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Comprehensive Review

Evaluation of the carcinogenicity of dichloromethane in rats, mice, hamsters and humans

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ABSTRACT

Dichloromethane (DCM) is a high production volume chemical (>1000 t/a) mainly used as an industrial solvent. Carcinogenicity studies in rats, mice and hamsters have demonstrated a malignant tumor inducing potential of DCM only in the mouse (lung and liver) at 1000–4000 ppm whereas human data do not support a conclusion of cancer risk. Based on this, DCM has been classified as a cat. 2 carcinogen. Dose-dependent toxicokinetics of DCM suggest that DCM is a threshold carcinogen in mice, initiating carcinogenicity via the low affinity/high capacity GSTT1 pathway; a biotransformation pathway that becomes relevant only at high exposure concentrations. Rats and hamsters have very low activities of this DCM-metabolizing GST and humans have even lower activities of this enzyme. Based on the induction of specific tumors selectively in the mouse, the dose- and species-specific toxicokinetics in this species, and the absence of a malignant tumor response by DCM in rats and hamsters having a closer relationship to DCM toxicokinetics in humans and thus being a more relevant animal model, the current classification of DCM as human carcinogen cat. 2 remains appropriate.

1. Introduction

Dichloromethane (DCM; Methylene Chloride; CH_2Cl_2; CAS no. 75-09-2), a liquid with a high vapor pressure, belongs to the family of chlorinated methanes, *e.g.*, carbon tetrachloride, chloroform, and methyl chloride, for which there is an extensive toxicology database demonstrating similarities and differences. They all can be metabolized by cytochromes P450 to reactive intermediates that are interacting with glutathione (GSH).

DCM is a high production volume chemical (>1000 tons/year), with current global industrial production of 0.8–1.3 million tons/year, of which less than 10% is used in Europe. DCM is also produced by natural processes in seawater and soils, and by biomass burning accounting for a release of approximately 80,000 tons/year globally (Ohligschläger et al., 2019). DCM is mainly used as a solvent in synthesis, extraction, and purification purposes, e.g. of pharmaceutically active substances such as antibiotics, vitamins, caffeine, and flavors, and in the manufacture of polycarbonate plastics for glasslike products. It is also applied as a co-foam-blowing agent in the production of soft polyurethane foams, as a solvent in metal cleaning machines, in paint strippers (mainly for industry), in special adhesives and cleansers, and as a laboratory solvent. It is also used as a raw material in the synthesis of difluoromethane, known as HFC-32 or R 32, which is used as a low-temperature refrigerant in blends such as R–407C and R-410A.

Paint strippers based on DCM have been restricted or banned in industrialized countries due to incidents and fatalities related to the uncontrolled exposure in these open applications of this highly volatile solvent and lack of appropriate ventilation and/or use of respiratory protection equipment. Use in cosmetic products (hair spray) has been banned for consumers and professionals, and the use of DCM in consumer products (special adhesives and cleansers) has become minimal if not vanished completely in the past decade. Given its physical/chemical properties, safe use requires that DCM is handled as much as possible in closed systems throughout its entire life cycle to prevent emissions and avoid worker exposure.

Owing to its short atmospheric lifetime and the consequent inefficiency of transport into the stratosphere, DCM is classed as VSLS (Very Short-Lived Substance). Such chemicals are excluded from controls under the Montreal Protocol. DCM is also insufficiently reactive in the atmosphere to be classified as a “significant photochemically active volatile organic compound” (VOC) pollutant under the treaty on long-range transport of air pollutants.

As expected for a high production volume chemical, DCM has a large

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Abbreviations

1,1,1-TCE	1,1,1-trichloroethane
1,2-DCP	1,2-Dichloropropane
CLP	Classification, Labelling and Packaging (of substances and mixtures)
CoRAP	Community Rolling Action Plan
DCM	Dichloromethane
ECHA	European Chemical Agency
EPA	Environmental Protection Agency (US)
GSH	Glutathione
GST	Glutathione-S-transferase
IARC	International Agency for Research on Cancer
MAK	Maximale Arbeitsplatz-Konzentration (Maximum Workplace Concentration; Germany)
NTP	National Toxicology Program (US)

body of toxicity data available, both from accidental/incidental/occupational exposures and from many short-term through chronic animal studies (for summaries see MAK (2015) and IARC (2017). Contact with liquid DCM is painful to the eyes and to skin, but only in case of prolonged contact with the skin allowing penetration to reach the nociceptors. Absorption through the skin is possible, but in practice of minor consequence as DCM is highly volatile and quickly evaporates, and skin contact is usually prevented by use of appropriate chemicals gloves. The principal effects of human exposure to high DCM-concentrations (in excess of 1000 ppm) are incoordination, drowsiness and dizziness, and at very high concentrations, unconsciousness and coma which can be fatal.

