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Absence of *in vivo* genotoxicity of 3-monochloropropane-1,2-diol and associated fatty acid esters in a 4-week comprehensive toxicity study using F344 *gpt* delta rats

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3-Monochloropropane-1,2-diol (3-MCPD) is regarded as a rat renal and testicular carcinogen and has been classified as a possible human carcinogen (group 2B) by International Agency for Research on Cancer. This is potentially of great importance given that esters of this compound have recently found to be generated in many foods and food ingredients as a result of food processing. There have been a few reports about their toxicity, although we have recently found that the toxicity profile of 3-MCPD esters was similar to that of 3-MCPD in a rat 13-week repeated dose study, except for the acute renal toxicity seen in 3-MCPD-treated females. In the present study, to examine *in vivo* genotoxicity we administered equimolar doses of 3-MCPD or 3-MCPD fatty acid esters (palmitate diester, palmitate monoester and oleate diester) to 6-week-old male F344 *gpt* delta rats carrying a reporter transgene for 4 weeks by intragastric administration. *In vivo* micronucleus, *Pig-a* mutation and *gpt* assays were performed, as well as investigations of major toxicological parameters including histopathological features. As one result, the relative kidney weights of the 3-MCPD and all three ester groups were significantly increased compared with the vehicle control group. However, the frequency of micronucleated reticulocytes and *Pig-a* mutant red blood cells did not differ among groups. Moreover, no changes were observed in mutant frequencies of *gpt* and *red/gam* (Spi$^-$) genes in the kidney and the testis of 3-MCPD and 3-MCPD-fatty-acid-esters-treated rats. In histopathological analyses, no treatment related changes were observed, except for decrease of eosinophilic bodies in the kidneys of all treated groups. These results suggest that 3-MCPD and its fatty acid esters are not *in vivo* genotoxins, although they may exert renal toxicity.

Introduction

Genotoxicity assays have been used for screening carcinogenic potential of various agents. Although *in vitro* experiments can be readily and quickly performed, they may not reflect the absorption, distribution, metabolism and excretion of test compounds *in vivo*. Data from *in vivo* assays may have more relevance to effects in humans, including mechanisms of carcinogenesis. Therefore, the *in vivo* genotoxicity assays have been considered as important for risk assessment of new materials. Several *in vivo* genotoxicity assays have become established, each with a different endpoint. The *in vivo* micronucleus (MN) assay using peripheral blood or bone marrow cells obtained from rodents after single exposure of the test compound is widely accepted. It was also recommended by the International Conference on Harmonisation (ICH) guidelines as one of the genotoxicity testing battery required for the development of new drugs (1). This assay is used to detect damage to chromosomes or the mitotic apparatus. Substances that cause cytogenetic damage may give rise to cells with micronuclei, which consist of lagging chromosome fragments (a result of clastogenic activity) or whole chromosomes (due to aneugenic activity) (2).

In contrast to the MN assay, which generally is sensitive only to damage produced in a relatively small window of time before the sampling time, the *Pig-a* mutation assay can take advantage of the accumulative damage produced during an extended animal exposure (3). This assay is used to detect damage to the phosphatidylinositol glycan complementation class A (*Pig-a*) gene. The product of X-linked *Pig-a* gene is essential for synthesis of the glycosylphatidylinositol (GPI) anchor that links a specific subset of proteins to the cell surface. Therefore, mutations in the *Pig-a* gene can cause deficiencies of GPI-anchored proteins and GPI-anchored markers (4). The CD59 antigen is attached to the cytoplasmic membrane of erythroid cells *via* GPI anchors in the rat. In this assay, the frequency of mutant phenotype erythrocytes (RBC^{CD59-}) is determined via flow cytometric analysis.

The MN and *Pig-a* mutation assays are based on detecting DNA damage to haematopoietic cells as a result of systemic exposure. On the other hand, the *gpt* assay can detect point mutations and deletion mutations that escape from repair mechanisms and become fixed in target cells of carcinogens in rats and mice (5). This assay permits the efficient and quantitative detection of mutations in any tissue of rodents, with analyses of the mutations at the molecular level.

In the present study, we evaluated genotoxicty comprehensively using these three assays (MN, *Pig-a* mutation and *gpt* assays) for 3-monochloropropane-1,2-diol (3-MCPD) and three kinds of associated fatty acid esters.

3-MCPD is a food processing contaminant that belongs to a group of compounds called chloropropanols. It was first detected in acid-hydrolysed vegetable proteins (6). Further studies showed that 3-MCPD may also occur in other various products such as thermally processed foods like cereal-derived products (bread, biscuits), malt-derived products, coffee, cheese, smoked food, meat and salted fish (7, 8). Several *in vitro* and *in vivo* genotoxicity studies of 3-MCPD have been reviewed (9). *In vitro*, 3-MCPD induced reverse mutations in various strains of *Salmonella typhimurium* (10–12), and DNA

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strand breaks in the comet assay with Chinese hamster ovary cells (13). On the other hand, no genotoxic effects of 3-MCPD were observed with the *in vivo* comet assay in various organs of SD and F344 rats (13), and there was no micronucleus formation in male Han Wistar rat bone marrow cells or unscheduled DNA synthesis in male Han Wistar rat hepatocytes (14).

