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

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

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

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

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

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

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

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Research Article

Evaluation of *In Vivo* Genotoxicity Induced by *N*-Ethyl-*N*-nitrosourea, Benzo[*a*]pyrene, and 4-Nitroquinoline-1-oxide in the *Pig-a* and *gpt* Assays

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The recently developed *Pig-a* mutation assay is based on flow cytometric enumeration of glycosylphosphatidylinositol (GPI) anchor-deficient red blood cells caused by a forward mutation in the *Pig-a* gene. Because the assay can be conducted in nontransgenic animals and the mutations accumulate with repeat dosing, we believe that the *Pig-a* assay could be integrated into repeat-dose toxicology studies and provides an alternative to transgenic rodent (TGR) mutation assays. The capacity and characteristics of the *Pig-a* assay relative to TGR mutation assays, however, are unclear. Here, using transgenic *gpt* delta mice, we compared the *in vivo* genotoxicity of single oral doses of *N*-ethyl-*N*-nitrosourea (ENU, 40 mg/kg), benzo[*a*]pyrene (BP, 100 and 200 mg/kg), and 4-nitroquinoline-1-oxide (4NQO, 50 mg/kg) in the *Pig-a* (peripheral blood) and *gpt* (bone marrow and liver) gene mutation assays. *Pig-a* assays were conducted at 2, 4, and 7 weeks after the treatment, while *gpt* assays were conducted on tissues collected at the 7-week terminal sacrifice. ENU increased both *Pig-a* and *gpt* mutant frequencies (MFs) at all sampling times, and BP increased MFs in both assays but the *Pig-a* MFs peaked at 2 weeks and then decreased. Although 4NQO increased *gpt* MFs in the liver, only weak, nonsignificant increases (two- or threefold above control) were detected in the bone marrow in both the *Pig-a* and the *gpt* assay. These findings suggest that further studies are needed to elucidate the kinetics of the *Pig-a* mutation assay in order to use it as an alternative to the TGR mutation assay. Environ. Mol. Mutagen. 54:747–754, 2013.

Key words: transgenic rodent mutation assays; glycosylphosphatidylinositol anchor; red blood cells; genotoxicity

INTRODUCTION

Since gene mutations are implicated in the etiology of cancer and other human diseases, *in vivo* genotoxicity tests are important as public health management tools. One such tool is the transgenic rodent (TGR) mutation assay, which quantitatively measures the accumulation of mutations in all organs, including germ cells [Nohmi et al., 2000]. The TGR mutation assay fulfills a need for a practical and widely available *in vivo* test for the assessment of gene mutation; the assay has been recommended by regulatory authorities for safety evaluations [COM, 2011; ICH, 2011] and international guidelines have been published describing the conduct of the assay [OECD488, 2011].

The recently developed *Pig-a* gene mutation assay is a powerful and potentially useful tool for evaluating *in vivo* genotoxicity that may complement the TGR assay [Miura et al., 2008a,b,2009]. Because the *Pig-a* gene is on the X-chromosome and involves the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, single mutations in the *Pig-a* gene can result in the loss of

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expression of GPI-anchored proteins, a phenotype that can be detected by flow cytometric evaluation with only a few μL of peripheral blood cells [Miura et al., 2009]. Additionally, *Pig-a* mutation appears to function in an apparently neutral manner, and the accumulated effects of repeat exposures can be evaluated. A standardized protocol for conducting and interpreting the assay, as well as the accuracy of the assay, however, have not been established, and the target organ for the assay currently is limited only to blood cells.

In this study, we performed the *Pig-a* assay and the *gpt* assay in the same animals and compared the performance of the two assays in detecting three known mutagens. We also developed a flow cytometric strategy for defining *Pig-a* mutant cells. This report describes the performance, effectiveness and advantages of the *Pig-a* assay in comparison with the *gpt* assay.

MATERIALS AND METHODS

Preparation of Chemicals

We dissolved *N*-ethyl-*N*-nitrosourea (ENU, Sigma-Aldrich Japan, Tokyo) in phosphate-buffered saline (PBS) (pH 6.0) at 10 mg/mL. Benzo[*a*]pyrene (BP, Wako Pure Chemical, Osaka) was suspended in olive oil at 10 mg/mL (for 100 mg/kg treatment) or 20 mg/mL (for 200 mg/kg treatment). 4-Nitroquinoline-1-oxide (4NQO, Sigma-Aldrich Japan, Tokyo) was suspended at 5 mg/mL in olive oil.

