEL= 12 ppm: Male kidney (proximal tubules necrosis) 25 ppm: Lesions of olfactory epithelium (metaplasia, desquamation, atrophy or disarrangement) and nasal cavity (oedema) 100 ppm: Female liver necrosis and cell atypia 200 ppm: Male liver swelling	Kasai et al., 2002
Mouse, BDF1	Not reported	6 h/day, 5 d/week, 13 weeks 5, 30 or 90 ppm (25, 149, 446 mg/m^3)	LOAEL= 30 ppm: Male dose-dependent increase in regenerating tubules within the renal cortex 90 ppm: Female Increased centrilobular to midzonal hepatocyte degeneration and vacuolation	Templin et al., 1998
Mouse, B6C3F1	Not reported	6 h/day, 5-7 d/week, 13 weeks 0.3, 2, 10, 30, and 90 ppm (1, 10, 50, 149, 446 mg/m^3)	LOAEL= 30 ppm: induced hepatic cell proliferation 30 ppm: Male induced renal histologic changes and regenerative cell proliferation	Larson et al., 1996
Rat, F344	10/sex/dose	Vapor, 6h/day, 5d/w, 13weeks 25, 50, 100, 200 or 400 ppm (124, 248, 496, 992, 1984 mg/m^3)	LOAEL= 25 ppm: mineralization and atrophy of the respiratory epithelium 100 ppm: Female liver collapse 200 ppm: Male liver collapse 200 ppm: Female kidney vacuolic changes	Kasai et al., 2002
Rat, F344	Not reported	6 h/day, 7 d/week, 13 weeks 0, 2, 10, 30, 90, or 300 ppm (10, 50, 149, 446, 1488 mg/m^3)	LOAEL= 2 ppm: generalized atrophy of the ethmoid turbinates 10 ppm: Enhanced bone growth and hypercellularity in the lamina propria of the ethmoid turbinates of the nose:	Templin et al., 1996

Conclusion

Considering renal and severe nasal effects on mice and rats at concentrations ≤ 250 mg/m^3, application of R48/20: danger of serious damage to health by prolonged inhalation exposure,exposure is indicated.

There is no need to maintain the specific concentration levels of the 19th ATP.

Classification R48/20 (CLP STOT Rep 1 –H372) was agreed at TC C&L in September 2007.

5.6.3 Repeated dose toxicity: dermal

No data

5.6.4 Summary and discussion of repeated dose toxicity:

Laboratory animal studies identify the liver and kidneys as the key target organs of chloroform's toxic potential. Oral LOAELs from 15 up to 50 mg/kg/day were reported in dogs, rats and mice. Nasal lesions have also been observed in rats and mice exposed by inhalation.

Classification R48/20 (CLP STOT Rep 1 –H372) was agreed at TC C&L in September 2007.

5.7 Mutagenicity

5.7.1 In vitro data summary

Ten gene mutation studies in *S. typhimurium* and *E. coli*, including tests done under condition designed to reduce evaporation, are negative, with or without metabolic activation with microsomes from liver or kidney of rats and mice. Two studies have showed positive results in bacteria (*S. typhimurium* transfected, *B. subtilis*), however the relevance is uncertain due to the use of ethanol as a diluent (causing formation of potent alkylating agents with chloroform) or the absence of reported concentration that caused effects.

Six tests are also negative in fungi and yeast. In all three of the positive studies (intrachromosomal recombination or chromosome malsegregation), doses that caused positive results also caused cell death, indicating that exposures were directly toxic to the test cells.

Mammalian gene mutation assays on mouse lymphoma cells gave weak positive results with metabolic activation at cytotoxic concentrations, the OECD 476 HGPRT assay on Chinese hamster lung cells was inconclusive with metabolic activation and negative without metabolic activation (Muller, 1987). Seven DNA repair assays reported negative results on S. typhimurium, E. coli, rat and mouse hepatocytes, human's lymphocyte or hepatocytes (umu test, SOS-chromotest and UDS), only one study gave positive results on S. typhimurium at the only dose tested: 1000 µg/l (Ono et al., 1991).

Primary DNA damage studies showed that CHCl3 induced sister-chromatid exchange (SCE) in a permanent leukaemia cell line (Fujie et al., 1993) and in meristematic cells of *Allium cepa* (Cortés et al., 1985). Induced Sister Chromatid Exchanges have been reported in human lymphocytes at cytotoxic concentration ($\geq 10^{-2}$ M) without exogenous activation (Morimoto and Koizumi, 1983).

Table 10 Summary of in vitro mutagenicity

Test system	Test object	Concentration	Results	Reference and year

Test system	Test object	Concentration	Results	Reference and year
Bacterial mutation assays	Salmonella typhimurium: TA 1535 and TA 1535 transfected with rat theta-class glutathione S-transferase T1-1	200-25600 ppm	Weak positive ≥ 19200 ppm on GST T1-1 transfected strain Corresponding to 226 mg/plate of $CHCl_3$ (5 mg/plate recommended in guidelines)	Pegram et al., 1997
	Bacillus subtilis Strains: H17 and M45	No data	Positive with S9	Matsui et al., 1989
Gene mutation assays on fungi and yeast	Saccharomyces cerevisiae Strain: D7	0, 21, 41, 54 mM	Positive Cytotoxic ≥ 41 mM	Callen et al., 1980
	Saccharomyces cerevisiae *Strain RS112*	0, 0.75, 1.49, 2.98, 4.47, 5.59 mg/ml	Positive Cytotoxic ≥ 4.47 mg/ml	Brennan & Schiestl, 1998
	Aspergillus nidulans	0.04, 0.08, 0.12, 0.16, 0.20 % v/v	Positive 0.20 % Cytotoxic 0.20 % v/v	Crebelli et al., 1988, 1992, 1995
Mammalian gene mutation assays	L5178Y mouse lymphoma cells	Without S9: 0.39 to 1.5 µl/ml With S9: 0.007 to 0.06 µl/ml	Weak positive with S9 Negative without S9 Cytotoxic ≥ 1.2 µl/ml without S9 Cytotoxic ≥ 0.04 µg/ml with S9	Mitchell et al., 1988
	L5178Y mouse lymphoma cells	Without S9: 15.6-1000 nl/ml With S9: 0.78-25.0 nl/ml	Weak positive with S9 Negative without S9 Cytotoxic Without S9: ≥500 nl/ml Cytotoxic With S9: > 6.25 nl/ml	Myhr and Caspary, 1988
Chromosomal aberration test	Meristematic cells of *Allium cepa*	0, 250, 500, 1000, 1500, 2500 and 5000 µg/ml	Positive ≥ 1500 µg/ml Cytotoxic ≥ 1500 µg/ml	Cortés et al., 1985
Assay for aneuploidy	V79 Chinese hamster lung cells	$6\ 10^{-3}$, 10^{-2} and $1.2\ 10^{-2}$ M	Positive Cytotoxic >$1.2\ 10^{-2}$ M	Onfelt, 1987
DNA repair assay	Salmonella typhimuriumn TA1535/pSK1002	1000 µg/ml	Positive	Ono et al., 1991
Primary DNA damage	Permanent leukemia cell line K3D	0, 2.10^{-3}, 2.10^{-4} and 2.10^{-5} M	Positive with S9	Fujie et al., 1993
	Human lymphocytes	$1.6\ 10^{-5}$, $8\ 10^{-5}$, $4\ 10^{-4}$, $2\ 10^{-3}$, $1\ 10^{-2}$, $5\ 10^{-2}$ M	Positive ≥ $1\ 10^{-2}$ M Concentrations ≥ $1\ 10^{-2}$ M induce a delay in the cell cycles	Morimoto and Koizumi, 1983
	Syrian hamster embryo cells	2.0, 1.0, 0.5, 0.25, 0.12 ml/chamber (equivalent to 640, 320, 160, 80, 40 mg/l air)	Positive ≥ 0.25 ml/chamber Cytotoxic ≥ 0.25 ml/chamber (160 mg/l air)	Hatch et al., 1983

Test system	Test object	Concentration	Results	Reference and year
	Meristematic cells of *Allium cepa*	0, 250, 500, 1000, and 1500 µg/ml	Positive Cytotoxic ≥ 1500 µg/ml	Cortés et al., 1985

5.7.2 In vivo data summary

Negative results were reported for transgenic female mice in a gene mutation assay in somatic cells. Results of bone marrow chromosomal aberration assays in male mice and OECD 475 in Chinese hamster were negative (Shelby and Witt, 1995; Hoechst AG, 1988). Fujie et al., (1990) reported positive results for chromosomal aberrations at 119 mg/kg in male rats (5 days oral administration, sacrifice at 6, 12, 18 or 24h after the last treatment) and 1.2 mg/kg in rats of both sexes (IP treatment at 0h, sacrifice at 6, 12, 18 or 24h).

Several micronucleus assays were negative in rats and mice, but two positive results were obtained with doses above the DL50 via intra peritoneal administration (3 treatments at 24 hr intervals, sacrifice 24 hr after the final injection) or after partial nephrectomy (treatment 72h, sacrifice at 74h) (Shelby and Witt, 1995; Robbiano et al., 1998).

Negative results for interchromosomal mitotic recombination in drosophila and positive results in aneuploidy assay in grasshopper embryos were reported in inhalation studies with high chloroform doses up to 620000 ppm (Liang et al., 1983). No effects on hepatocyte UDS were observed following oral administration of chloroform in male rats and female mice (Mirsalis et al., 1982; Larson et al., 1994).

Morimoto and Koizumi (1983) observed an increase in the frequency of sister chromatid exchange in bone marrow cells of mice (treatment D1-4, sacrifice D5) at dose of 50 mg/kg/day, but at 200 mg/kg/day, all of the mice died. Studies of DNA binding in liver and kidney of mice and rats exposed to chloroform orally or by inhalation showed no clear positive results at doses up to 240 mg/kg (Diaz Gomez and Castro, 1980; Reitz et al., 1982; Pereira et al., 1982).

Topham (1980) reported no effects on germ cells for male mice receiving 5 daily i.p. injections of vehicle alone (corn oil, 5 ml/kg/ day) or chloroform at 0.025, 0.05, 0.075, 0.1, 0.25 ml/kg/d (0.25 ml/kg is a lethal dose). No increase of abnormal sperm heads was observed 5 weeks after the last dose injection.

Table 11 Summary of in vivo mutagenicity

Test system	Method	Route of administration	Toxic dose	Result	Reference
Gene mutation assays in somatic cells - Studies reliable with or without restriction					
Female B6C3F1 LacI transgenic mice	Gene mutation assay at the lacI transgenic gene in liver	Inhalation 6 hr/day 0, 10, 30, or 90 ppm 10, 30, 90, or 180 days	≥30 ppm (liver toxicity)	Negative	Butterworth et al., 1998
Chromosomal aberration assays - Studies reliable with or without restriction					

Test system	Method	Route of administration	Toxic dose	Result	Reference
Male and female Chinese hamsters	Cytogenetic assay in bone marrow cells OECD TG 475	Oral 0, 40, 120, 400 mg/kg Treatment at 0h, sacrifice at 6, 24 or 48h	500 mg/kg	Weak positive (based on rarity of aberration observed)	Hoechst AG, 1988
Male Long-Evans rats	Cytogenetic assay in bone marrow cells	Oral 1.2, 11.9 and 119.4 mg/kg 5 days, sacrifice at 6, 12, 18 or 24h after the last treatment	No data	Positive 119 mg/kg	Fujie et al., 1990
Male and female Long-Evans rats	Cytogenetic assay in bone marrow cells	i.p. 1.2, 11.9 and 119.4 mg/kg Treatment at 0h, sacrifice at 6, 12, 18 or 24h	No data	Positive ≥ 1.2 mg/kg	Fujie et al., 1990
Male B6C3F1 mice	Cytogenetic assay in bone marrow cells	i.p. 0, 200, 400, 800, 1000 mg/kg/d single administration, sacrifice 17 and 36 hr later	No data	Negative	Shelby and Witt, 1995
Micronucleus assays - Studies reliable with or without restriction					
B6C3F1 mice	Micronucleus assay in bone marrow cells	i.p. 80% of the LD50 2 treatments (sampling times: 48, 72 and 96h) 1 treatment (sampling times: 36, 48, 60, 72h) 1 treatment (sampling time: 60h)	No data	Negative	Salamone et al., 1981
Male and female NMRI Mice	Micronucleus assay in bone marrow cells	i.p. 0, 238, 476, 952 mg/kg in olive oil Treatment at 0 and 24 h, sacrifice at 30h	No data	Negative	Gocke e t al., 1981
Male B6C3F1 mice	Micronucleus assay in bone marrow cells	i.p. 0, 200, 400, 600, 800 mg/kg/d 3 treatments at 24 hr intervals, sacrifice 24 hr after the final injection	No data	Positive ≥ 400 mg/kg/d	Shelby and Witt, 1995
Male and female CD1 mice	Micronucleus assay in bone marrow cells	i.p. 0, 0.015, 0.03 and 0.06 ml/kg (0, 22, 44 & 89 mg/kg) 2 treatments at 24 hr intervals, sacrifice 6 hr after the final injection	LD50 = 0.11 ml/kg (163 mg/kg)	Negative	Tsuchimoto & Matter, 1981
Male Sprague-Dawley rats	Micronucleus assay in kidney cells	Oral 480 mg/kg Treatment 72h after partial nephrectomy and sacrifice at 74h	> 480 mg/kg	Positive	Robbiano et al., 1998