In 2001, the Joint FAO/WHO Expert Committee on Food Additives established the provisional maximum tolerable daily intake (PMTDI) of 3-MCPD as 2 μg/kg body weight (B.W.)/day for human. This dose was based on the lowest observed effect level (LOEL) of 1.1 mg/kg B.W./day that induced renal tubular hyperplasia in a rat long-term study giving 3-MCPD by drinking water (15). The safety factor of 500 including additional factor of 5 for extrapolation from a LOEL to a no-observed-effect level (NOEL) was applied to establish the PMTDI (9). After rat carcinogenicity studies showed development of kidney and testicular carcinomas (15, 16), the International Agency for Research on Cancer classified 3-MCPD as a 'possible human carcinogen (group 2B)' (17).

Importantly, further analyses have revealed that 3-MCPD occurs in various foods either as a free form or more commonly esterified with long-chain fatty acids. High concentrations of 3-MCPD fatty acid esters have been reported in hydrogenated fats, palm oil and palm oil fractions solid frying fats (18,19). Moreover, occurrence of 3-MCPD fatty acid esters in human breast milk has been documented (20).

Recently, we conducted a rat 13-week subchronic toxicity study of three 3-MCPD fatty acid esters (palmitate diester: CDP, palmitate monoester: CMP, oleate diester: CDO) administered by gavage (21). Compared with 3-MCPD, the toxicities of the 3-MCPD fatty acid esters were lower in the acute phase regarding renal tubular necrosis and were equivalent in the subchronic phase, as evidenced by increase in male and female kidney weights and apoptosis in the initial segments of the epididymis. Liu *et al.* (22) also reported that renal tubular necrosis and protein casts in the kidneys were the major histopathological changes caused by acute oral toxicity of both CMP and CDP in Swiss mice. A 90-day toxicology study of 3-MCPD and CDP administered by daily oral gavage, conducted by Barocelli *et al.* (23), also revealed tubular epithelial hyperplasia or proliferation, basophilic or hyaline material and karyomegaly of tubular epithelial cells in CDP-treated rat kidneys.

For further risk assessment of 3-MCPD and 3-MCPD fatty acid esters, we here evaluated *in vivo* genotoxicity not only in haematopoietic cells but also in the potential target organs of carcinogenesis by *in vivo* MN, *Pig-a* mutation and *gpt* assays in male F344 *gpt* delta rats given these chemicals by gavage for 4 weeks.

Materials and methods

Test chemicals

Olive oil, 3-MCPD (98% pure), CDP (98% pure), (*sn1*)-CMP (1-palmitoyl-3-chloropropanediol; 98% pure) and CDO (98% pure) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Initially, (*sn2*)-CMP (2-palmitoyl-3-chloropropanediol) was also synthesised by Wako for analysis, but HPLC analysis revealed that (sn2)-CMP was unstable and spontaneously converting into (*sn1*)-CMP. For this reason, we analysed only (*sn1*)-CMP as a monoester.

Animals, diet and housing conditions

Six-week-old male F344 *gpt* delta rats carrying approximately 10 copies of the transgene lambda EG10 per haploid genome (24) were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in polycarbonate cages (five or six rats per cage) with softwood chips for bedding in a specific pathogen-free animal facility, maintained under conditions of controlled temperature (23±2°C), humidity (55±5%), air change (12 times per hour) and lighting (12 h light/dark cycle) were given free access to a CRF-1 basal diet (Charles River Japan, Kanagawa, Japan) and tap water. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences.

Experimental design

After a 1-week acclimatisation period, 35 male F344 *gpt* delta rats weighing 82.2–117 g were used in this experiment. Rats were allocated with body-weight-basis randomisation to six groups consisting of five rats in the control group and six rats each in the olive oil (5 ml/kg B.W.), 3-MCPD (40 mg/kg B.W.) and 3-MCPD fatty acid ester groups (CDP: 220 mg/kg B.W., CMP: 130 mg/kg B.W. CDO: 240 mg/kg B.W.). The dose of 3-MCPD was selected based on previous carcinogenicity tests (16). Dose of 3-MCPD fatty acid esters was the same molar concentration of 3-MCPD. 3-MCPD and 3-MCPD fatty acid esters were dissolved in olive oil at the time of dosing and administered 5 times a week by intragastric tube for 4 weeks. The animals were observed daily for any clinical signs and mortality. Body weights were measured weekly. For *Pig-a* mutation assay, peripheral blood samples were collected from the tail vein of each animal (five per group) at Day 0 (before the first administration) and 15 (2 weeks after start of administration). At Day 29, after an overnight fast, within 24 h after the final administration, all the animals were anaesthetised with isoflurane, weighed blood samples were collected from the abdominal aorta for serum biochemistry and *Pig-a* mutation assays. The animals were then sacrificed by exsanguination from the abdominal aorta. For the *Pig-a* mutation assay, 9 μl whole blood samples were transferred to tubes and the remaining blood samples were left out to clot at room temperature for 30 min. After centrifugation at 3000 rpm for 10 min, the resultant sera were stored at −20°C until shipping for analysis.

