Table 3 Quantitative data for GST-P positive liver cell foci found in F344 rats given estragole or DEN

	4 weeks	8 weeks	16 weeks
Number (No./cm^2)			
Control	ND	ND	0.08 ± 0.19
Estragole	9.48 ± 2.67[a]	60.57 ± 10.91	84.83 ± 34.41*
DEN	–[b]	–	34.91 ± 10.10*
Area (mm^2/cm^2)			
Control	ND	ND	0.00 ± 0.00[c]
Estragole	0.03 ± 0.02	0.33 ± 0.08	4.27 ± 3.50*
DEN	–	–	0.02 ± 0.01*

ND not detected

* $P < 0.01$ versus control

[a] Means ± S.D

[b] Not examined

[c] Actual value was about 0.0003 ± 0.0007 mm^2/cm^2

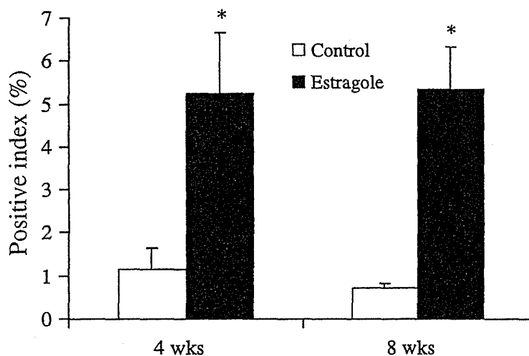

Fig. 2 PCNA-positive indices (%) of normal-looking hepatocytes in the livers of F344 rats treated with 600 mg/kg bw estragole for 4 and 8 weeks (mean ± SD). *$P < 0.01$ versus control

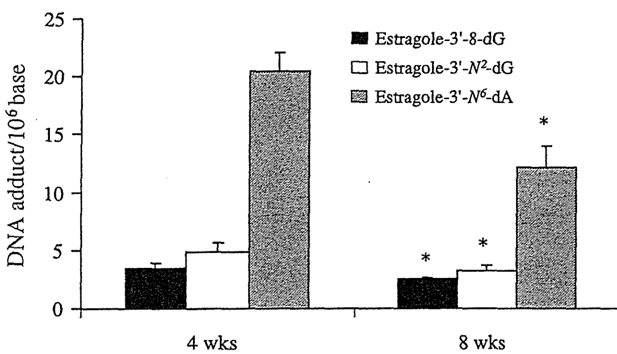

Fig. 3 Hepatic estragole-DNA adduct levels in F344 rats treated with 600 mg/kg bw estragole for 4 and 8 weeks. Each point represents the mean ± SD data from five rats. *$P < 0.01$ versus 4 weeks

Histopathologically, minimal lymphocytic aggregation was observed in three of five animals of the 200 mg/kg bw group. Immunohistochemical expression for the cell proliferation marker PCNA is summarized in Fig. 5. PCNA-positive indices (%) in the liver were significantly ($P < 0.01$) increased in the 200 mg/kg bw group as compared to the control group. Rats dosed with less than 66 mg/kg showed no increase in cell proliferative activity caused by treatment.

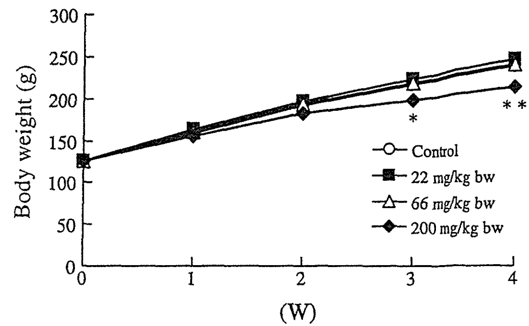

Fig. 4 Growth curves for *gpt* delta rats treated with 0, 22, 66 or 200 mg/kg estragole for 4 weeks. *, **$P < 0.01$, 0.05 versus control

The ES-specific DNA adducts, ES-3′-8-dG, 3′-N^2-dG and 3′-N^6-dA, in the rat liver treated with 0, 22, 66 or 200 mg/kg bw ES for 4 weeks were also assessed by the same method as in Experiment I. ES-DNA adducts were found in ES-treated rats, but were not detected in controls (Fig. 6). 3′-N^6-dA was predominant in the groups given more than 66 mg/kg bw, whereas 3′-N^2-dG was predominant only in the 22 mg/kg bw group. While 3′-8-dG and 3′-N^6-dA in the ES-treated rats were increased in a clear dose-dependent manner, 3′-N^2-dG was increased without dose dependence.

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 5. The MFs (1.09 ± 0.26 x 10^5) in the 200 mg/kg bw group were significantly ($P < 0.01$) higher than the control value (0.49 ± 0.18 × 10^5) but in a dose-dependent manner. As a reference data, *gpt* MFs from survived 2 rats in the 600 mg/kg bw group were much higher (2.96 × 10^5 and 2.89 × 10^5) as compared to the other groups. The characterization of *gpt* mutations induced by ES exposure was analyzed by DNA sequencing of mutant colonies (Table 6). In the 200 mg/kg bw groups, A:T base pair was the preferable site for mutation, accounting for 43.5 % (10/23) of the mutations. Specific mutation frequencies of AT:GC transitions were significantly ($P < 0.05$) higher in the 200 mg/kg bw group than in the control group.

Discussion

In the present study, GST-P-positive liver cell foci were increased in ES-treated rats in the early period, correlating well with increase in relative liver weights. Because GST-P-positive foci are established rat liver preneoplastic lesions detectable for the medium-term assay (Ito et al. 1988; Tsuda et al. 2003), our results suggest that ES may

Table 4 Mean body and liver weights of *gpt* delta rats treated with estragole for 4 weeks

Dose (mg/kg bw)	Rats	Body weights (g)	Absolute weights (g)	Relative weights (g%)
0	5	236.9 ± 10.6[a]	9.67 ± 0.53	4.09 ± 0.19
22	5	243.8 ± 12.1	10.43 ± 1.04	4.27 ± 0.24
66	5	237.0 ± 6.4	10.20 ± 0.30	4.30 ± 0.16
200	5	212.1 ± 5.0*	9.60 ± 0.35	4.53 ± 0.13*
		(−11 %)		(+11 %)

Values in the parenthesis indicate the percentage of change against the mean control values (+: increase, −: decrease)

* $P < 0.01$ versus control

[a] Means ± SD

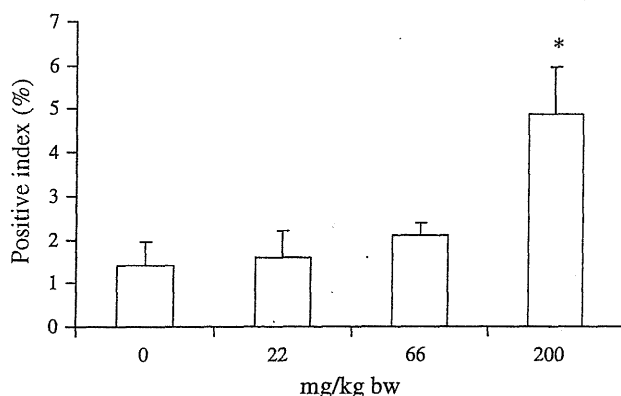

Fig. 5 PCNA-positive indices (%) in the livers of *gpt* delta rats treated with 0, 22, 66 or 200 mg/kg bw estragole for 4 weeks (mean ± SD). *$P < 0.01$ versus control

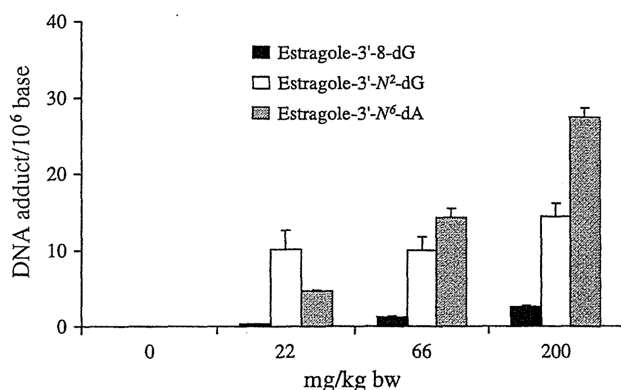

Fig. 6 Hepatic estragole-DNA adduct levels in *gpt* delta rats treated with 0, 22, 66 or 200 mg/kg bw estragole for 4 weeks. Each point represents the mean ± SD of data from five rats

also exert hepatocarcinogenicity in rats, in addition to mice. The fact that the cell proliferation activity assessed using PCNA immunohistochemistry was increased in the present study also indicates that ES could exhibit tumor-promotion activity. Interestingly, PCNA-positive cell ratios at week 4 were almost the same as those at week 8, implying sustained cell proliferation at least during this period. At the same time, ES-specific DNA adducts such as ES-3′-8-dG, 3′-N^2-dG and predominantly 3′-N^6-dA were consistently detected in rat livers treated with ES, suggestive of genotoxic initiation activity. Since 8-week-treated rats demonstrated lower DNA adduct levels than 4-week-treated rats, DNA adduct might not accumulate over time, rather being repaired.

The overall results in the present study using wild F344 rats are in good agreement with those in the NTP 3-month toxicity study of ES (NTP 2011) in terms of liver weight and histopathological changes. However, in comparison with wild-type rats, it is likely that *gpt* delta rats may be somewhat more susceptible to toxicity of ES judging from lethality, liver weight change and cell proliferative activity. It can be said that the duration of exposure is not long enough to fully assess the carcinogenicity, and the dose was somewhat too high to evaluate systemic toxicity. However, the medium-term bioassay for rat hepatocarcinogenesis using GST-P immunohistochemistry is aimed at screening potent hapetocarcinogenicity but not strictly assessing repeated dose toxicity. Therefore, it is suggested that ES could be a possible hepatocarcinogen.