Test system	Method	Route of administration	Toxic dose	Result	Reference
Larvae of Pleurodeles waltl	Micronucleus assay in blood	Dissolved in water 12.5, 25 and 50 µg/ml water 12 days	≥ 100 µg/ml	Negative	Le Curieux et al., 1995 Fernandez et al., 1993
Male and female Sprague Dawley Crl:CD® rats	Micronucleus assay in bone marrow cells	Oral 0, 120, 240 or 480 mg/kg/day 5 days treatment, sacrifice 24 hours post the last dose	> 480 mg/kg	Negative	Whitwell, 2009
Drosophila melanogaster **assay - Studies reliable with or without restriction**					
Drosophila melanogaster	Interchromosomal mitotic recombination	Inhalation 0, 2000, 4000, 8000, 16000 ppm 17 hours	No data	Negative	Vogel and Nivard, 1993
Assays for aneuploidy - Studies reliable with or without restriction					
Grasshopper embryos	Mitotic arrest and anaphase abnormalities	Inhalation 0, 31000, 155000, 310000 and 620000 ppm 16 hours	No data	Positive	Liang et al., 1983
DNA repair assays - Studies reliable with or without restriction					
Male Fischer 344 rats	Unscheduled DNA synthesis in hepatocytes	Oral 0, 40, 400 mg/kg Treatment at 0h, sacrifice at 2 and/or 12h	c.a. 400 mg/kg	Negative	Mirsalis et al., 1982
Female B6C3F1 mice	Unscheduled DNA synthesis in hepatocytes	Oral 0, 238, 477 mg/kg Treatment at 0h, sacrifice at 2 and 12h	477 mg/kg	Negative	Larson et al., 1994
Primary DNA damage assays - Studies reliable with or without restriction					
Male ICR/SJ mice	Sister chromatid exchange assay in bone marrow cells	Oral 0, 25, 50, 100, 200 mg/kg /day Treatment at D1-4, sacrifice at D5	No data	Positive ≥ 50 mg/kg/d	Morimoto and Koizumi, 1983
Female B6C3F1 mice	DNA binding in liver	Oral 119 mg/kg Treatment at 0h, sacrifice at 16-18h	No data	Negative	Pereira et al., 1982
Male Sprague-Dawley rat	DNA binding in liver and kidney	Oral 48 mg/kg Treatment at 0h, sacrifice at 16-18h	No data	Weak positive	Pereira et al., 1982
Male B6C3F1 mice	DNA binding in liver and kidney	Oral 240 mg/kg Treatment at 0h, sacrifice at 4h	No data	Negative (minimal)	Reitz et al., 1982

Test system	Method	Route of administration	Toxic dose	Result	Reference
Male A/J mice	DNA, RNA and nuclear protein binding in liver	i.p. Up to “toxic dose” Single or once daily for 4 days or twice a week for 2 weeks	-	Negative Positive (for nuclear proteins)	Diaz-Gomez and Castro, 1980
Male F 344 rats	DNA strand break in kidney	Gavage 1.5 mmol/kg (180 mg/kg) Daily for 7 days, sacrifice 1 day later	No data	Negative	Potter et al., 1996
Germ cells assays - Studies reliable with or without restriction					
Male (CBA x Balb/C)F1 mice	Mouse sperm abnormality test	i.p. 0, 0.025, 0.05, 0.075, 0.1, 0.25 ml/kg/d Treatment at D1-5, sacrifice at D35	0.25 ml/kg	Negative	Topham, 1980
Drosophila melanogaster	Sex-linked recessive lethal assay	Oral 25 mM Single	c.a. 25 mM	Negative	Gocke et al., 1981
Drosophila melanogaster	Sex-linked recessive lethal assay	Oral 0.1 and 0.2% 3 days	No data	Negative	Vogel et al., 1981

5.7.3 Detailed description of the key *in vivo* studies

This section aims at providing further information to determine whether chloroform is an *in vivo* mutagen and should be classified as Muta. Cat. 3; R68. *In vitro* data were summarised in Table 10 and are not further detailed here.

The *in vivo* key studies presented in this section were chosen based on their reliability (1 or 2). The reliability of these studies was evaluated using the scoring system of Klimisch.

5.7.3.1 Micronuclei formation

Robbiano *et al*., 1998, (Oral micronuclei evaluation in kidney cells):

The frequency of micronucleated kidney cells was evaluated in rats exposed to 6 halogenated anesthetics including Chloroform.

7 males Sprague-Dawley albino rats per group were injected i.v with 250 mg/kg of folic acid to increase the proliferative activity of kidney cells induced by nephrectomy. Chloroform was dissolved in corn oil and administered as a single p.o. dose of 472 mg/kg bw/day in corn oil (which was half of the LD_{50} of chloroform) 2 days after folic acid injection. The dose was administered by gastric intubation in a volume of 0.01 ml/g. NDMA (20 mg/kg) was used as a positive control. Results are presented in Table 12.

Chloroform induced a statistically significant increase in the average frequency of micronucleated kidney cells. The mean frequency of micronucleated cells in rats was $1.33.10^{-3}$ for the negative control. The ratio treated/control being 3.32, and the ratio for positive control being 6.52.

This test was conducted according to OECD guideline 474 with the following deviations:

- The study was realized on kidney cells instead of erythrocytes but kidney is the target organ
- Only one concentration was tested: 472 mg / kg bw/day whereas according to OECD guideline 474, three doses are recommended.

Table 12: Frequency of micronucleated kidney cells in rats treated with chloroform.

Treatment conditions	N° of cells scored	Frequency (x10^{-3}) of micronucleated cells	Frequency (x10^{-2}) of binucleated cells
Control	37046	1.33 ± 0.41	1.91 ± 0.37
Chloroform 4 mmol/kg	15995	4.42 ± 1.16*	2.15 ± 0.55
NDMA 20mg/kg	9038	8.68 ± 2.69*	1.62 ± 0.61

*Significantly different from the control group at p< 0.001 as determined by the Wilcoxon's two sample (two tail test).

Gocke *et al*., 1981, (Intraperitoneal mice bone-marrow micronucleus assay):

This study consisted in a micronucleus assay in bone marrow cells in male and female NMRI mice treated with chloroform.

Male and female NMRI Mice were injected intraperitoneally with 0, 238, 476 and 952 mg/kg in olive oil at 0 and 24 h with a sacrifice at 30 h. Results are presented in Table 13. This study was conducted according to OCDE guideline 474, no deviation was noted.

Table 13: Results of the micronucleus test on mouse bone marrow.

Compound	Surviving / treated mice	Dose mg/kg	Route of application	Micronucleated PE (‰)
Chloroform	4/4	2 x 952	ip	2.2
	4/4	2 x 476	ip	2.6
	4/4	2 x 238	ip	2.2
	4/4	0	ip	1.2
Hydroquinone	8/8	2 x 110	ip	10.0**
	8/8	2 x 55	ip	3.5
	4/4	2 x 22	ip	1.4
	4/4	0	ip	1.1

** Significantly different from control, p<0.01.

No statistically significant dose-related increase in micronuclei formation was observed with chloroform.

Tsuchimoto & Matter, 1981, (Intraperitoneal bone marrow micronucleus assay):

Activity of chloroform in the micronucleus test was assessed in male and female CD1 mice. Each group consisted of two males and two females.

Chloroform was administered i.p twice with 0, 0.015, 0.03 and 0.06 ml/kg (equivalent to 0, 22, 44 and 89 mg / kg bw/day) in DMSO, 24 h apart. The animals were killed 6 h after the second application. Femoral bone marrow cells were obtained and smears were prepared. The number of micronucleated polychromatic erythrocytes (MPE) was counted, but not the number of micronuclei per cell.

The data obtained were evaluated on the basis of the following criteria:

- Two or more mice per group with MPE frequencies above 0.40%
- One or more treated groups with mean MPE frequencies above 0.30%
- Statistical significance in one or more treated group.

This study was conducted according to OCDE guideline 474. Results are presented in Table 14.

Table 14: Frequencies of micronucleated polychromatic erythrocytes.

Compound	Doses	Micronucleated polychromatic erythrocytes (%)
Chloroform	0 ml/kg	0.12
	0.015 ml/kg	0.08
	0.03 ml/kg	0.08
	0.06 ml/kg	0.07
2-acetylaminofluorene	0 mg/kg	0.08
	280 mg/kg	0.70*
	560 mg/kg	0.65*
	1120 ml/kg	0.45*

* Significantly different from control, $p<0.05$.

A test substance was judged positive when all three of these criteria were met. The mutagenic compound 2-acetylaminofluorene was considered as positive.

In the conditions of this study, the authors concluded that no micronucleus formation was observed whatever the concentration of chloroform tested.

<u>**Shelby & Witt 1995,** (Micronucleus assay in bone marrow cells by intraperitoneal route):</u>

Tests for the induction of micronuclei (MN) in bone marrow cells of mice have been conducted on 65 chemicals including chloroform.

Groups of 5 or more male B6C3F1 mice were injected intraperitoneally (i.p.) chloroform at 200, 400, 600 and 800 mg/kg bw/day three times at 24 h intervals with the test chemical dissolved in corn oil (CO) in two independent trials. The total dosing volume per mouse was 0.4 ml (chloroform or solvent

control). A concurrent positive control group (including benzene, acrylamide and phenol) of mice was included in each of the micronucleus tests (data not presented). Twenty-four hours after the final injection, smears of the bone marrow cells from femurs were prepared and 2000 polychromatic erythrocytes (PCE) were scored per animal for frequency of micronucleated cells. The percentage of PCE among the total erythrocyte population in the bone marrow was scored for each dose group as a measure of toxicity (see Table 15). This study was conducted according to OCDE guideline 474, no major deviation was noted.

Table 15:

TABLE X. Chloroform (CAS No. 67-66-3) (MN+/ABS−)

Test[a] (solvent)	Tissue	Trend P value	Dose (mg/kg)	MN-PCE/1,000	Survival (No. scored)
Micronucleus (CO)	BM	0.011*	0	2.40 ± 0.45	10/10
			200	3.00 ± 0.39	10/10
			400	3.50 ± 0.72	10/10
			800	4.20 ± 0.47	10/10
		0.001*	0	2.10 ± 0.29	5/5
			400	4.00 ± 0.72*	5/5
			600	4.75 ± 1.20*	4/5

One trial gave a non statistically significant increase in MN but with a dose-response trend and the second trial gave a statistically significant dose-related increase in MN, although the highest effects observed were only about 2 times control value. The results of this study were considered as positive.

<u>**Salamone *et al*., 1981**, (Intraperitoneal bone marrow micronucleus assay):</u>

This study consisted in micronucleus assay in bone marrow cells in B6C3F1 mice treated with chloroform.

B6C3F1 mice were injected intraperitoneally with 80% of the LD_{50} of chloroform (exact dose not specified) as follow:

- P1: 2 treatments with 80% of LD_{50} at 0 and 24 h , sampling times: 48, 72 and 96 h.
- P2: 1 treatment with 80% of LD_{50}, sampling times 36,48, 60 and 72 h.
- CT: 1 treatment with 80% of LD_{50}, sampling time : 60h.

Results were presented in Table 16. Micronuclei formation was observed at 60 h for chloroform with a concentration of 80 % of LD_{50}. 2-acetylaminofluorene, known to be a mutagenic compound, was used as positive control. This study was conducted according to OECD guideline 474 with minor deviations:

- Only one concentration was tested for chloroform.
- This concentration was described as 80% LD_{50} but numerical data was not indicated.
- 500 PCE were counted per mouse instead of 1000.

Table 16: Number of micronuclei/500 PCE for a single mouse for each compound

Chemical	Phase P1, P2 or CT	Dose % LD_{50}	N° of treatments	Sampling time					
				30	36	48	60	72	96
Chloroform	P1	80	2			0,0,0,0		0,1,0,0	0,1
	P2	80	1		0,0,0		<u>2,3</u>	0,2	
	CT	80	1				0,0,1,1,1,1		

2-acetylaminofluorene	P2	50	1	0,2		1,0,1	5,2,11		
		50	1				0,0,0,0,1,2,3 3,4,6,8		
	CT	25	1				0,1,2,2,4		
		12.5	1				0,1,1,2,4		

Statistically significant positive groups are underscored.

In conclusion, as only 2 animals presented micronuclei formation in first experiment, which was not confirmed in the second trial. The results of this study were considered as negative.

Whitwell, 2009 , (Oral bone marrow micronucleus assay):

Chloroform was tested for its ability to induce micronuclei in the polychromatic erythrocytes (PCE) of the bone marrow of treated rats, following 5 days of repeated oral dosing.

Groups of six male and six female rats were treated for five consecutive days with the vehicle (corn oil) or chloroform (at 120, 240 or 480 mg/kg/day) via oral gavage. Doses were selected based on previous literature and tested in range finder study.

A group of six male and six female rats were treated once with cyclophosphamide (CPA 20 mg/kg) dissolved in saline, as a clastogen positive control 24 hours prior to necropsy. Two additional groups of six male and six female rats were treated with an aneugenic positive control, Carbendazim (CBZ), dosed twice (on Days 4 and 5). Carbendazim was dosed at 1500 mg/kg/day and 2000 mg/kg/day.

All animals were sacrificed on Day 6 (approximately 24 hours post the last dose) and bone marrow smears prepared. Polychromatic erythrocytes were analysed for micronuclei.

- Results

Clinical signs observed essentially in the Micronucleus Experiment at 480 mg/kg/day included ataxia, bradypnoea, tachypnoea, hunched posture, hypothermia, lethargy, mouth rubbing, decreased activity, ptosis, piloerection and tremors. Suppression of motility (under the form of ataxia) was observed in only one high-dose male and one high-dose female and this was observed only at 0.5 hours post-dose in the preliminary study, and in high-dose females at Day 1 (0.5 hours post-dose), at Day 2 (0.5 hours post-dose), Day 3 (0.5 to 2 hours), and Day 4 (0.5 to 1 hour); no serious CNS symptoms (convulsions or tremors) were observed except for the moribund animal for which causes of its state are unknown. As shown in table 18, high-dose male rats (according to our own calculations: - 9.1% based on the mean of five animals since male 470 died at Day 5) and in mid- and An increase in severity of observations was noted in female animals on Days 3 and 4 compared to males. One male animal was killed in extremis on Day 4 but was not necropsied. Clinical signs in both genders were noted to be less severe by Day 5. High-dose female rats (- 1.5% and - 8.3%, respectively) lost weight. Most of the males having lost weight gained weight from day 5 (not shown in the table).

Table 17: Mean group body weight evolution over the dosing period of the assay from day 1 to day 6 per sex compared to concurrent vehicle controls

Dose (mg/kg/day)	Sex (M/F)	Group mean % change in bodyweight (Day 1 to Day 6)

Vehicle	M	+15.8%
Vehicle	F	+4.1%
120	M	+11.2%
120	F	+2.0%
240	M	+3.6%
240	F	-1.5%
480	M	-10.5%
480	F	-8.3%

M Male

F Female

Modest reduction in temperature was noted on Day 1 at 240 and 480 mg/kg/day in several male and female animals compared to control values (more pronounced in male animals, although decreased by a factor up to 7.6 for male rats and by a factor up to 8.1 for female rats). This effect was not observed on Day 5.