In laboratory animals, DCM is moderately toxic after ingestion. Longer term exposures have resulted in liver and lung effects depending on the animal species and exposure conditions; chronic inhalation exposure to high concentrations of DCM induced malignant liver and lung tumors in mice but not in rats or hamsters. The overall NOAEC for toxic effects is 200 ppm (Burek et al., 1984; Serota et al., 1986a; Maltoni et al., 1988; JBRC, 2000a,b; Nitschke et al., 1988; Aiso et al., 2014).

At present, DCM is classified as carcinogen cat. 2 ("limited evidence of carcinogenicity") according to CLP (EC-Regulation, 2008). IARC had classified DCM in their cat. 2B ("possibly carcinogenic to humans") but changed its classification for DCM to their cat. 2A ("probably carcinogenic to humans"; preliminary report published as Benbrahim-Tallaa et al. (2014); full monograph published as IARC (2017)). IARC's change of the classification was mainly driven by human epidemiology data indicating an increased incidence of biliary tract tumors in workers exposed to DCM and 1,2-dichloropropane (1,2-DCP). DCM has also been included in ECHA's CoRAP 2016 program for substance evaluation which was ceased in May 2019 and in which a change of classification from carcinogen cat. 2 to 1B has been proposed.

This review integrates the animal toxicity data and the more recent occupational epidemiology in DCM-exposed workers into an overall weight-of-evidence assessment of the available data and existing uncertainties.

2. Animal studies conducted to evaluate the carcinogenicity of DCM

Carcinogenicity studies in experimental animals most often provide the most relevant information for hazard-based classification regarding carcinogenicity. As a high production volume chemical, the potential carcinogenicity of DCM has been assessed in a number of carcinogenicity studies following both oral administration and inhalation exposures (IARC, 2017). The following summaries only cover studies considered adequate regarding reporting of results on cancer and study conduct and design (i.e. oral or inhalation exposures) and covered by MAK (2015) and IARC (2017).

2.1. Studies in rats

2.1.1. Oral administration of DCM

In a drinking water study Fischer F344 rats were exposed to DCM doses of 5, 50, 125 and 250 mg/kg bw/day over 104 weeks; two control groups received drinking water without DCM (Serota et al., 1986a). In the liver of both male and female rats, non-neoplastic changes consisting of increased incidences of foci/areas of cellular alteration and of fatty change were observed at 50 mg/kg bw/day and higher. While an increased incidence of combined liver adenoma and carcinoma was observed in female rats in the 50 and 250 mg/kg bw/day groups when compared to one of the control groups, this increase remained within the range of the historical control incidences of the laboratory. As the incidence of hepatic tumors in this control group was unusually low and a dose-response was not observed due to the absence of an increased incidence of hepatic tumors in the 125 mg/kg bw/day group, the increased incidences were not considered treatment-related (Serota et al., 1986a). In conclusion, oral exposure of rats to DCM did not induce an increased incidence of tumors up to and including doses of 250 mg/kg bw/day.

2.1.2. Inhalation exposures to DCM

In rats, results of four well conducted and reported inhalation carcinogenicity studies with DCM are available (Burek et al., 1984; NTP, 1986; Nitschke et al., 1988; Aiso et al., 2014). However, in one of the studies (Nitschke et al., 1988) rats were only exposed to DCM-concentrations of up to 500 ppm and therefore this study has limited utility for hazard evaluation of DCM. Air concentrations of DCM in the three other studies were up to 4000 ppm over a duration of up to 104 weeks.

All studies reported inconsistent increases in benign mammary gland tumors in DCM-exposed groups. The findings consisted of a slight increase in the overall incidence or a slightly higher number of mammary tumors per animal. However, statistical significance was not always obtained and/or the incidences remained below the historical control incidence. In addition, the benign mammary tumors were not consistently seen in rats of both sexes, there was no progression of benign to malignant mammary tumors, and the SD rat used at that time had a high and variable background incidence of benign mammary tumors.

In contrast to the inhalation studies with DCM in mice (see below) none of these studies in rats demonstrated statistically significant increases in liver or lung adenoma and carcinoma. Only non-neoplastic liver effects were noted at DCM-concentrations $\geq$ 500 ppm (Burek et al., 1984; NTP, 1986; Nitschke et al., 1988; Aiso et al., 2014).

2.2. Studies in hamsters

In hamsters, one inhalation study with DCM is available. Hamsters exposed to 500, 1500 and 3500 ppm DCM by inhalation for two years (6 h/day, 5 days/week) did not show exposure-related increases in tumor incidences nor specific indications of target organ toxicity (Burek et al., 1984); in addition, DCM-exposed hamsters showed less extensive spontaneous geriatric changes and decreased mortality (females).