In the in vivo genotoxicity assay with *gpt* delta rats, MFs were significantly increased in rats treated with 200 mg/kg bw ES in a dose-dependent manner. Because normal ranges are relatively stable compared to the other models as reported previously (OECD 2011), statistically significant twofold increase in MFs in the 200 mg/kg was considered to be biologically meaningful. This is important, considering that ES-specific DNA adduct formation is implicated in hepatocarcinogenesis (Wiseman et al. 1985; Fennell et al. 1985). It is well known that DNA adducts can be repaired rather than becoming fixed as mutations. However, if the balance between DNA adduct formation and DNA damage removal is disturbed, the DNA adducts accumulate and may be fixed as mutations (de Vries et al. 1997). Taken together with the results of the mutation spectrum analysis and the quantitative ES-DNA adduct analysis, it is conceivable that increased MF in the 200 mg/kg bw group was due to the increase in AT-base pair mutation resulting from the ES-3′-N^6-dA adduct formation. This fact

Table 5 *gpt* mutant frequencies in the livers of *gpt* delta rats treated with estragole for 4 weeks

Dose (mg/kg bw)	Animal no.	Cm^R colonies ($\times 10^5$)	$6\text{-}TG^R$ and Cm^R colonies	Mutant frequency[a] ($\times 10^{-5}$)	Mean ± SD
0	101	3.29	2	0.61	
	102	6.98	3	0.43	
	103	ND	ND	ND	
	104	3.83	1	0.26	
	105	4.59	3	0.65	0.49 ± 0.18[b]
22	201	5.76	3	0.52	
	202	4.41	5	1.13	
	203	7.29	4	0.55	
	204	3.65	3	0.82	
	205	6.93	5	0.72	0.75 ± 0.25
66	301	4.05	4	0.99	
	302	7.61	3	0.39	
	303	3.24	3	0.93	
	304	6.89	4	0.58	
	305	3.29	4	1.22	0.82 ± 0.33
200	401	3.11	3	0.97	
	402	5.31	8	1.51	
	403	4.05	4	0.99	
	404	3.65	3	0.82	
	405	4.28	5	1.17	1.09 ± 0.26*

ND not detected

* $P < 0.01$ versus control

[a] Mutant frequency = the number of $6\text{-}TG^R$ and Cm^R colonies/the number of Cm^R colonies

[b] Means ± SD

Table 6 Mutation spectra of *gpt* mutant colonies detected in *gpt* delta rat livers given estragole for 4 weeks

	Control		22 mg/kg bw		66 mg/kg bw		200 mg/kg bw	
	No.	Mutation frequency[a] ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)
Base substitutions								
Transversions								
GC:TA	3[b]	0.13 ± 0.15[c]	9	0.35 ± 0.18	1	0.03 ± 0.06	4	0.21 ± 0.13
GC:CG	0	0.00 ± 0.00	0	0.00 ± 0.00	3	0.13 ± 0.21	3	0.13 ± 0.21
AT:TA	0	0.00 ± 0.00	0	0.00 ± 0.00	1	0.03 ± 0.06	3	0.13 ± 0.18
AT:CG	0	0.00 ± 0.00	1	0.05 ± 0.10	0	0.00 ± 0.00	0	0.00 ± 0.00
Transitions								
GC:AT	2	0.13 ± 0.15	6	0.22 ± 0.06	8	0.36 ± 0.19	3	0.16 ± 0.15
AT:GC	2	0.13 ± 0.15	1	0.03 ± 0.06	3	0.15 ± 0.26	7	0.34 ± 0.10*
Deletions								
Single bp	2	0.10 ± 0.13	1	0.03 ± 0.08	2	0.12 ± 0.17	1	0.05 ± 0.10
Over 2 bp	0	0.00 ± 0.00	1	0.05 ± 0.10	0	0.00 ± 0.00	0	0.00 ± 0.00
Insertions	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00
Complexes	0	0.00 ± 0.00	1	0.03 ± 0.06	0	0.00 ± 0.00	2	0.08 ± 0.17

* $P < 0.05$ versus control

[a] Specific mutation frequency = the number of specific mutations/the number of Cm^R colonies

[b] The number of colonies with independent mutations after clonal correction

[c] Means ± SD

is in line with statistically significant increase in MFs in the 200 mg/kg. Moreover, cell proliferation is thought to play an important role in fixation of DNA lesions into mutations (de Vries et al. 1997). Based on the results of histopathological analysis and PCNA immunohistochemical analysis, it is considered that the enhancement of cell proliferation subsequent to liver parenchymal damage due to ES treatment may have contributed to the significant increase in MF at higher doses of ES.

It has been reported that ES-3′-N^2-dG is a major adduct derived from ES exposure (Punt et al. 2007; Wiseman et al. 1985; Phillips et al. 1981), but in the current experiment, hepatic levels did not clearly correlate with the dose of ES. Rather, the liver DNA adduct level of ES-3′-N^6-dA was much higher than that of ES-3′-N^2-dG in the 200 mg/kg bw ES-treated rat, clearly indicating that ES-3′-N^6-dA is a predominant adduct formed under the present experimental conditions. However, the possibility that this difference may result from variation in the experimental design cannot be ruled out. Namely, previous studies used mouse livers after treatment with a single intraperitoneal injection of ES and assessed by a ^{32}P-post-labeling assay (Randerath et al. 1984; Phillips et al. 1984), whereas herein our study was investigated in rat livers repeatedly treated by gavage and then evaluated by an LC-MS/MS method. ES-3′-8-dG was consistently lower in level than other DNA adducts, but the dose-dependent increase may suggest some role in in vivo genotoxicity.

Although MFs of the *gpt* gene were not significantly increased at less than 66 mg/kg bw ES treatments, MFs tended to be elevated dose-dependently, suggesting possible genotoxicity even at low doses, given the DNA adduct levels clearly showed a dose-dependent increase in the prevailing paradigm of the absent threshold for genotoxic carcinogens. Interestingly, PCNA immunohistochemistry showed that proliferation was not affected by ES treatment in the lower-dose groups. It was similarly reported that DNA adduct formation but no mutations were detected in rats given a genotoxic carcinogen MeIQx at low doses (Fukushima et al. 2009). It is well known that DNA adducts and/or mutated cells are removed by the kinetics of DNA repair and apoptosis during cell turnover (Kuraoka 2008). Therefore, genotoxicity of ES at such lower doses may not be enough to cause mutation at least in 4-weeks administration.

When extrapolating the data from high-dose animal experiments to the low-dose human situation, it is important to take species differences in metabolism and metabolic activation into account (Rietjens et al. 2005). It was reported that there was a minor influence of species differences between human and rat on the ultimate overall bioactivation of ES to its ultimate carcinogenic DNA reactive metabolite 1′-sulfooxyestragole (Punt et al. 2009). The estimated average human daily intake of ES is evaluated as 0.01–0.07 mg/kg bw in FEMA and SCF. It has been reported that ES-specific DNA adduct formation is predicted to be liner with increasing dose of ES by a physiologically based biodynamic model (Paini et al. 2010). Taken together with our data showing that ES-specific DNA adduct levels were shown in a dose-dependent manner at 22 mg/kg bw to 200 mg/kg bw, ES-DNA adducts may be formed even in very low-dose human situation. However, the possible involvement of other factors in the fixation of DNA lesions to give mutations must be taken into consideration for human risk assessment. In conclusion, the results in the present study suggest that ES may be a possible genotoxic hepatocarcinogen in rats at high doses. However, further studies are needed to critically assess the human risk of ES-genotoxicity and/or carcinogenicity.

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Conflict of interest We declare that we have no conflict of interest.

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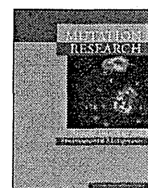

Possible involvement of sulfotransferase 1A1 in estragole-induced DNA modification and carcinogenesis in the livers of female mice

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ABSTRACT

Estragole (ES), a natural organic compound, is frequently used as a flavoring in food even though it is a hepatocarcinogen in mice. Although formation of ES-specific DNA adducts following conversion from ES to the nucleophilic metabolite by sulfotransferase 1A1 (SULT1A1) has been reported, the modes of action underlying ES-induced hepatocarcinogenesis remain uncertain because conventional genotoxicity tests for ES yield negative results. In the present study, taking notice of the fact that there is a sex difference in SULT1A1 activity in the mouse liver, we assessed the frequency of micronuclei in polychromatic erythrocytes and the mutant frequency (MF) of reporter genes in female *gpt* delta mice treated with ES at doses of 0 (corn oil), 37.5, 75, 150 or 300 mg/kg body weight (bw) by gavage for 13 weeks. Results were compared with those obtained in males. Since one female was found dead at week one, the highest dose was reduced to 250 mg/kg bw in females from week two. As reported previously in C57BL/6 mice, the mRNA levels of *Sult1a1* in female *gpt* delta mice were significantly higher than those in the males. The levels of ES-specific DNA adducts in the females were higher than those in the males at all doses except the highest dose. In addition, MFs of the *gpt* gene were significantly increased from doses of 75 mg/kg bw of females, but the increment was observed only at the highest dose in males. There were no changes in the micronucleus test among the groups. Thus, the overall data suggest that specific DNA modifications by the SULT1A1-mediated carbocation formation and the resultant genotoxicity are key events in the early stage of ES-induced hepatocarcinogenesis of mice.