Negative (vehicle) control male rats exhibited a group mean frequency of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE), ratio expressed as %PCE, that slightly exceeded the historical (vehicle) control (normal) range (62% PCE versus 39-59% range). However, this ratio was within normal values for control females.

The clastogen (CPA) positive control group exhibited increased numbers of MN PCE such that the frequency in the positive control group was significantly ($p \leq 0.001$) greater than in the concurrent controls. Significantly elevated aneugenic (CBZ) positive control responses were also noted, although these were of a lower magnitude than the clastogenic response with a degree of heterogeneity (both genders). However for both doses of CBZ analysed there was a clear upward shift in distribution of MN PCE with several individual animals exhibiting MN PCE values exceeding those expected from historical vehicle control distribution data.

The assay system was therefore considered as valid.

Rats treated with Chloroform showed group mean %PCE values that decreased in a dose dependent manner, with the highest dose (480 mg/kg/day) exhibiting 38% PCE (males) or 27% PCE (females). These were markedly lower than the concurrent vehicle control values of 62% or 44% PCE (males and females respectively). However, these data were within the historical control values (21-78%), confirming what was found during the dose range finding pre-experiment where high group mean percentage PCE values were not significantly altered (56% and 54%, for male and female respectively). These levels are considered normal when comparing with historical vehicle control (normal) ranges. As such, the bone marrow toxicity of the test article was not convincing.

Table 18: Number of micronuclei/500 PCE for a single mouse for each compound

Sexe	Treatment (mg/kg/day)	PCE scored	MN PCE observed	% MN PCE	Standard Deviation	Heterogeneity		2x2 contingency	
						X2	Significance	X2	Significance
Males	Vehicle	12000	8	0.07	0.09	13.01	$p \leq 0.05$		
	120	12000	13	0.11	0.05	2.23	NS	0.76	NS

	240	12000	6	0.05	0.03	2	NS	0.07	NS
	480	10000	10	0.1	0.09	7.01	NS	0.39	NS
	CPA,20+	12000	227	1.89	0.56	17.16	p ≤ 0.01	204.23	p ≤ 0.001
	CBZ, 1500#	12000	24	0.2	0.15	11.52	p ≤ 0.05	7.04	p ≤ 0.01
	CBZ, 2000#	12000	48	0.4	0.52			27.22	p ≤ 0.001
Females	Vehicle	12000	14	0.12	0.08	5.72	NS		
	120	12000	11	0.09	0.05	2.64	NS	0.16	NS
	240	12000	3	0.03	0.06	15	p ≤ 0.05	5.89	NS
	480	12000	20	0.17	0.1	6.41	NS	0.74	NS
	CPA,20+	12000	140	1.17	0.39	13.5	p ≤ 0.05	102.12	p ≤ 0.001
	CBZ, 1500#	12000	35	0.29	0.22	16.3	p ≤ 0.01	8.18	p ≤ 0.01
	CBZ, 2000#	12000	32	0.27	0.16			6.29	p ≤ 0.05

+ Administered as a single dose
# Administered twice on Days 4 and 5

The groups mean frequencies of MN PCE observed in test article treated groups (male and female data) were not significantly (p ≤ 0.05) different to the vehicle controls (see table 19). In addition, individual frequencies of MN PCE were generally similar to those seen in the vehicle control groups and consistent with the laboratory's historical (vehicle) control distribution data.

As no induction of MN PCE was observed in the polychromatic erythrocytes of the bone marrow of male and female rats treated up to 480 mg/kg/day for five consecutive days, the study was considered negative and no further mechanistic investigations were performed.

- Deviations from guideline

Study was conducted to meet the known requirements of the OECD guideline 474. Other minor deviations than those specified in the appendix 16 of the study report were observed and are reported below.

In the protocol, it is stated that slides from the CPA-treated rats were initially checked to ensure the system was operating satisfactorily implying that they were not blindly read. This is a deviation to the OECD guideline 474 where it is stated that "positive control doses should be chosen so that […] do not immediately reveal the identity of the coded slides to the reader".

- Limitations of the study

The mild clinical signs observed (behaviour, body weights, CNS symptoms, modest and reversible hypothermia observed after treatment) and the absence of necropsy of the moribund rat preventing to determine if morbidity was treatment-related, question the doses selection, in particular the choice of the highest dose used [see Annex V of the Directive 67/548/EEC (Part B; Methods for the determination of toxicity and other health effects; General Introduction)]. Moreover, the groups mean percentage PCE values for the different groups were within the historical control range (21-78%) showing no indication of a test article related effect on bone marrow toxicity.

Although previous studies performed at this dose level lead to positive findings, results obtained here (in both dose range finding study and micronucleus experiment) indicated that dose used is not the MTD and did not induce bone marrow toxicity.

Formulation analyses demonstrated variability in terms of achieved concentrations from all of the sampling points across the range of concentrations used and most particularly at the low dose-level. The groups mean results ranged from:

- 8.6 to 90.1 % of the nominal concentration of 12 mg/mL (low dose-level);
- 63.6 to 95.4 % of the nominal concentration of 24 mg/mL (medium dose-level);
- 79.7 to 97.3 % of the nominal concentration of 48 mg/mL (high dose-level).

Blood plasma analysis confirmed that animals were systemically exposed to Chloroform with increasing exposure with both concentration and time. The clinical toxicity data, consistent between range-finder and Micronucleus Experiments, supported also the correct exposure of the animals to Chloroform. However that exposure was highly variable and that level could not be defined.

Altogether, we consider this study valid although the limitations observed do not allow overruling the positive results obtained in previous studies.

5.7.3.2 Chromosomal aberration formation.

Shelby & Witt 1995, (Chromosomal aberration test in bone marrow by i.p route):

Tests for the induction of chromosomal aberrations (CA) in bone marrow cells of mice have been conducted on 65 chemicals including chloroform.

Chloroform was tested for induction of chromosomal aberrations in the mouse bone marrow cells using two different sacrifice times (17 h or 36 h). Male B6C3F1 mice (8 per dose group) received a single i.p. injection with chloroform dissolved in corn oil at doses: 200, 400, 800, 1000 mg/kg pending harvest time. The total dosing volume per mouse was 0.4 ml (chloroform or solvent control). A concurrent positive control group of mice was included for each test (data not presented). Fifty well-spread first-division metaphase cells from each animal per treatment group were scored for presence of chromosomal aberrations (see Table 19). This study was conducted according to OECD guideline 473, no major deviation was noted.

Table 19

	Harvest time (hr)	Trend P value	Dose (mg/kg)	% Cells with ABS	Survival
Chromosome aberrations (CO)	17	0.004*	0	0.25 ± 0.25	8/8
			200	1.75 ± 0.70	8/8
			400	2.50 ± 0.98*	8/8
			800	1.75 ± 0.45	8/8
	17	0.500	0	1.50 ± 0.73	8/8
			800	0.50 ± 0.33	8/8
			1,000	1.25 ± 0.37	8/8
	36	0.781	0	1.00 ± 0.53	8/8
			200	2.00 ± 1.00	8/8
			400	1.75 ± 0.70	8/8
			800	1.25 ± 0.53	8/8

[a]Tests performed at BNL.
*Significant positive effect.

One CA trial with a 17 h sample time gave a statistically significant effect at 400 mg/kg only but the concurrent solvent control value was very low, 0.25% aberrant cells (historical control value is 3.26%). This effect was not confirmed in a second trial with higher doses. Results of a trial with a 36 h sample time were also negative, so the final result was concluded to be negative.

Fujie *et al.*, 1990

- Chromosomal aberration test in bone marrow by intraperitoneal administration (i.p.):

Chloroform has been studied for its ability to induce chromosome aberrations (CA) *in vivo* in rats.

Chloroform was administered by intraperitoneal injection in water to male and female Long-Evans rats at doses of 1.2, 11.9 or 119.4 mg/kg body weight (10^{-2}, 10^{-1} or 1 mmole/kg). Non-diluted benzene (234.3 mg/kg or 3 mmole/kg) was administered i.p. as a positive control. Dose-response relationship was studied in cells sampled 12 h after i.p. administration. A significant increase in the incidence of aberrant cells was noted for chloroform at doses of 1.2 mg/kg bw and greater with a significant dose-response trend (see Table 20). This study was conducted according to OCDE guideline 475, no major deviation was noted.

Table 20: Relationship between dose and trialomethanes (THM)-induced CA 12h after i.p. injection

RELATIONSHIP BETWEEN DOSE AND THM-INDUCED CA 12 h AFTER INTRAPERITONEAL INJECTION

Chemical	Dose [a] (mmole/kg)	Sex [b]	Number of cells examined	Number of cells with		Number of aberrations/cell (mean ± SD) [c]	Incidence of aberrant cells (mean ± SD) [c]	χ^2-test	Trend test (P value) [d]
				gaps	breaks				
$CHCl_3$	10^{-2}	Male (3)	300	5	13	0.043 ± 0.005	4.3 ± 0.5 (%)	*	M 0.001
		Female (3)	300	3	10	0.033 ± 0.004	3.3 ± 0.5	*	F 0.001
		Total (6)	600	8	23	0.038 ± 0.007	3.8 ± 0.7	**	T 0.001
	10^{-1}	Male (3)	300	9	23	0.077 ± 0.012	7.7 ± 1.2	**	
		Female (3)	300	9	19	0.063 ± 0.004	6.3 ± 0.5	**	
		Total (6)	600	18	42	0.070 ± 0.011	7.0 ± 1.2	**	
	1	Male (3)	300	9	22	0.073 ± 0.005	7.3 ± 0.5	**	
		Female (3)	300	7	19	0.063 ± 0.013	6.3 ± 1.2	**	
		Total (6)	600	16	41	0.068 ± 0.011	6.8 ± 1.1	**	
Positive control (benzene)	3	Male (3)	525	14	70	0.133 ± 0.019	13.3 ± 1.9	**	
		Female (3)	525	10	38	0.072 ± 0.014	7.2 ± 1.4	**	
		Total (6)	1050	24	108	0.103 ± 0.035	10.3 ± 3.5	**	
Vehicle control (physiological saline)		Male (3)	300	4	3	0.010 ± 0.000	1.0 ± 0.0		
		Female (3)	300	1	2	0.007 ± 0.005	0.7 ± 0.5		
		Total (6)	600	5	5	0.008 ± 0.003	0.8 ± 0.4		

[a] Doses of 10^{-2}–1 mmole/kg body weight for each chemical are as follows: $CHCl_3$, 1.2–119.4 mg/kg; $CHCl_2Br$, 1.6–163.8 mg/kg; $CHClBr_2$, 2.1–208.3 mg/kg; $CHBr_3$, 2.5–253 mg/kg.
[b] Figures in parentheses indicate the number of animals examined.
[c] Not including the cells with gaps. Values indicate the mean and standard deviation of the results from 3 or 6 rats.
[d] Trend test indicates the significance of the dose response for each chemical at each P value. M indicates the value for males, F for females, and T for the total of male and female rats.
* Significantly different from untreated control at $P < 0.05$.
** Significantly different from untreated control at $P < 0.01$.

In a second experiment, the percentage of aberrant metaphase cells was determined for 6, 12, 18 and 24 h after i.p. injection of 11.9 mg/kg bw (see Table 21). Compared to the values for the untreated control, statistically significant increases were noted at 6, 12 and 18 h after chloroform i.p. injection. The incidence of aberrant cells reached the maximum level at 12 h, and decreased to the control level within 24 h.

Table 21: Variation over time of THM-induced CA

VARIATIONS OVER TIME OF THM-INDUCED CA IN RAT BONE MARROW CELLS AFTER INTRAPERITONEAL INJECTION

Chemical	Dose [a] (mmole/kg)	Time (h)	Sex [b]	Number of cells examined	Number of cells with		Number of aberrations/cell (mean ± SD) [c]	Incidence of aberrant cells (mean ± SD) [c]	χ^2-test
					gaps	breaks			
$CHCl_3$	10^{-1}	6	Male (3)	300	4	14	0.047 ± 0.005	4.7 ± 0.5 (%)	**
			Female (3)	300	4	9	0.030 ± 0.008	3.0 ± 0.8	*
			Total (6)	600	8	23	0.038 ± 0.011	3.8 ± 1.1	**
		12	Male (3)	300	9	23	0.077 ± 0.012	7.7 ± 1.2	**
			Female (3)	300	9	19	0.063 ± 0.004	6.3 ± 0.5	**
			Total (6)	600	18	42	0.070 ± 0.011	7.0 ± 1.2	**
		18	Male (3)	300	5	12	0.040 ± 0.008	4.0 ± 0.8	*
			Female (3)	300	4	11	0.037 ± 0.005	3.7 ± 0.5	*
			Total (6)	600	9	23	0.038 ± 0.007	3.8 ± 0.7	**
		24	Male (3)	300	4	3	0.010 ± 0.000	1.0 ± 0.0	
			Female (3)	300	4	4	0.013 ± 0.005	1.3 ± 0.5	
			Total (6)	600	8	7	0.012 ± 0.004	1.2 ± 0.4	
Vehicle control (physiological saline)		12	Male (3)	300	4	3	0.010 ± 0.000	1.0 ± 0.0	
			Female (3)	300	1	2	0.007 ± 0.005	0.7 ± 0.5	
			Total (6)	600	5	5	0.008 ± 0.003	0.8 ± 0.4	

[a] Doses of 10^{-1} mmole/kg body weight for each chemical are as follows: $CHCl_3$, 12.0 mg/kg; $CHCl_2Br$, 16.3 mg/kg; $CHClBr_2$, 20.8 mg/kg; $CHBr_3$, 25.3 mg/kg.
[b] Figures in parentheses indicate the number of animals examined.
[c] Not including the cells with gaps. Values indicate the mean and standard deviation of the results from 3 or 6 rats.
* Significantly different from untreated control at $P < 0.05$.
** Significantly different from untreated control at $P < 0.01$.