2.3. Studies in mice

2.3.1. Oral administration of DCM

In an oral study in B6C3F$_1$ mice, administration of DCM in drinking water at doses up to 250 mg/kg bw/day for 104 weeks did not result in an increased incidence of tumors) in female mice. A statistically significant increase ($p = 0.0114$) was seen for hepatocellular carcinoma incidence in male mice at the highest dose level of 250 mg/kg bw/day when compared to one of two control groups included in the study

design (Serota et al., 1986b). However, the tumor incidence in this highest dose group (18%) was essentially not different from the incidence in the other treatment groups, viz. 60 mg/kg bw/day (17%), 125 mg/kg bw/day (18%), and 185 mg/kg bw/day (17%). Control group 1 had an incidence of 8% while in control group 2 the incidence was 14%. In addition, neither the incidences of hepatocellular adenoma nor the combined incidence of hepatocellular adenoma and carcinoma were significantly increased in the male mice at any dose level. Therefore, the statistically significant increase seen in this study at the highest dose level of DCM in males is unlikely related to DCM-exposure due to absence of a dose-response relationship and a tumor incidence remaining within the historical control range. Increased incidences of other tumor types were not observed.

2.3.2. Inhalation exposures to DCM

In the inhalation studies performed in mice, animals were exposed to concentrations ranging from 1000 up to 4000 ppm (6 h/day, 5 days/week) for up to 102 weeks. Slikker et al. (2004) estimated the daily received doses to be as high as 3162 mg/kg bw/day.

NTP (1986) reported concentration-related increases in the incidence of bronchiolar-alveolar adenoma, carcinoma, and combined adenoma and carcinoma in both male and female B6C3F1 mice. In addition, concentration-related increases in the incidence of hepatocellular adenoma, carcinoma, and combined adenoma and carcinoma were seen in both males and females (Table 1).

Another study (Aiso et al., 2014) in a different mouse strain (BDF_1) confirmed the observation of NTP, viz. DCM-concentration related increases in the incidences of lung and liver adenomas and carcinomas. In males, a concentration-related increase in bronchiolar-alveolar carcinomas was seen while in females a statistically significant increase only occurred at 4000 ppm. A statistically significant increase in hepatocellular carcinomas was seen in males and females exposed to 4000 ppm; and an increased incidence of hepatocellular adenoma in females exposed to 4000 ppm (Table 1).

Incidences of hyperplasia in the terminal bronchioles of the lung and peripheral vacuolar changes in the liver were also increased in males and females at 4000 ppm (NTP, 1986; Aiso et al., 2014). Such changes were also seen in 90-day inhalation studies with DCM in mice (Foster et al., 1992, 1994).

3. Toxicokinetics and biotransformation of DCM

The relevance of toxicokinetics and biotransformation of DCM for formation of lung and liver tumors in mice and the role of species differences in tumor susceptibility has been intensively studied. After oral administration and following inhalation exposure, DCM is rapidly absorbed and widely distributed into tissues. Absorbed DCM is cleared partly by exhalation of unchanged DCM due to its high volatility and by biotransformation of DCM. At high exposure concentrations, most of the absorbed DCM is exhaled as unchanged parent compound.

Biotransformation is multifaceted and yields both stable and chemically reactive metabolites responsible for most toxic effects of DCM. Two competitive pathways of biotransformation of DCM have been identified in animals (Fig. 1), i) oxidation of DCM by cytochrome P450 and ii) conjugation of DCM by glutathione S-transferases (Kubic et al., 1974; Ahmed and Anders, 1976; Kubic and Anders, 1978; Green, 1983). Both pathways of DCM-biotransformation differ significantly in the chemical reactivity of the products formed and in their kinetic properties. Biotransformation of DCM by the high affinity/low capacity pathway catalyzed by cytochrome P450 (mainly by the CYP2E1 enzyme) results in dichloromethanol as the initial product of oxidative biotransformation. Dichloromethanol is not chemically stable and readily eliminates hydrochloric acid to give formyl chloride that is further hydrolyzed to the 1st chemically stable metabolite of the oxidative pathway carbon monoxide. CO may be further oxidized to CO_2; however, a significant part of the CO formed from DCM binds to hemoglobin in blood due to the high affinity of CO to the heme iron. Since carboxyhemoglobin (CO-Hb) can be readily quantified, levels of carboxyhemoglobin in experimental animals (and also in humans) may serve as biomarker for the oxidation of DCM by cytochromes P450s (Stewart et al., 1972a,b). Indeed, inhalation exposure of humans resulted in increased CO-Hb levels which shows that the CYP2E1 route is relevant up to and including a concentration level of 200 ppm (DiVincenzo and Kaplan, 1981). The 2nd pathway of DCM biotransformation - conjugation of DCM with glutathione - is catalyzed by a specific glutathione S-transferase designated GSTT1 and is a low affinity/high

Table 1
Results of inhalation carcinogenicity studies with DCM in mice.

