Table 6
Classification of the independent *gpt* mutations in the liver of *gpt* delta rats administered dietrary phenacetin for 26 and 52 weeks.

Type of mutation	Number of mutations[a]							
	26 weeks				52 weeks			
	Control		0.5% phenacetin		Control		0.5% phenacetin	
	No.	%	No.	%	No.	%	No.	%
Base substitutions								
Transitions								
G:C → A:T	5	33.3	44	77.2	16	72.7	32	64.0
A:T → G:C	0	0.0	2	3.5	0	0.0	3	6.0
Transversions								
G:C → T:A	6	40.0	3	5.3	2	9.1	7	14.0
G:C → C:G	1	6.7	2	3.5	0	0.0	0	0.0
A:T → T:A	0	0.0	0	0.0	0	0.0	3	6.0
A:T → C:G	0	0.0	0	0.0	0	0.0	0	0.0
Deletions								
−1 bp	2	13.3	3	5.3	1	4.5	3	6.0
>2 bps	1	6.7	2	3.5	1	4.5	1	2.0
Insertions	0	0.0	1	1.8	2	9.1	1	2.0
Total	15	100.0	57	100.0	22	100.0	50	100.0

[a] Number of mutations was calculated by adding the values of male and female rats.

who relied heavily upon phenacetin-containing analgesics and developed renal pelvic tumors had papillary necrosis (Johansson et al., 1974). In the epidemiologic analysis of 100 cases, the presence and severity of phenacetin-induced renal papillary scarring correlated phenacetin-related tumors of the renal pelvis with tumor progression (Stewart et al., 1999). These reports suggest that renal papillary injury plays an important role in the induction of cancer by phenacetin in humans. In the present study, we did not observe significant toxicological or histopathological changes such as inflammation or hyperplastic lesion of the kidney. Based on the amount of food consumed and body weight, we estimated the mean daily dose of phenacetin to be 202 and 246 mg/(kg · day) in male and female rats, respectively, when mixed in CRF-1 diet at a proportion of 0.5% for 52 weeks. The maximum recommended daily dose for phenacetin in humans was 2.4 g (Bergman et al., 1998). Based on a 60-kg human, the dose per body weight in humans equates to 40 mg/(kg · day). Therefore, the dose of phenacetin administered to *gpt* delta rats was estimated to be 5–6-fold higher than the maximal clinical dose in humans. Additionally, the relative systemic exposure ratio (rat area under the curve at 0.5% in diet/human area under the curve at 40 mg/kg) was calculated to be 7 and 15 (Bergman et al., 1998); thus, the systemic exposure of phenacetin in *gpt* delta rats was also greater than that in humans. When dose per body surface area was calculated using conversion factors (CDER, 2005), the maximal clinical dose of phenacetin was converted to 1480 mg/m^2 by multiplying by 37, and the dose of male rats in this study was converted to 1476 mg/m^2 by multiplying by 6 (1212 mg/m^2 in female rats). Considering the estimated dose per body surface area, the daily dose of phenacetin in this study was comparable with the maximum recommended clinical dose. If we had performed the genotoxicity study of phenacetin using a higher dose in *gpt* delta rats, we might have confirmed more definitive renal damage or the genotoxic potential in kidney. However, we consider that the present study was conducted at an appropriate dose for evaluating a risk of phenacetin for human. Phenacetin administration moderately increased *gpt* MFs in kidney, the genotoxic potential of phenacetin was judged to be weak. Nevertheless, it is possible that phenacetin induces mutations at specific sites of kidney, i.e., pyelic cells, more strongly because we measured average MFs in whole kidney (see below for more detail). Therefore, we suggest that phenacetin has the potential to induce genotoxicity in human kidney. Furthermore, we postulate that phenacetin genotoxicity may partially be associated with the induction of renal cancer. The combined action of genotoxicity and renal papillary injury may account for cancer in humans who consumed a large quantity of phenacetin.

