Research article

Journal of Applied Toxicology

Received: 7 July 2016, Revised: 13 October 2016, Accepted: 21 October 2016 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jat.3416

Lack of *in vivo* mutagenicity of 1,2-dichloropropane and dichloromethane in the livers of *gpt* delta rats administered singly or in combination

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ABSTRACT: 1,2-Dichloropropane (1,2-DCP) and dichloromethane (DCM) are possible causative agents associated with the development of cholangiocarcinoma in employees working in printing plant in Osaka, Japan. However, few reports have demonstrated an association between these agents and cholangiocarcinoma in rodent carcinogenicity studies. Moreover, the combined effects of these compounds have not been fully elucidated. In the present study, we evaluated the *in vivo* mutagenicity of 1,2-DCP and DCM, alone or combined, in the livers of *gpt* delta rats. Six-week-old male F344 *gpt* delta rats were treated with 1,2-DCP, DCM or 1,2-DCP + DCM by oral administration for 4 weeks at the dose (200 mg kg^{-1} body weight 1,2-DCP and 500 mg kg^{-1} body weight DCM) used in the carcinogenesis study performed by the National Toxicology Program. *In vivo* mutagenicity was analyzed by *gpt* mutation/Spi$^-$ assays in the livers of rats. In addition, gene and protein expression of CYP2E1 and GSTT1, the major enzymes responsible for the genotoxic effects of 1,2-DCP and DCM, were analyzed by quantitative polymerase chain reaction and western blotting. *Gpt* and Spi$^-$ mutation frequencies were not increased by 1,2-DCP and/or DCM in any group. Additionally, there were no significant changes in the gene and protein expression of CYP2E1 and GSTT1 in any group. These results indicated that 1,2-DCP, DCM and 1,2-DCP + DCM had no significant impact on mutagenicity in the livers of *gpt* delta rats under our experimental conditions. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: 1,2-dicloropropane; dichloromethane; *gpt* delta rat; *in vivo* mutagenicity; liver

Introduction

Cholangiocarcinoma was reported to occur frequently in employees of printing company in Osaka, Japan. 1,2-Dichloropropane (1,2-DCP) and dichloromethane (DCM) are suspected to be causative agents for cholangiocarcinoma because these compounds are used in large amounts and may be inhaled at high concentrations over a prolonged time (Kubo *et al*., 2014; Kumagai *et al*., 2013). In 1999, the International Agency on Research on Cancer (IARC) had classified 1,2-DCP as a group 3 carcinogen because of the lack of epidemiological evidence for carcinogenicity in humans and DCM as a group 2B carcinogen (IARC, 1999a,b). Recently, 1,2-DCP and DCM were reclassified as group 1 and group 2A carcinogens, respectively (Benbrahim-Tallaa *et al*., 2014). Several reports have investigated the carcinogenesis of 1,2-DCP and DCM in rodents (Matsumoto *et al*., 2013; NTP, 1986a,b; Umeda *et al*., 2010). For example, administration of 1,2-DCP by gavage causes hepatocellular adenomas in male and female B6C3F1 mice. Additionally, administration of 1,2-DCP by inhalation causes nasal cavity tumors in male and female F344 rats, bronchiolo-alveolar adenomas and carcinomas in female B6D2F1 mice, and marginal increases in the incidence of Harderian gland adenomas in male B6D2F1 mice (Matsumoto *et al*., 2013; NTP, 1986b; Umeda *et al*., 2010). DCM also increases the incidence of mammary gland fibroadenomas in male and female F344 rats and bronchiolo-alveolar adenomas/carcinomas and hepatocellular adenomas/carcinomas in male and female B6C3F1 mice when administered by inhalation (NTP, 1986a). In addition, several *in vitro* and *in vivo* genotoxicity studies of 1,2-DCP and DCM have been performed. *In vitro*, both 1,2-DCP and DCM induce reverse mutations in various strains of *Salmonella typhimurium* (De Lorenzo *et al*., 1977; Graves *et al*., 1994; Principe *et al*., 1981). Moreover, both 1,2-DCP and DCM cause chromosome aberrations, and 1,2-DCP causes sister chromatid exchange (De Lorenzo *et al*., 1977; Galloway *et al*., 1987; Graves *et al*., 1994; Principe *et al*., 1981; Thilagar & Kumaroo, 1983; von der Hude *et al*., 1987). *In vivo*, the genotoxic effects of DCM have been demonstrated using *in vivo* comet assays in the livers of rats and mice (Kitchin & Brown 1989; Sasaki *et al*., 1998). Additionally, significant increases in the frequencies of sister chromatid exchange and chromosome aberrations have been observed in the lungs,