Histopathology

The liver, kidneys and spleen were weighed and relative organ weights were calculated as the ratios between organ and body weights. The right kidney and testis were stored at −80°C for *gpt* assays. In addition, the liver, left kidney, spleen and left testis were fixed in 10% neutral buffered formalin embedded in paraffin, sectioned to a thickness of 3 μm stained with haematoxylin and eosin (HE) for microscopic examination. Kidney sections were also stained with PAS and Masson's trichrome staining. Histopathological examinations were carried out for all animals. Those found dead were also analysed as far as possible.

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 shown in Table II were analysed.

Micronucleus assays

For the Micronucleus (MN) assays, proximal and distal ends of the left femurs were cut and syringes with 23G needles were used to flush the bone marrow tissue with 0.5 ml foetal bovine serum (FBS) into 1.5 ml tubes. Cell suspensions kept on ice were centrifuged at 1000 rpm for 5 min and cell pellets were re-suspended in almost the same volume of FBS using Pasteur pipettes. Aliquots of 5 μL of cell suspension were spread on standard microscope slides. After air-drying for overnight, these smear slides were fixed with absolute methanol for 10 min and stored in a slide box until staining with acridine orange (Wako Pure Chemical Industries, Ltd.) and washing with phosphate buffer (pH 6.8). Micronuclei were immediately counted per at least 2000 reticulocytes (RETs) per animal using a fluorescence microscope. In addition, at least 1000 erythrocytes (RETs and mature) were scored to determine %RETs as a measure of chemical-induced bone marrow toxicity.

Pig-a *mutation assays*

Peripheral blood collection and staining of total red blood cells (RBCs) were conducted according to the previous report with slight modifications (25). Peripheral blood samples of 9 μl were promptly mixed with 1 μl of 12 mg/ml K_2-EDTA. Three microlitres of blood/EDTA mixture were suspended in 200 μL of phosphate-buffered saline (PBS) the cells were labelled with 1 μg of FITC-conjugated anti-rat CD59 (BD Biosciences, Tokyo, Japan) and 0.125 μg of biotinylated HIS49 antibody (erythroid marker; BD Biosciences). After incubation for 1 h in the dark at room temperature, the samples were washed with PBS, centrifuged for 5 min at 1000 g re-suspended in 200 μL of PBS. Then the samples were mixed with 0.2 μg of APC-conjugated streptavidin (BD Biosciences) and incubated for 15 min in the dark at room temperature. They were finally centrifuged for 5 min at 1000 g and re-suspended in 1 mL of PBS. The frequency of *Pig-a* mutant RBCs was determined as the number of RBC^{CD59-} per 1 million total RBCs using a FACS Canto II flow cytometre (BD Bioscience). For determinations of the

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frequency of RBC^{CD59-}, single cells, including RBCs and white blood cells, were gated by light scatter. HIS-49-positive cells from this population were analysed further for the presence of the GPI-anchored CD59 antigen on the cell surface.

gpt *assays*

Following the method of Nohmi *et al.* (5), 6-thioguanine (6-TG) and Spi$^-$ selections were performed. Briefly, genomic DNA was extracted from the kidney and testis, reported as target organs of 3-MCPD (15, 16), 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. In order to determine the ratio of mutation-carrying plasmids, infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol with/without 6-TG. The plates were then incubated at 37°C for selection of 6-TG-resistant colonies the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction for the number of rescued phages. *gpt* mutations were characterised by amplifying a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene (5). 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 sterilised 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. To confirm the efficacy of the procedure for the *gpt* assay, positive DNA samples extracted from rat liver treated with a known genotoxic hepatocarcinogen, diethylnitrosamine (DEN), were also assessed.

Statistical analysis

Variance in the data for body weights, organ weights, serum biochemistry, frequencies of MN-RETs, percent RETs among total erythrocytes and frequencies of RBC^{CD59-} 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, Dunnett's multiple test was employed for comparison between olive oil and treated groups. P values less than 0.05 were considered statistically significant in both analyses.

Results

General conditions

Body weights. Three rats, one each treated with CDP, CMP or CDO died with haemorrhage of the oesophagus–thoracic cavity without histological change of kidneys, the cause being accidental related to gavage problems. There was no deterioration in the general conditions observed in any of the groups. The body weights of all groups increased gradually and progressively with age. No significant differences in body weight gain were detected among treatment groups (Figure 1).