Study design	Tumor types	Tumor incidences in exposure groups							
		Females				Males			
		0 ppm	1000 ppm	2000 ppm	4000 ppm	0 ppm	1000 ppm	2000 ppm	4000 ppm
NTP (1986)	Bronchiolo-alveolar adenoma	2/50	n.a.	23/48	28/48	3/50	n.a.	19/50	24/50
$B6C3F_1$		(4%)		(48%)***	(58%)***	(6%)		(38%)***	(48%)***
0, 2000, 4000 ppm, 6 h/day, 5	Bronchiolo-alveolar	1/50	n.a.	13/48	29/48	2/50	n.a.	10/50	28/50
d/wk for 102 wk	carcinoma	(2%)		(26%)***	(58%)***	(4%)		(20%)*	(56%)***
50 mice/sex/group	Hepatocellular adenoma	2/50	n.a	6/48	22/48	10/50	n.a.	14/49	14/49
		(4%)		(13%)	(46%)***	(20%)		(29%)	(29%)
	Hepatocellular carcinoma	1/50	n.a.	11/48	32/48	13/50	n.a.	15/49	26/49
		(2%)		(23%)**	(67%)***	(26%)		(31%)	(53%)*
	Combined hepatocellular	3/50	n.a.	16/48	40/48	22/50	n.a.	24/49	33/49
	adenoma/carcinoma	(6%)		(33%)**	(83%)***	(44%)		(49%)	(67%)*
Aiso et al. (2004)	Bronchiolo-alveolar adenoma	2/50	4/50	5/49	12/50	7/50	3/50 (6%)	4/50 (8%)	14/50
$Crj:BDF_1$		(4%)	(8%)	(10%)	(24%)***	(14%)			(28%)
0, 1000, 2000, 4000 ppm, 6 h/	Bronchiolo-alveolar	3/50	1/50	8/49	20/50	1/50	14/50	22/50	39/50
day, 5 d/wk, for 104 wk	carcinoma	(6%)	(2%)	(16%)	(40%)***	(2%)	(28%)***	(44%)***	(78%)***
50 mice/sex/group	Combined bronchiolo-alveolar	5/50	5/50	12/49	30/50	8/50	17/50	26/50	42/50
	adenoma/carcinoma	(10%)	(10%)	(24%)*	(60%)***	(16%)	(34%)*	(52%)***	(84%)***
	Hepatocellular adenoma	1/50	7/49	4/49	16/50	10/50	13/50	14/50	15/50
		(2%)	(14%)*	(8%)<	(32%)***	(20%)	(26%)	(28%)	(30%)
	Hepatocellular carcinoma	1/50	1/49	5/49	19/50	10/50	9/50	14/50	20/50
		(2%)	(2%)	(10%)	(38%)***	(20%)	(18%)	(28%)	(40%)*
	Combined hepatocellular	2/50	8/49	9/49	30/50	15/50	20/50	25/50	29/50
	adenoma/carcinoma	(4%)	(16%)*	(18%)*	(60%)***	(30%)	(40%)	(50%)*	(58%)*

n.a. not applicable (NTP did not test the 1000 ppm concentration); *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (given by IARC).

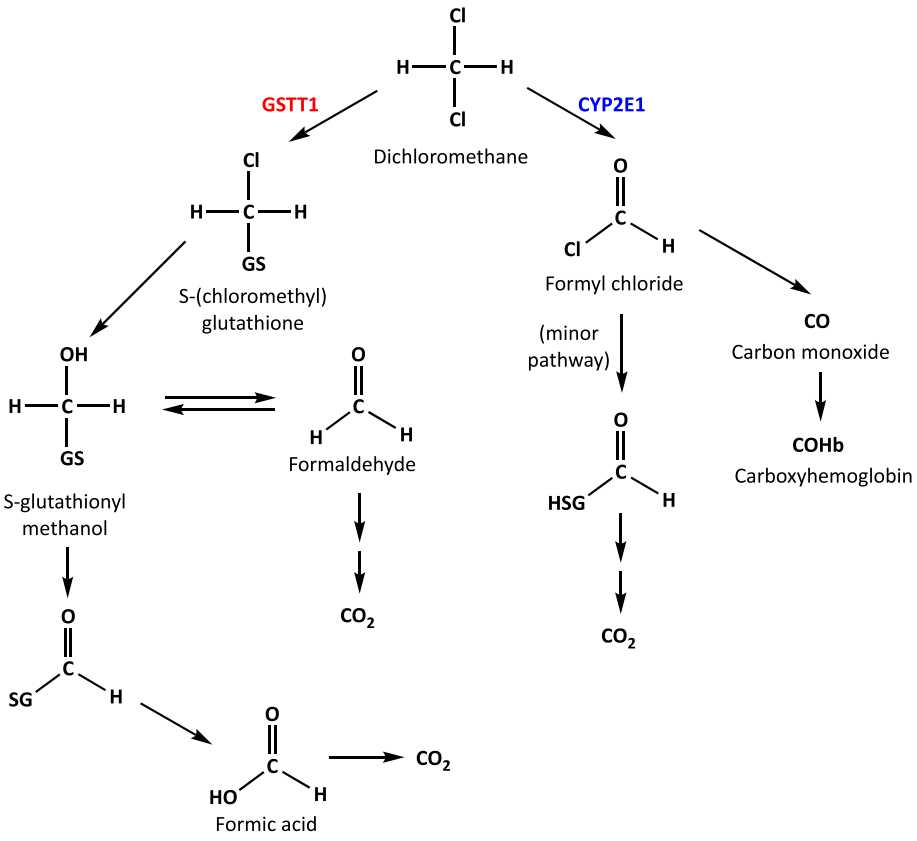

Fig. 1. Biotransformation of DCM by cytochrome P450-catalyzed oxidation (blue) and by glutathione S-conjugate formation (red). The cytochrome P450 pathway is a high affinity/low capacity pathway and thus predominant at lower exposure concentrations. The glutathione S-conjugate formation pathway has a low affinity/high capacity and therefore becomes relevant at very high exposure concentrations. Mice have a much higher activity of the glutathione S-conjugation pathway than rats, hamsters, or humans. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