Antibodies

We obtained anti-mouse TER119 antibody for erythroid cell staining (clone TER-119, PE-Cy7-conjugated) and anti-mouse CD24 antibody (clone M1/69, FITC-conjugated) from BioLegend Japan (Tokyo).

Treatment of Mice

Animal experiments were conducted humanely according to the regulations of the Animal Care and Use Committee of the National Institute of Health Sciences (NIHS), Tokyo, and with their permission. *gpt* Delta C57BL/6J transgenic male mice were bred and maintained at the NIHS animal facility. They were housed individually under specific pathogen-free conditions with a 12-hr light–dark cycle and given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co., Tokyo) *ad libitum*. At 8 weeks of age, five mice per group were given a single oral administration of ENU (40 mg/kg), BP (100 mg/kg or 200 mg/kg), 4NQO (50 mg/kg), or PBS (the negative control). Peripheral blood (18 μL) was withdrawn from a tail vein 2, 4, and 7 weeks after the treatments and used for the *Pig-a* assay. At 7 weeks, all mice were killed and bone marrow and liver samples were collected for the *gpt* assay.

Pig-a Mutation Assay

The *Pig-a* assay was performed with some modification of previously described methods [Miura et al., 2008a; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Briefly, EDTA (dipotassium salt) was dissolved in distilled water to make a 12% solution and used as an anticoagulant. Peripheral blood (18 μL) was mixed with 2 μL EDTA solution. Two microliters of the blood/EDTA mixture was suspended in 0.2 mL PBS, and labeled with 1 μg each of anti-mouse TER119 and anti-mouse CD24 antibodies. The cells were incubated for 1 hr in the dark at room temperature, centrifuged (1000*g*, 5 min), resuspended in 2 mL PBS, and examined using a FACS Canto II flow cytometer (BD Biosciences Japan, Tokyo). After gating for single cells, about 1×10^6 TER119-positive cells were analyzed for the presence of surface CD24, and the *Pig-a* mutant frequency (MF) was calculated as previously described [Horibata et al., 2011].

Gating Strategy for *Pig-a* Assay

Red blood cells (RBCs) were stained with anti-TER119, an antibody that specifically recognizes RBCs, and anti-CD24, an antibody used to detect GPI-anchored protein, as previously reported [Keller et al., 1999; Phonethepswath et al., 2008; Horibata et al., 2011; Kimoto et al., 2011]. Single cells, including RBCs and white blood cells (WBCs), were gated by light scatter (Fig. 1A). TER119-positive cells from this population (Fig. 1B) were analyzed further for the presence of the GPI-anchored CD24 antigen on the cell surface. The FITC-fluorescence intensities of RBCs without FITC-conjugated anti-CD24 were distributed as shown in Figure 1C, defining a gate that included 100% of lower FITC intensities of RBCs as "*Pig-a* mutant RBCs." This gating most likely included a number of events that were not true RBC *Pig-a* mutants, and therefore, to avoid artifactually inflating *Pig-a* MFs, we refined the gate for "*Pig-a* mutant RBCs" as the area encompassing a maximum of 99.0% of the lower RBC FITC staining intensities only (Figs. 1D and 1E).

gpt Mutation Assay

We extracted high molecular weight genomic DNA from liver and bone marrow cells using a Recover Ease DNA Isolation Kit (Agilent Technologies, Santa Clara, CA), rescued lambda EG10 phages using Transpack Packaging Extract (Agilent Technologies) and conducted the *gpt* mutation assay as previously described [Nohmi et al., 2000]. *gpt* MFs were calculated by dividing the number of confirmed 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids [Nohmi et al., 2000].

Statistical Analyses

The Kruskal–Wallis nonparametric test was used for comparisons among multiple groups at each time point. When significant differences were observed in the Kruskal–Wallis test, the Steel test was performed as a *post hoc* analysis for comparisons between the responses in the negative control and each treated group. Statistical analyses were performed using GraphPad Prism6 (GraphPad Software, La Jolla, CA) and Excel Statistics 2012 (Social Survey Research Information, Tokyo, Japan). For these analyses, a P-value of <0.05 was considered significant and two-tailed tests were performed.