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1. Introduction

Estragole (ES) is an allylbenzene compound that is a natural constituent of several herbs, including basil, fennel, anise and tarragon and has been used as a flavoring agent. However, it has been found to be a hepatocarcinogen in various strains of mice [1–3]. Following intraperitoneal injections of ES, specific DNA adducts were detected in the livers of adult CD-1 female mice or newborn $B6C3F_1$ male mice [4,5]. It has been reported that allylbenzene analogues are mainly catalyzed by CYP1A2 and sulfotransferase 1A1 (SULT1A1), in the liver and metabolized to the ultimate carcinogenic metabolite that is capable of binding covalently to DNA [6,7]. Nevertheless, ES has been shown to be non-mutagenic in reverse mutation assays using *Salmonella typhimurium*, *Escherichia coli* WP2 uvrA and *Bacillus subtilis* [8,9] except for TA1535 of *S. typhimurium* without metabolic activation [10]. In *in vivo* assays, it was found that the UDS (unscheduled DNA synthesis) assay in rats was positive while a rat bone marrow micronucleus test was negative [11]. Thus, ES genotoxicity has remained equivocal. However, the fact that a potent sulfotransferase inhibitor, pentachlorophenol (PCP), strongly inhibits hepatocarcinogenicity in mice along with covalent binding of the ultimate carcinogenic metabolite to hepatic macromolecules [12,13] suggests a possible participation of DNA modification in ES-induced hepatocarcinogenesis.

We used *gpt* delta mice in the present study. The assays with this transgenic mouse permit quantitative measurements of mutant frequencies (MFs) of the two transgenes, *i.e. gpt* and *red/gam*, in multiple tissues/organs and molecular analysis of induced and spontaneous mutations by DNA sequencing analysis [14]. Additionally, since the assays can include *in vivo* metabolism, it may be a

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useful tool to explore *in vivo* mutagenicity of chemicals requiring metabolic activation to exert their genotoxicities. Moreover, the levels of ES-specific DNA adducts in the livers can be quantified with our established method using LC–MS/MS [15] together with an *in vivo* mutation assay.

In the present study, to clarify the modes of action underlying ES-induced hepatocarcinogenesis, male and female *gpt* delta mice were given ES at carcinogenic doses for 13 weeks. We assessed the quantitative levels of ES-specific DNA modifications with LC–MS/MS methods, MFs of reporter genes and frequencies of micronuclei in polychromatic erythrocytes in the bone marrows of mice. Considering that there are sex differences in sulfotransferase activity in mouse liver, we compared the results obtained in male and female mice.

2. Materials and methods

2.1. Chemicals and animals

ES (CAS No. 140-67-0) was purchased from Tokyo Kasei (Tokyo, Japan). Male and female B6C3F$_1$ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome status were raised by mating C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Fifty male and 50 female B6C3F$_1$ *gpt* delta mice were each randomized by weight into five groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature, 23 ± 2 °C; relative humidity, 55 ± 5%; ventilation frequency, 18 times/h; 12 h light:12 h dark cycle, with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo) and tap water.

2.2. Animal treatment and sample collection

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Starting at six weeks of age, the mice received ES in corn oil by gavage at doses of 37.5, 75, 150, or 300 mg/10 mL/kg body weight (bw) (250 mg/10 mL/kg bw from week two in the females) five days per week for 13 weeks. Vehicle control animals (0 mg/10 mL/kg bw) received corn oil only. The doses of 150 and 300 mg/kg bw were carcinogenic doses for female mice reported in a 20-month carcinogenicity study [2]. All mice were euthanized 24 h after the last administration. Ten *gpt* delta mice were used to compare the toxicity of ES to that in the wild type mice observed in the previous report [16]. For other analyses (DNA adducts, micronuclei, *gpt* and Spi$^-$ mutations, hepatic *Cyp1a2* and *Sult1a1* mRNA levels), the first five animals each group were selected.

The livers were immediately removed and weighed; slices were fixed in buffered formalin for hematoxylin and eosin (H&E) staining. Histopathological examination was performed using non-coded slides. A part of the liver was preserved with an ISOGEN kit (Nippon Gene, Tokyo) and stored at −80 °C until used to isolate total RNA. Remaining pieces of the liver were frozen with liquid nitrogen and stored at −80 °C until performance of mutation assays and DNA adduct measurements. Bone marrow, from epiphyseal cartilage decapitated femurs, was flushed out with fetal bovine serum (Invitrogen, Carlsbad, CA) for the micronucleus (MN) test.

2.3. Quantitative real time RT-PCR

Total RNA from the liver was isolated with the ISOGEN kit according to the manufacturer's instructions. RNA reverse transcription with random hexamers was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR was performed on an Applied Biosystems 7900HT FAST Real-Time PCR Systems (Applied Biosystems) with TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), TaqMan® Gene Expression Assays (Applied Biosystems) and TaqMan® Rodent *GAPDH* control reagents (Applied Biosystems). The assayed genes included cytochrome P450 family 1, subfamily A, polypeptide 2 (*Cyp1a2*) and sulfotransferase family 1A, phenol-preferring, member 1 (*Sult1a1*). Rodent glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The expression levels of the target genes *Cyp1a2* and *Sult1a1* were evaluated by a relative standard curve method.

2.4. Quantification of ES-DNA adduct levels in livers

The levels of ES-specific DNA adducts in livers were quantified with our newly established method using LC–MS/MS [15]. Briefly, samples were homogenized with the lysis buffer included in a commercial DNA isolation kit. The mixture was centrifuged, and the deposit was dissolved in enzyme reaction buffer. After treatment with RNase and protease K, a DNA pellet was obtained. The dried DNA pellet was dissolved in surrogate standard solution containing sodium acetate buffer, pH 4.8, and incubated with nuclease P1. This was followed with the addition of Tris–HCl buffer, pH 8.2, and the solution was incubated with alkaline phosphatase. After the addition of sodium acetate buffer, pH 5.1, 1 μL of digested samples were diluted with an equal volume of methanol and injected into the LC–MS/MS. One millimolar solutions of ES-3′-C8-dG, ES-3′-N^2-dG and ES-3′-N^6-dA were prepared in methanol and immediately diluted with methanol/HPLC grade water (50/50, v/v) to 10 μM (stock solution). Working solutions for calibration (0.1–10 nM) were prepared by the addition of an adequate amount of surrogate standard and diluted with methanol/HPLC grade water (50/50, v/v) to appropriate concentrations. LC–MS/MS analysis was performed using a Quattoro Ultima (Micromass, Beverly, MA, USA) coupled to a HEWLETT PACKARD 1100 series (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated using an electrospray ionization source in the positive ion mode (ESI$^+$) for multiple reaction monitoring (MRM).

2.5. In vivo mutation assays

6-Thioguanine (TG) and Spi$^-$ selections were performed as previously described [14,17–21]. Briefly, genomic DNA was extracted from the livers, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *E. 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. In order to determine the total number of rescued plasmids, 9000-fold diluted phages were used to infect YG6020, and poured on the plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day three and collected on day four. The MF was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For the Spi$^-$ selection, the 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 pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2 or WL95 P2 strains were spread with soft agar. Real Spi$^-$ mutants, which made clear plaques on every plate, were counted.

To characterize the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously [19]. Sequencing analysis was done at Takara Bio Inc. (Mie, Japan).

2.6. In vivo mouse bone marrow MN test

The MN test was performed as described by Hayashi et al. [22]. Briefly, bone marrow was collected by mild centrifugation in centrifuge tubes, and pelleted marrow cells were resuspended in a small amount of serum, spread on clean microscope slides, air-dried, methanol fixed, and were finally stained with acridine orange (Wako Pure Chemical Industries, Ltd., Osaka, Japan). To determine MN frequencies, 2000 polychromatic erythrocytes (PCEs) were analyzed for each treatment and animal. PCEs are developmentally immature erythrocytes still containing ribosomes and therefore can be distinguished from mature, normochromatic erythrocytes (NCEs) by selective ribosome staining. In addition, 1000 total (immature and mature) erythrocytes were scored for PCE frequency for each treatment and animal.

2.7. Statistical evaluation

The significance of differences in the body and liver weights and the results for the mutation assays were evaluated with ANOVA, followed by Dunnett's multiple comparison test. MN–PCEs were compared using Fisher's exact test. PCE frequencies were analyzed using Dunnett's *t*-test for group mean comparisons. Significant differences of RT-PCR analysis were evaluated by Student's *t*-test.

3. Results

3.1. Body weights, liver weights

Final body weights and liver weights are shown in Table 1. In the females treated with 300 mg/kg bw, a dead mouse appeared at week one and body weights decreased from week two. Therefore, the highest dose in females was reduced to 250 mg/kg bw from week two. One each dead mouse appeared in that group at weeks two, four, or 11. One male in the 37.5 mg/kg bw group died accidentally at week one. Body weights in the 150 and 250 mg/kg bw female group were significantly decreased as compared with the control group. Absolute and relative liver weights at doses greater than 75 mg/kg bw in males and 250 mg/kg bw females were significantly increased.

Table 1
Body and liver weights of B6C3F$_1$ *gpt* delta mice treated with estragole for 13 weeks.

	Dose (mg/kg bw)	Number of mice	Final body weights (g)	Liver weights	
				Absolute (g)	Relative (g%)
Male	0	10	32.5 ± 1.7[d]	1.41 ± 0.07	4.33 ± 0.13
	37.5	9[b]	31.6 ± 1.7	1.45 ± 0.11	4.59 ± 0.15
	75	10	33.1 ± 2.4	1.57 ± 0.09**	4.74 ± 0.23*
	150	10	31.6 ± 1.3	1.53 ± 0.10*	4.85 ± 0.28**
	300	10	30.9 ± 1.9	1.57 ± 0.13**	5.09 ± 0.26**
Female	0	10	25.8 ± 1.6	1.08 ± 0.07	4.17 ± 0.07
	37.5	10	25.7 ± 1.5	1.03 ± 0.09	4.00 ± 0.13
	75	10	24.6 ± 1.0	1.03 ± 0.07	4.18 ± 0.19
	150	10	24.4 ± 1.2*	1.08 ± 0.07	4.42 ± 0.30
	250[a]	6[c]	23.5 ± 0.5**	1.25 ± 0.05**	5.29 ± 0.17**

[a] The highest dose was changed from 300 to 250 mg/kg bw after week two.
[b] One mouse was found dead during week one accidentally.
[c] Four mice were found dead during weeks one, two, four or 11.
[d] Mean ± S.D.
* $p < 0.05$ *vs.* control (0 mg/kg bw).
** $p < 0.01$ *vs.* control (0 mg/kg bw).