Organ weights

Final body weights and the absolute and relative organ weights are shown in Table I. Compared with vehicle control group, significant increase in absolute and relative kidney weights

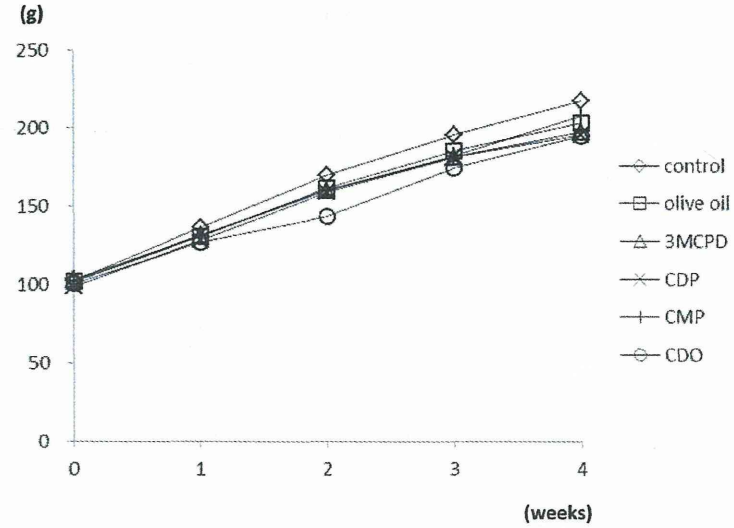

Fig. 1. Body weight curves for F344 *gpt* delta rats treated with 3-MCPD fatty acid esters for 4 weeks.

Table I. Organ weight for F344 *gpt* delta rats treated with 3-MCPD fatty acid esters for 4 weeks

Treatment	Control	Olive oil	3-MCPD	CDP	CMP	CDO
No. of animals	5	6	6	5	5	5
Body weight (g)	218±23	203±10	197±13	196±14	208±11	195±11
Absolute						
Spleen (g)	0.49±0.04	0.46±0.03	0.5±0.04	0.47±0.03	0.5±0.04	0.48±0.02
Liver (g)	8.4±1	7.4±0.5	7.5±0.5	7.7±0.9	8.1±0.6	7.6±0.3
Kidneys (g)	1.6±0.15	1.4±0.1	1.6±0.12*	1.7±0.10**	1.7±0.07**	1.7±0.06**
Relative						
Spleen (%)	0.23±0.013	0.23±0.01	0.25±0.009**	0.24±0.004	0.24±0.017	0.25±0.006*
Liver (%)	3.9±0.2	3.7±0.1	3.8±0.1	3.9±0.2	3.9±0.1	3.9±0.2
Kidneys (%)	0.72±0.02	0.69±0.04	0.81±0.03**	0.87±0.02**	0.83±0.04**	0.86±0.02**

Each value represents the mean ± SD.
Significantly different from the olive oil group at *P < 0.05 and **P < 0.01.

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was noted in the 3-MCPD, CDP, CMP and CDO groups. With spleen, only relative weights were increased in the 3-MCPD and CDO groups.

Serum biochemistry

The results of serum biochemical analysis are summarised in Table II. Compared with the vehicle control group, significant increases in glucose of all 3-MCPD fatty acid ester groups and inorganic phosphorus (IP) of the CMP and CDO groups were observed. Significant decrease in aspartate aminotransferase (AST) of the CMP and CDO groups and creatinine (Cre) of the 3-MCPD and all 3-MCPD fatty acid ester groups was also noted.

Histopathology

Eosinophilic bodies, negative for PAS reaction and positively stained with Masson's trichrome staining (data not shown), seen in the renal proximal tubular epithelium were decreased in the 3-MCPD fatty acid ester groups compared with control and olive oil groups (Table III and Figure 2). No treatment-related changes were observed in the liver, spleen and testis.

MN assay

The frequency of micronucleated reticulocytes (MN-RETs) (Figure 3A) and the percentage RETs among total erythrocytes (Figure 3B) did not differ significantly in the 3-MCPD-treated and 3-MCPD-fatty-acid-ester-treated groups compared with the control groups.

Pig-a *mutation assay*

The frequency of *Pig-a* mutant RBCs did not differ among groups at any time point (Figure 4).

gpt *assay*

In the samples from the DEN-treated liver performed as the positive control, the MFs of *gpt* (more than 40-folds) and of Spi$^-$ (more than 5-folds) were significantly elevated (data not shown), MFs in the kidney and testis in the 3-MCPD and its ester-treated groups (Figure 5) were within background levels reported previously (26). There were no significant differences in the *gpt* MFs as well as Spi$^-$ MFs among the groups.

Discussion

The results of the present study do not provide any evidence that 3-MCPD or the esters exert genotoxity or other major toxicity.

In organ weight data, the absolute and relative kidney weights in the 3-MCPD, CDP, CMP and CDO groups were significantly increased. This change was observed in our previous 13-week study of these chemicals in both male and female wild F344 rats (21), and in other groups' 13-week studies of 3-MCPD in B6C3F1 mice (27) and of 3-MCPD or CDP in Wistar rats (23). However, in histopathological analysis of kidney, treatment-related findings including tubular necrosis were not observed, except for decrease of eosinophilic bodies. In general, an increase of such bodies in male rat kidney has rather been

Table II. Serum biochemistry for F344 *gpt* delta rats treated with 3-MCPD fatty acid esters for 4 weeks