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peripheral blood lymphocytes and bone marrow cells of B6C3F1 male mice that inhaled DCM (Allen *et al.*, 1990). In studies examining the *in vivo* genotoxic effects of 1,2-DCP, inhalation of 1,2-DCP but not DCM was shown to increase DNA damage measured by comet assays in the livers of C57BL/6 J mice bearing a reporter gene; furthermore, co-exposure by inhalation of 1,2-DCP and DCM was shown to increase the mutation frequency (MF) of the reporter gene in the liver compared with that of exposure to 1,2-DCP alone (Suzuki *et al.*, 2014). These data indicated that the genotoxic effects of 1,2-DCP are enhanced by DCM. However, the mechanisms through which DCM enhances the genotoxic effects of 1,2-DCP are unclear. Researchers have speculated that this mechanism may involve the shared metabolic pathway of these two agents. Both 1,2-DCP and DCM are metabolized by CYP2E1 and conjugated with glutathione (Guengerich *et al.*, 1991, 1992). Thus, saturation with CYP and subsequently being predominant in a glutathione-conjugation pathway might be important for these test compounds to induce genotoxicity. Although the glutathione *S*-transferase (GST) specific for the metabolism of 1,2-DCP has not been identified, GSTT1–1, which metabolizes DCM and many other haloalkanes, also reacts with 1,2-DCP in glutathione conjugation (Meyer *et al.*, 1991; Oda *et al.*, 1996). In particular, metabolism of DCM and other haloalkanes in the glutathione-conjugation pathway has been shown to be associated with the genotoxicities of the compounds (Gisi *et al.*, 1999; Oda *et al.*, 1996). Thus, it is necessary to determine whether exposure to 1,2-DCP or DCM results in alterations in the expression levels of related metabolic enzymes. However, information regarding the correlation between the *in vivo* genotoxicity and the expression of metabolic enzymes for 1,2-DCP and DCM in rodents is lacking.

In the present study, we performed *gpt* mutation assays and Spi$^-$ assays to assess the mutagenicity of 1,2-DCP and DCM, alone and in combination, in the livers of *gpt* delta rats after administration by gavage for 4 weeks. In addition, to clarify the changes in the expression of metabolic enzymes, we performed quantitative polymerase chain reaction (PCR) and western blotting to assess mRNA and protein expression of CYP2E1 and GSTT1–1.

Materials and methods

Test chemicals

Corn oil, 1,2-DCP (98% pure) and DCM (99.5% pure) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Experimental animals and housing conditions

Six-week-old male F344 *gpt* delta rats carrying approximately 10 copies of the transgene lambda EG10 per haploid genome (Hayashi *et al.*, 2003) were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in polycarbonate cages with softwood chips for bedding in a specific pathogen-free animal facility, maintained under conditions of controlled temperature (23 ± 2 °C), humidity ($55\% \pm 5\%$), air change ($12 \times h^{-1}$), and lighting (12 h light/dark cycle), and were given free access to a CRF-1 basal diet (Charles River Japan, Kanagawa, Japan) and tap water.

Experimental design

After a 1 week acclimatization period, 50 male F344 *gpt* delta rats weighing 95.2–126.5 g were used in this experiment. Rats were randomized into seven groups based on body weight (BW) as follows: control group, administered corn oil (5 ml kg^{-1} BW; $n = 7$); 1,2-DCP groups ($n = 7$ each), administered 100 or 200 mg kg^{-1} BW 1,2-DCP; DCM groups ($n = 7$ each), administered 250 or 500 mg kg^{-1} BW DCM; and 1,2-DCP + DCM groups, administered 100 mg 1,2-DCP plus 250 mg DCM kg^{-1} BW ($n = 7$) or 200 mg 1,2-DCP plus 500 mg DCM kg^{-1} BW ($n = 8$). The doses of each compound were selected based on previous carcinogenicity tests (NTP, 1986b, 1994). Test chemicals were dissolved in corn oil at the time of dosing and administered by gavage every day for 4 weeks as recommended by OECD test guidelines (TG488) (OECD, 2013). The animals were observed daily for any clinical signs and mortality. BWs were measured weekly. At the end of the study, all the animals were anesthetized with isoflurane and weighed, and blood samples were collected from the abdominal aorta for serum biochemistry. The animals were then killed by exsanguination from the abdominal aorta. The livers were collected as described in our previous study (Onami *et al.*, 2014) and used for *gpt* mutation/Spi$^-$ assays, quantitative PCR and western blotting. In addition, the liver specimens from the left and median lobe were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned to a thickness of 3 μm and stained with hematoxylin and eosin for microscopic examination. Histopathological examinations were carried out in all animals. The present experimental protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences.