Power analyses were performed using GraphPad StatMate2 software. GraphPad StatMate2 estimates the statistical power of detecting a difference between two sets of observations using an unpaired t-test (two-tailed). For these estimates, observations from our historical vehicle control data ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) were used to generate the power estimates (at the 80 and 95% level) of detecting true increases of 2-, 3-, etc., fold over the historical vehicle control MF at an $\alpha = 0.05$. SigmaPlot 12.5 also was used to estimate the effect of increasing the number of animals assayed on the power.

We also hypothesized that the power of the assay would be influenced by the number of erythrocytes interrogated for each sample, with the variability in the measurements, reflected in the SDs of mean *Pig-a* MFs for groups. In order to evaluate this effect, we determined assay power when the number of animals per group was 5 and the SDs of the mean control MF ranged from 0.1 to 15, comparing to the historical vehicle control ($n = 95$, SD = 0.91). Again, the historical vehicle control

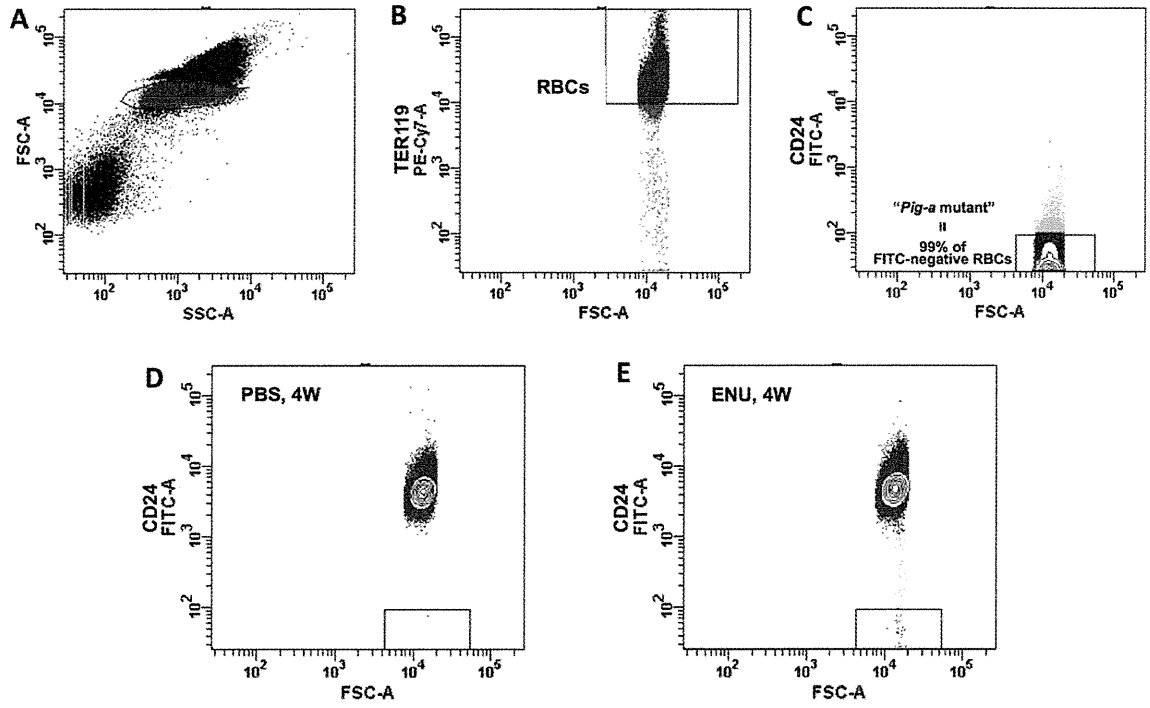

Fig. 1. Flow cytometeric analysis of mouse peripheral blood. **A**: Single cell populations were gated and further analyzed with anti-TER119 antibody. **B**: TER119-negative WBCs were excluded from the cell population gated in (A). TER119-positive RBCs were further analyzed with and without anti-CD24 antibody. **C**: TER119 positive cells were analyzed without anti-CD24 antibody staining so as to mimic *Pig-a* mutant RBCs. *Pig-a* mutant RBCs were defined by a gate encompassing at least 99% of lower intensities of FITC fluorescence associated with RBCs without anti-CD24 staining. **D**,**E**: Typical cytograms detecting *Pig-a* mutants. Approximately 1×10^6 TER119-positive cells derived from PBS- (D) or ENU- (E) treated mice were analyzed for CD24 expression.