Treatment	Control	Olive oil	3-MCPD	CDP	CMP	CDO
No. of animals	5	6	6	5	5	5
TP (g/dL)	6.1±0.2	6.1±0.2	5.9±0.2	5.8±0.2	5.9±0.1	5.8±0.1
Alb (g/dL)	4±0.1	4.1±0.2	4±0.1	3.9±0.2	4±0.1	4±0.1
A/G	1.9±0.1	2.1±0.2	2.2±0.1	2.1±0.4	2.2±0.1	2.2±0.1
Glucose (mg/dL)	183±39	169±27	168±21	219±25**	228±16**	248±12**
Bil (mg/dL)	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01	0.03±0	0.03±0.01
T-Cho (mg/dL)	65±5	54±4	54±3	57±8	55±5	57±3
TG (mg/dL)	191±18	117±51	68±24	67±26	96±34	88±23
γGTP (IU/L)	<3	<3	<3	<3	<3	<3
AST (IU/L)	87±13	85±10	74±6	65±6	62±1*	59±5**
ALT (IU/L)	33±3	32±4	29±1	28±4	29±2	28±1
ALP (IU/L)	1152±73	1336±91	1275±111	1190±233	1233±98	1229±117
BUN (mg/dL)	18.7±1.9	14.2±2.2	11.3±1.0*	11.7±1.2	12.8±1.5	12.7±1.7
Cre (mg/dL)	0.28±0.01	0.27±0.02	0.23±0.01**	0.22±0.01**	0.22±0.01**	0.22±0.02**
Ca (mg/dL)	10.2±0.2	10.3±0.3	10.2±0.2	10.2±0.1	10.2±0.1	10.3±0.2
IP (mg/dL)	6.1±0.2	5.9±0.2	6.9±0.4	6.7±0.9	7.1±0.5*	7.2±0.3*
Na (mg/dL)	141±2	143±3	143±1	142±1	141±1	141±1
K (mg/dL)	4.7±0.2	4.3±0.2	4.5±0.2	4.2±0.1	4.3±0.4	4.5±0.2
Cl (mg/dL)	100±1	102±2	104±1	103±2	102±1	101±2

Each value represents the mean ± SD. TP, total protein; Alb, albumin; Bil, bilirubin; T-Cho, total cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; Cre, creatinine.
Significantly different from the olive oil group at $*P < 0.05$ and $**P < 0.01$.

Table III. Histopathological change in kidney for F344 *gpt* delta rats treated with 3-MCPD fatty acid esters for 4 weeks

	Control	Olive oil	3-MCPD	CPD	CPM	CDO
No. of rats examined	5	6	6	5	5	5
Eosinophilic body, cortex, proximal tubular (−/±/+)[a]	0/1/4	0/4/2	6/0/0	5/0/0	5/0/0	5/0/0

[a]Ratio of proximal tubules with eosinophilic body :− < 5%, ± < 25%, + < 50%.

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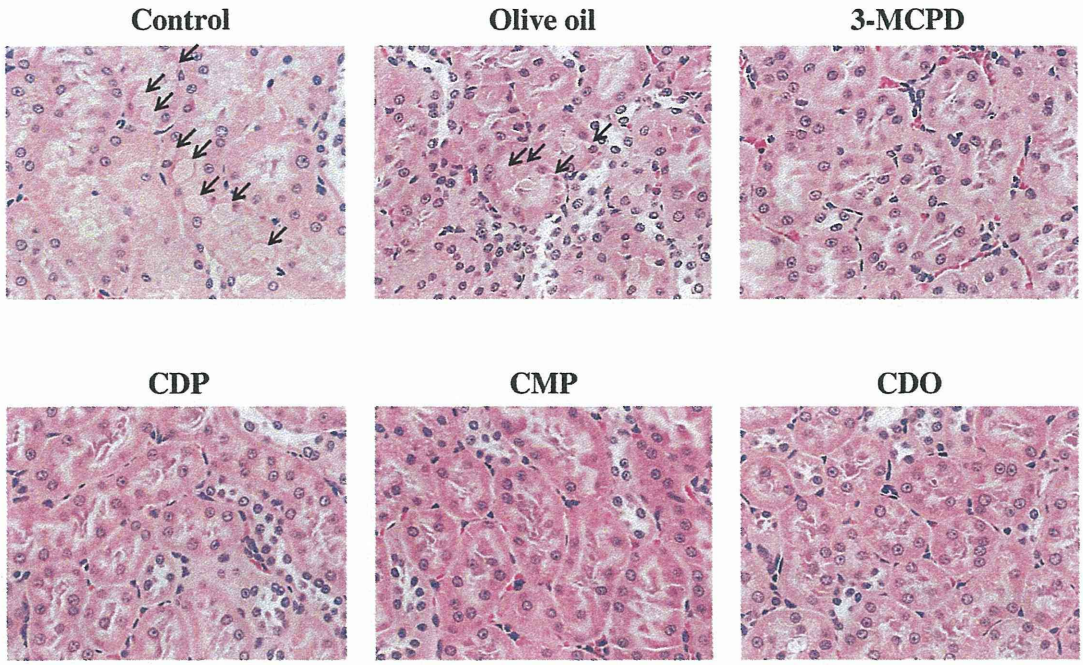

Fig. 2. Photomicrograph of kidneys of F344 *gpt* delta rats (HE staining). Black arrows indicate eosinophilic bodies in the renal proximal tubular epithelium in control and olive oil groups.