Table 1. Terminal body weights and liver weights of male *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks

Treatment	No. of animals	Terminal body weight (g)	Liver weight	
			Absolute (g)	Relative (g%)
Control (corn oil)	7	196.4 ± 12.8	5.83 ± 0.58	2.96 ± 0.16
1,2-DCP 100 mg kg^{-1} BW	7	188.3 ± 11.0	5.33 ± 0.27	2.93 ± 0.11
1,2-DCP 200 mg kg^{-1} BW	7	180.0 ± 12.5	5.54 ± 0.43	3.18 ± 0.20
DCM 250 mg kg^{-1} BW	7	208.6 ± 12.8	5.86 ± 0.50	2.93 ± 0.12
DCM 500 mg kg^{-1} BW	7	207.8 ± 10.1	5.89 ± 0.27	2.96 ± 0.08
1,2-DCP 100 + DCM 250 mg kg^{-1} BW	7	192.5 ± 8.2	5.56 ± 0.32	2.98 ± 0.09
1,2-DCP 200 + DCM 500 mg kg^{-1} BW	8	188.4 ± 10.5	5.74 ± 0.22	3.16 ± 0.11

BW, body weight; DCM, dichloromethane; 1,2-DCP, dichloropropane.

Table 2. Blood chemistry parameters of male *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks

Treatment	Control (corn oil)	1,2-DCP		DCM		1,2-DCP + DCM	
		100 mg kg^{-1} BW	200 mg kg^{-1} BW	250 mg kg^{-1} BW	500 mg kg^{-1} BW	100 + 250 mg kg^{-1} BW	200 + 500 mg kg^{-1} BW
No. of animals	7	7	7	7	7	7	8
TP (g dl^{-1})	5.7 ± 0.1	5.6 ± 0.1	5.8 ± 0.2	5.6 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	5.6 ± 0.2
Alb (g dl^{-1})	4.2 ± 0.1	4.1 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.1	4.2 ± 0.1
A/G	2.8 ± 0.1	2.8 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	3.0 ± 0.2	2.9 ± 0.1	3.0 ± 0.2
Glucose (mg dl^{-1})	167 ± 15	159 ± 33	151 ± 13	144 ± 15	135 ± 14*	139 ± 13	141 ± 16
Bil (mg dl^{-1})	0.04 ± 0.01	0.04 ± 0.01	0.06 ± 0.01**	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
T-Cho (mg dl^{-1})	59 ± 4	49 ± 5**	43 ± 4**	54 ± 3	54 ± 3	48 ± 6**	40 ± 8**
TG (mg dl^{-1})	65 ± 5	38 ± 6*	30 ± 8**	55 ± 32	46 ± 8	35 ± 11**	29 ± 8**
γ-GTP (IU l^{-1})	<3	<3	<3	<3	<3	<3	<3
AST (IU l^{-1})	67 ± 4	74 ± 5	74 ± 5	68 ± 5	69 ± 4	72 ± 4	67 ± 4
ALT (IU l^{-1})	41 ± 5	34 ± 2**	27 ± 3**	40 ± 1	40 ± 4	34 ± 4**	25 ± 2**
ALP (IU l^{-1})	1006 ± 101	1045 ± 108	952 ± 109	929 ± 111	920 ± 126	975 ± 139	871 ± 89
BUN (mg dl^{-1})	19 ± 2	18 ± 1	20 ± 2	19 ± 2	18 ± 2	19 ± 2	19 ± 3
Cre (mg dl^{-1})	0.28 ± 0.02	0.29 ± 0.02	0.27 ± 0.03	0.28 ± 0.03	0.28 ± 0.01	0.29 ± 0.03	0.28 ± 0.02
Ca (mg dl^{-1})	10.2 ± 0.2	9.9 ± 0.2	10.2 ± 0.2	10.0 ± 0.3	10.1 ± 0.2	10.1 ± 0.2	10.2 ± 0.2
IP (mg dl^{-1})	7.0 ± 0.2	6.9 ± 0.2	6.9 ± 0.3	7.3 ± 0.2	7.2 ± 0.3	7.3 ± 0.3	7.6 ± 0.3**
Na (mg dl^{-1})	143 ± 0	144 ± 1	145 ± 1	143 ± 1	144 ± 0	144 ± 1	144 ± 0
K (mg dl^{-1})	4.6 ± 0.2	4.7 ± 0.1	4.7 ± 0.1	4.7 ± 0.2	4.5 ± 0.1	4.6 ± 0.2	4.7 ± 0.2
Cl (mg dl^{-1})	104 ± 1	105 ± 1	105 ± 1	103 ± 1	103 ± 1	105 ± 1	104 ± 1

