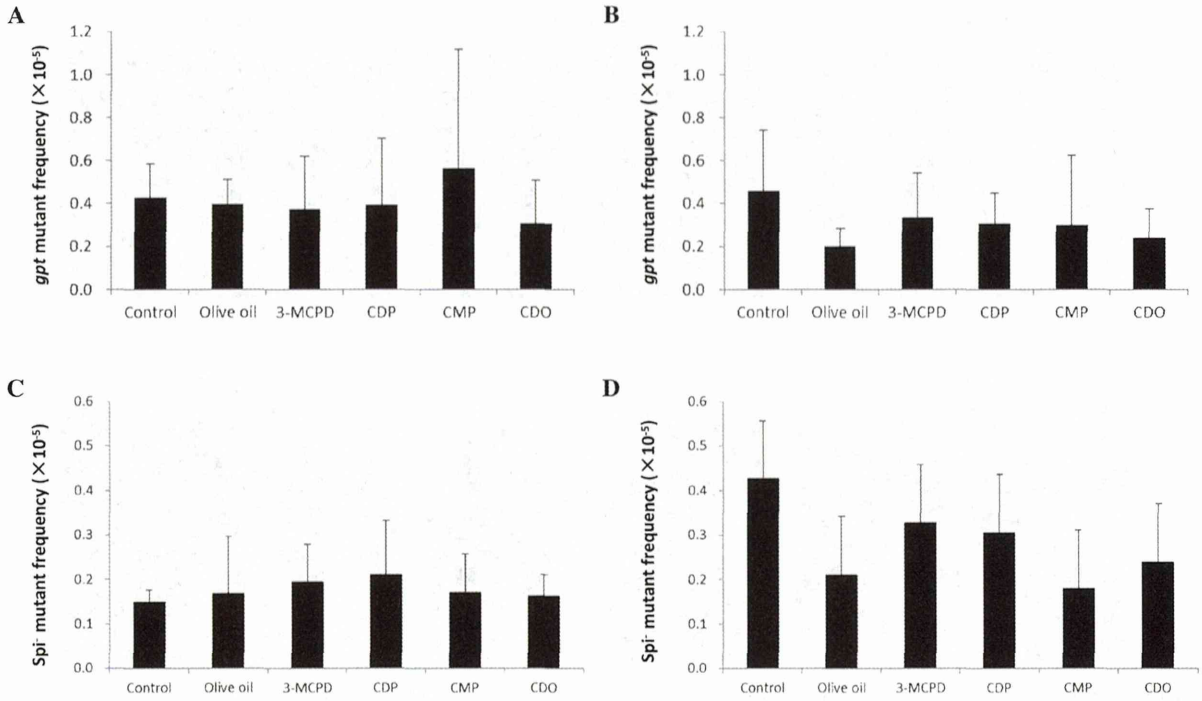

Fig. 5. *gpt* mutant frequencies in the (**A**) kidney and (**B**) testis, Spi⁻ mutant frequencies in the (**C**) kidney and (**D**) testis of F344 *gpt* delta rats treated with 3-MCPD or 3-MCPD esters for 4 weeks. The values represent the means of experiments ± standard deviations.

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.

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.

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