On the other hand, *gpt* and Spi$^-$ MFs were also increased in a nontarget organ (liver) of carcinogenicity in rats treated with phenacetin. Accordingly, phenacetin was judged to have genotoxic potential in the liver. However, in other genotoxicity studies in the liver of rats, phenacetin did not increase DNA fragmentation (De Flora et al., 1985) or micronucleus (Takasawa et al., 2010) with a single oral administration. We consider that repeated-dose treatment is required to detect the genotoxic potential of phenacetin in the liver. In a bacterial mutation assay, phenacetin displayed mutagenic potential in the presence of hamster liver S9 fraction and phenacetin was negative in the presence of rat liver S9 fraction (De Flora et al., 1985). Phenacetin is metabolized to its direct-acting mutagens, *N*-hydroxyphenetidine and *p*-nitrosophenetole, via *N*-hydroxylation and deacetylation, and the latter reaction is the rate-limiting step of species-specific mutagenicity in vitro (Nohmi et al., 1983). Deacetylation activities are significantly higher in hamsters than in rats (Nohmi et al., 1984). Given that we observed increases in MF in the liver, we consider that phenacetin was metabolized to its reactive metabolites in vivo and these metabolites were responsible for continuously damaging DNA over a long period. In fact, it has been reported that phenacetin induced a gene expression signature of oxidative stress related to reactive metabolites in rats (Leone et al., 2014). Meanwhile, phenacetin was negative in the bacterial mutation assay in the presence of hamster kidney S9 fraction (De Flora et al., 1985). This result suggests that phenacetin metabolism to its active metabolites occurs more in the liver than in the kidney. Indeed, we observed greater increases in phenacetin-induced MFs in the liver than in the kidney. From a viewpoint of cell populations in the organ, we may have underestimated the phenacetin-induced genotoxicity in the renal pelvis because the assay was conducted using the part of kidney which included not only pyelic cells (the toxic target cells of phenacetin) but also other major types of renal cells (the nontarget cells of phenacetin). In contrast, the assay in the liver was conducted using the part of the organ mainly consisting of hepatocytes. Similarly, aristolochic acid-induced MF in the liver (a nontarget organ of carcinogenesis) was higher than that in the kidney (a target organ of carcinogenesis) in *gpt* delta rats in a 28-day repeated-dose study

(Kawamura et al., 2012). Aristolochic acid also needs to be converted to reactive metabolites to induce genotoxicity and carcinogenicity (Arlt et al., 2002). In cancer bioassays of aristolochic acid in rats, tumors were found in the renal cortex and renal pelvis (Mengs et al., 1982). Considering the findings of these 2 compounds, the *gpt* delta rat assay is more sensitive in detecting liver rather than kidney genotoxicity in the case of assessments for compounds more effectively metabolized to their reactive metabolites in the liver than in the kidney. It is interesting to note that phenacetin does not induce liver tumors despite possessing stronger genotoxic potential toward that organ compared with the kidney. This suggests that factors other than genotoxicity, such as accelerating cell proliferation, may play an important role in the phenacetin-induced cancer.

In our longest (52-week) study, *gpt* MFs in the kidney of male rats receiving phenacetin treatment increased 3.5-fold compared with the control group. However, we did not observe a change in our 26-week study. Moreover, the increase in *gpt* MF in the liver of male rats given phenacetin for 52 weeks was greater than in the 26-week study (17.6-fold vs. 11.9-fold) (Table 3 and 4). Together, these data suggest that MFs are associated with the total amount of phenacetin administered. The findings from a study performed by Thybaud et al. (2003) suggest that caution should be applied when setting treatment periods longer than 28 days since this may lead to an apparent increase in MF due to clonal expansion of early fixed mutations. We do not think this is the case in the present study because fewer than 12% of *gpt* mutations were identical in the same animals even within our longest study. Therefore, we suggest that independent MFs increase in accordance with the total dosage and administration period within 52-weeks least in the present study.

The sequence analysis indicated that most of the phenacetin-induced mutations in the kidney and liver were a guanine base substitution; that is, G:C to A:T (Tables 5 and 6). In the statistical analysis of mutational spectra, the pattern of phenacetin-induced mutations was different from that of spontaneous mutations in the liver in the 26- and 52-week studies (Supplementary Table 2). We identified hot spots in the liver at position 26 and 416 of the *gpt* gene created from a tryptophan codon (TGG) to a stop codon (TAG) (Supplementary Table 2). In the kidney, no clear hot spot was noted and there were no differences in the pattern of mutations between control groups and phenacetin-treated groups (Supplementary Table 1). However, the small number of mutants analyzed in the kidney may explain this finding. In addition, the finding that no significant clonal expansion of mutations occurs within 52 weeks suggests that the *gpt* delta rat mutation assay can be integrated into other repeat-dose toxicological studies. For example, a 13-week treatment period is frequently used for dose-finding studies for 2-year rodent cancer bioassays. Thus, we propose that the transgenic mutation assay can be integrated into a 13-week dose-finding study without the results being confounded by clonal expansion. Evaluation of genotoxicity along with general toxicity in 13 weeks would more clearly delineate the role of genotoxicity in carcinogenesis by chemicals, compared to the currently recommended treatment period of 4 weeks of transgenic mutation assays (OECD TG488).

In summary, phenacetin is weakly genotoxic in the kidney, the target organ of carcinogenesis, and strongly genotoxic in the liver, the nontarget organ. Results from *gpt* delta rat assays following repeated-dose treatments are extremely useful to elucidate the relationship between gene mutations and carcinogenesis in the target organ induced by cancer-causing agents.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2014.07.003.

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