A/G, albumin globulin ratio; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bil, bilirubin; BUN, blood urea nitrogen; BW, body weight; Cre, creatinine; DCM, dichloromethane; 1,2-DCP, dichloropropane; γ-GTP, γ-glutamyl transpeptidase; IP, inorganic phosphorus; T-Cho, total cholesterol; TG, triacylglycerol; TP, total protein.
Each value represents the mean ± SD.
Significantly different from the vehicle control group at $^{*}P < 0.05$ and $^{**}P < 0.01$.

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Table 3. Histopathological findings in the liver of *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks

Treatment	Control (corn oil)	1,2-DCP		DCM		1,2-DCP + DCM	
		100 mg kg^{-1} BW	200 mg kg^{-1} BW	250 mg kg^{-1} BW	500 mg kg^{-1} BW	100 + 250 mg kg^{-1} BW	200 + 500 mg kg^{-1} BW
No. of animals	7	7	7	7	7	7	8
Eosinophilic changes	0	0	0	1	0	0	2
Cell infiltration	6	6	7	7	7	7	8
Microgranuloma	2	3	1	5	5	5	4
Single cell necrosis	3	3	4	3	1	3	2
Karyocytomegaly	0	0	0	0	1	0	0
Mitosis	3	0	0	2	1	0	0

BW, body weight; DCM, dichloromethane; 1,2-DCP, dichloropropane.

Serum biochemistry

The frozen serum samples were packed with dry ice and shipped within a few hours to SRL, Inc. (Tokyo, Japan), where the parameters for serum biochemistry were analyzed.

RNA isolation and quantitative polymerase chain reaction for analysis of mRNA expression

Total RNA was extracted using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen N.V., Hilden, Germany) according to the manufacturer's instructions. cDNA copies reverse transcribed from total RNA were obtained using a SuperScript VILO cDNA Synthesis Kit and Master Mix (Life Technologies, Carlsbad, CA, USA). PCR was performed with primers for rat Cyp2e1 (Rn00580624_m1) and Gstt1 (Rn00583932_m1), and Rat GAPD (GAPDH) Endogenous Control (Life Technologies) was used as an endogenous reference. PCR was carried out using an Applied Biosystems 7900HT FAST Real-Time PCR System with TaqMan Gene Expression Assays Master Mix and TaqMan Gene Expression Assays (Life Technologies). The expression levels of target genes were determined by normalization to GAPDH expression and calculated by the comparative C_t method. Data are presented as the fold change of treated samples relative to the vehicle controls.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

The samples were homogenized using a Mixer Mill (Qiagen N.V.) with RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing Protease Inhibitor Cocktail (Roche Applied Science, Penzberg, Upper Bavaria, Germany) and PhosSTOP Phosphatase inhibitor cocktail (Roche Applied Science). Samples were centrifuged at 15 000 *g* for 15 min, and the supernatants were used. Protein concentrations were determined using BCA Protein Assay Reagent (Thermo Fisher Scientific Inc.). Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels and transferred on to PVDF membranes (Millipore, Billerica, MA, USA). For the detection of target proteins, membranes were incubated with anti-CYP2E1 monoclonal antibodies, anti-GSTT1 monoclonal antibodies (Abcam, Cambridge, UK) or anti-GAPDH monoclonal antibodies (Santa Cruz, Dallas, TX, USA) at room temperature. Secondary antibody incubation was performed using horseradish peroxidase-conjugated secondary antirabbit or mouse antibodies (GE Healthcare, Little Chalfont, UK) at room temperature. Protein detection was facilitated by chemiluminescence using ECL Prime (GE Healthcare).

