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In Vivo Genotoxicity of Methyleugenol in *gpt* Delta Transgenic Rats Following Medium-Term Exposure

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Methyleugenol (MEG), which is commonly used as a fragrance and flavoring agent, has been shown to induce hepatocellular tumors in rodents. However, the role of genotoxicity as a possible mechanism of action is not fully understood even though the DNA-reactive metabolite of MEG has been identified. In this study, a *gpt* delta transgenic rat model was used to clarify whether genotoxic mechanisms are involved in MEG-induced hepatocarcinogenesis following medium-term exposure. F344 *gpt* delta rats were subjected to repeated oral administration of MEG at dosages of 0, 10, 30, or 100 mg/kg (a carcinogenic dose) for 13 weeks. The relative weight of the liver of the male and female rats that were administered 100 mg/kg MEG and the absolute weight of the liver of the male rats that were administered 100 mg/kg MEG were significantly increased. In addition, the number and area of glutathione S-transferase placental form (GST-P) positive foci and proliferating cell nuclear antigen (PCNA) positive cell ratios in the hepatocytes were significantly increased in the male and female rats that were administered 100 mg/kg MEG compared with the control animals. In the *in vivo* mutation assays, a significant increase in the *gpt* and Spi$^-$ mutant frequencies was observed in both sexes at the carcinogenic dose. These results suggest the possible participation of genotoxic mechanisms in MEG-induced hepatocarcinogenesis.

Key Words: **methyleugenol; *in vivo* genotoxicity; *gpt* delta rats.**

Methyleugenol (3,4-dimethoxyallylbenzene; MEG) is a natural flavor present in many herbs and spices consumed at low levels in the human diet and has been approved for commercial use as a fragrance and flavoring agent. MEG is a member of the alkoxy-substituted allylbenzene family of chemicals, which include other naturally occurring substances, such as estragole and safrole. MEG is a hepatocarcinogen in F344 rats and B6C3F$_1$ mice at doses higher than 37 mg/kg, which is similar to the carcinogenic dose of other alkoxy-substituted allylbenzenes (National Toxicology Program [NTP], 2000). At the 69th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), a safety assessment of flavoring substances, including alkoxy-substituted allylbenzenes, was reported to be pending because the underlying mechanisms of their carcinogenic effects remained unclear (FAO/WHO, 2008). Data regarding the underlying modes of action in MEG-induced hepatocarcinogenesis are important for establishing an overall risk assessment of the flavoring agents classified as alkoxy-substituted allylbenzenes.

Zhou *et al*. (2007) reported that MEG forms adducts in human cellular DNA. Other alkoxy-substituted allylbenzenes, such as safrole and estragole, also form specific DNA adducts (Ishii *et al*., 2011; Miller and Miller, 1983), which may trigger genotoxicity in the rat liver (Jin *et al*., 2011; Suzuki *et al*., 2012). As such, it is highly probable that MEG has the potential to be genotoxic *in vivo* in the rat liver. However, MEG was not shown to be mutagenic in *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 (NTP, 2000). Several MEG metabolites have been reported to be activated to DNA-reactive mutagens in *S. typhimurium* following incorporation of increased sulfation capacity and may be involved in carcinogenicity, organ toxicity, and immune reactions (Cartus *et al*., 2012; Herrmann *et al*., 2012). The results of conventional genotoxicity tests, such as sister chromatid exchange (SCE), the micronucleus test, and the comet assay, have not been consistent (Ding *et al*., 2011; NTP, 2000).

The *gpt* delta transgenic rat developed by Nohmi *et al*. (1996) is recognized as an *in vivo* mutation assay model that is capable of detecting point mutations by 6-thioguanine (6-TG) selection and deletion mutations by Spi$^-$ selection (Hayashi *et al*., 2003; Masumura *et al*., 2003; Umemura *et al*., 2009). Quantitative analyses of glutathione S-transferase placental form (GST-P) positive foci can be performed in *gpt* delta transgenic rats and conventional non-transgenic rats (Toyoda-Hokaiwado *et al*., 2010). The *gpt* delta rat animal model may be a promising tool for investigating the comprehensive toxicities, including the genotoxicity and carcinogenicity, of chemical agents in target organs. We confirmed the availability of a medium-term animal model using *gpt* delta rats that demonstrated hepatotoxicity, hepatocarcinogenicity, and genotoxicity following exposure to safrole in the target organ (Jin *et al*., 2011).