3.2. *Histopathological changes in the livers*

No histopathological changes in the livers were found in males. In females, very slight hepatocellular hypertrophy was observed in two of 10, five of 10 and five of six animals in the 75, 150 and 250 mg/kg bw groups, respectively. These results are in good agreement with those in the NTP 3-month toxicity study of ES using wild-type B6C3F$_1$ mice [16].

3.3. *Comparison of mRNA expression between male and female livers*

Expression levels of *Cyp1a2* and *Sult1a1* in the livers of male and female mice are shown in Fig. 1. Expression levels of *Cyp1a2* were not affected by sex differences, but those of *Sult1a1* were significantly higher in female groups as compared to male groups at the same doses of administration (except the highest dose). Expression levels were not affected in any of the treated groups in either sex.

3.4. *The levels of ES-DNA adducts in mouse livers*

The levels of ES-DNA adducts, ES-3′-8-dG, 3′-N^2-dG and 3′-N^6-dA, in liver DNA were measured by the LC–MS/MS method after ES-treating mice for 13 weeks (Fig. 2). The DNA adducts were detected in ES-treated livers, whereas no ES-DNA adducts were detected in controls. ES-specific DNA adduct formation from ES-treated mice increased in a linear dose-dependent manner, except

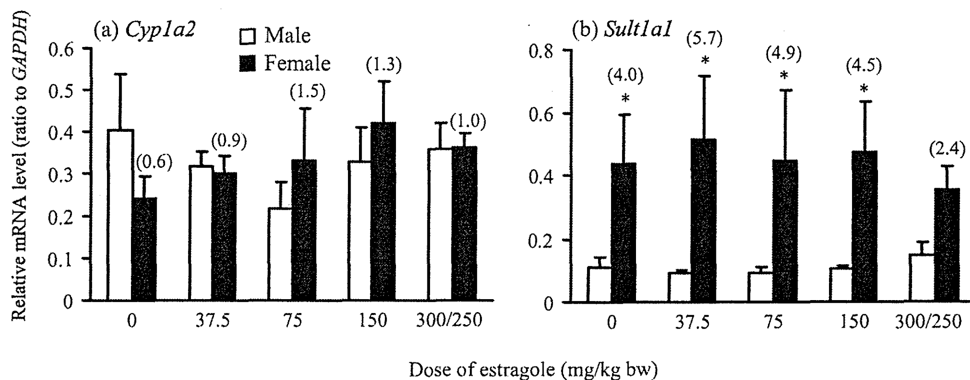

Fig. 1. *Cyp1a2* (a) and *Sult1a1* (b) mRNA expression determined by real-time RT-PCR in the livers of male and female mice. Data in the parentheses are shown as the fold change in the female mice relative to the expression levels of male mice. *$p < 0.01$, significantly different from corresponding male values.

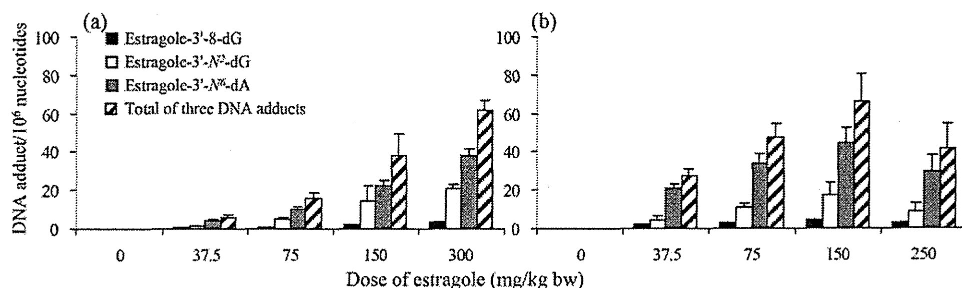

Fig. 2. Hepatic estragole DNA adduct levels in *gpt* delta (a) male and (b) female mice treated with 0, 37.5, 75, 150 and 300 or 250 mg/kg bw for 13 weeks. Each point represents the mean ± S.D. of five mice.

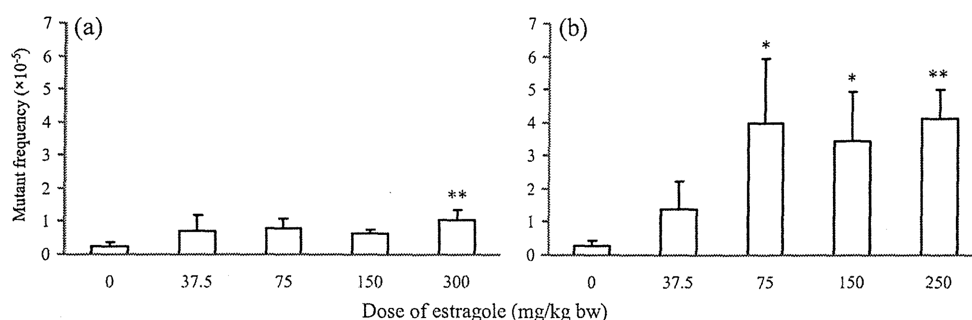

Fig. 3. Data for *gpt* mutant frequencies in livers of *gpt* delta (a) male and (b) female mice given estragole at concentrations of 0, 37.5, 75, 150 and 300 or 250 mg/kg bw for 13 weeks. Values are mean ± S.D. of data for five mice. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from controls (0 mg/kg bw groups in males and females).

in the 250 mg/kg bw group in females. DNA adduct levels in females were higher than those of males treated with the same doses except at the highest dose.

3.5. In vivo mutation assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Fig. 3. The MFs in males at the highest doses and in females at doses greater than 75 mg/kg bw were significantly higher than in the respective control group. In males, the MFs in the highest group were significantly higher than those in the control group, but with a slight change. The MF values of females were higher than those of males treated with the same doses. To characterize *gpt* mutations in both sexes at the highest doses due to ES exposure, the mutant colonies were analyzed by DNA sequencing (Table 2). In male and female mice, G:C mutations were predominant, accounting for 62.1% (18/29) and 58.5% (69/118) of the mutations, respectively. In males, the specific mutation frequency of GC:TA transversion was significantly higher than in the control group. In females, the specific mutation frequency of GC:TA and GC:CG transversions, GC:AT transitions and complex mutations were significantly higher than the control group.

As shown in Fig. 4, while there were no significant differences in the Spi^{-} MFs between the male groups, the MF in the 250 mg/kg bw treated females was 1.16 ± 0.72, which was significantly higher than the control value (0.28 ± 0.11). Since inactivation of the red/gam protein requires a disruption spanning the two genes, a positive result in the Spi^{-} assay is indicative of an increase in deletion mutations [14], ES might cause deletion mutations as well as point mutations in the mouse liver DNA.

3.6. MN test in mouse bone marrow

The results are tabulated in Table 3. No significant increases in the frequencies of micronucleated PCEs were observed in male or female mice, and no significant changes in PCE frequency were shown, which indicates that ES induces no chromosomal aberration in the bone marrow. This is consistent with the fact that the bone marrow is not implicated as a target organ.

4. Discussion

ES is a natural constituent and human exposure level is estimated to be 0.01–0.07 mg/kg/day [23,24]. Although it has been reported that ES is hepatocarcinogenic and is capable of forming specific DNA adducts in the liver [1–5], genotoxic tests for ES have been equivocal [8–11] and the mechanism of ES-induced hepatocarcinogenicity is unclear. However, in our previous study, ES could be a possible genotoxic hepatocarcinogen in the rat, at least when given at high doses [25]. It has been proposed that ES carcinogenicity is linked to its metabolic conversion [1–3]. ES is metabolized to 1′-hydroxyestragole mainly by cytochrome P450 enzymes [6] followed by formation of 1′-sulfooxyestragole, the ultimate carcinogenic metabolite, by sulfotransferases

Table 2
Mutation spectra of *gpt* mutant colonies.

	Male				Female			
	0 mg/kg bw		300 mg/kg bw		0 mg/kg bw		250 mg/kg bw	
	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)	No.	Mutation frequency ($\times 10^{-5}$)
Base substitution								
Transversions								
GC:TA	2[a]	0.04 ± 0.05[b]	9	0.37 ± 0.23*	4	0.05 ± 0.07	32	1.06 ± 0.44**
GC:CG	2	0.04 ± 0.05	1	0.02 ± 0.04	0	0.00 ± 0.00	15	0.40 ± 0.24**
AT:TA	0	0.00 ± 0.00	2	0.09 ± 0.12	2	0.02 ± 0.03	11	0.29 ± 0.21*
AT:CG	0	0.00 ± 0.00	0	0.00 ± 0.00	1	0.01 ± 0.03	3	0.10 ± 0.17
Transitions								
GC:AT	5	0.08 ± 0.08	8	0.26 ± 0.29	9	0.12 ± 0.10	22	0.68 ± 0.30**
AT:GC	0	0.00 ± 0.00	2	0.04 ± 0.09	0	0.00 ± 0.00	14	0.58 ± 0.47*
Deletion								
Single bp	1	0.02 ± 0.04	2	0.06 ± 0.08	0	0.00 ± 0.00	4	0.19 ± 0.20
Over 1 bp	0	0.00 ± 0.00	1	0.07 ± 0.15	0	0.00 ± 0.00	0	0.00 ± 0.00
Insertion	0	0.00 ± 0.00	1	0.05 ± 0.11	2	0.02 ± 0.03	4	0.08 ± 0.14
Complex	1	0.01 ± 0.03	3	0.09 ± 0.12	3	0.04 ± 0.06	13	0.46 ± 0.33*

[a] The number of colonies with independent mutations.
[b] Mean ± S.D.
* $p < 0.05$ vs. control (0 mg/kg bw).
** $p < 0.01$ vs. control (0 mg/kg bw).