MF (0.41×10^{-6}) was used as the control MF: minimal detectable fold-increases over vehicle control were calculated by dividing each estimated minimal detectable increase by the mean value of our historical vehicle control MF.

RESULTS

Pig-a Assay

The *Pig-a* mutant (CD24-negative) RBC population identified by flow cytometry after PBS or ENU treatment is shown in Figure 1D and 1E. MFs in the ENU-treated mice were significantly increased and the increase was modestly dependent upon time (mean ± SD for the PBS: 2 weeks after treatment, $0.80 \pm 0.45 \times 10^{-6}$; 4 weeks after treatment, $0.40 \pm 0.55 \times 10^{-6}$; and 7 weeks after treatment, $0.80 \pm 1.30 \times 10^{-6}$; mean ± SD for mice treated with 40 mg/kg ENU: 2 weeks after treatment, $23.00 \pm 6.96 \times 10^{-6}$; 4 weeks after treatment, $29.40 \pm 6.11 \times 10^{-6}$; and 7 weeks after treatment, $29.60 \pm 13.58 \times 10^{-6}$) (Fig. 2A).

In the case of 4NQO-treated mice, the average frequencies were more than twofold greater than controls at each of the time points studied (mean ± SD for mice treated with 50 mg/kg 4NQO: 2 weeks after treatment, $2.60 \pm 4.72 \times 10^{-6}$; 4 weeks after treatment, $1.00 \pm 1.00 \times 10^{-6}$; and 7 weeks after treatment, $2.20 \pm 4.38 \times 10^{-6}$) (Fig. 2B). Although there were no significant differences between the control and treated mice, there was relatively large range for the *Pig-a* MFs in individual 4NQO-treated mice (Fig. 2B, e.g. minimum MF was 0×10^{-6} and maximum MF was 11×10^{-6}). These results prompted us to conduct power analyses. At the 2-, 4-, and 7-week sampling times, the power of the assay to detect a significant increase in *Pig-a* MF after 4NQO-treatment was only 12%, 18%, and 10%, respectively, whereas the statistical power of the assay to reject the null hypothesis (i.e. no difference between control and treatment groups) and detect a significant increase in MF in the ENU-treated group was >95–99% at each of the sampling points.

Significant increases in *Pig-a* MF were observed for both BP doses at 2 weeks after treatment (Figs. 2C and 2D). For the mice treated with 100 mg/kg BP, the frequencies diminished with time, and no increase was evident 7 weeks after treatment (mean ± SD for mice treated with 100 mg/kg BP: 2 weeks after treatment, $9.25 \pm 2.63 \times 10^{-6}$; 4 weeks after treatment, $3.00 \pm 2.16 \times 10^{-6}$; and 7 weeks after treatment, $0.50 \pm 1.00 \times 10^{-6}$; with