In this study, the *gpt* delta rat animal model was used to determine the comprehensive carcinogenicity and *in vivo* genotoxicity of MEG at three different doses, including the reported carcinogenic dose, following medium-term exposure.

MATERIALS AND METHODS

Chemicals. MEG and methylcellulose were purchased from Wako Pure Chemical Industries (Osaka, Japan). The MEG solutions were prepared in 0.5% aqueous methylcellulose.

Animals and housing conditions. The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences (Tokyo, Japan). Five-week-old male and female F344 *gpt* delta rats carrying approximately five tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan). The rats were housed in polycarbonate cages (three or four rats per cage) with hardwood chips for bedding in a conventional animal facility. The animals were maintained in a controlled environment with constant temperature (23 ± 2°C), humidity (55 ± 5°C), air changes (12 times per hour), and lighting (12 h light/dark cycle). The animals were allowed free access to a basal diet of CRF-1 commercial pellets (Charles River Japan, Kanagawa, Japan) and tap water.

Experimental design. After a 1-week acclimatization period, the animals were divided into four groups consisting of 10 male and 10 female F344 *gpt* delta rats per group. The rats were administered an intragastric dose of 0, 10, 30, or 100 mg/kg MEG daily for 13 weeks.

The clinical signs and general appearance of the rats were observed once per day. Body weight and food consumption were measured once per week. At the end of each 13-week treatment cycle, the animals were euthanized under deep anesthesia. The left lobes of the liver were fixed in neutral-buffered formalin for histopathological and immunohistopathological examination. The remaining liver was stored at −80°C for *in vivo* mutation assays.

In vivo *mutation assays.* 6-TG and Spi⁻ (insensitive P2 interference) selection assays were performed as previously described (Nohmi *et al*., 1996, 2000). Briefly, genomic DNA was extracted from the liver tissue, and lambda EG10 DNA (48 kb) was rescued as the lambda phage through *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *Escherichia coli* YG6020, which expresses Cre recombinase and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured onto plates containing chloramphenicol without 6-TG. The plates were incubated at 37°C for selection of 6-TG-resistant colonies, and the *gpt* mutant frequency (MF) was calculated by dividing the number of *gpt* mutants after clonal correction by the number of rescued phages. To characterize the *gpt* mutations, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified using PCR as previously described. The PCR products were analyzed with an Applied Biosystems 3730x1 DNA Analyzer (Applied Biosystems, Japan Ltd).

For Spi⁻ selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. The infected cells were mixed with molten lambda-trypticase agar plates. The following day, plaques (Spi⁻ candidates) were punched out with sterilized glass pipettes, and the agar plugs were suspended in SM buffer. To confirm the Spi⁻ phenotype of the candidates, the suspensions were spotted on three types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and spread with soft agar. The number of mutants that exhibited clear plaques on each plate was counted as confirmed Spi⁻ mutants. The Spi⁻ MF was calculated by dividing the number of Spi⁻ mutants by the number of rescued phages. In all of the *in vivo* mutations assays, positive DNA samples were included to ensure that the assay was functioning properly.

Immunohistochemical staining. Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical and Biological Laboratories Co., Ltd, Nagoya, Japan), which is a marker of preneoplastic lesions in the rat liver, and monoclonal anti–mouse proliferating cell nuclear antigen (PCNA) antibodies (1:100; Dako, Glostrup, Denmark) to evaluate cell proliferation activity using the avidin–biotin peroxidase complex method. The number (No./cm^2) and area (mm^2/cm^2) of the GST-P-positive foci (> 0.01 mm^2) and the total area of each liver section were measured using an IPAP image analyzer (Sumika Technos, Osaka, Japan) (Watanabe *et al*., 1994). The numbers of PCNA-positive cells per 600–800 intact liver cells from 10 different areas per animal were counted to determine the PCNA-positive ratio.