capacity pathway (Reitz et al., 1988; Mainwaring et al., 1996). The reaction of DCM with glutathione initially results in the formation of S-chloromethyl (glutathione). While this compound has a very short half-life in aqueous media, it is an electrophile that may bind to nucleophilic sites present in DNA-constituents. Formation of this electrophile has been associated with the GSTT1-mediated genotoxicity of DCM in bacteria and positive responses in some genotoxicity assays in lung and liver of mice exposed to DCM by inhalation. Therefore, glutathione conjugation of DCM has been implicated as a mechanism to explain the induction of lung and liver tumors in mice after inhalation exposures to high DCM concentrations. However, formation of DNA-adducts derived from DCM could not be demonstrated. Due to the high reactivity of S-chloromethyl (glutathione) with the nucleophile water in biological media, this compound is rapidly hydrolyzed to hydroxymethyl (glutathione) followed by the release of formaldehyde (Hashmi et al., 1994). Formaldehyde can be further oxidized to formic acid and carbon dioxide or may be used for biosynthetic processes. In 2010, Evans and Caldwell proposed a different explanation for DCM metabolism in which only CYP2E1 is involved (Evans and Caldwell, 2010). However, this proposal was refuted (Anders et al., 2010), and IARC (2017) also concluded there were no data supporting biotransformation reactions of DCM that exclude glutathione S-conjugate formation, particularly at higher DCM concentrations.

The presence of a high affinity/low capacity and a low affinity/high capacity biotransformation pathway, the species differences in GSTT1 regarding kinetic parameters, and the distribution and activity of GSTT1 in different species create very pronounced species differences in DCM biotransformation. As a consequence, the relative contributions of the two pathways to the overall DCM-biotransformation are highly dose- and species-dependent (Andersen et al., 1987, 1991; Reitz et al., 1988, 1989; Pemble et al., 1994; Slikker et al., 2004).

The dose and species-dependent biotransformation of DCM is difficult to directly assess from determination of metabolites formed by the two pathways due to the absence of a stable metabolite of DCM that may serve as biomarker for the extent of glutathione conjugation. Moreover, investigations of human biotransformation of DCM by glutathione conjugation would require experiments at very high inhalation exposure concentrations. Therefore, physiologically based pharmacokinetic (PBPK) modeling has been applied to predict species differences in the fate of DCM and the amounts of metabolites formed by the different pathways ("dose surrogates"). The initial PBPK model well predicted the measured kinetics of DCM in animals and in humans (Andersen et al., 1987, 1991; Reitz et al., 1988, 1989). The model has been refined over time integrating new information (Slikker et al., 2004) and new methodologies (David et al., 2006; Marino et al., 2006). All applications of the PBPK models consistently predict extensive glutathione *S*-conjugate formation from DCM at the air concentrations of DCM used in the carcinogenicity studies and systemic doses of DCM examined in the mouse studies.

There is also a highly non-linear extent of formation of the glutathione S-conjugates in rodents. At lower oral DCM-doses, <250 mg/kg bw, or following inhalation of DCM at concentrations below approximately 500 ppm for 6 h, the cytochrome P4502E1-pathway dominates both in mice and rats. At higher concentrations, the P4502E1-pathway (high affinity/low capacity) becomes saturated and more DCM is available to be metabolized by glutathione conjugation. In mice, the predicted metabolic flux through the hepatic glutathione-conjugation pathway is increased by 56-fold when the systemic dose increases by app. 9-fold. In lung, the predicted increase in metabolic flux of DCM through glutathione conjugation is app. 120-fold when the systemic dose of DCM is increased by 9-fold (Andersen et al., 1987).

Regarding species differences, the extent of glutathione-dependent biotransformation is dependent upon the activity of the specific GSTT1 in the different species. Mouse lung and liver contain significantly higher activities of GSTT1 compared to rat lung (almost 4-fold higher activity) and liver (7-fold higher activity). While human tissues also contain an orthologue of GSTT1, this enzyme is present only in very low concentrations in liver and lung, is much less efficient in catalyzing

glutathione-dependent biotransformation of DCM, and has a different subcellular location (Sherratt et al., 1997, 2002). Thus, regarding capacity for biotransformation, neither the mouse nor the rat is a good model regarding the human hazard of DCM. However, the rat is the more appropriate model for human DCM-biotransformation and its biological consequences compared to the mouse due to the large differences in toxicokinetics (Reitz et al., 1988, 1989; Slikker et al., 2004). It is, therefore, highly unlikely that glutathione conjugation contributes to the biotransformation of DCM in the liver and lung in humans. The species differences in the capacity for DCM biotransformation to possible toxic metabolites are further supported by the absence of DNA-reactivity of DCM metabolites seen in rat and human cells (Casanova and Conolly, 1996; Graves and Green, 1996) and the absence of formaldehyde formation (a product of the GSTT1-mediated biotransformation of DCM) in human hepatocytes (Hashmi et al., 1994).