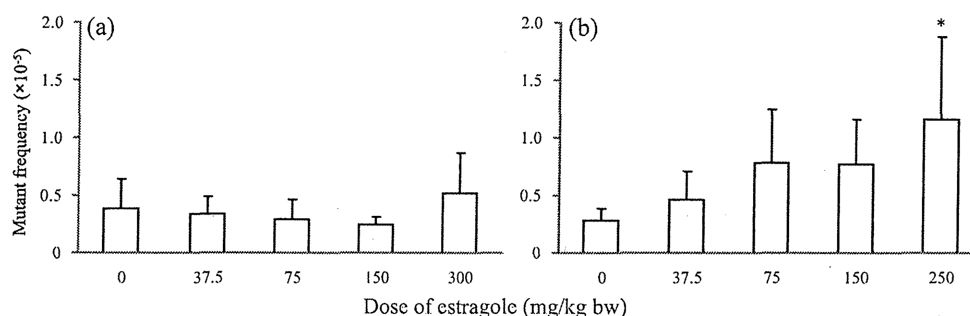

Fig. 4. Data for Spi⁻ mutant frequencies in livers of *gpt* delta mice given estragole at concentrations of 0, 37.5, 75, 150 and 300 or 250 mg/kg bw in (a) males and (b) females for 13 weeks. Values are mean ± S.D. of data for five mice. *$p < 0.05$, significantly different from the controls (0 mg/kg bw groups in males and females).

Table 3
Frequency of micronuclei in bone marrow of mice following treatment with estragole by gavage for 13 weeks.

	Dose (mg/kg bw)	Number of mice	Micronucleated PCEs/PCEs (%)	PCEs/PCEs + NCEs (%)
Male	0	5	0.18 ± 0.08[a]	46.72 ± 11.65
	37.5	5	0.13 ± 0.08	46.52 ± 7.74
	75	5	0.19 ± 0.07	40.28 ± 4.65
	150	5	0.15 ± 0.05	40.28 ± 5.71
	300	5	0.27 ± 0.16	43.00 ± 15.32
Female	0	5	0.07 ± 0.06	50.54 ± 7.62
	37.5	5	0.14 ± 0.10	52.08 ± 12.31
	75	5	0.14 ± 0.07	49.58 ± 5.83
	150	5	0.12 ± 0.08	57.94 ± 8.90
	250	5	0.11 ± 0.07	59.42 ± 10.63

NCE: normochromatic erythrocyte; PCE: polychromatic erythrocyte.
[a] Mean ± S.D.

(SULT). 1′-Sulfooxyestragole is unstable and subjected to degradation in an aqueous environment, consequently leading it to form a reactive carbocation that is capable of binding covalently to DNA [6,26,27]. As SULT activity and mRNA expression were reported to vary with sex [28,29], we made an attempt to compare changes in various parameters relating to carcinogenesis between male and female mice following ES exposure. Certainly, the present study demonstrated that mRNA levels of *Sult1a1* in female *gpt* delta mice were significantly higher than those in the males, in line with other strains of non-transgenic mice [28].

Quantification of DNA adduct levels by LC–MS/MS clearly showed that the levels of ES-specific DNA adducts in females were higher than those in the males. The expression levels of *Cyp1a2* catalyzing ES into proximate carcinogenic metabolites were comparable between females and males. Thus, it is highly probable that the sex difference of ES-specific DNA adduct levels resulted from the differences in SULT activity, supporting the proposed etiology of ES-specific DNA modifications. In addition, it has been reported that pre-exposure of mice to pentachlorophenol, a known inhibitor of SULT, inhibited ES-induced hepatocarcinogenicity as well as DNA adduct formation [12,13]. In the present *gpt* assay, the MFs of females were significantly higher at the low dose while those of males increased significantly only at the highest dose. Therefore, the overall data strongly suggest that ES-specific DNA adducts are formed by SULT, and consequent metabolites contribute to ES genotoxicity.

Although the recent *in vitro* data demonstrated that the amount of DNA adduct at dG was more abundant than that at dA [30,31], the present *in vivo* study showed that the predominant DNA adduct in any group in both sexes was ES-3′-N^6-dA and the predominant mutations in the *gpt* assay were GC base-pair mutations. Considering that point mutations occur mainly at bases opposite to the modified one [32,33], it is likely that ES-3′-N^6-dA could induce mutations at AT base-pairs. Thus, the amount of DNA adducts did not always result in mutations at the site. Additionally, in females, obvious increases in MF values were observed in all dosing groups except at the lowest dose in which DNA adduct formation appeared to some extent, but MF values in males showed no change even though DNA adduct level of 150 mg/kg males was comparable to 75 mg/kg females. Also, the MF of males receiving the highest doses was significantly increased, but obviously lower than that of 150 mg/kg females in spite of equivalent DNA adduct levels. Therefore, it is suggested that ES-induced mutagenicity is related not only to DNA adduct formation mediated by SULT metabolic activation but also to other factors. For example, the cell proliferation progressed the cell cycle or Y-family polymerase required the error-prone replication of damaged DNA [32,34].

It has been reported that ES is hepatocarcinogenic in adult female mice [1–3] and SULT activity may contribute to the hepatocarcinogenicity [4,5]. In the present study, females (having higher *Sult1a1* expression level) showed higher levels of DNA adducts and MFs of the *gpt* gene compared to males. Therefore, it is suggested that the genotoxic mechanism is related to the carcinogenic mechanism of ES. It is likely that ES-specific DNA adduct formation by SULT mediated metabolites is responsible for its genotoxicity.

Conflict of interest statement

The authors declare no conflict of interests.

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GENOTOXICITY AND CARCINOGENICITY

Flumequine enhances the in vivo mutagenicity of MeIQx in the mouse liver

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Abstract The combined effects of various carcinogens found in food products are a concern for human health. In the present study, the effects of flumequine (FL) on the in vivo mutagenicity of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) in the liver were investigated. Additionally, we attempted to clarify the underlying mechanisms through comprehensive gene analysis using a cDNA microarray. Male *gpt* delta mice were fed a diet of 0.03 % MeIQx, 0.4 % FL, or 0.03 % MeIQx + 0.4 % FL for 13 weeks. The effects of cotreatment with phenobarbital (PB) were also examined. Treatment with MeIQx alone increased *gpt* and Spi^- mutant frequencies, and cotreatment with FL, but not with PB, further exacerbated these effects, despite the lack of in vivo genotoxicity in mice treated with FL alone. FL caused an increase in *Cyp1a2* mRNA levels and a decrease in *Ugt1b1* mRNA levels, suggesting that the enhancing effects of FL may be due in part to modification of MeIQx metabolism by FL. Moreover, FL induced an increase in hepatocyte proliferation accompanied by hepatocellular injury. Increases in the mRNA levels of genes encoding cytokines derived from Kupffer cells, such as *Il1b* and *Tnf*, and cell cycle-related genes, such as *Ccnd1* and *Ccne1*, suggested that FL treatment increases compensatory cell proliferation. Thus, the present study clearly demonstrated the combined effects of 2 different types of carcinogens known as contaminants in foods.

Keywords MeIQx · Flumequine · In vivo mutagenicity · *gpt* delta mouse · Combined effects

K. Kuroda and A. Kijima contributed equally to this work.

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Introduction

A variety of carcinogens are produced in foods or have been found to accidentally contaminate foods. Many studies have reported the toxicities of individual carcinogens. Although some carcinogens are known to cause either synergistic or antagonistic effects (Takayama et al. 1989; Hasegawa et al. 1991), the effects of many combinations of carcinogens have not yet been determined. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is a heterocyclic amine produced in cooked meat and has been shown to induce tumors in the liver, Zymbal glands, clitoral glands, and skin in rats and in the liver, lungs, hematopoietic system, and colon in mice (Ohgaki et al. 1987; Sugimura et al. 2004; Nishikawa et al. 2005). The in vitro genotoxicity of MeIQx has been demonstrated, and MeIQx has also been shown to exhibit in vivo mutagenicity in the livers of transgenic mice (Itoh et al. 2000; Masumura et al. 2003). Recently, we reported that the in vivo mutagenicity of MeIQx in the liver was enhanced by cotreatment with carbon tetrachloride (CCl_4) in *gpt* delta mice (Okamura et al. 2010). These data suggested that the genotoxicity of carcinogens could be enhanced by injury or inflammation in the target organs.

Flumequine (FL) is a fluoroquinolone compound with antimicrobial activity against gram-negative organisms and has been used in the treatment of enteric infections in domestic animals and livestock (Greenwood 1998); FL is

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thought to persist in food products made from such livestock (Choma et al. 1999). FL induces hepatotoxicity, characterized by hepatocyte vacuolation and inflammatory cell infiltration, in both rats and mice and induces liver tumors in mice (JECFA 1998; Pronk 2004). Based on the negative results of genotoxicity tests, JECFA concluded that FL is a nongenotoxic hepatocarcinogen and that hepatocellular necrosis-regeneration cycles caused by hepatotoxicity are mechanistically relevant to its induction of liver tumors in mice (JECFA 2004). FL and MeIQx, both of which possibly exist in foods, could be ingested by humans on a long-term basis. Moreover, given that FL exhibits hepatotoxicity similar to CCl_4, FL indeed may exert additional effects on the genotoxicity of MeIQx.

Therefore, in the present study, we investigated the effects of FL on the in vivo mutagenicity of MeIQx in the mouse liver. *gpt* delta mice were given MeIQx and FL for 13 weeks. To clarify the relationship between changes in reporter gene mutations and molecular mechanisms, mouse livers were used for histopathological examination, bromodeoxyuridine (BrdU) immunostaining, and analysis of reporter gene mutations (*gpt* and Spi^- assays) as well as comprehensive gene expression analysis by cDNA microarray. In addition, cotreatment with phenobarbital (PB), a nonhepatotoxic tumor promoter for hepatocarcinogenesis, was examined as a comparative control.