Statistics. The body weight measurements, food and water consumption, weight of the liver, GST-P-positive foci, PCNA-LI, *gpt* and Spi⁻ MFs, and various mutation frequencies in the spectrum analysis were expressed as the mean ± SD. Significant differences between the control and treated groups were determined using Dunnett's multiple comparison test (Dunnett, 1955) after ANOVA. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Body and Liver Weights and Histopathological Examination

The final body and liver weights are shown in Table 1. There was no suppression of body weight gain in the treated groups

TABLE 1
Final Body and Liver Weights of *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Number of animals	Final body weight (g)	Liver weight	
				Absolute (g)	Relative (g/100 g bw)
Male					
	Control	10	300.0 ± 17.4[a]	8.09 ± 0.49	2.45 ± 0.07
	10 mg/kg MEG	10	325.0 ± 10.4	8.22 ± 0.41	2.53 ± 0.08
	30 mg/kg MEG	10	322.4 ± 13.7	8.54 ± 0.41	2.65 ± 0.08
	100 mg/kg MEG	10	310.2 ± 35.4	9.43 ± 0.71*	3.09 ± 0.45*
Female					
	Control	10	189.0 ± 5.6	4.26 ± 0.22	2.26 ± 0.12
	10 mg/kg MEG	9	190.3 ± 8.3	4.34 ± 0.22	2.28 ± 0.14
	30 mg/kg MEG	9	187.6 ± 5.3	4.29 ± 0.22	2.29 ± 0.11
	100 mg/kg MEG	9	176.5 ± 9.7	4.38 ± 0.29	2.48 ± 0.08[#]

[a]Mean ± SD.
*Significantly different from the male control group at the levels of $p < 0.001$ (Dunnett's test).
**Significantly different from the female control group at the levels of $p < 0.001$ (Dunnett's test).

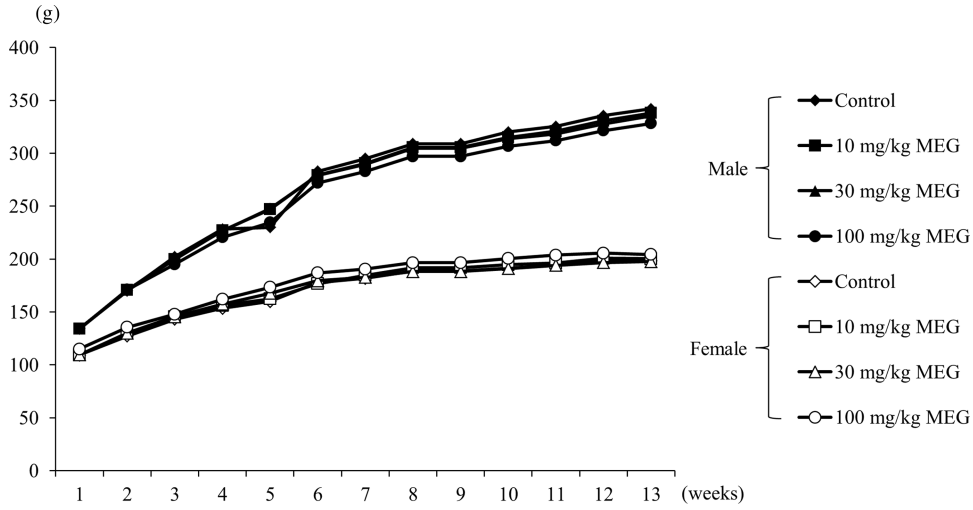

FIG. 1. Body weights of male and female *gpt* delta rats treated with MEG for 13 weeks.

TABLE 2
***gpt* MFs in the Liver of Male *gpt* Delta Rats Administered MEG for 13 Weeks**

Sex	Groups	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Male	Control	1	3.4	2	0.59	0.53 ± 0.18
		2	4.5	2	0.44	
		3	4.5	3	0.67	
		4	2.9	2	0.69	
		5	7.6	2	0.27	
	10 mg/kg MEG	11	9.5	4	0.42	0.79 ± 0.34
		12	4.8	6	1.25	
		13	3.0	2	0.66	
		14[a]	1.9	2	1.03[a]	
		15	5.3	3	0.57	
	30 mg/kg MEG	21	2.5	1	0.40	0.79 ± 0.42
		22	5.5	8	1.45	
		23	4.9	3	0.61	
		24	7.3	7	0.96	
		25	3.6	2	0.55	
	100 mg/kg MEG	31	6.0	12	2.20	1.35 ± 0.60*
		32	4.1	3	0.73	
		33	8.3	8	0.96	
		34	3.5	4	1.14	
		35	3.6	6	1.69	