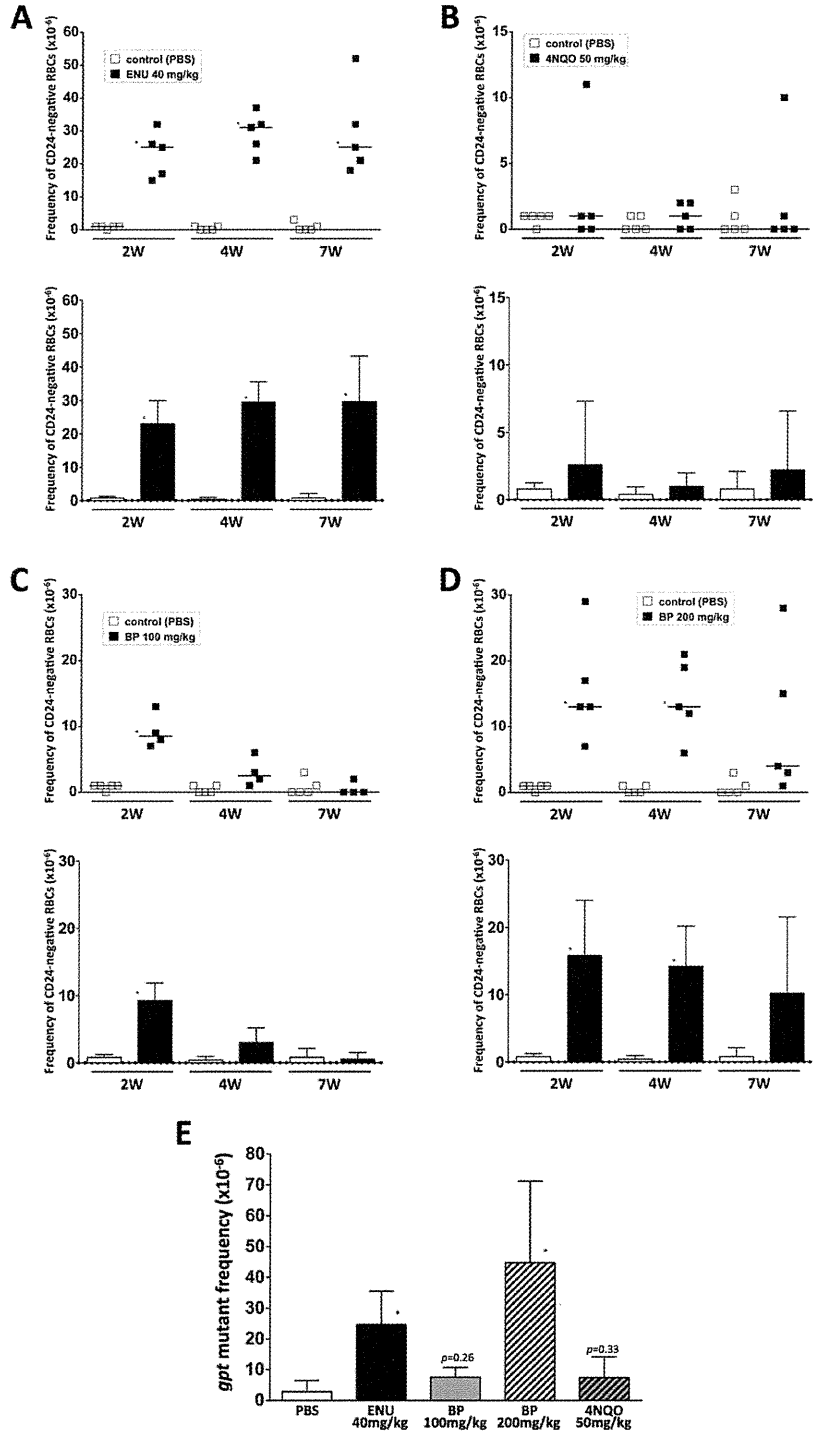

Fig. 2. Comparative analyses of *Pig-a* mutation in peripheral blood RBCs and *gpt* mutation in bone marrow cells. At 2, 4, and 7 weeks after treatment with (**A**) 40 mg/kg ENU, (**B**) 50 mg/kg 4NQO, (**C**) 100 mg/kg BP, or (**D**) 200 mg/kg BP, or PBS solvent, peripheral blood was withdrawn from the tail vein and analyzed by flow cytometry for the presence of CD24 on the surface of RBCs. Upper panels of (A)–(D): scatter plots with median bars. Lower panels of (A)–(D): the mean ± SD. (**E**) Seven weeks after treatment, all mice were killed and their bone marrow cells were isolated for the *gpt* assay. Frequencies are the mean ± SD of five animals per treatment group. $^{*}P < 0.05$.

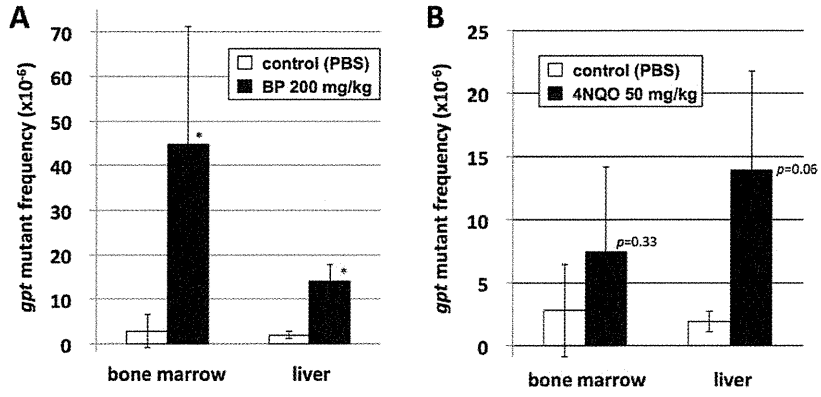

Fig. 3. Comparative analyses of *gpt* mutation in bone marrow and liver. Seven weeks after treatment, all mice were killed and liver samples were collected and analyzed by the *gpt* assay (bone marrow data are the same as in Fig. 2). The frequencies are the mean ± SD of data from five animals. $^*P < 0.05$.