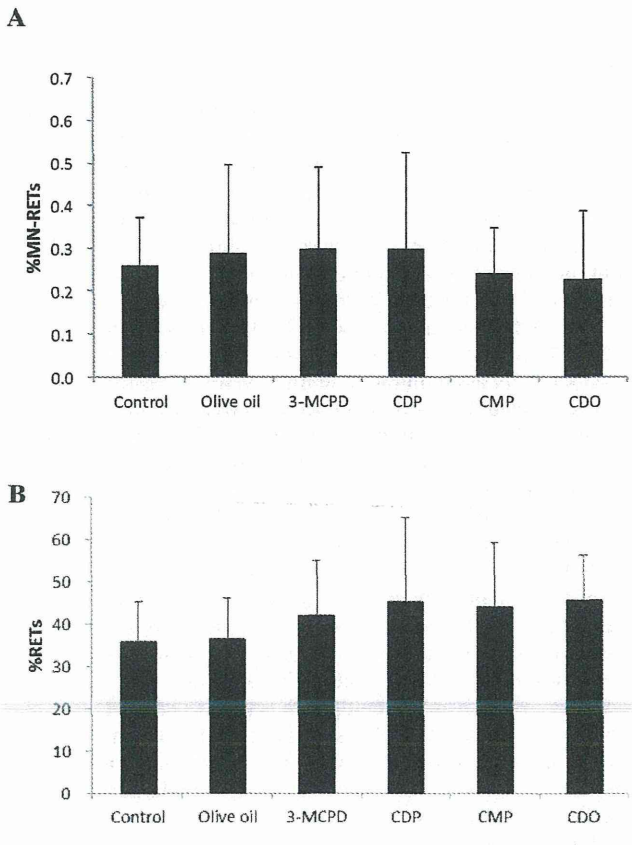

Fig. 3. (**A**) Frequency of MN-RETs in rat bone marrow after 4 weeks treatment. (**B**) Percentage RETs among total erythrocytes in rat bone marrow after 4 weeks treatment. The values represent the means of experiments ± standard deviations.

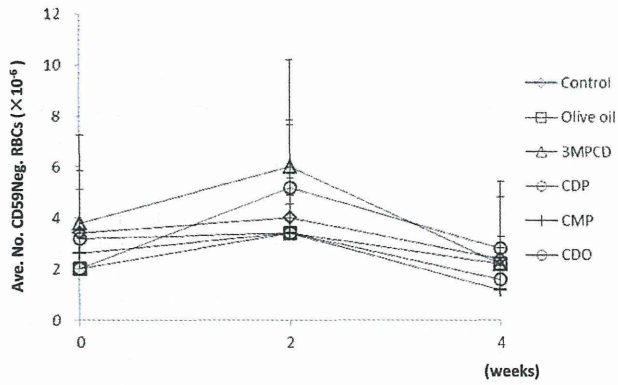

Fig. 4. Average frequencies of RBC^{CD59-} following treatment of F344 *gpt* delta rats for 4 weeks. The values represent the means of experiments ± standard deviations.

reported as an adverse change associated with the accumulation of alpha 2u-globulin, a male-specific protein synthesised in the liver (28,29). No significant change in serum biochemical analysis related to liver toxicity was confirmed. Furthermore, any toxicological implication of decrease of eosinophilic bodies in kidney has not been elucidated. Although the mode of action was not clear, it was confirmed that the increase of the absolute and relative kidney weight indicated the renal effects of 3-MCPD and its fatty acid esters in the present 4 weeks *gpt* delta rat study.

The relative spleen weight increased in 3-MCPD and CDO groups but the absolute spleen weight did not change. The difference was about 10% and there were no significant histological lesions in the spleens of these groups. Therefore, the relative spleen weight changes were considered as incidental.