[a]Data from animal No. 14 was excluded for the calculation of MF because of the poor packaging efficiency of the transgene.
*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

during the experiment (Fig. 1). The relative weight of the liver of the male and female rats treated with 100 mg/kg and the absolute weight of the liver of the males treated with 100 mg/kg MEG were significantly increased compared with the rats in the control group. There were no histopathological changes observed in the liver of the rats treated with MEG.

In Vivo *Mutation Assays*

The *gpt* and Spi^- MFs in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks are summarized in Tables 2–5. A significant increase in the *gpt* and Spi^- MFs was observed in the male and female rats treated with the carcinogenic dose of 100 mg/kg. We determined the *gpt* mutation spectra in the *gpt* mutant colonies to characterize the types of *gpt* mutations caused by exposure to MEG. A GC→CG transversion mutation in the treated male rats and an AT→TA transversion mutation in the treated female rats were observed, but the incidence rates were not statistically significant compared with the controls (Table 6).

Effects of MEG on GST-P-Positive Foci and Cell Proliferation

Under immunohistochemical examination, treatment with MEG increased the number and area of GST-P-positive foci

TABLE 3
***gpt* MFs in the Liver of Female *gpt* Delta Rats Administered MEG for 13 Weeks**

Sex	Groups	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ± SD
Female	Control	41	10.5	1	0.10	0.21 ± 0.13
		42	14.9	2	0.14	
		43	13.1	5	0.38	
		44	13.3	4	0.30	
		45	18.0	2	0.11	
	10 mg/kg MEG	51	6.0	3	0.50	0.38 ± 0.18
		52	7.5	4	0.53	
		54	6.2	3	0.48	
		55	10.7	3	0.28	
		56	8.8	1	0.11	
	30 mg/kg MEG	61	9.5	4	0.42	0.53 ± 0.19
		62	7.8	2	0.25	
		63	10.3	7	0.68	
		64	8.8	6	0.68	
		65	4.8	3	0.62	
	100 mg/kg MEG	71	8.9	10	1.12	1.23 ± 0.59*
		72	3.1	7	2.25	
		73	5.0	4	0.81	
		74	5.6	5	0.90	
		75	5.7	6	1.05	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

TABLE 4
Spi^- MFs in the Liver of Male *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Male	Control	1	7.3	1	0.14	0.32 ± 0.27
		2	4.1	1	0.25	
		3	5.9	1	0.17	
		4	8.4	6	0.72	
		5	8.6	1	0.12	
	10 mg/kg MEG	11	9.5	4	0.42	0.31 ± 0.11
		12	7.6	2	0.27	
		13	6.9	3	0.44	
		14	8.8	2	0.23	
		15	10.4	2	0.19	
	30 mg/kg MEG	21	7.8	3	0.38	0.41 ± 0.24
		22	8.8	2	0.23	
		23	6.8	1	0.15	
		24	7.2	5	0.70	
		25	6.7	4	0.60	
	100 mg/kg MEG	31	7.0	5	0.72	0.85 ± 0.40*
		32	6.9	2	0.29	
		33	7.8	10	1.29	
		34	4.2	5	1.20	
		35	4.1	3	0.74	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

in a dose-dependent manner compared with the control group, although the differences were not statistically significant at doses lower than 30 mg/kg (Fig. 2). The effects of MEG on cell proliferation were evaluated using immunohistochemistry for PCNA (Fig. 3). The ratio of PCNA-positive hepatocytes was significantly increased in the male and female rats treated with 100 mg/kg MEG.