gpt mutation/Spi$^-$ assays

Five samples randomly selected from all groups were used in the *gpt* mutation/Spi$^-$ assay. Following the method of Nohmi *et al.* (2000), 6-thioguanine (6-TG) and Spi$^-$ selections were performed. Briefly, genomic DNA was extracted from the liver, and lambda EG10 DNA (48 kb) was rescued as phages by *in vitro* packaging. For 6-TG selection, packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase converted to plasmids carrying the *gpt* and chloramphenicol acetyltransferase genes. To determine the ratio of mutation-carrying plasmids, infected cells were mixed with molten soft agar and poured on to agar plates containing chloramphenicol with/without 6-TG. The plates were then incubated at 37 °C for

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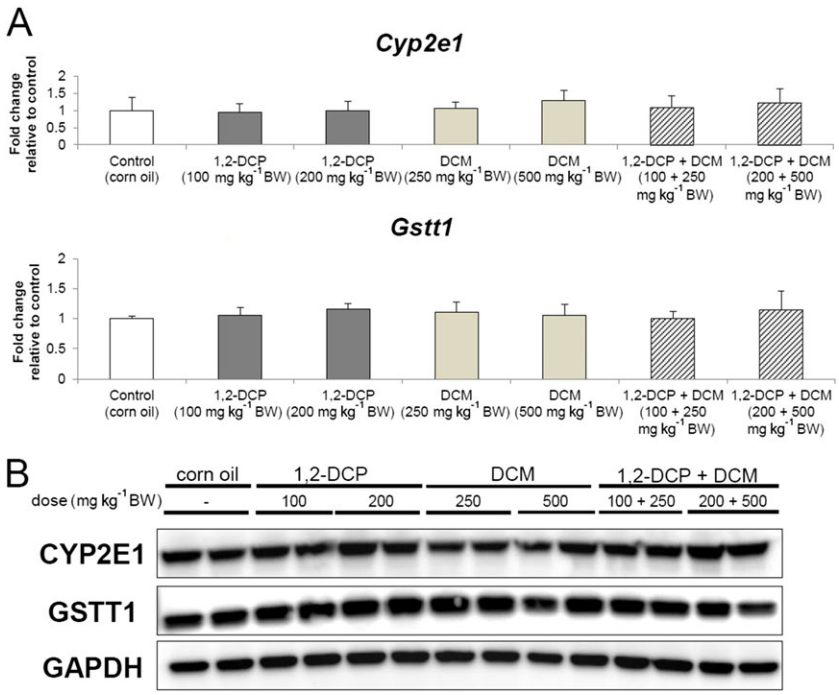

Figure 1. (A) Changes in the mRNA levels of the 1,2-DCP and/or DCM metabolic enzyme genes *Cyp2e1* and *Gstt1* in the livers of *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks. Each value is the mean ± SD of data for seven or eight rats. Data were normalized to *GAPDH* mRNA levels. (B) Western blotting analysis of CYP2E1 and GSTT1 in the livers of *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks. 1,2-DCM, dichloromethane; 1,2-DCP, dichloropropane.

selection of 6-TG-resistant colonies and the *gpt* (MF) was calculated by dividing the number of *gpt* mutants after clonal correction for the number of rescued phages. *Gpt* mutations were characterized by amplifying a 739 BP DNA fragment containing the 456 BP coding region of the *gpt* gene (Nohmi *et al*., 2000). For Spi$^-$ selection, packaged phages were incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. The next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in sucrose mannitol buffer. The Spi$^-$ phenotype was confirmed by spotting the suspensions on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2 or WL95 P2 strains were spread with soft agar. Spi$^-$ mutants, which made clear plaques on every plate, were counted.