In conclusion, positive results were obtained for chloroform in dose-dependent manner after intraperitoneal injection in rat bone marrow cells

- Chromosomal aberration test in bone marrow by oral administration:

Chloroform was administered by gastric intubation to male Long-Evans rats at doses of 1.2, 11.9 or 119.4 mg/kg bw/day with 24-h interval for 5 days. Potassium bromate (250.5 mg/kg or 1.5 mmole/kg) was administered orally as a positive control. Dose-response relationships were studied in cells sampled 18 h after the last day of treatment. For oral treatment, male rats were used because they showed a slightly higher sensitivity to the chemicals than female rats with i.p. treatment. A statistically and dose-related significant increase in the incidence of aberrant cells and of the number of aberration / cells was noted with 119.4 mg/kg chloroform (6%) compared to the untreated control (1%) (see Table 22). This study was conducted according to OCDE guideline 475, no major deviation was noted.

Table 22: Relationship between dose and THM-induced CA

RELATIONSHIPS BETWEEN DOSE AND THM-INDUCED CA AFTER ORAL TREATMENT [a]

Chemical	Dose [b] (mmole/kg)	Time (h)	Sex [c]	Number of cells examined	Number of cells with		Number of aberrations/cell (mean ± SD) [d]	Incidence of aberrant cells (mean ± SD) [d]	χ^2-test	Trend test [e]
					gaps	breaks				
$CHCl_3$	10^{-2}	24 h × 5 + 18 h	Male (3)	300	5	6	0.020 ± 0.008	2.0 ± 0.8 (%)		
	10^{-1}		Male (3)	300	6	10	0.033 ± 0.004	3.3 ± 0.5		$P < 0.001$
	1		Male (3)	300	7	18	0.060 ± 0.008	6.0 ± 0.8	**	
Positive control ($KBrO_3$)	1.5	24 h × 5 + 18 h	Male (3)	525	16	41	0.078 ± 0.018	7.8 ± 1.8	**	
Vehicle control (physiological saline)		24 h × 5 + 18 h	Male (3)	300	2	3	0.010 ± 0.000	1.0 ± 0.0		

The percentage of aberrant metaphase cells over time was determined 6, 12, 18 and 24 h after the last day of oral treatment with 119.4 mg/kg chloroform (see Table 23). A slight but statistically significant increase in the incidence of CA were observed at 12h and clearly confirmed at 18h.

Table 23: Variation of THM-induced CA

VARIATION OF THM-INDUCED CA AT VARIOUS TIMES AFTER ORAL TREATMENT [a]

Chemical	Dose [b] (mmole/kg)	Time (h)	Sex [c]	Number of cells examined	Number of cells with gaps	Number of cells with breaks	Number of aberrations/cell (mean ± SD) [d]	Incidence of aberrant cells (mean ± SD) [d]	χ^2-test
$CHCl_3$	1	24 h × 5 + 6 h	Male (3)	300	14	10	0.033 ± 0.004	3.3 ± 0.5 (%)	
		24 h × 5 + 12 h	Male (3)	300	9	11	0.037 ± 0.005	3.7 ± 0.5	*
		24 h × 5 + 18 h	Male (3)	300	7	18	0.060 ± 0.008	6.0 ± 0.8	**
		24 h × 5 + 24 h	Male (3)	300	6	3	0.010 ± 0.000	1.0 ± 0.0	
Vehicle control (physiological saline)		24 h × 5 + 18 h	Male (3)	300	2	3	0.010 ± 0.000	1.0 ± 0.0	

[a] 1 mmole/kg body weight of each THM was administered orally (gastric intubation) 5 times at 24-h intervals. The rats were killed at various times after the last treatment.
[b] These figures indicate the amounts of each THM administered once daily. The total dose volumes were as follows: $CHCl_3$, 119.4 × 5 mg/kg; $CHCl_2Br$, 163.8 × 5 mg/kg; $CHClBr_2$, 208.3 × 5 mg/kg; $CHBr_3$, 253 × 5 mg/kg.
[c] Figures in parentheses indicate the number of animals examined.
[d] Not including the cells with gaps. Values indicate the mean and standard deviation of the results from 3 rats.
* Significantly different from untreated control at $P < 0.05$.
** Significantly different from untreated control at $P < 0.01$.

In conclusion, chloroform did not produced chromosomal rearrangements in any of the aberrant cells, the type of damage being largely limited to chromatid-type aberrations. The study shows a positive result at 119.4 mg/kg for 12 and 18h after last day of treatment.

Hoechst *et al*., 1988, (Chromosomal aberration assay):

Chloroform was evaluated for clastogenicity in Chinese Hamsters (5/sex/treatment group) exposed by oral gavage to single dose of 0 (solvent control), 40, 120, and 400 mg/kg bw with subsequent harvest, preparation and analysis of metaphase bone marrow cells (100 cells/animal) at 6 (high dose), 24 (all doses), and 48 (high dose) hours post-treatment.

Results are presented in Table 24. When male and female results are combined, the slight enhancement of chromosomal aberrations was statistically significant (Mann-Whitney-U-test) at 6 and 24 hours after doses of 400 mg/kg, although the rate was still within the range of historical negative controls. In a second study, exposing groups of hamsters to doses of 0 (solvent control), 120, and 400 mg/kg bw, 24-hour cytogenetic assay again revealed a slight but statistically significant increase in chromosome aberrations in association with 400 mg/kg doses, failing again to demonstrate a dose-response relationship for rates of damage (chromosome breaks) beyond the range of historical controls. However, when the results are individually analysed for both sexes, no reproducible increase of chromosomal aberrations was observed.

The study authors noted an inference of chloroform mutagenicity, based on the nature of marked damage (multiple aberrations, chromosomal disintegration, and exchanges) associated with oral chloroform at doses of 120 and 400 mg/kg (6-, 24-, and 48-hour assessments).

The authors concluded that chloroform can induce rare but heavy structural chromosome alterations as analysed in bone marrow cells of the Chinese hamster under the experimental conditions described in this report. Therefore a mutagenic potential of the test substance cannot be excluded.

Table 24

Dose mg/kg	Time (hours)	Aberration rate excluding gaps (%)

First experiment		
Negative control	24	1.3
Positive control (CPA, 30mg/kg)	24	9.7*
40	24	1.4
120	24	1.7
400	6 24 48	2.4* 1.6* 1.0
.Second experiment		
Negative control	24	0.2
Positive control (CPA, 30mg/kg)	24	11.4*
120	24	0.6
400	24	0.9*

*Significantly different from control, p<0.05.

5.7.3.3 Sister chromatide exchange (SCEs)

Morimoto & Koizumi, 1983, (Sister chromatide exchange (SCEs)):

Trihalomethanes (THMs) including chloroform have been investigated for their ability to induce sister chromatid exchanges (SCEs) in mouse bone marrow cells *in vivo*.

Chloroform, dissolved in olive oil, was administered orally to male ICR/SJ mice (0, 25, 50, 100, 200 mg/kg /day) once a day for 4 days (see Figure 1). In bone marrow cells, an increase in SCE frequencies was observed from 50 mg/kg with a significant increase in the SCE frequency (P< 0.05). Administration of 200 mg/kg of chloroform led to an increase of about 3 SCEs per cell above the control value.

Figure 1

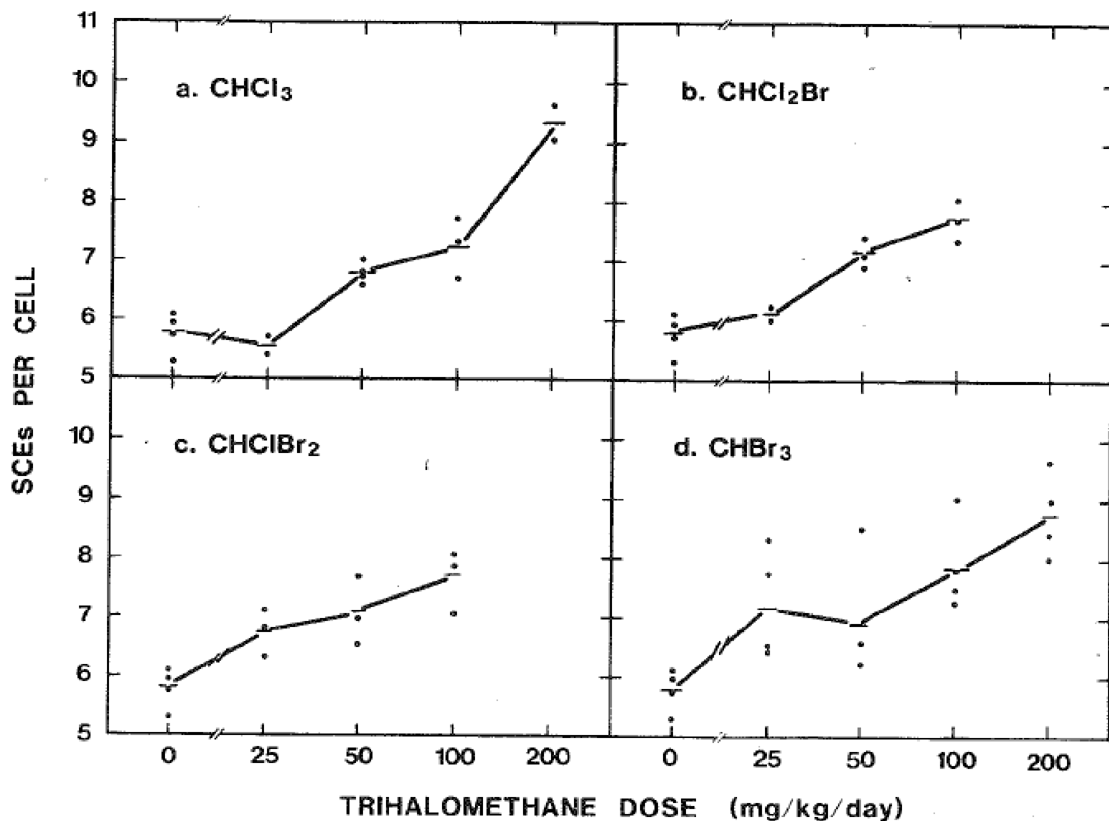


FIG. 3. The frequencies of SCEs in bone marrow cells from mice orally ingesting each of the trihalomethanes for 4 days. Each point represents the mean SCE frequency of 25 second-division cells from each animal. The bar indicates the average of the mean SCE frequencies in each dose group.

The authors suggest that the formation of SCE after chloroform exposure could be due to the formation of phosgene described as the major toxicologically relevant metabolite of chloroform (Gemma *et al*., 2003; Golden *et al*., 1997; Pohl and Krishna, 1978). Indeed, chloroform is known to be metabolically converted into trichloromethanol Cl_3OH and then converted into phosgene $COCl_2$, by mixed-function oxidases (MFOs). Phosgene is thus believed to be an active metabolite that might be responsible for the toxicity of chloroform.

5.7.3.4 Gene mutation

Butterworth *et al*., 1998, (Gene mutation in hepatocytes of B6C3F1 lacI mice):

Female B6C3F1 lacI mice were exposed daily for 6 hr/day 7 days/week up to 180 days to 0, 10, 30 or 90 ppm (equivalent to 0, 50, 166 and 500 mg/kg bw/ day) chloroform by inhalation. Results are presented in Table 25..

Table 25: LacI mutant frequencies in Chloroform-treated Mice.

TABLE III. *LacI* Mutant Frequencies in Chloroform-Treated Mice

Chloroform exposure (ppm)	Timepoint (days)[a]	Mutant frequency ($\times 10^{-5}$)[b]
0	10	10.1 ± 5.1
10	10	11.7 ± 2.4
90	10	12.7 ± 4.4
0	30	9.5 ± 2.3
90	30	10.4 ± 3.5
0	90	13.0 ± 3.1
90	90	14.7 ± 6.1
0	180	12.3 ± 0.8
90	180	13.7 ± 3.6

[a]Duration of exposure to chloroform. Exposures were 6 hr/day 7 days/week. Animals were held for 10 days after completion of exposures to allow for fixation of mutations and for complete clearance of test chemical.
[b]Mutant frequency is calculated as the number of mutant plaques isolated per total plaques screened. Values are the mean ± SD (animal-to-animal variation) from five animals per dose group for each timepoint. At least 200,000 plaques were screened per animal. As chloroform clearly did not induce an increase in mutant frequency, the remaining five animals in the group were not analyzed because of cost limitations.

The results presented here show that chloroform administered by inhalation does not increase mutant frequency in the *lacI* assay.

5.7.3.5 DNA binding – DNA damage

Pereira *et al*., 1982, (DNA binding):

Trihalomethanes used as initiators and promoters of carcinogenesis were evaluated in this study. The authors attempted to determine whether chloroform increases the incidence of cancer in the NCI bioassay by genetic, epigenetic or both mechanisms. The authors evaluated the DNA binding capability of chloroform.

Male Sprague-Dawley rats and female B6C3/F1 mice were administered intragastrically ^{14}C-chloroform (47.2 mg / kg bw for rats and 118 mg/kg bw for mice) dissolved in corn oil. The animals were sacrificed by cervical dislocation 16-18 hr later.

In rat liver and kidney, a definite peak of radioactivity representing chloroform was found associated with the ultraviolet-absorbing peak containing the DNA, whereas no association was found for chloroform in mouse liver.

Chloroform was demonstrated to bind rat liver and kidney DNA but there was no evidence for binding to mouse liver DNA within the sensitivity of the assay. The binding index of chloroform to rat liver and kidney DNA was 0.017 and 0.0055, respectively, which represents 0.05-0.15% the binding index for DMN (11.4) used as positive control.

The low level of DNA binding by chloroform indicated that the contribution of the genetic or initiating component of the carcinogenicity of the chloroform was much less than the genetic component of DMN.

Diaz-Gomez and Castro, 1980, (Binding to DNA, RNA or nuclear proteins):

This work aims to find evidence of covalent binding of chloroform or its metabolites to rat or mouse liver DNA, RNA or nuclear proteins.

Male strain A/J mice or Sprague-Dawley male rats were injected i.p with [^{14}C]$CHCl_3$ 22.72µCi/ml (spec. act. 5.4 Ci/mol) (estimated to 4.96 mg/kg bw/ day) and toxic dose (spec. act. 13.15 µCi/mmol,

conc 10% in olive oil) (estimated to 730 mg/kg/day). Mice were sacrificed 6h after the last chloroform injection and their liver processed for DNA or RNA isolation, purification and counting. Results are presented in Table 26for covalent binding to mouse liver DNA or RNA.