DISCUSSION

MEG, a flavoring agent classified as an alkoxy-substituted allylbenzene, is present in a variety of foods, spices, teas, and essential oils. Several flavoring agents that are classified into the same chemical category, such as estragole and safrole, have been reported to be hepatocarcinogenic in rodents (Borchert

TABLE 5
Spi⁻ MFs in the Liver of Female *gpt* Delta Rats Administered MEG for 13 Weeks

Sex	Groups	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2)	MF ($\times 10^{-5}$)	Mean ± SD
Female	Control	41	17.9	2	0.11	0.15 ± 0.07
		42	24.8	2	0.08	
		43	16.5	4	0.24	
		44	20.9	4	0.19	
		45	35.1	4	0.11	
	10 mg/kg MEG	51	9.2	0	0.00	0.15 ± 0.10
		52	18.6	4	0.22	
		54	11.3	2	0.18	
		55	18.7	2	0.11	
		56	16.1	4	0.25	
	30 mg/kg MEG	61	18.9	5	0.27	0.20 ± 0.09
		62	13.1	4	0.31	
		63	20.1	2	0.10	
		64	14.0	2	0.14	
		65	11.2	2	0.18	
	100 mg/kg MEG	71	9.5	7	0.74	0.33 ± 0.26*
		72	9.3	1	0.11	
		73	6.9	3	0.43	
		74	10.0	2	0.20	
		75	10.5	2	0.19	

*Significantly different from the control group at $p < 0.05$ (Dunnett's test).

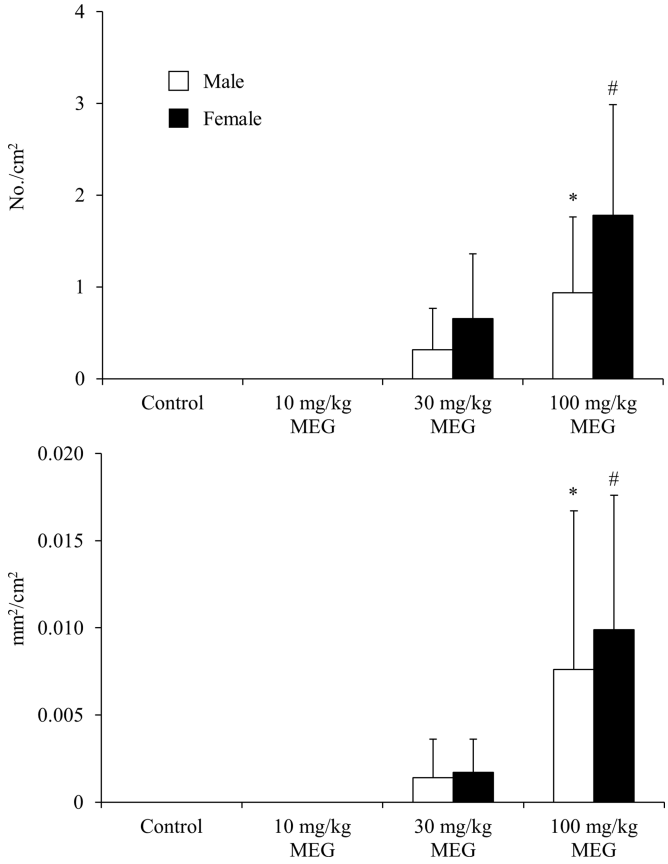

FIG. 2. Number and area of GST-P-positive foci (more than three cells) in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks. Data represent the mean ± SD. * indicates a significant difference from the male controls ($p < 0.05$) using Dunnett's test. # indicates a significant difference from the female controls ($p < 0.05$) using Dunnett's test.

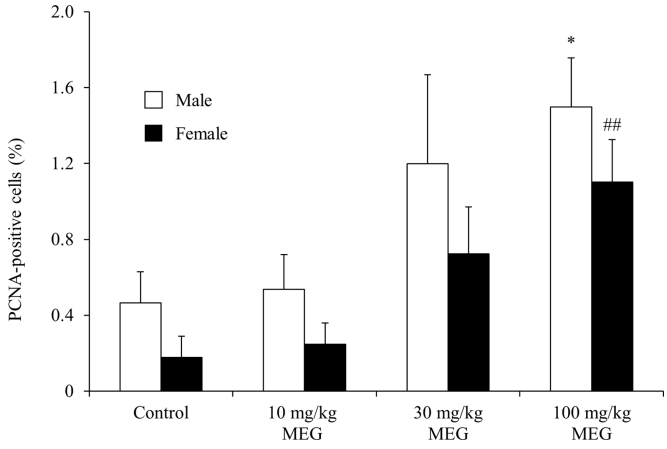

FIG. 3. Immunohistochemical staining of PCNA in the liver of male and female *gpt* delta rats treated with MEG for 13 weeks. Data represent the mean ± SD. * indicates a significant difference from the controls ($p < 0.05$) using Dunnett's test. ## indicates a significant difference from the female controls ($p < 0.01$) using Dunnett's test.