Materials and methods

Chemicals

FL, a white crystallized powder (purity: 99.3 %), was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). MeIQx and PB were purchased from Toronto Research Chemicals (North York, Canada) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. BrdU was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Male $B6C3F_1$ *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome were raised by mating C57BL/6 *gpt* delta and nontransgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Thirty male $B6C3F_1$ *gpt* delta mice were randomized by weight into 6 groups. Animals were housed in a room with a barrier system and maintained under the following constant conditions: temperature of 24 ± 1 °C, relative humidity of 55 ± 5 %, ventilation frequency of 18 times/h, and a 12-h light/dark cycle. The animals were housed in plastic cages (5 mice/cage) on soft chips (Sankyo Labo-Service, Tokyo, Japan). Throughout the experimental period, chips were renewed every 3 or 4 days, and mice were provided tap water ad libitum.

Starting at 6 weeks of age, *gpt* delta transgenic mice were fed a diet containing 0.03 % MeIQx, 0.4 % FL, 0.05 % PB, 0.03 % MeIQx and 0.4 % FL, or 0.03 % MeIQx and 0.05 % PB for 13 weeks. Animals in the control group received the basal diet only. General signs were observed daily, and body weight and food consumption per cage were measured once a week. After 13 weeks, all mice were killed, and a part of the left lateral lobe of the liver was stored at −80 °C for in vivo mutation assays and cDNA microarray analysis. At autopsy, the body and liver weights were measured.

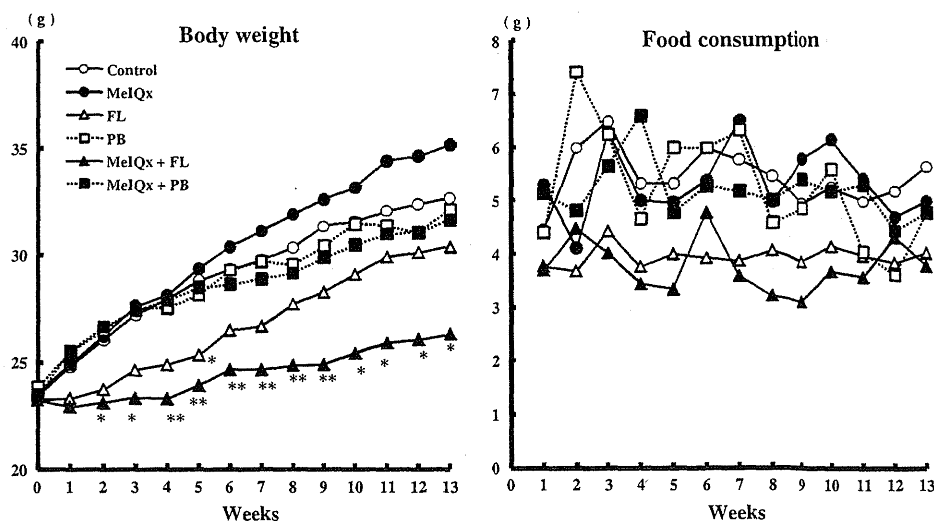


Fig. 1 Body weight and food consumption for $B6C3F_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQX + PB for 13 weeks. *, **Significantly different from control group at $p < 0.05$ and 0.01, respectively

Table 1 Final body and liver weights in B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Final body weight (g)	Liver weight	
		Absolute (g)	Relative (g/100 g BW)
Control	32.7 ± 3.9[a]	1.40 ± 0.15	4.29 ± 0.23
MeIQx	35.4 ± 4.4	1.63 ± 0.21	4.63 ± 0.40
FL	31.1 ± 1.7	1.60 ± 0.15	5.13 ± 0.23**
PB	32.4 ± 2.9	1.57 ± 0.07	4.88 ± 0.30
MeIQx + FL	26.4 ± 2.1*	1.33 ± 0.10	5.02 ± 0.15*
MeIQx + PB	31.7 ± 2.2	1.64 ± 0.16	5.20 ± 0.47**

*, ** Significantly different from control group at $p < 0.05$, 0.01, respectively

[a] Mean ± SD

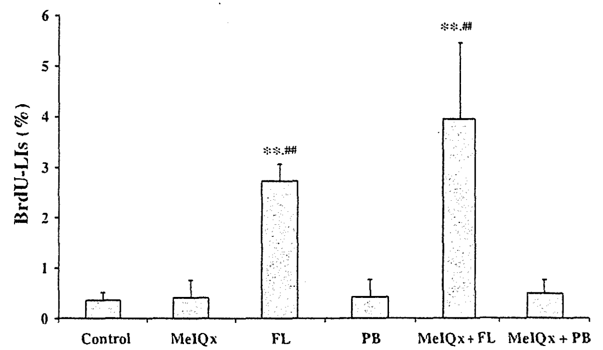


Fig. 3 BrdU-LIs for hepatocytes from B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Values are the mean ± SD of data for 5 mice. **, ## Significantly different from control and MeIQx groups, respectively, at $p < 0.01$

Quantification of hepatocyte proliferation

In order to examine the proliferative activity of hepatocytes, all animals were given BrdU (100 mg/kg) by intraperitoneal (i.p.) injection once a day for the final 2 days before killing and once on the day of killing at 2 h before being euthanatized at autopsy. For immunohistochemical staining of BrdU, after activation of the antigen by autoclaving, tissue sections were treated with rat anti-BrdU (AbD Serotec Inc., NC, USA) diluted by Dako Antibody Diluent (Dako, Glostrup, Denmark), followed by incubation with a high polymer stain (HISTOFINE Simple Stain, NICHIREI, Japan). At least 2,000 hepatocytes in each liver were counted, and labeling indices (LIs) were calculated as the percentage of cells positive for BrdU incorporation.

In vivo mutation assays

The 6-TG and Spi$^-$ (insensitive P2 interference) selections were carried out 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 *Escheichia coli* YG6020, expressing Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol

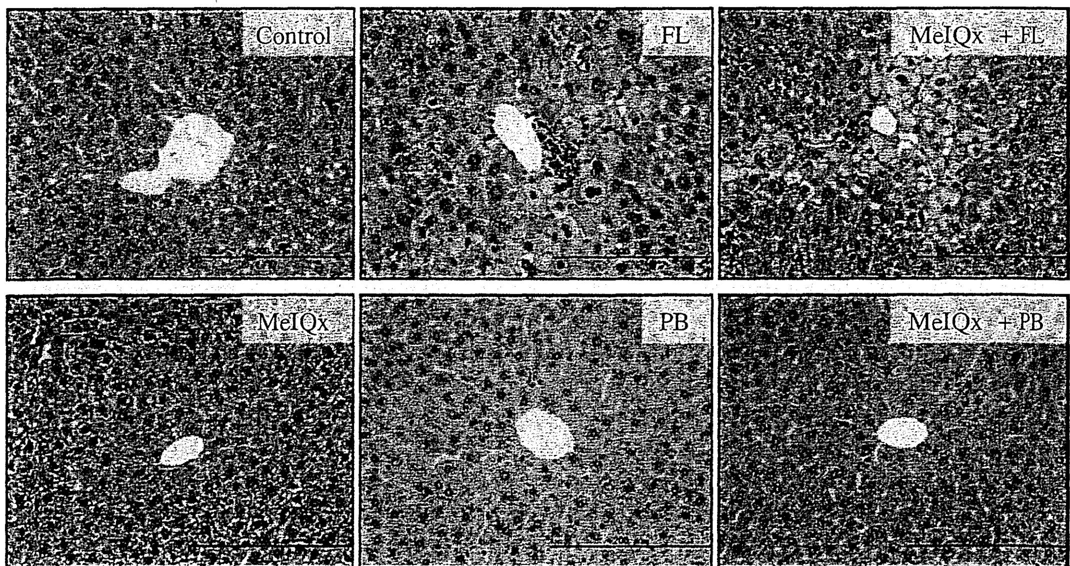


Fig. 2 Histopathological features in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Note that no obvious alterations (MeIQx group), centrilobular hypertrophy of hepatocytes with vacuolation (FL and MeIQx + FL groups), or centrilobular hypertrophy of hepatocytes (PB and MeIQx + PB groups) are evident. *Bar* represents 200 μm

Table 2 *gpt* mutant frequencies (MFs) in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Control	1	26.7	4	0.15	0.20 ± 0.12
	2	12.5	3	0.24	
	3	26.7	2	0.07	
	4	1.7	0	0.00[b]	
	5	26.2	9	0.34	
MeIQx[a]	6	0.7	3	4.33	5.47 ± 2.03
	7	3.2	18	5.56	
	8	3.5	10	2.85	
	9	2.3	19	8.18	
	10	3.1	20	6.44	
FL	11	25.6	7	0.27	0.29 ± 0.15
	12	20.2	8	0.40	
	13	20.4	10	0.49	
	14	22.4	4	0.18	
	15	7.9	1	0.13	
PB	16	26.6	4	0.15	0.24 ± 0.12
	17	17.2	7	0.41	
	18	23.9	7	0.29	
	19	18.5	2	0.11	
	20	22.4	5	0.22	
MeIQx + FL[a]	21	3.0	21	7.11	11.92 ± 6.26**,#
	22	3.4	37	10.93	
	23	5.1	28	5.51	
	24	1.9	29	15.06	
	25	1.0	20	20.96	
MeIQx + PB[a]	26	5.7	5	0.88	2.57 ± 2.60
	27	3.2	7	2.17	
	28	5.3	7	1.32	
	29	2.3	3	1.33	
	30	1.3	9	7.14	

** Significantly different from control group at $p < 0.01$

Significantly different from the MeIQx group at $p < 0.05$

[a] For mice treated with MeIQx alone and for cotreatment groups, *gpt* mutants were analyzed only on one selected plate whose colony numbers were closest to the average number of 5 plates, and MFs were calculated using the packaging efficiency values (Cm^R colonies) divided by 5

[b] Data of animal No. 4 were excluded for the calculation of the MF because of the poor packaging efficiency of the transgene

acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, infected cells were also poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the 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 *gpt* mutations, a 739-bp DNA fragment containing the 456-bp coding region of the *gpt* gene was amplified by PCR as previously described, and the PCR products were analyzed with an Applied Biosystems 3,730 × 1 DNA Analyzer (Applied Biosystems Japan Ltd.). For the group of mice treated with MeIQx alone and for cotreatment groups, *gpt* mutants were analyzed only on one selected plate based on the assumption that the colony number on the selected plate was closest to the average number of 5 plates, and MFs were calculated by the packaging efficiency value (Cm^R colonies) divided by 5.