Table 26: Studies on possible covalent binding of ^{14}C from [14C]$CHCl_3$ to mouse liver DNA or RNA.

Experimental conditions	**^{14}C from [^{14}C]$CHCl_3$ in dpm/mg**	
	DNA	**RNA**
Control	12 ± 3	11 ± 3
Phenobarbital	8 ± 2	20 ± 6
3-Methylchloanthrene	13 ± 3	15 ± 4
730 mg/kg 1 admin.	16 ± 4	15 ± 4
730 mg/kg x 4 days	6 ± 2	9 ± 3
730 mg/kg x 2 weeks	3 ± 1	8 ± 3

Under the experimental conditions, results failed to detect any significant covalent binding of $CHCl_3$ or its reactive metabolites to DNA or RNA in mouse liver. However, positive controls (phenobarbital and 3-methylcholanthrene) did not showed high DNA or RNA binding. $CHCl_3$ or its reactive metabolites have no direct effect on DNA.

Rats were sacrificed 6h after the last chloroform injection and their liver processed for separation of nuclear protein fraction. Details of protocol were not described in the study.

^{14}C from [^{14}C]$CHCl_3$ was detected in all fractions of nuclear protein analysed. The authors concluded that nuclear proteins covalently bind ^{14}C from $^{14}CHCl_3$ and that all the fractions isolated (acidic, histone, deoxyribonucleo-protein and residual) participated in the interaction.

Reitz *et al*., 1982, (DNA binding/DNA repair *in vivo* assay):

The potential of chloroform to induce genetic damage and/or organ toxicity at the site where tumors have been observed (liver and kidney) in the various bioassays was evaluated in male B6C3F1 mice and male Sprague-Dawley rats.

To evaluate DNA binding, male mice (B6C3F1 strains) were exposed to ^{14}C-chloroform (240 mg/kg bw, *Per Os*).

The capacity of ^{14}C-chloroform to bind DNA isolated from the liver and kidneys of B6C3F1 mice was given based on data from a previous publication. Chloroform had a Chemical Binding Index (CBI) of 1.5 µmol/mol DNA, with a detection limit of 1 µmol/mo. For comparison, chemicals which strongly bind to DNA such as aflatoxine or dimethylnitrosamine have a CBI of 17,000 µmole/DNA and 6,000 µmole/mole DNA, respectively.

DNA repair was estimated by administering non radioactive chloroform to animals and subsequently determining the rate of incorporation of ^{3}H-thymidine into DNA in animals receiving doses of hydroxyurea sufficient to depress normal DNA synthesis. Details of this procedure was not described in the study. Results are presented in Figure2.

Figure 2: DNA repair in the liver of mice treated with dimethylnitrosamine (DMN) or chloroform (CHCl3) relative to control group.

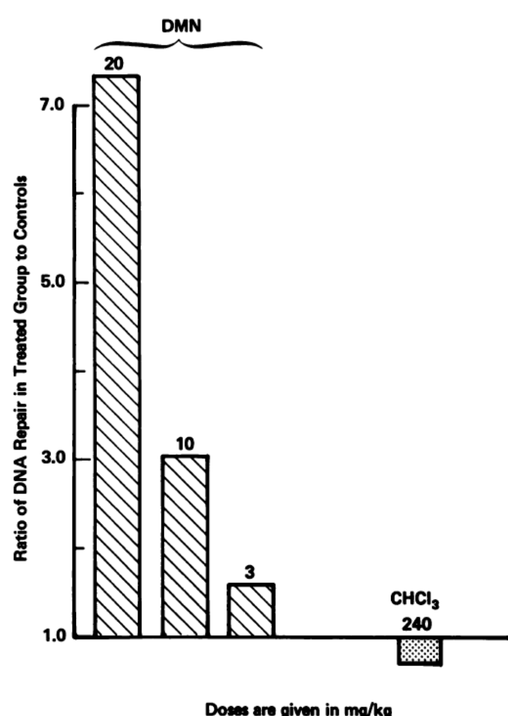


FIGURE 1. DNA repair in the liver of mice treated with dimethylnitrosamine (DMN) or chloroform ($CHCl_3$) relative to control groups.

Intraperitoneal administration of dimethylnitrosamine (DMN) cause a large increases in DNA repair in the liver of B6C3F1 mice, but chloroform was inactive in this system. Thus these data fail to indicate any significant repair of DNA (estimated as hydroxyurea-resistant incorporation of ^{3}H-thymidine into DNA) for orally administered chloroform. The very low alkylation of DNA observed after chloroform administration suggests that the genotoxic potential of chloroform is minimal.

Potter *et al.*, 1996, (Induction of DNA strand breaks):

Effects of four trihalomethanes including chloroform on DNA strand breaks in kidneys were evaluated in male F344 rats by an alkaline unwinding procedure.

Male F344 rats were administered chloroform daily by oral gavage equimolar doses (0.75 or 1.5 mmole / kg body weight equivalent to 88.5 mg / kg bw or 177 mg / kg bw respectively) in vegetable oil for 7 days. Induction of DNA strand break was evaluated by the fraction of double stranded DNA. The decrease of this fraction suggests the induction of DNA strand break as observed for positive controls diethylnitrosamine and dimethylnitrosamine.

Results are presented in Table 27.

Table 27: DNA strand break induction by THMs.

Treatment	Fraction of double stranded DNA remaining after 45 min unwinding
Vehicle control	0.83± 0.02
Chloroform	0.87± 0.01

Diethylnitrosamine	0.79± 0.003*
Dimethylnitrosamine	0.55 ± 0.02*

* Significantly different from control, $p<0.05$.

The fraction of double stranded DNA for chloroform was equivalent to fraction observed for negative control which suggests that chloroform did not induce DNA strand breaks in rat kidneys.

Mirsalis *et al*., 1982, (UDS assay) :

Unscheduled DNA synthesis (UDS) was evaluated in hepatocytes of male Fischer 344 rats orally administered with a single dose of 0, 40 or 400 mg/kg of chloroform. Rats were treated at 0h and sacrificed at 2 and/or 12h. This study was conducted according to OECD guideline 486 without major deviations; except that the cells were stained with solution of methyl-green Pyronin Y. Results were presented in Table 28.

Table 28: Induction of UDS by chemicals in the in vivo – in vitro hepatocyte DNA repair assay.

Chemical	Dose mg/kg	Sacrifice Time (h)	Number of treated animals	NG ± SE
Corn oil		2	7	-5.1 ± 0.5
		12	13	-4.4 ± 0.5
DMN	10	2	4	55.8 ± 3.3
CCl_3	40	2	3	-4.1 ± 0.4
	400	2	3	-4.4 ± 0.8
	400	12	3	-2.7 ± 0.3

Net Grain (NG) formation was not observed in chloroform treated cells by comparison to negative control. Positive control (DMN) leads to a significant increase in Net Grain formation.

5.7.3.6 Cell proliferation

Larson e*t al*., 1994, (Regenerative cell proliferation in livers and kidneys):

This study was designed to determine the dose-relationships for chloroform-induced cell proliferation in the male F344 rat kidney and liver. The labeling index (LI) was evaluated as the percentage of S-phase cells in livers and kidneys of males F344 rats given chloroform by gavage or in drinking water.

In the gavage study: (i) in kidney, an increase of labelling index was observed only with 180 mg/kg bw/day at 4 days; (ii) in liver, an increase of labeling index was detected from 90 mg/kg bw/day at 4 days and with 180 mg/kg bw/day after 3 weeks of treatment.

In the drinking water study, chloroform exposure caused no increase in LI in any region of the kidney at any exposure either at 4 days or 3 weeks. The range of exposure in drinking water was lesser (0-90 mg/kg bw/ day) than exposure by gavage.

The authors concluded that dose-dependent increases in cell proliferation were associated with the mild hepatotoxic effects of chloroform administered in corn oil.

This study described the regenerative cell proliferation in liver and kidney of rats and the relevance of the results presented in this study to evaluate the mutagenicity of chloroform is unclear.

5.7.3.7 Summary of key studies

To compare the different data, all of these studies are summarized in Table 29.

Table 29: Summary of key studies.

Species	End Point	Doses	Exposure	Vehicle	Route of administration	Results	Reliability	Guideline Deviations	References
Micronucleus assay									
Male and female Sprague Dawley rat	MN Bone marrow	0, 120, 240 or 480 mg/kg/day	5 days treatment, sacrifice 24 hours post the last dose	Corn oil	Oral	Negative	1	OCDE 474 Highest dose<MTD Positive controls not blindly read.	Whitwell, 2009
Sprague Dawley rat	MN Kidney	472 mg / kg bw / d	Single dose	Corn oil	Oral	+ 472 mg /kg bw/d	2	OCDE 474 Rat kidney cells instead of erythrocytes	Robbiano *et al.*, 1998
Mice	MN Bone marrow	0; 238; 476; 952 mg / kg bw	Treatment at 0 and 24 h	Olive oil	i.p	-	2	OCDE 474	Gocke *et al.*, 1981
Male and female mice	MN Bone marrow	0; 22; 44; 89 mg / kg bw	2 treatments at 24 h sacrifice 6 h after the final injection	DMSO	i.p	-	2	OCDE 474 Route of administration was not adequate	Tsuchimoto and Matter, 1981
B6C3F1 mice	MN Bone marrow	200, 400, 800 mg / kg bw	3 daily inject	Corn oil	i.p	+	2	OCDE 474 No deviation	Shelby and Witt 1995
B6C3F1 mice	MN Bone marrow	80% of LD_{50}	½ daily doses	DMSO	i.p	+/- 60 h	2	Only one concentration was tested (80% LD_{50})	Salamone *et al.*, 1981

								500 PCE counted per mouse	
Chromosomal aberration									
B6C3F1 mice	CA Bone marrow	200, 400, 800 mg / kg bw	single injection	Corn oil	i.p	-	2	OCDE 473 no major deviation	Shelby and Witt 1995
Long Evans rat	CA Bone marrow	1.2, 11.9 and 119.4 mg / kg bw	5 days	Distilled water	Oral	+ 119 mg / kg	2	OCDE 473 no deviation	Fujie *et al.*, 1990
Long Evans rat	CA Bone marrow	1.2, 11.9 and 119.4 mg / kg bw	Treatment at 0h, sacrifice at 6, 12, 18 or 24 h	Distilled water	i.p	+ 1.2mg / kg	2	OCDE 473 no deviation	Fujie *et al.*, 1990
Male and female hamsters	CA Bone marrow	0; 40; 120; 400 mg / kg bw	6, 24, 48 h	Paraffin oil	Oral	+/- 400 mg / kg bw	1	OCDE 475 No deviation	Hoechst *et al*, 1988 Not published
Sister chromatide exchange									
ICR/SJ mice	SCE Bone marrow	25, 50, 100, 200 mg / kg bw	4 days	Olive oil	Oral	+ ≥ 50 mg /kg bw / d	2	OCDE 479 No deviation	Morimoto and Koizumi 1983
Mutations									
B6C3F1 mice	Mutation Liver	0; 50; 166; 500 mg / kg bw	6h / 7 days Sacrifice at 24 after treatment	Unspecified	Inhalation	-	2	No guideline	Butterworth *et al.*, 1998

DNA damage – DNA binding									
Sprague Dawley rat	DNA binding Liver, kidney	47.2 mg / kg bw /d	Single dose	Corn oil	Oral	+/- 47.2 mg /kg bw/d	2	No Guideline	Pereira *et al*., 1982
B6C3F1 mice	DNA binding Liver, kidney	118 mg / kg bw / d	Single dose	Corn oil	Oral	-	2	No Guideline	Pereira *et al*., 1982
B6C3F1 mice	DNA binding Liver, kidney	240 mg / kg bw / d	Single dose	Unspecified	Oral	+/- 240 mg / kg bw / d	2	No Guideline	Reitz *et al*., 1982
B6C3F1 mice	DNA repair Liver, kidney	240 mg / kg bw / d	Single dose	Unspecified	Oral	-	2	No Guideline	Reitz *et al*., 1982
F344 rats	DNA strand break Kidney	88.5 ; 177 mg /kg bw /d	7 days	Vegetable oil	Gavage	-	2	No guideline	Potter *et al*., 1996
Male F344 rats	UDS DNA repair Liver	0; 40; 400 mg / kg bw /d	Single dose	Corn oil	Gavage	-	2	OCDE 486 No deviation	Mirsalis *et al*., 1982
Male A/J mice	DNA binding Liver	Up to toxic dose	Single or once daily for 4 days or twice a week for 2 weeks	Olive oil	i.p	- [+ for nuclear proteins]	2	No guideline	Diaz-Gomez and Castro, 1980

5.7.4 Human data

5.7.5 Other relevant information

5.7.6 Summary and discussion of mutagenicity

A. Data review at international level

Data on the mutagenicity of chloroform have recently been reviewed and evaluated by several groups: IARC, US EPA, ILSI and WHO. Most of the reviews concluded that chloroform is not a strong mutagen but a weak genotoxic effect was not excluded:

The International Life Sciences Institute (ILSI, 1997) performed a review of the available data on the mutagenicity of chloroform. ILSI committee concluded that no subset of observations points unequivocally to a specific genotoxic mode of action associated with chloroform, and that the preponderance of the evidence indicates that chloroform is not strongly mutagenic. The conclusion of IARC study on carcinogenic chemicals (1999) is that no data were available on the genetic and related effects of chloroform in humans. There is weak evidence for the genotoxicity of chloroform in experimental systems *in vivo* and in mammalian cells, fungi and yeast *in vitro*. It was not mutagenic to bacteria.

US EPA (2001) concluded that the weight of evidence indicates that even though a role for mutagenicity cannot be excluded with certainty, chloroform is not a strong mutagen and that neither chloroform nor its metabolites readily bind to DNA.

CICAD (2004) based on Environment Canada (2001) source document, concluded that most studies did not identify genotoxic potential for chloroform. Results from a few, non-standard studies indicate the possibility of a weak positive response in rats. Overall, however, the weight of evidence indicates that chloroform does not have significant genotoxic potential.

B. Summary of Data

In vitro, positive results appear sporadically and are outnumbered by negative results in other tests in the same system.