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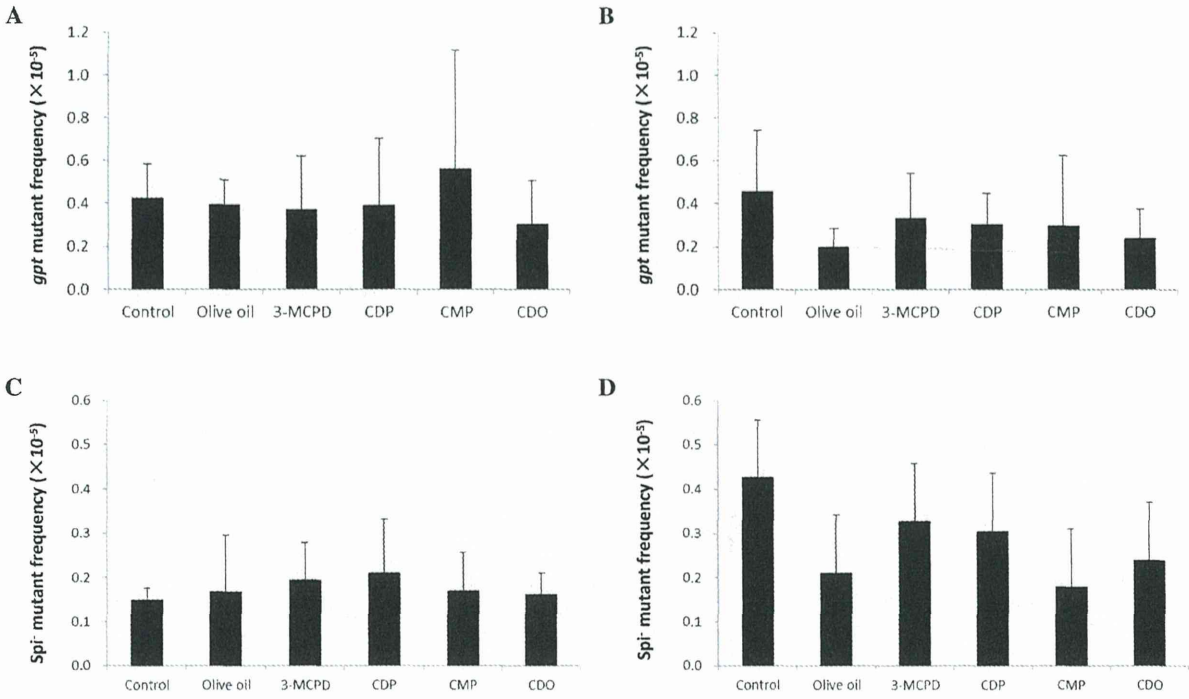

Fig. 5. *gpt* mutant frequencies in the (**A**) kidney and (**B**) testis, Spi⁻ mutant frequencies in the (**C**) kidney and (**D**) testis of F344 *gpt* delta rats treated with 3-MCPD or 3-MCPD esters for 4 weeks. The values represent the means of experiments ± standard deviations.

In serum biochemistry, glucose, IP, AST and Cre were significantly altered compared with vehicle control group. In this study, we could not confirm dose dependence; therefore, we could not discuss the meaning of these changes in detail. Although, increase of glucose was not observed in our previous 13-week study (21) and there was no related histological change, clear increase limited to the 3-MCPD fatty acid ester groups might be related to the treatment. Decrease of AST usually does not indicate toxicity and minimal changes of IP without other ionic change may not be related to any toxic outcome. Significant decrease of Cre in the 3-MCPD and its all ester groups was observed not only in the present study but also in the previous 13-week study without loss of body weight or muscle mass (21). It could be related to the treatment, although the mechanism and impact are not clear.

In this experiment, we investigated the *in vivo* genotoxicity of 3-MCPD and its fatty acid esters in rat non-target (blood and bone marrow) and target (kidney and testis) organs identified in the carcinogenicity studies of 3-MCPD published or submitted to WHO for its evaluation (15,16). The dose of administration was equimolar to the carcinogenic dose of 3-MCPD (40 mg/kg B.W./day) (16) and was equivalent to 26% of the LD_{50} (152 mg/kg B.W.) of 3-MCPD in the rat oral administration study (30). Although this dose might be lower than the maximum tolerated, renal toxicity was evident and 50% of female rats died within 4 weeks with this dose in our 13-week study (21). Moreover, 50% of female rats did not survive in another 90-day study in which 3-MCPD was given at 29.5 mg/kg B.W./day by bolus gavage (23). On the other hand, following the Organization for Economic Cooperation and Development (OECD) guideline 488 of transgenic rodent somatic and germ cell gene mutation assays including the *gpt* assay, we selected the duration of the experiment as 29 days. The OECD guideline 474 for *in vivo* MN assay recommends a single exposure and an appropriate sampling time. However, evidence continues to accumulate that the *in vivo* MN assay is sensitive for genotoxicants administered over protracted durations, despite the fact that the frequencies do not tend to rise with repeat dosing (31). Taking account that the basic schedule of *Pig-a* mutation assay was also 29 days, we considered that the present dosage and the duration were reasonable and adequate for evaluation of *in vivo* genotoxicity.

In our MN assay, all three 3-MCPD fatty acid esters as well as 3-MCPD were negative. Robjohns *et al.* also showed negative results in MN assay in rat bone marrow and liver for 3-MCPD and concluded that 3-MCPD does not possess genotoxic activity *in vivo* (14). Although the percentage of RETs among total erythrocytes in rat bone marrow is reported to show a relatively wide range (controls were 48.0–83.6%) (32), the present 35.8–45.7% were similar to our previous study (control was 54%) (33). Therefore all three 3-MCPD fatty acid esters as well as 3-MCPD did not indicate clear cytotoxicity to RETs.