While polymorphisms of expression of the DCM-metabolizing GST in humans have been identified, the GSTT1-activities of all assessed human liver samples remained well below those of rat liver and lung. Rats do not show liver and lung tumors as a consequence of life-time exposure of up to 4000 ppm DCM and most genotoxicity assessments with DCM using rat or hamster tissues were negative. Moreover, the polymorphic human GSTT1 is mostly present in erythrocytes and conjugation of DCM in erythrocytes may further decrease the availability of DCM in lung and liver for GST-mediated biotransformation (Pemble et al., 1994).

4. Genotoxicity studies with DCM

DCM has been assessed for genotoxicity in a large number of assays in mammals *in vivo*, in mammalian cells *in vitro*, and in bacteria (see IARC, 2017, Tables 4.3–4.5 in this document list all studies performed up to 2014 and their results).

4.1. Effects of DCM-exposures on genotoxicity endpoints in rodents after in vivo exposures

Consistently, in mice, DCM induced effects such as DNA-single strand breaks, DNA-protein crosslinks, and sister chromatid exchanges in liver and lung tissues. However, these findings were only noted following high concentration inhalation exposures of mice (1000–4000 ppm, 6 h/day for up to 5 days) (Casanova et al., 1992; Graves et al., 1994a; Graves et al., 1994b; Casanova and Conolly, 1996). Effects of the modulation of glutathione concentrations support a role of glutathione conjugation of DCM to a reactive glutathione *S*-conjugate in the positive responses in mice. Effects of DCM on chromosome aberrations and micronucleus induction in lung cells, erythrocytes, and bone marrow of mice were less consistent. Negative results include absence of an induction of unscheduled DNA synthesis, negative gene mutation and DNA-damage assays in mouse liver and several negative bone marrow/erythrocyte micronucleus assays after repeated DCM-exposures (IARC, 2017). In addition, a more recent study did not observe genomic signatures indicative of a cytotoxic or DNA-reactive metabolite response in lung and liver from female B6C3F1 mice exposed to 1000–4000 ppm DCM for 90 days (Andersen et al., 2017).

In contrast to the mixed results in the *in vivo* genotoxicity testing of DCM in mice, mostly negative results were seen in assays for a variety of genotoxicity endpoints in tissues from rat and hamster exposed to high concentrations of DCM (IARC, 2017).

4.2. Effects of DCM-exposures on genotoxicity endpoints in freshly isolated or in cultured mammalian cells

DCM gave mixed results regarding genotoxicity endpoints in mammalian cells *in vitro* with clear positive effects seen only in mouse hepatocytes and in V79 cells transfected with GSTT1 and some positive outcomes in mouse lung Clara cells. Assays using rat and hamster-derived cells were mostly negative or inconclusive (IARC, 2017). In general, results of genotoxicity testing with DCM obtained in cultured cell systems typically used for assessment of genotoxic responses such as Chinese hamster ovary cells or non-transfected V79 cells have to be treated with caution since it is not known if the specific biotransformation pathways that account for the unique sensitivity of the mouse to DCM are expressed in these systems and if added metabolic systems such as rat liver S9 have sufficient activity of the GSTT1 enzyme relevant for DCM-bioactivation. Rat liver contains much lower activities of DCM-metabolizing GSTs as compared to mouse liver. Moreover, DCM has a low water solubility and is highly volatile.

4.3. Effects of DCM-exposures on genotoxicity endpoints in bacteria

In bacteria, DCM fairly consistently induced reverse mutations in several Salmonella strains (TA 100, TA 1535, TA 98) and in *E. coli* at high nominal exposure concentrations without metabolic activation. The mutagenic responses to DCM in bacteria could be modulated by changes in GSTT1-activity and in glutathione levels in these bacterial testing systems (Dillon et al., 1992; DeMarini et al., 1997; Akiba et al., 2017). This is consistent with the expression of the specific glutathione S-transferases that metabolize DCM in mice (IARC, 2017).

5. Human data

A large number of human epidemiology studies with DCM have been conducted, usually in groups with occupational exposures. As with other widely used chemicals, the available database is inconsistent with some studies showing associations of confirmed/possible exposures to DCM with tumor outcomes and others not showing such associations. This resulted in opposing conclusions regarding a human cancer hazard by DCM due to different approaches in the evaluation of the epidemiology studies.