In vivo, studies conducted to evaluate **DNA binding** suggest that chloroform or its metabolites does not bind strongly to DNA (Pereira *et al*., 1982; Reitz *et al*., 1982; Butterworth *et al*., 1998; Mirsalis *et al*., 1982; Diaz-Gomez and Castro, 1980; Rosenthal *et al*., 1987). However, it binds covalently to nuclear proteins (Diaz-Gomez and Castro, 1980). No DNA strand breaks were observed in kidneys of F344 rats treated with 88.5 or 177 mg / kg bw during 7 days (Potter *et al*., 1996).

Chloroform is able to induce **micronucleus formation** or **chromosomal aberrations** when the compound was orally administered in studies of good quality in rats and mice (Robbiano *et al*., 1998; Morimoto and Koizumi, 1983;Fujie *et al*., 1991) and minimally in hamster (Hoechst *et al*., 1988). Results in rats were not reproduced in a well conducted study (Whitwell, 2009). By i.p route, chromosomal aberrations were induced in rats (Fujie *et al*., 1990). In mice, no effect was induced in studies at low dose (Tsuchimoto and Matter, 1981) or with single administration (Shelby and Witt, 1995 ; Gocke et al., 1981) but a positive effect was seen after repeated administration of high doses in Shelby and Witt (1995). The increase for micronucleus formation was about 3.3 fold and 50 % of

positive control in Robbiano *et al*., (1998) and about 1.75 fold in Shelby and Witt., (1995), no information is available on positive control. The increase of micronucleus formation after treatment with chloroform was between 1.75 and 3.32 fold when compare to negative control.

The chromosomal aberration formation was increased about 6 and 8.5 fold in Fujie *et al*., 1990 by oral and intraperitoneal route, respectively.

C. Metabolism of chloroform

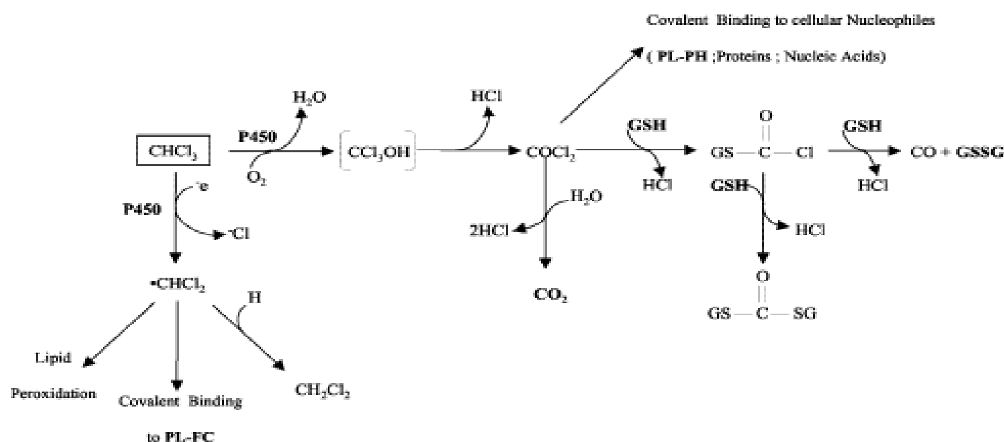
Chloroform can undergo both **oxidative** and **reductive metabolism** in the human liver (Figure 3), depending on oxygen and substrate concentration. The required step for $CHCl_3$-induced toxicity is the **cytochrome P450** (P450)-mediated bioactivation to reactive metabolites. Extensive *in vitro* and *in vivo* studies on rodents have demonstrated that chloroform may be metabolized oxidatively to trichloromethanol, which spontaneously decomposes to the electrophilic **phosgene** ($COCl_2$). $COCl_2$ is highly reactive and binds covalently to cell components containing nucleophilic groups, including proteins, phospholipid's polar heads, and reduce gluthatione (Gemma *et al*., 2003).

At low levels, reflecting human exposure through the use of chlorinated waters, $CHCl_3$ is metabolized primarily to phosgene by CYP2E1. When the CYP2E1-mediated reaction is saturated CYP2A6 can also produce phosgene, efficiently even in highly hypoxic conditions (1% pO_2). Phosgene is the **major toxicologically relevant** metabolite produced by the human liver (Gemma *et al*., 2003; Golden *et al*., 1997).

At high concentrations, chloroform is believed to increase the half-life of phosgene with the electrophilic chlorine atoms of chloroform. The stabilisation could prevent a direct reaction with water and allow phosgene to reach more reactive compounds (Potts *et al*., 1949) such as glutathione and other critical cell components.

Moreover, the reductive metabolism of chloroform produces $\cdot CHCl_2$ which is highly reactive and then could lead to lipid peroxidation. The lipid peroxidation could also contribute to radical peroxide formation.

Figure 3: The two pathways of chloroform bioactivation.



PL-FC= Adducts to Phospholipids Fatty Acyl Chains ; **PL-PH**= Adducts to Phospholipids Polar Heads;
GSH=reduced glutathione; **GSSG**= oxidated glutathione; **P450**= cytochrome P450)

FIG. 1. *The two pathways of chloroform bioactivation.*

D. Glutathione

Acute chloroform toxicity is associated with glutathione depletion (Brown et al., 1974), and it has been reported that glutathione levels decrease in a dose dependent manner prior to microscopic evidence of liver pathology (Brown et al., 1974; Docks and Krishna, 1976).

Ammann *et al*., (1998) demonstrated that chloroform and phosgene induce a moderate **glutathione (GSH) depletion**, (Sciuto *et al*., 2004; Jaskot *et al*., 1991). GSH is produced by cells for its antioxidant properties but this function could be saturated. The decrease of GSH levels by chloroform and / or phosgene will decrease protective levels of GSH. This could increase **oxidative stress** and probably reactive oxygen species production. These free radicals generation could bind to DNA and contribute to genotoxicity at high or repeated dose.

E. Role of vehicle

The results of some animal studies have suggested that the vehicle used to administrate chloroform may affect the toxicity (US EPA 2001). Indeed, Larson *et al*., (1994a and b) indicated that dose-related increases in renal damage were observed in male rat F344 administered with chloroform in corn oil and not with chloroform in drinking water. However, the range of exposure in drinking water (0-90 mg / kg bw/ day) was lower than the exposure in corn oil (0-180 mg / kg bw / day). However, from the results presented in this report, this hypothesis was not confirmed. Indeed, Fujie *et al*., (1990) observed chromosomal aberration when chloroform was administered in distilled water whereas, Pereira *et al*., (1982), Potter *et al*., (1996), Gocke *et al*., 1981 and Mirsalis *et al*., (1982) presented negative results while chloroform was administered in oil.

F. Role of phosgene

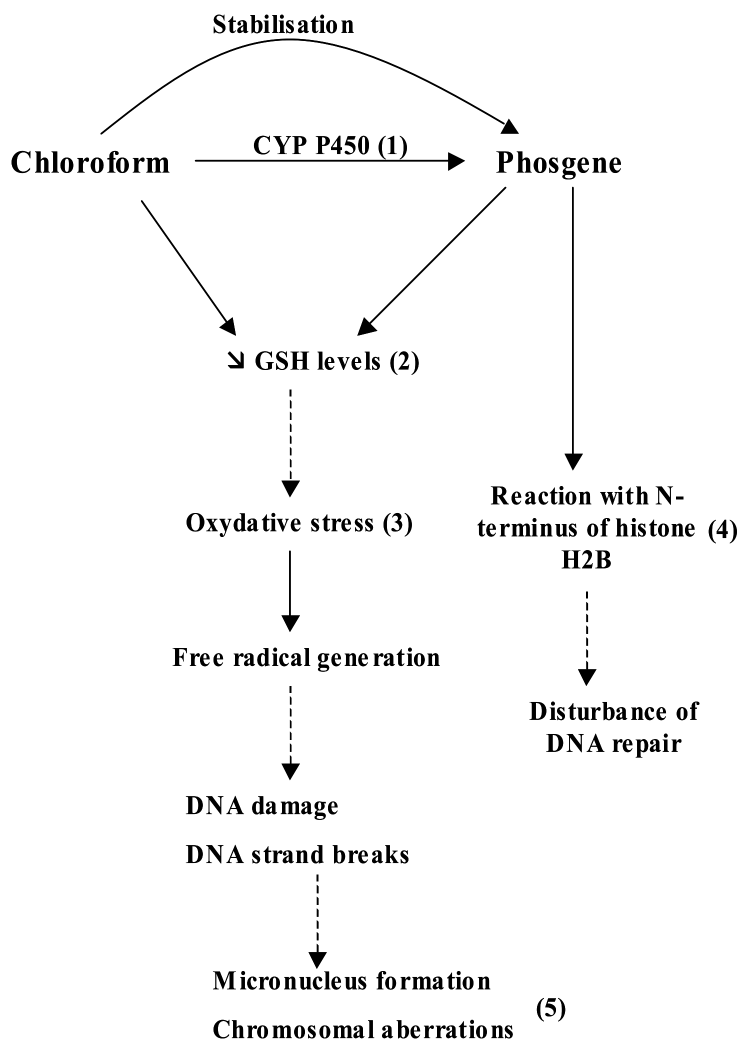
ILSI (1997) noted that **phosgene is highly reactive** and might be expected to have the capacity to interact directly with DNA, but that phosgene has not been tested in any standard mutagenicity test system. The committee also noted that, because of its high reactivity, phosgene formed in the cytosol following chloroform metabolism would likely react with cellular components prior to reaching the cell nucleus, and concluded that direct effects on DNA would be unlikely. However, it is contradictory with a recent finding of Fabrizi *et al*., (2003) which demonstrated that phosgene is able to reach cell nucleus, since phosgene can react with the N-terminus of human **histone H2B**, especially with proline and serine residues. Histone H2B is one of the 5 main histone proteins involved in the structure of chromatin in eukaryotic cells. H2B bear a main globular domain and a long N terminal tail and is involved with the structure of the nucleosomes of the 'beads on a string' structure. Histone plays a role in chromatine folding, stabilization of DNA and double DNA strand breaks repair. Moreover, Diaz-Gomez *et al*., (1980) demonstrated that chloroform or its metabolites is able to bind to nuclear protein such as histone.

G. Mechanistic hypothesis

The data presented herein indicate that chloroform does probably not bind to DNA itself. Previous studies (Brown *et al*., 1974; Gopinath and Ford, 1975; Constant *et al*., 1999; Pohl and Krishna, 1978) and results presented in this report support the conclusion that metabolism of chloroform is required for toxicity (**CYP P450 (1)**).

Data indicates that chloroform and phosgene induce **glutathione (GSH) depletion (2)** which could contribute to **oxidative stress (3)**. Moreover, it was shown by Fabrizi *et al*., (2003) that phosgene could react with **Histone H2B (4)** which could lead to disturbance of DNA repair based on indirect genotoxic mechanisms. These results are summarized in in Figure 4.**Erreur ! Source du renvoi introuvable.**.

Figure 4: Hypothesis for micronucleus formation and chromosomal aberration after exposure to chloroform.



H. Conclusion on mutagenicity

Studies presented in this section were chosen based on their reliability (1 or 2) according to Klimish scoring system. Although negative *in vivo* results are reported, several well conducted *in vivo* tests presented herein demonstrated that chloroform could induce micronuclei and chromosomal aberrations. Positive results are observed in the target organ (kidney) or after at least three administration in bone marrow cells which might be consistent with a mechanism of oxidative damage due to glutathione depletion.

Mentioned results and chloroform metabolism via oxidative or reductive pathways suggest that chloroform is a slightly genotoxic compound *in vivo*, based on indirect genotoxic mechanism. Therefore, it is proposed to classify chloroform for mutagenicity in Category 3 with the risk phrases R68 possible risk of irreversible effects (CLP Muta 2 –H341) .

5.8 Carcinogenicity

5.8.1 Carcinogenicity: oral

Effects of combined inhalation and oral exposures to chloroform on carcinogenicity and chronic toxicity in male F344 rats were examined by Nagano et al. (2006). A group of 50 male rats was exposed by inhalation to 0 (clean air), 25, 50, or 100 ppm (v/v) of chloroform vapor-containing air

for 6 h/d and 5 d/wk during a 104 w period, and each inhalation group was given chloroform-formulated drinking water (1000 ppm w/w) or vehicle water for 104 wk, ad libitum. Renal-cell adenomas and carcinomas and atypical renal-tubule hyperplasias were increased in the combined inhalation and oral exposure groups, but not in the oral- or inhalation-alone groups. The results from this study revealed that renal tumors found in the combined-exposure groups were greater in size (16-17 mm in average size, with a maximum of 40-50 mm) and incidence than those reported previously in gavage-only or drinking water-only administration studies. It was concluded that combined inhalation and oral exposures markedly enhanced carcinogenicity and chronic toxicity in the proximal tubule of male rat kidneys, suggesting that carcinogenic and toxic effects of the combined exposures on the kidneys were greater than the ones that would be expected under an assumption that the two effects of single route exposures through inhalation and drinking were additive.

Table 30 Dose-Response Relationships for the Incidences of Renal Tumors Induced by Chloroform Exposures in the Male Rat Study (Nagano et al., 2006).

Drinking-water exposure 1000 ppm (Estimated uptake)	Inhalation exposure	Estimated amount of chloroform uptake (mg/kg/d)	Renal tumor incidencea
0	0		0/50
	25 ppm	20	0/50
	50 ppm	39	0/50
45 mg/kg/d		45	0/49
53 mg/kg/d	25 ppm	73	4/50 (8%)
	100 ppm	78	1/50 (2%)
54 mg/kg/d	50 ppm	93	4/50 (8%)
57 mg/kg/d	100 ppm	135	18/50 (36%)*

Note. Data in the combined-exposure groups are indicated in italics.
[a] Incidence of renal-cell adenoma and carcinoma.
* significantly different from the untreated control group, the oral-alone group, and each inhalation-alone group with matching concentrations, respectively, at $p \leq 0.05$ by Fisher's exact test.