power analyses indicating that the assay had probabilities of >99%, >60%, and <10% of rejecting the null hypothesis, respectively, i.e. to distinguish between the negative control and BP-treated group). Although significant increases were measured at all sampling times, a similar reduction with time was detected in the mice treated with 200 mg/kg BP (mean ± SD for mice treated with BP: 2 weeks after treatment, $15.80 \pm 8.20 \times 10^{-6}$; 4 weeks after treatment, $14.20 \pm 5.97 \times 10^{-6}$; and 7 weeks after treatment, $10.20 \pm 11.34 \times 10^{-6}$; with power analysis indicating that the assay had probabilities of >95%, >99%, and >30%, respectively, to distinguish between the treated and control groups) (Figs. 2C and 2D).

gpt Assay on Bone Marrow and Liver

Compared with the solvent control animals (MF for PBS control group, $2.83 \pm 3.68 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU- and high-dose BP-treated mice (ENU, $24.70 \pm 10.49 \times 10^{-6}$; BP 200 mg/kg, $44.86 \pm 26.37 \times 10^{-6}$) (Fig. 2E), but not in low-dose BP- or in 4NQO-treated mice (100 mg/kg BP, $7.64 \pm 3.12 \times 10^{-6}$; 4NQO, $7.45 \pm 6.75 \times 10^{-6}$) (Figs. 2B and 2C). *gpt* MFs also were increased in the liver of high-dose BP- and 4NQO-treated mice (MF for PBS control group, $1.97 \pm 0.83 \times 10^{-6}$; 200 mg/kg BP, $14.04 \pm 3.76 \times 10^{-6}$; 4NQO, $13.92 \pm 7.83 \times 10^{-6}$) (Fig. 3).

DISCUSSION

TGR mutation assays, such as the *gpt* gene mutation assay, are an established method for monitoring *in vivo* genotoxicity in multiple tissues concurrently [for review, see Nohmi et al., 2000; Lambert et al., 2005; OECD488, 2011]. While the *Pig-a* gene mutation assay analyzes only one type of cells, i.e., blood cells, it has the advantage of not requiring the use of transgenic animals [Miura et al., 2008a,b]. The *Pig-a* assay has been undergoing extensive development and validation studies are presently being conducted [see the special issue of *Environmental and Molecular Mutagenesis* 52, 2011]. Based on the recent studies, it has been suggested that the *Pig-a* assay could be integrated into repeat-dose toxicology studies. The majority of *Pig-a* work to date has been performed with rats, with very little data being generated in mice, and this data gap needs to be addressed. Therefore, in this study, we analyzed both transgene and *Pig-a* mutation using *gpt*-delta transgenic mice.

Our results using a single oral administration of ENU were consistent with those of previously reported mouse studies [Bhalli et al., 2011a; Horibata et al., 2011; Kimoto et al., 2011]. The ENU-induced MFs detected by the *Pig-a* and *gpt* assays were similar, suggesting that both assays were able to detect ENU genotoxicity equally well.

We also detected dose-dependent increases of *Pig-a* MF in BP-treated mice (Figs. 2C and 2D). The MFs in the mice treated with both low- and high- doses of BP peaked at 2 weeks after treatment and declined thereafter. These time-dependent reductions in *Pig-a* MF differed from the kinetics of ENU–induced *Pig-a* mutation. Phonethepswath et al. reported on the kinetics of *Pig-a* mutation in RBCs from Wister rats treated with ENU, 7,12-dimethyl-1,2-benz[*a*]anthracene, N-methyl-N-nitrosourea, 4NQO and BP [Phonethepswath et al., 2010]. While erythroid progenitors normally mature over a period of 7–14 days *in vitro* [Iscove and Sieber, 1975], it takes 4–6 months to re-establish stable hematopoietic stem cell numbers following bone marrow transplantation [Jordan and Lemischka, 1990]. These observations imply that ENU may effectively mutate hematopoietic stem cells, and that a large proportion of *Pig-a* mutants in BP-treated mice may be due to mutations induced in erythroid progenitors.