et al., 1973; Drinkwater *et al.*, 1976; IARC, 1976; Wislocki *et al.*, 1977). However, conventional mutagenicity tests have failed to demonstrate definitive results due to exposure to these chemicals (Drinkwater *et al.*, 1976; IARC, 1976; Natarajan and Darroudi, 1991; NTP, 2000; To *et al.*, 1982) even though positive results were reported using *S. typhimurium* that was modified to contain human and/or murine sulfotransferases (Herrmann *et al.*, 2012). As a result, the safety assessment of flavoring agents, including the alkoxy-substituted allylbenzenes, was pending at the 69th meeting of the JECFA. However, the Scientific Committee on Food concluded that alkenylbenzenes, such as safrole, MEG, and estragole, have genotoxic and carcinogenic effects (van den

TABLE 6
Mutation Spectra of *gpt* Mutant Colonies in the Liver of F344 *gpt* Delta Rats Administered MEG

Sex		Control		10 mg/kg MEG		30 mg/kg MEG		100 mg/kg MEG	
		Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Male	*Base substitution*								
	Transversions								
	GC-TA	3[a] (37.5)	0.16±0.16	2 (18.2)	0.08±0.17	3 (15.0)	0.09±0.13	4 (15.4)	0.19±0.17
	GC-CG	0	0	2 (18.2)	0.12±0.22	1 (5.0)	0.04±0.08	5 (19.2)	0.20±0.23
	AT-TA	0	0	0	0	3 (15.0)	0.10±0.16	0	0
	AT-GC	1 (12.5)	0.04±0.10	1 (9.1)	0.02±0.05	0	0	1 (3.8)	0.04±0.08
	Transitions								
	GC-TA	3 (37.5)	0.12±0.11	3 (27.3)	0.15±0.21	7 (35.0)	0.31±0.09	8 (30.8)	0.29±0.21
	AT-GC	1 (12.5)	0.04±0.10	2 (18.2)	0.06±0.09	4 (20.0)	0.16±0.10	4 (15.4)	0.15±0.17
	Deletion								
	Single base pair	0	0	1 (9.1)	0.07±0.15	2 (10.0)	0.06±0.09	1 (3.8)	0.06±0.13
	Over 2 bp	0	0	0	0	0	0	1 (3.8)	0.04±0.08
	Insertion	0	0	0	0	0	0	0	0
	Complex	0	0	0	0	0	0	2 (7.7)	0.09±0.13
	Total	8	0.53±0.18	11	0.79±0.34	20	0.79±0.42	26	1.35±0.60*
Female	*Base substitution*								
	Transversions								
	GC-TA	2 (18.2)	0.03±0.04	1 (9.1)	0.03±0.07	3 (16.7)	0.19±0.25	5 (17.9)	0.23±0.32
	GC-CG	1 (9.1)	0.01±0.03	2 (18.2)	0.06±0.08	0	0	2 (7.1)	0.13±0.29
	AT-TA	0	0	0	0	2 (11.1)	0.04±0.09	2 (7.1)	0.10±0.15
	AT-GC	0	0	0	0	1 (5.6)	0.02±0.04	0	0
	Transitions								
	GC-TA	4 (36.4)	0.06±0.07	3 (27.3)	0.08±0.08	9 (50.0)	0.23±0.18	6 (21.4)	0.21±0.17
	AT-GC	1 (9.1)	0.02±0.03	2 (18.2)	0.06±0.08	2 (11.1)	0.05±0.06	8 (28.6)	0.24±0.28
	Deletion								
	Single base pair	2 (18.2)	0.06±0.07	0	0	1 (5.6)	0.03±0.06	0	0
	Over 2 bp	0	0	2 (18.2)	0.06±0.08	0	0	2 (7.1)	0.07±0.16
	Insertion	0	0	1 (9.1)	0.02±0.04	0	0	1 (3.6)	0.04±0.09
	Complex	1 (9.1)	0.02±0.03	0	0	0	0	2 (7.1)	0.10±0.15
	Total	11	0.21±0.13	11	0.38±0.18	18	0.53±0.19	28	1.23±0.59*

[a]Number of colonies with independent mutations.
*Significantly different from the control group at $p < 0.05$.