Statistical analysis

Variance in the data for BWs, organ weights, serum biochemistry and *gpt* mutation/Spi$^-$ assays was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance was applied. In the heterogeneous cases, the Kruskal–Wallis test was used. When statistically significant differences were indicated, Tukey's multiple test was employed for comparison between corn oil and treated groups. $P < 0.05$ was considered statistically significant in both analyses.

Results

General signs and changes in body and liver weights

No deaths or marked changes in general signs were observed in any group. Final BWs and absolute and relative liver weights are shown in Table 1. Compared with the vehicle control group, although there were no significant changes in BW or absolute and relative liver weight among groups, 1,2-DCP tended to reduce the BW.

Serum biochemistry

The results of serum biochemistry analysis are shown in Table 2. Compared with the vehicle control group, significant increases in bilirubin (Bil) were observed in the 1,2-DCP (200 mg kg^{-1} BW) group. Additionally, inorganic phosphorus was significantly increased in the 1,2-DCP + DCM (200 + 500 mg kg^{-1} BW) group. Significant decreases in glucose in the DCM (500 mg kg^{-1} BW) group and in total cholesterol, triacylglycerol and alanine aminotransferase (ALT) in all 1,2-DCP and all 1,2-DCP + DCM groups were also noted.

Histopathology

The results of histopathological examination are shown in Table 3. Compared with the vehicle control group, no treatment-related changes were observed in any group.

Quantitative polymerase chain reaction and western blotting analysis

Next, the expression levels of mRNAs encoding CYP2E1 and GSTT1, enzymes involved in the metabolism of 1,2-DCP and DCM, were examined. Compared with the vehicle control group, there were no significant differences in *Cyp2e1* and *Gstt1* mRNA expression levels in any group (Fig. 1A). In addition, protein expression levels of CYP2E1 and GSTT1 were examined by western blotting analysis. There were also no significant changes in CYP2E1 and GSTT1

Table 4. *gpt* MFs in the livers of male *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks

Treatment	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
	1	7.8	4	0.51	
	2	6.0	10	1.67	
	3	6.4	4	0.62	
	4	5.9	8	1.35	
Control (corn oil)	5	7.2	6	0.83	1.0 ± 0.5
	8	7.9	3	0.38	
	9	5.8	4	0.69	
	10	6.9	3	0.44	
	11	8.3	3	0.36	
1,2-DCP (100 mg kg^{-1} BW)	12	4.0	5	1.26	0.63 ± 0.38
	15	6.1	3	0.49	
	16	5.2	1	0.19	
	17	8.5	4	0.47	
	18	10.8	3	0.28	
1,2-DCP (200 mg kg^{-1} BW)	19	3.6	3	0.82	0.47 ± 0.23
	22	5.1	5	0.97	
	23	9.1	1	0.31	
	24	6.4	2	0.11	
	25	10.3	3	0.29	
DCM (250 mg kg^{-1} BW)	26	8.1	3	0.37	0.41 ± 0.33
	29	6.9	2	0.29	
	30	9.0	7	0.78	
	31	5.9	2	0.34	
	32	7.7	4	0.52	
DCM (500 mg kg^{-1} BW)	33	5.4	2	0.37	0.46 ± 0.20
	36	6.9	1	0.15	
	37	5.2	6	1.16	
	38	7.1	5	0.70	
	39	9.0	7	0.78	
1,2-DCP + DCM (100 + 250 mg kg^{-1} BW)	40	9.5	1	0.11	0.70 ± 0.42
	43	3.1	4	1.31	
	44	7.5	10	1.33	
	45	11.6	6	0.52	
	46	6.7	3	0.45	
1,2-DCP + DCM (200 + 500 mg kg^{-1} BW)	47	7.9	1	0.13	0.75 ± 0.54

BW, body weight; Cm^R, chloramphenicol resistant; DCM, dichloromethane; 1,2-DCP, dichloropropane; 6-TG^R, 6-thioguanine resistant; MF, mutation frequency.

protein expression levels in any group (Fig. 1B). The quantity of both mRNA and protein samples were normalized with internal controls and were equivalent to other samples responded normally.

gpt mutation/Spi^- assays

Point mutations detected by *gpt* mutation assays using the livers of *gpt* delta rats treated with 1,2-DCP and DCM are summarized in Table 4. There were no significant increases in MFs in any group. In addition, the deletion mutations detected by Spi^- assays using the livers of *gpt* delta rats treated with 1,2-DCP and DCM are summarized in Table 5. There were also no significant increases in MFs in any group. As system control samples, we also analyzed rat liver tissues treated with n-nitrosodiethylamine, which is a well-known genotoxic reagent. There were significant increases in *gpt* MFs and Spi^- MFs in the present study (data not shown). In addition, negative control data in the present study was almost equivalent compared to our historical control data (Matsushita et al., 2014).