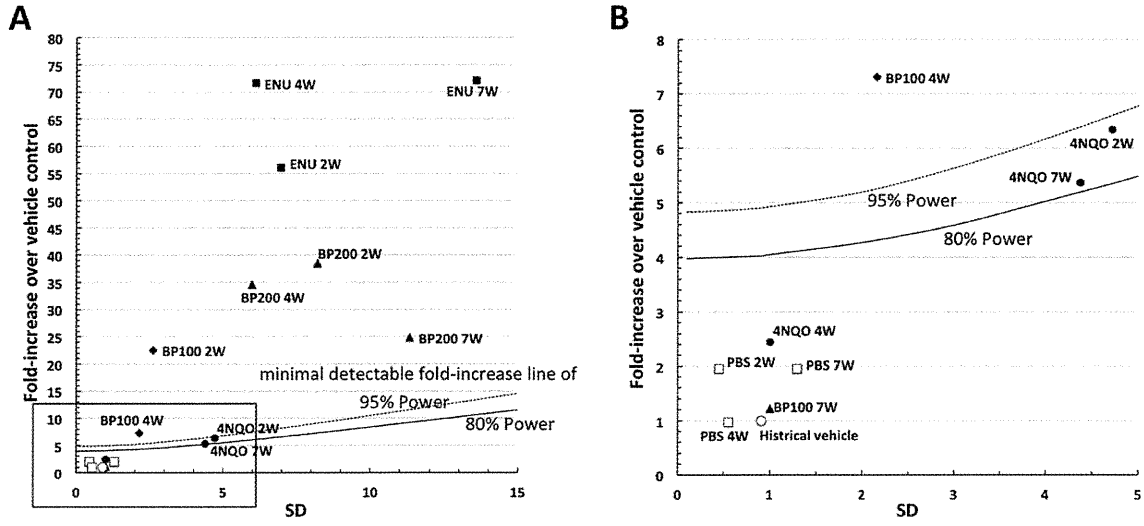

Fig. 4. Relationships between the power to detect fold-increases in *Pig-a* mutant frequency (MF) over the historical vehicle control MF and the effect of the SD of the mean measurements on the power of detection. The solid and dotted lines indicate the fold-increase over the historical vehicle control data detected with 80% and 95% power, respectively. All power estimates were based on an $n = 5$ comparing to the historical vehicle control ($n = 95$, SD = 0.91), α5%, and use an unpaired two-tailed t-test. Closed square, closed triangle, closed diamond, closed circle, open square, and open circle indicate the fold-increase produced by treatment with ENU, high-dose BP, low-dose BP, 4NQO, and PBS and the historical vehicle control, respectively. The symbols located above each boundary line indicate that the fold-increases for these groups are detected with 95 and or 80% power by an unpaired t-test (two-tailed). Open-boxed area indicated in (**A**) is expanded in (**B**).

TABLE I. Power Analysis Using the Historical Control Data[a]

The number of animals of		Power to detect increase of				
experimental group	control group	twofold change over control	threefold change over control	fourfold change over control	fivefold change over control	sixfold change over control
5	5	10%	24%	47%	71%	88%
10	10	16%	48%	82%	97%	>99%
15	15	22%	66%	95%	>99%	>99%
20	20	28%	79%	>99%	>99%	>99%
25	25	35%	88%	>99%	>99%	>99%
80	80	81%	>99%	>99%	>99%	>99%

[a]The historical control data of *Pig-a* MF were $n = 95$, male mice of C57BL/6, 3–12 weeks of age and mean ± SD: 0.41 ± 0.91.

In this study, we found no significant increases in *Pig-a* MF in 4NQO-treated mice using the Steel multiple comparison test, although at each sampling time, the mean MF for the treated mice was greater than the negative control (Fig. 2B). Power analysis employing our historical background *Pig-a* MF ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) indicated that the assays conducted in this present study (sample size of 5, interrogating 1×10^6 cells/sample) lacked the ability to distinguish these two- to threefold differences between the treated and control groups. The plots shown in Figure 4 indicate that the assays had, at best, an 80–95% power to detect a true four- to fivefold increase of the control and that power depended on the variability of the measurements. When the SD for the MF measurement is small, that is, when the range in MFs for the individuals in the treatment group is small, the assay is capable of detecting about a fourfold increase in *Pig-a* MF with 80% of power (Fig. 4). But when the variability in within-group measurements increased, as it did for many of the treated groups and even some of the controls, then the power of the assay to detect true differences decreased. For instance, the range of MFs for the individual 4NQO-treated mice was relatively large, which contributed to the uncertainty in this measurement. It should be noted that the 2- and 7-week 4NQO responses fell into the 'gray area' for detection based on our power analysis. Performing unpaired t-tests comparing the historical vehicle control data and the responses produced by the 4NQO-treated groups indicated that the assays on 4NQO-

treated mice at 2 and 7 weeks were able to detect significant increases in *Pig-a* MF with over 80% of power (Fig. 4).