Berg *et al*., 2011). It has been reported that chronic oral intake of more than 37 mg/kg of MEG induced neoplasms in the liver and stomach of F344/N rats and B6C3F$_1$ mice (NTP, 2000). MEG has the potential to form a 1′-hydroxy metabolite and an epoxide on the alkenyl side chain, which are similar to the chemical changes induced by safrole. Al-Subeihi *et al*. (2012) reported that 1′-hydroxymethyleugenol glucuronide, 1′-oxomethyleugenol, and 1′-sulfooxymethyleugenol in the liver of humans and male rats were observed to be metabolites of MEG. In addition, the use of physiologically based biokinetic models to evaluate safrole, estragole, and MEG showed that the three alkenylbenzenes used similar processes to bioactivate their ultimate carcinogenic 1′-sulfoxy metabolites (Martati *et al*., 2011). Furthermore, Chan and Caldwell (1992) reported that 1′-hydroxymethyleugenol, a metabolite of MEG, is a strong inducer of unscheduled DNA synthesis in cultured rat hepatocytes. The 1′-hydroxymetabolite and corresponding sulfate esters of allyl alkoxybenzene substances have been shown to form DNA adducts *in vivo* and *in vitro*. Herrmann *et al*. (2012) reported that hydroxylated metabolites of MEG formed the same DNA adducts as high levels of N(2)-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine and modest levels of N(6)-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyadenosine in *S. typhimurium* TA100-hSULT1A1 and that positive results were demonstrated in the Ames test. However, these adducts did not form in the wild-type strain, which is consistent with previous results of the micronucleus assay and several Ames tests (NTP, 2000; Sekizawa and Shibamoto, 1982). In this study, we demonstrated a significant increase in the *gpt* and Spi$^-$ MFs in male and female rats treated with 100 mg/kg MEG, which is considered a carcinogenic dose. GC→CG and AT→TA transversion mutations were observed in the treated rats, although the incidence or mutation frequencies were not significant compared with the control group. In contrast to the outcome of conventional mutagenicity tests, the positive results of the reporter gene mutation assay indicate that *in vivo* metabolism at the target site is necessary to induce MEG genotoxicity. The numbers of DNA modifications in Herrmann

et al. (2012) were not directly reflected in the mutation spectra in the *gpt* mutant colonies, which may be due to the use of different experimental systems.

In this study, the mean number and area of GST-P-positive foci were significantly increased in the liver of male and female rats treated with 100 mg/kg MEG compared with the controls. The mean number and area of GST-P-positive foci in rats in the 30 mg/kg group were slightly increased, but the results were not statistically significant. A previous study reported that the incidence of hepatocellular carcinoma was significantly increased in F344 rats and B6C3F1 mice treated with MEG at doses of 37 mg/kg and higher (NTP, 2000). The data on the quantitative analysis of GST-P-positive foci using *gpt* delta transgenic rats appear to be similar to the carcinogenicity data previously reported by NTP (2000). The quantitative analysis of PCNA-positive hepatocytes, which are an indicator of cell proliferation activity, demonstrated that there were significant increases in the ratio of PCNA-positive hepatocytes at a dose of 100 mg/kg in male and female rats, but not at doses of 30 mg/kg and lower. Similar dose-related changes were shown using the *in vivo* mutation assay. According to a previous report (Smith *et al*., 2002), MEG-specific DNA adducts have not been detected at doses lower than 10 mg/kg. These data suggest that DNA modifications under conditions of increased cell proliferation may be required to induce gene mutations and lead to tumor formation. Although alternate modes of action have not been examined, the present data show that genotoxic mechanisms may contribute to MEG-induced hepatocarcinogenesis.

In conclusion, the MEG dose that induces preneoplastic lesions in the liver resulted in *in vivo* genotoxicity in the reporter gene mutation assay. The data presented in this study provide valuable information regarding the development of risk assessments for the flavoring agents classified as alkoxy-substituted allylbenzenes.

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