Discussion

The results of the present study showed the lack of *in vivo* genotoxicity of 1,2-DCP and DCM, alone or in combination, in the livers of rats treated by gavage. Additionally, there were no changes in the gene and protein expression levels of the metabolic enzymes CYP2E1 and GSTT1.

In serum biochemistry, compared with the vehicle control, there were some significant changes in all groups except the DCM (250 mg kg^{-1} BW) group. Significant increases in Bil were observed in the 1,2-DCP (200 mg kg^{-1} BW) group. This result was similar to the findings of a previous report, which showed a significant increase in Bil levels in rats administered 1,2-DCP (250 or 500 mg kg^{-1} BW) by gavage for 4 weeks (Bruckner *et al.*, 1989). Total cholesterol, triacylglycerol and ALT in the 1,2-DCP and 1,2-DCP + DCM groups decreased in a dose-dependent manner. Although the decrease in ALT was not considered related to the toxicity of the compound, 1,2-DCP was speculated to be the cause of abnormalities in lipid metabolism. However, there were no

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Table 5. Spi$^-$ MFs in the livers of male *gpt* delta rats treated with 1,2-DCP and/or DCM for 4 weeks

Treatment	Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within WL95 (P2)	MF ($\times 10^{-5}$)	Mean ± SD
	1	18.0	4	0.22	
	2	15.4	12	0.78	
	3	17.4	14	0.81	
	4	9.7	7	0.72	
Control (corn oil)	5	12.0	16	1.34	0.77 ± 0.40
	8	9.7	5	0.51	
	9	13.5	12	0.89	
	10	9.0	7	0.78	
	11	14.8	13	0.88	
1,2-DCP (100 mg kg^{-1} BW)	12	3.8	4	1.06	0.82 ± 0.20
	15	7.7	3	0.39	
	16	6.0	4	0.66	
	17	10.0	5	0.50	
	18	18.8	30	1.59	
1,2-DCP (200 mg kg^{-1} BW)	19	6.0	5	0.83	0.80 ± 0.48
	22	12.2	7	0.57	
	23	10.6	12	1.13	
	24	10.6	4	0.38	
	25	17.6	22	1.25	
DCM (250 mg kg^{-1} BW)	26	7.7	6	0.78	0.82 ± 0.37
	29	8.6	6	0.70	
	30	19.6	10	0.51	
	31	10.3	7	0.68	
	32	9.7	13	1.34	
DCM (500 mg kg^{-1} BW)	33	6.0	10	1.66	0.98 ± 0.49
	36	10.1	3	0.30	
	37	7.8	10	1.28	
	38	12.6	3	0.24	
	39	10.7	17	1.59	
1,2-DCP + DCM (100 + 250 mg kg^{-1} BW)	40	16.3	15	0.92	0.85 ± 0.68
	43	9.0	3	0.33	
	44	12.0	8	0.67	
	45	13.0	8	0.62	
	46	12.8	14	1.10	
1,2-DCP + DCM (200 + 500 mg kg^{-1} BW)	47	15.6	17	1.09	0.76 ± 0.33

BW, body weight; DCM, dichloromethane; 1,2-DCP, dichloropropane; MF, mutation frequency.

significant changes in absolute and relative liver weights and in histopathology in these groups. Thus, unlike by inhalation or intraperitoneal injection in rats (Di Nucci *et al.*, 1990; Trevisan *et al.*, 1989), these results revealed a lack of significant changes in the livers of rats orally administered 1,2-DCP or DCM, including the combination groups, except for a slight increase in Bil seen in 1,2-DCP (200 mg kg^{-1} BW) group.

In histopathology, there were no significant changes in the liver of *gpt* delta rats treated with these compounds although microgranuloma was observed in all groups. In a previous report, microgranuloma is one of the spontaneous non-tumor changes in F344/DuCrj rats (Maekawa *et al.*, 1983). Therefore, we assumed that microgranulomas observed in the present study showed spontaneous changes in F344 rats, as there was no statistical significance.