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. 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 resulting agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on 3 types of plates containing XL-1 Blue MRA, XL-1 Blue MRA P2, or WL95 P2 strains and were spread with soft agar. The numbers of mutants that made clear plaques on each plate were counted as confirmed Spi$^-$ mutants. The Spi$^-$ MF was calculated by dividing the number of Spi$^-$ mutants by the number of rescued phages. For the group of mice treated with MeIQx alone and for the cotreatment groups, confirmation of Spi$^-$ phenotype candidates

Table 3 Mutation spectra of *gpt* mutant colonies in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Mutation	Control		MeIQx		FL		PB		MeIQx + FL		MeIQx + PB	
	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)	Number (%)	Specific mutation frequency ($\times 10^{-5}$)
Transversions												
GC-TA	4[a] (22.2)	0.03 ± 0.05[b]	36 (51.4)	2.85 ± 0.77	6 (20.0)	0.05 ± 0.05	6 (24.0)	0.05 ± 0.06	74 (54.8)	6.90 ± 4.36**,#	16 (51.6)	1.21 ± 1.15
GC-CG	0	0	2 (2.9)	0.15 ± 0.21	1 (3.3)	0.01 ± 0.02	1 (4.0)	0.01 ± 0.02	2 (1.5)	0.14 ± 0.23	1 (3.2)	0.04 ± 0.08
AT-TA	1 (5.6)	0.01 ± 0.02	3 (4.3)	0.19 ± 0.28	0	0	0	0	5 (3.7)	0.48 ± 0.34*	0	0
AT-CG	0	0	1 (1.4)	0.06 ± 0.14	2 (6.7)	0.02 ± 0.03	0	0	0	0	0	0
Transisions												
GC-AT	9 (50.0)	0.08 ± 0.05	2 (2.9)	0.35 ± 0.63	9 (30.0)	0.10 ± 0.06	13 (52.0)	0.13 ± 0.11	11 (8.1)	0.77 ± 0.83	7 (22.6)	0.67 ± 0.98
AT-GC	0	0	0	0	3 (10.0)	0.03 ± 0.03	3 (12.0)	0.03 ± 0.03	0	0	0	0
Deletion												
Single bp	3 (16.7)	0.04 ± 0.07	22 (31.4)	1.55 ± 1.24	6 (20.0)	0.06 ± 0.06	2 (8.0)	0.02 ± 0.03	37 (27.4)	3.14 ± 1.36**,#	5 (16.1)	0.50 ± 0.63
Over 2 bp	0	0	1 (1.4)	0.09 ± 0.19	2 (6.7)	0.02 ± 0.03	0	0	1 (0.7)	0.21 ± 0.47	0	0
Insertion	0	0	2 (2.9)	0.15 ± 0.21	1 (3.3)	0.01 ± 0.02	0	0	2 (1.5)	0.12 ± 0.26	1 (3.2)	0.06 ± 0.14
Complex	1 (5.6)	0.01 ± 0.02	1 (1.4)	0.09 ± 0.19	0	0	0	0	3 (2.2)	0.16 ± 0.26	1 (3.2)	0.09 ± 0.20

*, ** Significantly different from control group at $p < 0.05$ and 0.01, respectively

Significantly different from the MeIQx group at $p < 0.05$

[a] Number of colonies with independent mutations

[b] Mean ± SD

Table 4 Spi$^-$ mutant frequencies (MFs) in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks

Group	Animal no.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within WL95 (P2)	Mutant frequency ($\times 10^{-5}$)	Mean ± SD
Control	1	34.6	8	0.23	0.15 ± 0.07
	2	14.9	1	0.07	
	3	49.4	8	0.16	
	4	6.0	2	0.33	
	5	21.1	3	0.14	
MeIQx[a]	6	3.2	4	1.27	3.53 ± 1.97*
	7	5.7	12	2.12	
	8	7.3	24	3.28	
	9	9.6	58	6.07	
	10	12.0	59	4.91	
FL	11	39.8	6	0.15	0.17 ± 0.03
	12	25.6	5	0.20	
	13	31.3	5	0.16	
	14	41.4	6	0.14	
	15	20.2	4	0.20	
PB	16	46.6	7	0.15	0.20 ± 0.09
	17	45.7	8	0.17	
	18	30.4	4	0.13	
	19	17.3	6	0.35	
	20	32.9	7	0.21	
MeIQx + FL[a]	21	4.9	27	5.48	7.66 ± 3.02**,#
	22	5.5	30	5.49	
	23	8.0	43	5.40	
	24	6.5	70	10.80	
	25	4.0	44	11.11	
MeIQx + PB[a]	26	7.2	3	0.42	1.72 ± 1.74
	27	5.7	4	0.71	
	28	8.3	7	0.84	
	29	6.0	12	1.99	
	30	3.0	14	4.64	

*, ** Significantly different from the control group at $p < 0.05$ and 0.01, respectively

Significantly different from the MeIQx group at $p < 0.05$

[a] For mice treated with MeIQx alone and for cotreatment groups, confirmation of Spi$^-$ phenotype candidates was performed only on the first of 2 plates, and MFs were calculated using the packaging efficiency values (plaques within XL-1 Blue MRA) divided by 2

was performed only on the first of 2 plates, and MFs were calculated using the packaging efficiency value (plaques within XL-1 Blue MRA) divided by 2.

RNA isolation

The livers from all animals were soaked overnight in RNAlater-ICE (Applied Biosystems/Ambion, Austin, TX) at −20 °C, and total RNA was then isolated using RNeasy Mini Kits (Qiagen GmbH, Hilden, Germany). The concentration and quality of total RNA were analyzed using a UV–VIS spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA microarray

In 3 mice from the control, MeIQx, and MeIQx + FL groups, purified total RNA was labeled with cyanine-3 dye using a Quick Amp Labeling Kit (Agilent Technologies). RNA concentration, dye incorporation, and quality were analyzed using a UV–VIS spectrophotometer and an Agilent 2100 Bioanalyzer. Fluorescently labeled cRNA was hybridized to Agilent 4 × 44 K whole mouse genome microarray gene expression chips following the manufacturer's protocol (Agilent Technologies). Hybridized microarray chips were then scanned using an Agilent Microarray Scanner (Model G2565BA, Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for imaging analysis and data extraction processes. Using

Table 5 Genes extracted from cDNA microarray analysis that were up- or downregulated by FL treatment in the livers of B6C3F$_1$ *gpt* delta mice

Gene symbol	Gene name	Fold change	GO categorize
Ccna2	Cyclin A2	3.7	Cell cycle
Ccnb1	Cyclin B1	12.7	Cell cycle
Ccnb2	Cyclin B2	4.0	Cell cycle
Ccnd1	Cyclin D1	3.1	Cell cycle
Cdk1	Cyclin-dependent kinase 1	8.6	Cell cycle
Chek1	Checkpoint kinase 1 homolog (S. pombe)	3.2	Cell cycle, DNA damage
Fos	FBJ osteosarcoma oncogene	8.0	Cell proliferation
Jun	Jun oncogene	4.1	Cell proliferation, Apoptosis
Tnf	Tumor necrosis factor	4.4	Cell proliferation, Apoptosis, Immune system
Exo1	Exonuclease 1	6.7	DNA repair
Rad18	RAD18 homolog (S. cerevisiae)	2.4	DNA repair
Rad51	RAD51 homolog (S. cerevisiae)	5.4	DNA repair
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1	23.5	Drug metabolism
Cyp2b10	Cytochrome P450, family 2, subfamily b, polypeptide 10	6.2	Drug metabolism
Ccl2	Chemokine (C–C motif) ligand 2	19.6	Immune system
Ccl3	Chemokine (C–C motif) ligand 3	9.3	Immune system
Ccl4	Chemokine (C–C motif) ligand 4	7.6	Immune system
Ccl7	Chemokine (C–C motif) ligand 7	10.2	Immune system
Ccr2	Chemokine (C–C motif) receptor 2	3.7	Immune system
Ccr7	Chemokine (C–C motif) receptor 7	3.7	Immune system
Il1b	Interleukin 1 beta	2.8	Immune system
Il1f8	Interleukin 1 family, member 8	−3.7	Immune system
Nfkb2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	1.8	Immune system
Ugt2b1	UDP glucuronosyltransferase 2 family, polypeptide B1	−2.5	Drug metabolism
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1	−3.1	Drug metabolism

Listed genes were extracted under the cutoff condition of $p < 0.05$ and exhibiting at least 1.5-fold change in expression when comparing the MeIQx group with the MeIQx + FL group