5.1. Evaluation of the epidemiology database on DCM by the German MAK

The German MAK-committee concluded in its 2015 update of its justification of the MAK-value for DCM – based on a scientific evaluation of the epidemiology database applying weight of evidence and the Bradford-Hill criteria (Bradford Hill, 1965) - that no clear association between exposure to DCM and the development of tumors was demonstrated for humans. According to MAK, there was “sporadic evidence for an association of DCM-exposure with different tumor types in humans”, but there was “no concordance between the various study outcomes that supports causality”. In addition, MAK noted that all studies suffered from small numbers of cases, insufficient assessment of confounders including exposures to other solvents, and absence of a dose–response (MAK, 2015). MAK (2015) also concluded that none of three case–control studies (Miligi et al., 2006; Seidler et al., 2007; Wang et al., 2009) published after the previous MAK evaluation of DCM in 2000, provided evidence of an association between exposure to DCM and the occurrence of NHL. In addition, in two cohort studies with workers employed in the production of cellulose triacetate film (Hearne and Pifer, 1999; Tomenson, 2011), no increased risk of dying from cancer of all types or from tumors of the lungs, liver, brain and pancreas or from leukemia, NHL, or multiple myelomas was found.

5.2. Evaluation of the epidemiology database on DCM by IARC

In 2014, an IARC working group concluded that there was “*limited evidence*” in humans for the carcinogenicity of DCM (Benbrahim-Tallaa et al., 2014).

IARC’s conclusion was mainly based on one cohort study in a small printing company in Japan indicating an association between an exceptionally high incidence of cholangiocarcinomas and exposure to DCM and 1,2-dichloropropane (1,2-DCP; Kumagai et al., 2013). .A high

incidence of cholangiocarcinomas was observed in former and current workers; all 11 observed cases were exposed to 1,2-DCP, but 10 of them were also exposed to DCM. Later, two additional cases of cholangiocarcinoma were described in workers employed in two different printing shops in Japan. One of the two had been co-exposed to DCM and to 1,1,1-trichloroethane (1,1,1-TCE), the other had been exposed only to 1,2-DCP (Kumagai, 2014).

IARC considered the rarity of cholangiocarcinoma, the very high relative risk, the young ages of the patients, the absence of reported non-occupational risk factors, and the intensity of the exposure as indications that the excess of cholangiocarcinoma was unlikely to be the result of chance, bias, or non-occupational confounding. Most of the IARC working group concluded that 1,2-DCP was the causative agent responsible for the large excess of cholangiocarcinoma and a minority concluded that the association between 1,2-DCP and cholangiocarcinoma was credible, but that the role of other agents, mainly DCM, could not be separated with complete confidence (Benbrahim-Tallaa et al., 2014; IARC, 2017).

In contrast to MAK, IARC concluded that positive associations for non-Hodgkin lymphoma (NHL) with assumed DCM exposures were "*consistent among studies using different designs and in several countries*". However, like MAK, IARC also noted that "*most subjects were exposed to several solvents (some of which have been previously associated with NHL) and the risk estimates were based on small numbers*".

5.3. Recent epidemiology studies related to exposure of DCM

After both IARC's and MAK's evaluations of DCM, additional studies confirmed the role of 1,2-DCP in the development of cholangiocarcinoma (Kubo et al., 2014; Yamada et al., 2014; Sobue et al., 2015; Yamada et al., 2015a,b; Kumagai et al., 2016) based on a demonstration of a dose-response relationship between 1,2-DCP exposures and the incidence of cholangiocarcinomas in these cohorts.

Regarding the association of DCM-exposures with NHL as stated by IARC in 2014 (Benbrahim-Tallaa et al., 2014; IARC, 2017), Schlosser et al. (2015) suggested that based on four studies (Gold et al. 2011; Miligi et al. 2006; Seidler et al. 2007; Wang et al. 2009) a focus on non-Hodgkin lymphoma or multiple myeloma is warranted in future research. It was, however, noted that in all studies there was occupational exposure to solvents other than DCM. MAK (2015), again, concluded that none of these three case–control studies (Miligi et al., 2006; Seidler et al., 2007; Wang et al., 2009) provided conclusive evidence of an association between exposure to DCM and the occurrence of NHL due to presence of confounding by co-exposures to other chemicals.

Also, in other recent studies no conclusive associations were found between occupational exposure to DCM and cancer (Park et al., 2017; Talibov et al., 2017; Makris and Voniatis, 2018; Niehoff et al., 2019).

6. Discussion and conclusions

In this review, the currently available database on the carcinogenicity of DCM is discussed based on experimental and epidemiological studies. IARC based its most recent assignment of DCM to their group 2A ("*probably carcinogenic to humans*") (Benbrahim-Tallaa et al., 2014; IARC, 2017) on i) the possible association of DCM with cholangiocarcinoma and NHL in humans, ii) the "*sufficient evidence*" for the carcinogenicity of DCM in animals based on the observation of tumors in mice following inhalation exposure to high concentrations of DCM, and iii) the conclusion that biotransformation of DCM to potentially genotoxic intermediates could occur in humans.