In gene and protein expression analysis of their metabolic enzymes, no significant increases or decreases in CYP2E1 and GSTT1 in the livers were observed in all groups, including the combination groups. DCM is metabolized in the CYP pathway at lower concentrations, determined to be under approximately 500 ppm in rats (US EPA, 2011). At higher concentrations, the CYP pathway becomes saturated, and the GST pathway begins to become predominant (US EPA, 2011). In the GST pathway, *S*-(chloromethyl) glutathione and formaldehyde, which cause liver toxicity and genotoxicity, are produced from the results of DCM metabolism (US EPA, 2011). It is known that 1,2-DCP is also metabolized in the CYP pathway. Although GST, which metabolizes 1,2-DCP, has not been identified, 1,2-DCP is also considered metabolized by GSTT1–1 based on the analyses of several dihalides, which are similar in structure and reactivity to 1,2-DCP (Oda *et al.*, 1996). Therefore, at higher concentrations, the CYP pathway becomes saturated, and the GST pathway begins to become predominant in 1,2-DCP metabolism, possibly involving the production of a reactive episulfonium ion, which may form DNA adducts and induce genotoxicity (Kim & Guengerich, 1990; Zoetemelk *et al.*, 1986). From the results of a previous report, simultaneous exposure to 1,2-DCP and DCM results in saturation of the CYP pathway, and the GST pathway begins to become the predominant metabolic

route, suggesting that the genotoxicity of 1,2-DCP is enhanced by DCM (Suzuki *et al*., 2014). In the present study, the gene and protein expression levels of CYP2E1 and GSTT1 were not affected by exposure to the test chemicals, suggesting that the CYP pathway was not saturated and that the GST pathway did not begin to become predominant. Based on the hypothesis that the genotoxic effects of 1,2-DCP and DCM are closely associated with the expression of their metabolic enzymes, the results of *gpt* mutation/Spi$^-$ assays and the results of the GSTT1 gene and protein expression analyses were correlated. The reasons for this difference between the present study and previous reports may include species differences and route of administration, among others. From carcinogenicity tests of 1,2-DCP by gavage, liver tumors are increased in treated mice, but not in rats (NTP, 1986b). In particular, the expression and activity of GSTT1–1 are higher in the livers of mice than in those of rats (Mainwaring *et al*., 1996; Quondamatteo *et al*., 1998). Thus, this species difference in GSTT1–1 protein expression and activity could potentially result in a difference between the present study and the previous report. If the genotoxic effects of 1,2-DCP are associated with the GST metabolic pathway, species differences may be important. In the case of patients with cholangiocarcinoma in Japan, they might inhale a large amount of 1,2-DCP and/or DCM. We should take the route of administration into account because there were no significant increases of MFs in the liver of *gpt* delta rats treated with these compounds by gavage. However, it was considered that patients might be exposed to strong mutagens from period of onset and age of patient of cholangiocarcinoma and these compounds administrated by inhalation would not directly distribute to liver and bile duct. In addition, from a previous report, 1,2-DCP administrated by inhalation caused not liver tumor but nasal cavity tumors in rat (Umeda *et al*., 2010). Therefore, there might be also possibly species difference of the genotoxic effects of these compounds.

In conclusion, the *in vivo* mutagenicity of 1,2-DCP and DCM in the livers of *gpt* delta rats orally administered alone or in combination, was not observed, and the expression levels of CYP2E1 and GSTT1 were not altered under the current test conditions. Therefore, further studies are required to determine the genotoxic effects of 1,2-DCP and DCM in the livers of other species and the metabolic activities of 1,2-DCP and DCM. In addition, further studies are required to elucidate the relationships between cholangiocarcinoma and these agents.

Acknowledgments

We thank Ms. Ayako Saikawa and Ms. Yoshimi Komatsu for their expert technical assistance with the processing of histological materials. This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

Conflict of interest

Tadashi Hirata is an employee of Japan Tobacco. Inc., Kanagawa, Japan. Isamu Suzuki is an employee of BoZo Research Center Inc., Shizuoka, Japan. The authors including two mentioned above declare no conflicts of interest associated with this manuscript.

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