Table 31 Summary of oral carcinogenicity

Animal species & strain	**Number of animals**	**Doses, vehicle, duration**	**Result**	**Reference**
Rat, Osborne-Mendel	50/sex/dose	90 or 180 mg/kg bw/d (♂) 100 or 200 mg/kg bw/d (♀) gavage in corn oil, 78 weeks	significant increase (24%) in the incidence of kidney epithelial tumors in males at 180 mg/kg: Control, 0/99; matched controls, 0/19; 90mg, 4/50, 8%; 180mg 12/50, 24%	NCI, 1976

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference
Rat, Osborne-Mendel	50-330	200, 400, 900, 1800 mg/l chloroform in drinking water 104 weeks Estimated uptake: 19, 38, 81, or 160 mg/kg/day	Significant increase of kidney tumors (tubular cell adenomas and adenocarcinomas) in males at highest dose: control, 5/301, 1.7%; matched controls, 1/50, 2%; 19mg, 6/313, 1.9%; 38mg, 7/148, 4.7%; 81mg, 3/48, 6.3%; 160mg, 7/50, 14%	Jorgenson et al., 1985
Rat, F344	50 Male/dose	25, 50, or 100 ppm in air 1000 ppm in water Combined exposure inhalation and drinking water, 6 h/d and 5 d/wk during a 104 wk Estimated uptake: 20, 39, 45, 73, 78, 93, 135 mg/kg/d	Significant increase of renal-cell adenomas and carcinomas and atypical renal-tubule hyperplasias at highest dose: Control, 0/50; 20mg, 0/50; 39 mg, 0/50; 45 mg, 0/49; 73 mg, 4/50, 8%; 78 mg, 1/50, 2%; 93 mg, 4/50, 8%; 135 mg, 18/50, 36%	Nagano et al., 2006
Mouse, B6C3F1	50/sex/dose	138 or 277 mg/kg bw/d (♂) 238 or 477 mg/kg bw/d (♀) gavage in corn oil, 78 weeks	Significant increased incidence of hepatocellular carcinomas in males and females at low and high doses: (♂) control, 5/77, 6.5%; matched controls, 1/18, 5.6%; 138mg, 18/50, 36%; 277mg, 44/45, 97.8% (♀) control, 1/80, 1.3%; matched controls, 0/20; 238mg, 36/45, 80%; 477mg, 39/41, 95.1%	NCI, 1976
Mouse, ICI	1st study 35-72 2nd study 48-237 3rd study 47-83	17, 60 mg/kg bw Gavage in toothpaste or arachis oil, 6d/week for 80 weeks	Increased kidney adenomas and carcinomas in males at 60 mg/kg bw (LOAEL)	Roe et al., 1979

5.8.2 Carcinogenicity: inhalation

Yamamoto et al. (2002) conducted a study on chloroform carcinogenicity in BDF1 mice and F344 rats (50 animals/sex/dose). Inhalation exposure concentrations to chloroform were 5, 30 or 90 ppm for mice and 10, 30 or 90 ppm for rats, 6h/day, 5days/week, for 104 weeks. Due to the acute lethality of the 30 and 90 ppm doses in mice, an adaptation period with lower doses was performed. Mice in the 30 and 90 ppm groups were first exposed to 5 ppm for two weeks then 10 ppm for two weeks (then 30 ppm for two weeks in the 90 ppm group) before the 30 and 90 ppm concentrations were maintained. Statistically significant increases in the incidence of overall renal cell adenomas

and carcinomas were observed in the male mice exposed to 30 and 90 ppm (control, 0/50; 5 ppm, 1/50; 30 ppm, 7/50 90 ppm, 12/48). The incidence rates of renal cell carcinoma were statistically increased in male mice in the 90 ppm group when compared with controls (control, 0/50; 90 ppm, 11/48). There were no statistically significant changes in tumor incidence for female mice or for rats of either sex in any exposure group. Nasal lesions including thickening of the bone and atrophy and respiratory metaplasia of the olfactory epithelium were observed for rats of both sexes or female mice exposed to 5 ppm and above. The NOAEL= 5 ppm for the kidney adenoma/carcinoma endpoint in mice, for nasal lesions a LOAEL= 5 ppm was determined.

Table 32 Summary of inhalation carcinogenicity

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference
Mouse, BDF1	50/sex/dose	5, 30 or 90 ppm inhalation 6h/day, 5days/week, for 104 weeks	Significant increased incidence of overall renal cell adenomas + carcinomas in male mice at 30 and 90 ppm (control, 0/50; 5 ppm, 1/50; 30 ppm, 7/50; 90 ppm, 12/48) Significant increased incidence of renal cell carcinoma in male mice at 90 ppm (control, 0/50; 5 ppm, 1/50; 30 ppm, 4/50; 90 ppm, 11/48)	Yamamoto et al., 2002
Rat, F344	50/sex/dose	10, 30 or 90 ppm inhalation 6h/day, 5days/week, for 104 weeks	No statistically significant changes in tumor incidence for female mice or for rats of either sex in any exposure group	Yamamoto et al., 2002

5.8.3 Carcinogenicity: dermal

No data

5.8.4 Carcinogenicity: human data

There have been no studies of toxicity or cancer incidence in humans chronically exposed to chloroform (alone) via drinking water. Chlorinated drinking water typically contains chloroform, along with other trihalomethanes and a wide variety of other disinfection by-products. It should be noted that humans exposed to chloroform in drinking water are likely to be exposed both by direct ingestion and by inhalation of chloroform gas released from water into indoor air.

Although some studies have found increased risks of bladder cancer associated with long-term ingestion of chlorinated drinking-water and cumulative exposure to trihalomethanes, results were inconsistent between men and women and between smokers and non-smokers. Moreover, relevant studies contain little information on specific exposure, and it is not possible to attribute any excess risk specifically to chloroform. Specific risks may be due to other disinfection by-products, mixtures of by-products, other water contaminants, or other factors for which chlorinated drinking-water or trihalomethanes may serve as a surrogate (CICAD, 2004; IARC, 1999).

To conclude, the current human data are insufficient to establish a causal relationship between exposure to chloroform in drinking water and increased risk of cancer.

5.8.5 Other relevant information

5.8.6 Summary and discussion of carcinogenicity

Studies in animals reveal that chloroform can cause an increased incidence of kidney tumors in male rats or mice and an increased incidence of liver tumors in mice of either sex. These induced tumors responses are postulated to be secondary to sustained or repeated cytotoxicity and secondary regenerative hyperplasia, according to the dose levels tested. The weight of evidence in genotoxicity studies is consistent with the hypothesis that the liver and kidney tumors induced depend on persistent cytotoxic and regenerative cell proliferation responses. The persistent cell proliferation presumably would lead to higher probabilities of spontaneous cell mutation and subsequent cancer (US EPA, 2001).

Conclusion

The proposed classification for carcinogenicity of chloroform is Category 3 with the risk phrases R40 limited evidence of carcinogenic effects.

Classification Category 3; R40 (CLP Carc Cat 2 – H351) for carcinogenicity was agreed at TC C&L in September 2007.

5.9 Toxicity for reproduction

5.9.1 Effects on fertility

Table 33 Summary of effects on fertility

Animal species & strain	Number of animals	Doses, vehicle, duration	Result	Reference
Mouse, CD1	20 mated/sex/ dose	6.6, 15.9, 41.2 mg/kg-day gavage in corn oil, 31 weeks	Reduced body weight at the delivery of the 4th litter and on post natal day 14 of the 5th litter at 41.2 mg/kg males, absolute and relative weights of the right epididymis were increased at 41.2 mg/kg ($p<0.05$)	Chapin et al., 1997
Mouse, C57B1/C3H	5/dose	0.04%, 0.08 % chloroform (400, 800 ppm) Inhalation, 4 hr/day, 5 days	Significant increased frequency of abnormal sperm morphology at 0.04 ($p<0.05$) and 0.08% ($p<0.01$) Control, 1.42%; 0.04, 1.88%; 0.08, 2.76%	Land et al., 1979, 1981

Conclusion

No classification is required for effects on fertility.

5.9.2 Developmental toxicity

Timed mated Sprague-Dawley rats were exposed to chloroform by inhalation, 7 hr/day on each gestation days 6 through 15, at concentration levels of 30, 100 or 300 ppm; a starved control group

was also added to the experiment due to the marked anorexia observed (Schwetz *et al.*, 1974). No dams died during the study but statistically significant decreases of percent pregnant, maternal weight gain and food consumption were observed (see Table 34).

Table 34 Main maternal parameters following exposure to chloroform by inhalation

Parameters	air control	air starved	30 ppm	100 ppm	300 ppm
% pregnant	88	100	71	82	15*
body weight (g) ± SD					
GD 6	275 ± 21	274 ± 13	266 ± 14	274 ± 17	284 ± 9
GD 13	310 ± 17	223 ± 13*	280 ± 14*	274 ± 18*	192 ± 9*
GD 21	389 ± 28	326 ± 24*	381 ± 23*	365 ± 22*	241 ± 29*
feed (g/day)					
GD 6-7	19 ± 3	starved	5 ± 3*	13 ± 4*	1 ± 1*
GD 12-13	22 ± 2	starved	20 ± 1	15 ± 2*	1 ± 1*
GD 18-19	26 ± 3	24 ± 8*	29 ± 5	33 ± 3*	not done

* statistically different from controls at $p<0.05$

Changes in serum glutamic-pyruvic transaminase (SGPT) were measured as a means of evaluating liver function and to assess the degree of liver toxicity in rats. No statistically difference was observed between controls and rats exposed to 300 ppm of chloroform. In addition, livers for pregnant and nonpregnant rats, evaluated 6 days after the cessation of the treatment, were considered to have a normal appearance. Relative liver weights were affected only in the 300 ppm group of nonpregnant rats, showing a significant increase in comparison to the controls ($p<0.05$). Considering pregnant rats, relative liver weights were increased over control values at 100 and 300 ppm of chloroform, and in starved control ($p<0.05$).

In the 300 ppm group, only three dams of 20 were found to be pregnant; for these litters, as compared to controls, litter size was reduced, resorption frequency and percentage of litters with resorption were increased ($p<0.05$) (see Table 35).

Table 35 Main fetal parameters following exposure to chloroform by inhalation

Parameters	air control	air starved	30 ppm	100 ppm	300 ppm
litters	68	8	22	23	3
foetus/litter	10 ± 4	10 ± 4	12 ± 2	11 ± 2	4 ± 7*
resorptions	8 %	7 %	8 %	6 %	61 %*
litters with resorptions	57 %	25 %	68 %	52 %	100 %
sex ratio M:F	53:47	45:55	53:47	55:45	34:66*
mean fetal weight/litter (g)	5.69 ± 0.36	5.19 ± 0.29*	5.51 ± 0.2	5.59 ± 0.24	3.42 ± 0.02*
CRL (mm)	43.5 ± 1.1	42.1 ± 1.1*	42.5 ± 0.6*	43.6 ± 0.7	36.9 ± 0.2*
skeletal anomalies	68 %	38 %	90 %*	74 %	100 %

(% litters)					
soft tissue anomalies (% litters)	48 %	38 %	45 %	65 %	100 %

* statistically different from controls at p<0.05

At a concentration of 100 ppm, three out of 23 litters showed gross malformations, 3/23 had fetuses with acaudia or short tail and 3/23 had fetuses with imperforate anus: as the control malformation rate was 1/68, the increase was significant over the control. Otherwise, it is not stated how many fetuses were affected among the litters or if the same fetuses were affected by the anomalies. At 30 ppm, skeletal malformations were increased with delayed ossification of the skull (16/22), wavy ribs (4/22) and split sternebrae (2/22).

Thompson *et al.* (1974) exposed rabbits (15/group) to 0, 20, 35 or 50 mg/kg-day of chloroform, in corn oil by gavage, daily on gestation days 6-18. Seven dams died during the study and deaths in the high dose group were attributed to hepatotoxicity. Body weight gain decreased in dams of the top dose group. Complete abortions were seen in all groups (3 in the control group, 2 at 20 mg/kg-day, 1 at 35 mg/kg-day and 4 at 50 mg/kg-day). Mean fetal weights were significantly lower than controls for the 20 and 50 mg/kg-day groups. No visceral malformation was observed; only incomplete ossification of skull bones was observed in all groups with fetal incidence significant at 20 and 35 mg/kg-day (p<0.05).

Table 36 Summary for developmental toxicity

Reference	Protocol	Doses	Maternal effects	Developmental effects
Schwetz *et al.*, 1974	Sprague-Dawley rats *Inhalation* 0, 30, 100, 300 ppm 7 hr/day, gd 6-15	30 ppm 100 ppm 300 ppm	Reduced feed consumption 100 & 300 ppm; only on gd 6-7 for 30 ppm Reduced bw on gd 13 at 30, 100, & 300 ppm; on gd 21 at 300 ppm Absolute liver weight increased 300 ppm Relative liver weight decreased 100 & 300 ppm	30 ppm Increased skeletal anomalies 100 ppm Increased gross anomalies 300 ppm Reduced pregnancy rate, decreased litter size, increased resorptions, altered sex ratio and decreased fetal weight and CRL
Baeder & Hoffman, 1988	Wistar rats *Inhalation* 0, 30, 100, 300 ppm 7 hr/day, gd 7-16	All concentrations	Reduced food consumption, reduced body weight	Increased in completely resorbed litters, decreased CRL Decreased fetal weight (300 ppm only)
Baeder & Hoffman, 1991	Wistar rats *Inhalation* 0, 3, 10, 30 ppm 7 hr/day, gd 7-16	3 ppm 10 ppm 30 ppm	Reduced food consumption Reduced body weight	Increased ossification variations Decreased fetal weight and CRL
Thompson *et al.*, 1974	Sprague-Dawley rats Gavage 0, 20, 50, 126 mg/kg-day	50 mg/kg-day 126 mg/kg-day	Decreased food consumption, decreased weight gain	Increased implantations, decreased fetal weight

Reference	Protocol	Doses	Maternal effects	Developmental effects
	gd 6-15			
Ruddick *et al.*, 1983	Sprague-Dawley rats Intubation 0, 100, 200, 400 mg/kg-day gd 6-15	All doses 400 mg/kg/d	Decreased body weight, increased liver weight, decreased hematocrit, hemoglobin and red blood cells count Increased kidney weight	Decreased fetal weight, increased of sternebrae aberrations and runting
Murray *et al.*, 1979	CF-1 mice *Inhalation* 0, 100 ppm 7 hr/day, gd 6-15, 1-7 or 8-15		Decreased weight gain, gd 1-7 or 8-15 Increased relative liver weight, gd 6-15 or 8-15	Decreased pregnancy rate, gd 1-7 or 6-15 Increased resorptions, gd 1-7 Decreased fetal weight and CRL, gd 1-7 or 8-15 Increased cleft palate, gd 8-15 Increased delayed ossification of sternebrae, gd 1-7 or 8-15
Thompson *et al.*, 1974	Rabbits Gavage 0, 20, 35, 50 mg/kg/d gd 6-18	All doses 20 mg/kg-day 50 mg/kg-day	 Death, decreased body weight gains	Complete abortions Decreased fetal weight

5.9.3 Human data

Fertility

One case study of occupational exposure to chloroform and its effect on male reproductive toxicity was available (Chang *et al.*, 2001). A 34-year-old male laboratory worker was exposed to solvents at work for 1 year, due to the shutdown of the ventilation system. Before the exposure, his complete fertility test (semen appearance, volume and sperm count) showed no abnormality; after the exposure, asthenospermia was diagnosed (Table 4.2). An investigation was hence performed to determine the worker's possible exposure level to chemical hazards: the worker was exposed to chloroform levels approximately 10 times higher than the permissible exposure limit of 50 ppm (OSHA, 1997) and 50 times higher than the threshold limit value of 10 ppm (ACGIH, 2001), during 8 months. The worker was also exposed to other chemicals like isooctane and tetrahydrofuran but no study of male reproductive effects in association with exposure to isooctane was identified and no adverse effect of tetrahydrofuran on male fertility was reported in studies.