In *Pig-a* mutation assay, the frequency of *Pig-a* mutant RBCs did not differ among groups at all time points. Therefore, the cumulative damage reflecting *in vivo* genotoxicity from the exposure of 3-MCPD and 3-MCPD fatty acid esters for 4 weeks to rats was not evident in erythroid cells. This is the first time to conduct this assay with 3-MCPD and 3-MCPD fatty acid esters. It is documented that mutant phenotype RBC responses are modest on Day 29 and require additional time to reach their maximal values. In contrast, mutation responses in RET occur earlier (31). In the case of a 29 days experiment, although results assessed for RET are preferable, our sequential observation of RBC did not show any tendency for accumulation of mutations.

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Thus, in our two genotoxicity assays, 3-MCPD and 3-MCPD fatty acid esters did not appear to exert genotoxicity for blood and bone marrow with systemic exposure.

Previously, we have found that estragole (ES), a mouse liver carcinogen, was negative in the MN assay but positive in the *gpt* assay with C57BL/6 *gpt* delta mouse liver (34). Moreover, we showed that the *gpt* mutation frequency in the liver and the GST-P positive foci that have been considered to be a rat liver preneoplastic lesion were significantly increased in the F344 *gpt* delta rat by ES administration (35). ES is an allylbenzene compound that is a natural constituent of several herbs. The predominant ES-specific DNA adduct in these livers was ES-3'-N^6-dA and the predominant mutation in the *gpt* assay included AT:GC transition. This fact indicated that ES-specific DNA adducts in the liver may partly be related to genotoxicity (34, 35). Thus, it is desirable to conduct *in vivo* genotoxicity assays with target organs. As the organs tested in the MN and *Pig-a* mutation assays were different from the target organs of carcinogenicity, the *gpt* assay (5) was conducted to investigate if organ-specific genotoxic mechanisms could be involved in subchronic toxicity of 3-MCPD fatty acid esters and/or carcinogenicity of 3-MCPD in rats. In the present study, there were no significant treatment related increases in the *gpt* MFs in either kidney or testis. Furthermore, Spi^- MFs also did not significantly differ from those in the relevant control groups.

Since *in vivo* genotoxicity was not detected in these analyses, 3-MCPD and 3-MCPD fatty acid esters (CDP, CMP and CDO) were suggested to be non *in vivo* genotoxic agents. Scientific opinion from European Food Safety Authority recommends a step-wise approach for assessment of genotoxicity and states that normally, if the results of appropriate and adequately conducted *in vivo* tests are negative, then it can be concluded that the substance is not an *in vivo* genotoxin (36). Because of the presence of enzymatic reactions for metabolism and homeostatic or other epigenetic mechanisms, it has been generally accepted that non-genotoxic agents should have a threshold for toxicity, even if there is a possibility of carcinogenicity (37). As an example, fluensulfone (CAS No. 318290-98-1) used as nematicide, increased incidences of alveolar/bronchiolar adenomas and carcinomas in female mice and showed one positive result and two negative results *in vitro* Ames assays and a negative result in an *in vivo* MN assay in mice. A Joint FAO/WHO Meeting on Pesticide Residues evaluated this chemical as a non-genotoxic carcinogen and established an acceptable daily intake (ADI) on the basis of the no-observed-adverse-effect level (NOAEL) for chronic interstitial inflammation in the lungs and oesophageal hyperkeratosis and decreased body weight from the rat chronic toxicity and carcinogenicity studies with a safety factor of 100 (38). Severe renal toxicity characterised by renal tubular necrosis observed in 13-week toxicity studies (21, 23) may be related to the development of renal carcinomas induced in carcinogenicity tests (15, 16). Further experiments elucidating the mode of action of non-genotoxic carcinogenic 3-MCPD should be performed.

3-MCPD fatty acid esters have various forms with different fatty acids and are thought to be metabolised to 3-MCPD in the body (39–41). Because hydrolysis processes may take time so that increase the serum concentration of 3-MCPD is gradual (39), this might explain why acute renal toxicity of 3-MCPD was more severe than that of 3-MCPD esters (21). Two different metabolic pathways of 3-MCPD have been proposed in the rat (42). One is detoxification by conjugation with glutathione, yielding S-(2,3-dihydroxypropyl) cysteine, N-acetyl-S-(2,3-dihydroxypropyl) cysteine and mercapturic acid. The other is oxidation to beta-chlorolactic acid and then to oxalic acid. Beta-chlorolactic acid, negative in the comet assay on Chinese hamster ovary cells (13), and mercapturic acid are known to be excreted into urine in rats (23).

As a further concern, it has been reported that 3-MCPD might be metabolised to genotoxic carcinogen glycidols, although this reaction is characteristically observed in bacteria (43). However, the target organs of carcinogenicity are not the same between 3-MCPD and glycidoe in either rats (15, 16, 44) or mice (44, 45). Thus, the possible effect of glycidol as a metabolite may be negligible.

In conclusion, the present findings suggest that 3-MCPD fatty acid esters, at least CDP, CMP and CDO, as well as 3-MCPD are not *in vivo* genotoxins. For risk assessment of these compounds, it is therefore considered that ADI or tolerable daily intake values should be established.

Acknowledgements

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Conflict of interest: The authors declare that they have no conflict of interest.

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