The statistical power of the assays can be increased by increasing either the number of mice per group, the number of RBCs interrogated per sample, or both. For instance, increasing the number of mice per group to 20 by itself will enable the assay to detect a threefold increase with nearly 80% power (Table I). Interrogating additional erythrocytes will reduce the number mice with "0" MFs (as can be seen in Fig. 2) and, potentially, the mouse to mouse variability in MF [Nowosiad et al., 2011]. With sufficient sample sizes, it is possible that 4NQO will test positive in the mouse *Pig-a* assay, even if the statistical analyses are conducted using multiple comparison tests. Similar to the *Pig-a* assay results, the mean bone marrow *gpt* MF in 4NQO-treated mice was only about two times higher than the frequency in the negative control, and these frequencies were not significantly different.

In contrast to the bone marrow *gpt* findings, we did detect a marginally significant increase in 4NQO-induced *gpt* MF in liver (Fig. 3B). Significant increases in liver mutagenicity also have been detected in MutaMouse following a single oral administration of 4NQO [Nakajima et al., 1999; Suzuki et al., 1999]. In these studies, the *lacZ* MF in the liver increased with time, but the MF in bone marrow peaked at 1 week and then decreased with time. These findings suggest that the peak bone marrow *gpt* MF in our present study could have been missed because the tissues were analyzed only 7 weeks after treatment. Additionally, in the previous MutaMouse studies, the *lacZ* MFs induced by BP and 4NQO were much higher in the bone marrow than in the liver [Hakura et al., 1998; Nakajima et al., 1999; Suzuki et al., 1999; Lemieux et al., 2011]. We also observed a greater response for BP in the bone marrow than liver. For 4NQO, however, we observed a higher *gpt* MF in the liver than in the bone marrow. The reason for these disparate results is unknown, but they may be related to the use of different TGRs.

Both the *Pig-a* and TGR assays have unique strengths and weaknesses. TGR assays are costly but they can be used for surveying mutation in various tissues. In this study, in fact, the *gpt* assay detected 4NQO genotoxicity in liver whereas the *Pig-a* assay did not detect the mutagenicity of 4NQO in peripheral blood. This differential response may, at least in part, be due to the target tissues for 4NQO mutagenesis because 4NQO also did not significantly increase *gpt*-mutation in bone marrow (although it is recognized that sampling times in the current study were not optimal for bone marrow mutation assessment).

The OECD guideline for TGR assays recommends a tissue sampling time of 3 days after 28 consecutive daily treatments [OECD488, 2011], making it difficult to integrate TGR assays it into standard repeat-dose toxicology studies. Since the *Pig-a* gene is an endogenous gene, the *Pig-a* assay does not require TGR animals. Thus, while the *Pig-a* assay can be combined with a TGR assay, as was done in this present study, it also potentially can be integrated into repeat-dose toxicology studies that do not use TGRs [Dertinger et al., 2010; Dobrovolsky et al., 2010; Bhalli et al., 2011b; Cammerer et al., 2011; Dertinger et al., 2011; Lemieux et al., 2011; Lynch et al., 2011; Schuler et al., 2011; Shi et al., 2011]. Currently, however, we need additional studies that compare mutational responses in the *Pig-a* gene and TGR transgenes in order to help validate the *Pig-a* assay.

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AUTHOR CONTRIBUTIONS

Drs. Horibata and Honma designed the study. Drs. Horibata, Kimoto, Masumura, Nohmi, and Honma critically discussed the study. Dr. Horibata, Ms. Ukai, Dr. Suzuki, and Ms. Kamoshita collected the data. Dr. Horibata and Ms. Ukai analyzed the data and prepared draft figures. Dr. Horibata prepared the manuscript draft with important intellectual input from Dr. Honma. All authors approved the final manuscript. Drs. Horibata and Honma had completed access to the study data.

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