Table 37 Semen analysis after 1 year exposure (Chang et al.,2001)

Parameters	July 1997	August 1997	October 1997
Volume (ml)	4	5.5	3
Count (million/ml)	68.6	73.8	90.6
Motility 30 min after ejaculation:			

rapid	17 %	10 %	32 %
medium	6 %	1 %	6 %
slow	3 %	0 %	2 %
static	74 %	89 %	30 %
Path velocity (m/sec)	35	40	50

Developmental toxicity

Only one study studied exposure to chloroform in laboratory or non laboratory department for 1 year, in association with pregnancy outcomes (Wennborg *et al.*, 2000). A cohort of Swedish women (n=697, births=1417), born in 1945 or later, was studied. No effect was reported between laboratory work and reported spontaneous abortion, small gestation age or variations in birth weight. However, limitations are various: lack of exposure measurements, possible exposure to other solvents, long time between pregnancies and administration of the questionnaire.

As chloroform is a water disinfection byproduct, many studies have examined the relation between trihalomethanes (THMs), including chloroform, in drinking water and pregnancy outcomes.

A population-based case-control study was conducted in Iowa, between 1987 and 1990, to evaluate the relation between exposures to chloroform via drinking water and low birth weight (case=159, controls=795), prematurity (case=342, controls=1710) and intrauterine growth retardation (case=187, controls=935) (Kramer *et al.*, 1992). The results showed that exposure to chloroform at concentration ≥ 10 µg/l was associated with an increase risk of intrauterine growth retardation (odd ratio = 1.8, 95% CI, 1.1 – 2.9).

King *et al.* (2000) conducted a retrospective cohort study to determine the association between exposure to specific disinfectant by-products, including chloroform, and the risk of stillbirth, in Nova Scotia between 1988 and 1995 (perinatal database n= 49842). Exposure of chloroform ≥ 100 µg/l leads to a relative risk for stillbirth about 1.56; the risk estimate was higher for asphyxia-related deaths and increased with increasing levels of chloroform exposure. However, the lack of individual data on chloroform exposure could be a limitation of this study.

Dodds and King (2001) conducted a retrospective cohort study to determine the association between exposure to chloroform and birth defects, in Nova Scotia between 1988 and 1995 (perinatal database n= 49842). An increased risk of chromosomal abnormalities was observed with exposure to chloroform at levels 75-99 µg/l (relative risk = 1.9) and at levels ≥ 100 µg/l (relative risk = 1.4). An increased risk of cleft defects was reported too for exposure to chloroform ≥ 100 µg/l (relative risk = 1.5).

Dodds *et al.* (2004) conducted a case-control study to identify the association between exposure to THMs, including chloroform, in public water supplies and the risk of stillbirth. This study was performed in Nova Scotia and Eastern Ontario, between 1999 and 2001 (cases=112, controls=398). The results showed that the odds ratios for stillbirths were increased at the 1-49 µg/l level (OR=1.8, 95% CI, 1.1 – 3.0) and at the ≥ 80 µg/l level (OR=2.2, 95% CI, 1.0 – 4.8). There was no evidence of a monotonic increase.

Wright *et al.* (2004) conducted a retrospective cohort study to determine the effect of maternal third trimester exposure to chloroform on birth weight, gestational age, small for gestation age and

preterm delivery. This study was based on birth certificate data from 1995-1998 (n=196000) in Massachusetts. Reductions in mean birth weight were observed for chloroform concentrations > 20 µg/l. In addition, exposure to chloroform was associated too with an increase in mean gestational duration and a decreased risk for preterm delivery.

5.9.4 Other relevant information

5.9.5 Summary and discussion of reproductive toxicity

Regarding fertility, only one author reported increased mice abnormal sperm following exposure to an air concentration of 0.04 or 0.08 % chloroform (Land *et al.*, 1979-1981). Otherwise, animal findings were epididymal lesions or increased right epipidymis weight. As well, one occupational study reported asthenospermia in association to chloroform exposure. No other adverse reproductive effect has been evidenced in the 90 days studies.

Conclusion

Based on the data available for fertility, effects are not sufficiently severe to justify a classification.

Concerning developmental toxicity, epidemiological studies of chloroform in drinking water suggest an association between exposure to chloroform and reduced fetal weight, stillbirth, chromosomal abnormalities and cleft defects. Otherwise, we need to keep in mind that many epidemiological studies present limitations like the use of water concentration as the measure of exposure, co-exposure with other THM or Disinfection By-Product, which can lead to exposure misclassification.

By inhalation, the effects of chloroform on the various animals tested include effects on pregnancy rate, resorption rate, litter size and live fetuses, fetal weight and CRL, as well as skeletal and gross abnormalities or variations. However, maternal toxicity has been evidenced with the developmental effects reported in these studies.

Conclusion

Considering the effects evidenced in human and animal studies, chloroform should be classified as Category 3 with the risk phrase R63 possible risk of harm to the unborn child.

Classification Category 3; R63 for developmental toxicity (CLP Repr 2 – H361d) was agreed at TC C&L in September 2007.

5.10 Other effects

5.11 Derivation of DNEL(s) or other quantitative or qualitative measure for dose response

Not relevant for this type of dossier.

6 HUMAN HEALTH HAZARD ASSESSMENT OF PHYSICO-CHEMICAL PROPERTIES

No classification required

7 ENVIRONMENTAL HAZARD ASSESSMENT

This section is not covered in this dossier. For further information can be found in the transitional dossier.

JUSTIFICATION THAT ACTION IS REQUIRED ON A COMMUNITY-WIDE BASIS

Chloroform was on the 2nd priority list of the Existing Substances Regulation and its classification was reviewed in the context of the Risk Assessment procedure as it is a requirement to harmonise classification for all endpoints.

The need to revise the current harmonised classification on effects other than CMR was identified including a revision of the specific concentration limits applied (i.e. Xn; R22 (>5%) and Xn; R48/20/22 (>5%)).

Revision of the health classification of chloroform was discussed and agreed by the TC C&L in september 2007. However, no agreement could be reach by the TC C&L on mutagenicity and the classification for this endpoint is submitted to ECHA.

Environmental classification of chloroform was discussed and no classification was agreed by the TC C&L in January 2007. Therefore, this endpoint is not presented in this dossier. Further information can be found in the transitional dossier.

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OTHER INFORMATION

Revision of the health classification of chloroform was discussed and agreed by the TC C&L in september 2007 (see Annex 1):

The TC C&L agreed on addition of classifications Xn; R20, Xi; R36 and Repr. Cat. 3; R63 based on the FR proposal. They also agreed not to revise existing classifications Xn; R22, Xi; R38 and Carc. Cat. 3; R40 and not to classify chloroform with Xi; R37 (initially proposed by France) as the nasal effects reported were rather covered by Xn; R48/20. Further, the TC C&L agreed that R48/22 could be deleted as effects were only seen at high doses. They also agreed on classification with Repr. Cat. 3; R63 based on the FR proposal. The narcotic effects that are covered by Xn; R20 under Directive 67/548 trigger classification with STOT Single 3 under the CLP Regulation. The follow-up of the discussion having taken place in the TC C&L regarding R20 and R22 (see annex 1) do not mention any discussion regarding specific concentration limits. It is supposed that the agreement on the corresponding classifications R22 and R48/20 imply agreement on the withdrawal of these specific concentration limits

No agreement could be reach by the TC C&L on mutagenicity and the classification for this endpoint is submitted to ECHA.

For records, chloroform was a substance in the 2nd priority list of Regulation 793/93/EEC. TCNES I'08 did not succeed in taking a decision on a conclusion on the endpoint mutagenicity as for a conclusion (ii) or (iii) there was not enough evidence which could be supported by the majority of the member states and for a conclusion (i) no test proposal could be supported. Therefore the risk assessment of chloroform was not finalized for this endpoint under the ESR program and the conclusion was left open with regard to mutagenicity of chloroform.

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ANNEX 1: EXTRACT FROM “FOLLOW-UP III OF THE MEETING OF THE TECHNICAL COMMITTEE ON CLASSIFICATION AND LABELLING” IN ARONA, 26-28 SEPTEMBER 2007C067(F)	A new classification proposal was provided by FR in ECBI/42/07, circulated with Revision 2 of the September agenda. ***In September 2007*** TC C&L agreed not to classify chloroform with Xi; R37 as the nasal effects reported were rather covered by Xn; R48/20. Further TC C&L agreed that R48/22 could be deleted as effects were only seen at high doses. They also agreed on classification with Repr. Cat. 3; R63 based on the FR proposal. The narcotic effects that would be covered by Xn; R20 under the current system would trigger classification with STOT Single 3 under the CLP Regulation.

Chloroform (Trichloromethane) **602-006-00-4** **CAS: 67-66-3** **EC: 200-663-8** **Classification:** **Carc. Cat. 3; R40** ***Agreed 0907*** **[Muta Cat. 3; R68]** **Repr. Cat. 3; R63** ***Agreed 0907*** **Xn; R20/22-48/20** ***Agreed 0907*** **NC Xn; R48/22** ***Agreed 0907*** **Xi; R36/38** ***Agreed 0907*** **NC Xi; R37** ***Agreed 0907*** **NC for the ENV** ***Agreed 0107*** *Current classification (19 ATP): Xn; R22-48/20/22 - Xi; R38 - Carc. Cat. 3; R40* **Labelling:** Xn R: 20/22-36/38-40-48/20-63-[68] **S: (2-)36/37-46** **Classification assigned in accordance with the CLP Regulation:**	**Mutagenicity:** No agreement could be reached on mutagenicity. 5 of the present MS experts were in favour of Muta. Cat. 3: R68, 10 experts preferred no classification and 4 experts did not have a final position. FR will revise their proposal with more justification for Muta. Cat. 3 R68 and provide this to the ECB prior 7 November. MS changing their position from the one expressed at the meeting or MS not present at the meeting are then asked to react during FU II. A final decision whether the discussion on mutagenicity must be handed over to ECHA will be made only at the end of the Follow-up period. ECB has updated the S-phrases in accordance with the classification agreed at the meeting (i.e. added S46). Comments with a proposal for Muta. Cat. 3; R68 were sent by **SE** in ECBI/42/07 Add.1. A new proposal for Muta Cat. Cat. 3; R68 was submitted by **FR** after TCNES discussion in ECBI/42/07 Add.2. **After FUI:** Mutagenicicity **DE** still supports R68. **FR** provided further additional information to determine whether chloroform is an *in vivo* mutagen and should be classified as Muta. Cat. 3; R68 (ECBI/42/07 Add. 3). **ECB**: On the bases of the additional information on mutagenicity provided by **FR** (ECBI/42/07 Add. 3), MS especially those who have changed their position from the one put forward at the TC C&L meeting or who were not present at the meeting are welcome to react during FUII. **After FUII:** **NL:** agrees with Muta Cat. 3 R68

Carc. 2; H351 **[Muta. 2; H341]** **Repr. 2; H361d** **Acute Tox. 3; H331** **Acute Tox. 4; H302** **STOT Rep. 2; H373** **Eye Irrit. 2; H319** **Skin Irrit. 2; H315** **STOT Single 3; H336**	**IRL**: has considered the summary data presented in this document and we believe that there is insufficient evidence to classify chloroform as Mut. Cat 3: R68. Many of the positive effects seen appear to be species specific, and appear to be mediated by cyp450 metabolism to phosgene in certain target organs. Despite these results the overwhelming body of evidence is negative and on this basis we considered that chloroform should not be classified. **ECB/FR: Dec 2007 TECNES meeting decided that further testing for mutagenicity is necessary before any conclusion can be drawn.** **⇨ Hand-over to ECHA**

ANNEX 2: EXTRACT FROM "SUMMARY RECORD - TECHNICAL COMMITTEE ON CLASSIFICATION AND LABELLING OF DANGEROUS SUBSTANCES – MEETING ON ENVIRONMENTAL EFFECTS OF EXISTING CHEMICALS, PESTICIDES & NEW CHEMICALS -ISPRA, JANUARY 25, 2007"

C067 **Chloroform** 602-006-00-4 CAS: 67-66-3 EC : 200-663-8

F has sent in ECBI/12/07 and ECBI/12/07 Add. 1 (Classification proposal & ENV RAR).

Follow-up:

Since there was no reaction from the Committee the proposed classification is considered as agreed.

Final Conclusion:

C067 **Chloroform** 602-006-00-4 CAS: 67-66-3 EC : 200-663-8

Classification/ S -phrases	Toxicity	Degradation	Bioaccumulation	Escape clause
No Classification	$10 < L(E)C_{50} \leq 100$	Not readily degradable (based on data)	BCF < 100 log $K_{ow} < 3$	NOEC > 1 mg/l
Specific concentration limits:	Not applicable			