The majority of the new datasets considered by IARC in 2014 were studies on an association of cancer in humans with potential exposures to DCM. The "limited evidence" conclusion was mainly based on "*positive associations between exposure to DCM and cancer of the biliary tract*". However, new information on the exposure situation of the worker cohorts published after the IARC-evaluation (Suzuki et al., 2014; Yamada et al., 2014, Yamada et al., 2015ab; Sobue et al., 2015; Kumagai et al., 2016) confirms that exposure to 1,2-DCP and not to DCM is the basis for the positive associations between alleged DCM-exposures and biliary tract cancer. As stated above, an association of NHL with DCM exposure remains doubtful.

The animal carcinogenicity database on DCM has essentially not changed since IARC's last classification in 1999 (IARC, 2017) and DCM-induced tumors were consistently seen in mice in two independent studies. However, inhalation of DCM induces malignant tumors selectively in mouse liver and lung, but not in rats or hamsters indicating a species-specific effect. In mice, the species-specific tumor induction is most likely due to the very high capacity of mouse liver and lung to form reactive metabolites from DCM by glutathione-conjugation. Biotransformation by glutathione-conjugation of DCM even at high concentrations is much lower in rats and hamsters compared to mice; in humans, determined rates of glutathione conjugation of DCM and activity of the DCM-metabolizing GSTT1 are even lower compared to rats and hamsters.

While a mutagenic mode of action in mice is supported by the mutagenicity of DCM in bacteria (expressing the specific glutathione S-transferases) and some positive assays for genotoxicity following inhalation exposure *in vivo*, even the database on the genotoxicity of DCM in mice remains inconsistent since higher tier genotoxicity assays with DCM in mice are negative and an assessment of genomic changes in mice exposed by inhalation up to 4000 ppm DCM for 90 days did not show a DNA-damage response in lung and liver (Andersen et al., 2017). Moreover, mice are generally sensitive to lung (and liver) tumor induction by chemicals through non-genotoxic modes of action (Green et al., 1997; Banton et al., 2019; Cohen et al., 2020) and a wide range of chemicals can cause cancer under the "right" experimental circumstances many of which having no relevance to humans or achievable exposure levels (Boobis et al., 2016).

It is, however, clear that rats and hamsters did not show tumors in response to DCM concentrations up to 4000 ppm, and genotoxicity testing of DCM in these species overall gave negative results. At the high doses received by inhalation in the carcinogenicity studies (when recalculated these are above the testing limit dose of 1000 mg DCM/kg bw/day, and although higher in mice than in rats (Table 2), the highest level in rats is still higher than the 2000 ppm equivalent in mice that induced tumors), the very high activity of a specific glutathione S-transferase in mouse liver and lung results in very high doses of a reactive glutathione S-conjugate to these target organs.

While GSTT1 biotransformation of DCM leads to formation of reactive intermediates in mouse liver and lung, a role for a GSTT1-like biotransformation of DCM in humans remains doubtful given the known species differences in tissue distribution of the GSTT1 orthologue and enzyme kinetics, and the above conclusion that the mouse is not a model for human DCM-biotransformation and biotransformation-dependent toxicity.

Table 2
Calculation of intake of rats, mice and hamster following exposure to DCM, assuming 100% absorption after inhalation.

Exposure concentrations by inhalation	Rat 3600 min/day Minute ventilation 240 mL/min BW 250 g	Hamster 3600 min/day Minute ventilation 60.9 mL/min BW 60 g	Mouse 3600 min/day Ventilation 1.46 mL/g BW BW 25 g
1000 ppm	1210 mg/kg bw	1280 mg/kg bw	1840 mg/kg bw
2000 ppm	2420 mg/kg bw	2560 mg/kg bw	3680 mg/kg bw
4000 ppm	4840 mg/kg bw	5120 mg/kg bw	7360 mg/kg bw

Note: 1000 ppm = 3500 mg/m^3 = 3.5 mg/L; *ECHA guidance; Chapter R.7c: Endpoint specific guidance Version 3.0 – June 2017.

No new data regarding animal cancerogenicity or mode of action has been published since DCM was classified as a cat. 2 carcinogen according to CLP. Comparing the available information on DCM (tumors only in mice and only after inhalation of doses way above the testing limit dose of 1000 mg/kg bw/day) with those of the CLP criteria for carcinogenicity classification and labelling of chemicals (CLP, 2008), the available data continue to support classification as carcinogen cat. 2.

CRediT authorship contribution statement

Wolfgang Dekant: Writing - original draft, preparation, Writing - review & editing. **Paul Jean:** Writing - review & editing. **Josje Arts:** Writing - review & editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: WD received a honorarium from the Chlorinated Solvents REACH Consortium. PJ works for Olin Corporation and JA for Nouryon Industrial Chemicals which manufacture and sell methylene chloride (dichloromethane). Both companies are member of the Chlorinated Solvents Reach Consortium.

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