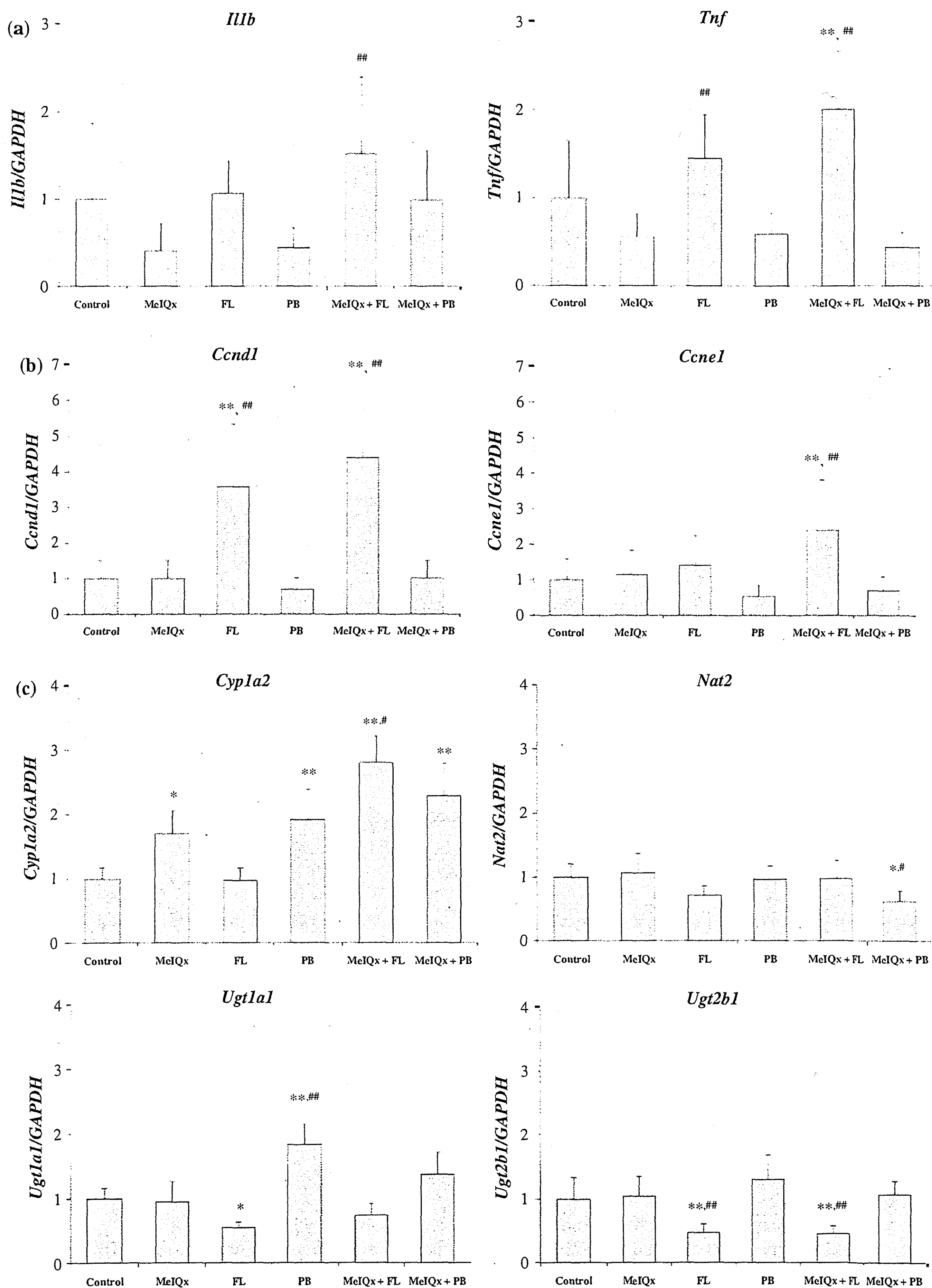
the analysis software GeneSpring (Agilent Technologies), normalization of gene expression data and filtering probe sets by expression levels, flags, and errors were performed. Differences in gene expression between the MeIQx group and the MeIQx + FL group were analyzed by analysis of variance (t test; cutoff value: $p < 0.05$; multiple testing corrections: Benjamini-Hochburg false discovery rate [FDR]). Extracted genes were analyzed by a gene ontology approach using GeneSpring software.

Quantitative real-time PCR for mRNA expression

In all animals, cDNA copies of total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan Ltd.). PCR was performed on an Applied Biosystems 7900HT FAST Real-Time PCR System (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Rodent GAPDH control reagents (Applied Biosystems). The primers for assayed genes in TaqMan Gene Expression Assays (Applied Biosystems) were used. Expression levels of target genes were calculated by the relative standard curve method. GAPDH levels were used as an endogenous control, and data were presented as fold-change values of treated samples relative to controls.

Statistical analysis

The significance of differences for body and liver weights, in vivo mutation assays, and real-time PCR analyses were evaluated using Turkey's multicomparison test. A p value of less than 0.05 was considered significant.


(a)
Il1b
Il1b/GAPDH
Tnf
Tnf/GAPDH
(b)
Ccnd1
Ccnd1/GAPDH
Ccne1
Ccne1/GAPDH
(c)
Cyp1a2
Cyp1a2/GAPDH
Nat2
Nat2/GAPDH
Ugt1a1
Ugt1a1/GAPDH
Ugt2b1
Ugt2b1/GAPDH
Control
MeIQx
FL
PB
MeIQx + FL
MeIQx + PB

◀ **Fig. 4** Changes in mRNA level of cytokines (*Il1b* and *Tnf*) (**a**), cell cycle-related factors (*Ccnd1* and *Ccne1*) (**b**), and enzymes related to MeIQx metabolism (*Cyp1a2*, *Nat2*, *Ugt1a1*, and *Ugt2b1*) (**c**) in the livers of B6C3F$_1$ *gpt* delta mice treated with MeIQx, FL, PB, MeIQx + FL, or MeIQx + PB for 13 weeks. Values are the mean ± SD of data for 5 mice. Values were normalized to the mRNA levels of GAPDH and are presented as fold-change values of treated samples relative to controls. *,**Significantly different from the control at $p < 0.05$ and 0.01, respectively; #,##Significantly different from the MeIQx group at $p < 0.05$ and 0.01, respectively

Results

General signs, body weight, food consumption, and liver weight

No deaths and no remarkable changes in general signs were observed in all of the treated groups. In both the FL- and MeIQx + FL-treatment groups, reduced body weight gain and decreased food consumption were observed (Fig. 1). Data for final body and liver weights are summarized in Table 1. Final body weight was significantly decreased in the MeIQx + FL group, and liver weight was significantly increased in the FL, MeIQx + FL, and MeIQx + PB groups.

Histopathological examinations and BrdU analysis of the liver

Histopathologically, hypertrophy of centrilobular hepatocytes with vacuolation was observed in all of the FL-treated groups (Fig. 2). Slight cell infiltration was also observed in these groups. In all of the PB-treated groups, only hypertrophy of centrilobular hepatocytes was observed (Fig. 2). The number of BrdU-positive liver cells was increased in the FL and MeIQx + FL groups, and the number of BrdU-positive cells was significantly higher in the MeIQx + FL group than in the MeIQx group (Fig. 3).

In vivo mutation assays

Data for *gpt* and Spi$^-$ MFs in the liver are summarized in Tables 2, 3, and 4. A clear elevation of *gpt* MFs was observed in the MeIQx group. In the MeIQx + FL group, *gpt* MFs were further increased, and this increase was statistically significant compared with the MeIQx group. There were no significant increases in *gpt* MFs in the FL and PB groups. Cotreatment with PB and MeIQx did not alter the *gpt* MF compared to MeIQx treatment alone (Table 2). In the *gpt* mutation spectra, increases in GC:TA transversions and single base pair deletions were observed in the MeIQx and MeIQx + FL groups (Table 3). In Spi$^-$ assays, Spi$^-$ MFs increased in the MeIQx group and were further elevated in the MeIQx + FL group, exhibiting a statistically significant difference (Table 4).

cDNA microarray analysis

When comparing the MeIQx group with the MeIQx + FL group, 2,224 genes (upregulated: 1,176; downregulated: 1,068) were modulated by FL treatment under the cutoff condition of $p < 0.05$ and with at least a 1.5-fold change in expression. A gene ontology approach was conducted, and apoptosis-, cell cycle/proliferation-, DNA damage/repair-, immune system-, and drug metabolism-related genes were extracted. Representative data for extracted genes are summarized in Table 5. The molecular functions of genes altered by FL treatment were induction of apoptosis (*Tnf* and *Jun*), cell cycle progression (*Ccnd1*, *Ccne1*, *Cdk1*, *Jun*, and *Fos*), cytokines (*Tnf*, *Il1b*, and *Ccl*), DNA repair (*Rad51*, *Rad18*, and *Exo1*), and drug metabolism (*Cyp1a1*, *Cyp2b10*, *Cyp7b1*, and *Ugt2b1*).

Quantitative real-time PCR

In order to confirm our cDNA microarray analysis data, representative genes altered by FL treatment were analyzed by quantitative real-time PCR. The expression of cytokines (*Il1b* and *Tnf*) and cell cycle progression factors (*Ccnd1* and *Ccne1*) were increased in the MeIQx + FL group as compared with the control and/or the MeIQx group (Fig. 4). In addition, we also investigated the mRNA expression of several enzymes related to MeIQx metabolism. The expression levels of transcription factors such as *Jun*, *Fos*, and *Nfkb2* did not change among all treated groups (data not shown). In contrast, the expression of *Cyp1a2* was increased in the MeIQx + FL group as compared with the MeIQx group, while the expression of *Ugt2b1* was decreased in the FL and MeIQx + FL groups as compared with the control. The expression of *Nat2* was not altered in any treatments (Fig. 4).

Discussion

In the present study, 13-week feeding of MeIQx caused clear increases in *gpt* and Spi$^-$ MFs in the livers of *gpt* delta mice. In mutation spectrum analysis of *gpt* mutant colonies, characteristic mutational patterns of MeIQx exposure such as GC:TA transversions and single base pair deletions were observed, in line with previous reports (Masumura et al. 2003; Okamura et al. 2010). Although no changes in *gpt* or Spi$^-$ MFs were observed in mice treated with FL alone, cotreatment with MeIQx and FL significantly increased *gpt* and Spi$^-$ MFs, reaching twofold that of MeIQx treatment alone. In addition, the mutation patterns observed in