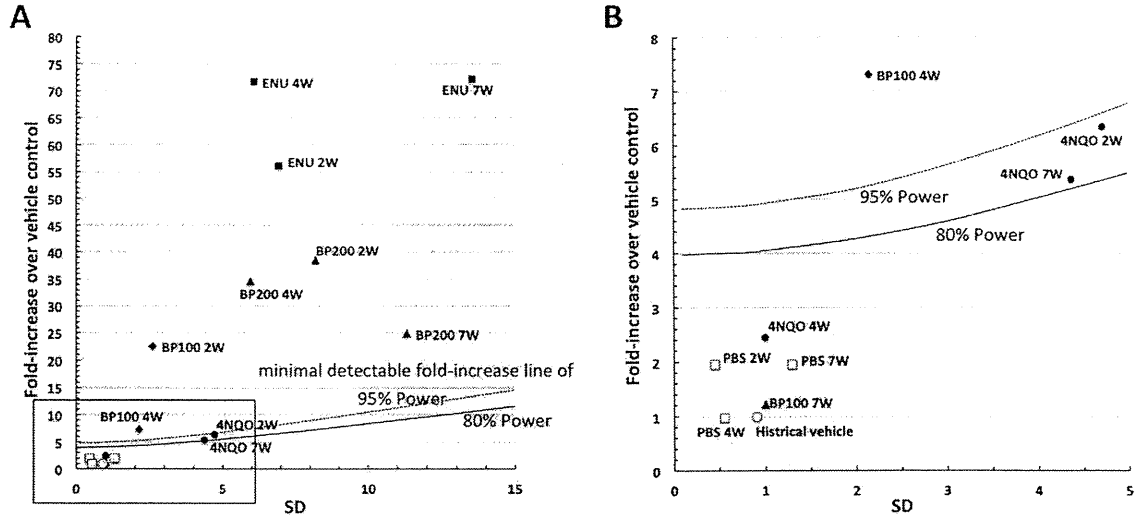

Fig. 4. Relationships between the power to detect fold-increases in *Pig-a* mutant frequency (MF) over the historical vehicle control MF and the effect of the SD of the mean measurements on the power of detection. The solid and dotted lines indicate the fold-increase over the historical vehicle control 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), $\alpha 5\%$, 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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Development of a repeated-dose liver micronucleus assay using adult rats (II): Further investigation of 1,2-dimethylhydrazine and 2,6-diaminotoluene

Hironao Takasawa[a,*], Rie Takashima[a], Akiko Hattori[a], Kazunori Narumi[a,b], Kazufumi Kawasako[a], Takeshi Morita[c], Makoto Hayashi[d], Shuichi Hamada[a]

[a] Mitsubishi Chemical Medience Corporation, 14-1 Sunayama, Kamisu-shi, Ibaraki 314-0255, Japan
[b] Yakult Honsha Co., Ltd., 1796 Yaho, Kunitachi-shi, Tokyo 186-8650, Japan
[c] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[d] Biosafety Research Center, Foods, Drugs, and Pesticides, 582-2, Shioshinden, Iwata, Shizuoka 437-1213, Japan

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ABSTRACT

Detecting genotoxicity in the liver is considered an effective approach for predicting hepatocarcinogenicity, as many genotoxic chemicals *in vivo* may act as hepatocarcinogens in rodents. Here, a genotoxic rodent hepatocarcinogen, 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), and a genotoxic (Ames positive) noncarcinogen, 2,6-diaminotolunene (2,6-DAT), were administered orally to rats for up to 28 days, and liver samples were then examined in a repeated-dose liver micronucleus (MN) assay, and additionally tested in the bone marrow (BM) MN assay concurrently. We recently established a simple method to isolate hepatocytes without *in situ* liver perfusion procedures, and applied this method in the liver MN assay. As a result, 1,2-DMH increased the proportion of micronucleated hepatocytes in both a dose- and duration-dependent manner at relatively low-dose levels that are routinely used in repeated-dose toxicity studies. In contrast to 1,2-DMH, 2,6-DAT did not have a detectable effect. In addition to these two chemicals, two genotoxic rodent hepatocarcinogens, diethylnitrosamine and 2,4-diaminotoluene, which gave positive responses in the liver MN assay in our previous investigation [Narumi et al., Mutat. Res. 747 (2012) 234-239], were subjected to the BM MN assay and histopathological evaluation. All four test chemicals gave negative responses in the BM MN assay. Furthermore, the three hepatocarcinogens displayed hepatotoxicity, including hepatocellular hypertrophy and anisokaryosis, but no abnormal findings were observed in the liver of rats treated with 2,6-DAT. Taken together, the present results indicate that the liver MN assay is effective for predicting hepatocarcinogenicity and may be integrated into repeated-dose toxicity studies without disturbing routine examinations, such as histopathology. Furthermore, with repeat-dose treatment protocols, our findings indicate that the liver MN assay is superior to the BM MN assay for detecting genotoxic or carcinogenic chemicals in rats.

1. Introduction

Most previous micronucleus (MN) assays in the liver have been conducted using partial hepatectomy (PH) [1,2], mitogenic treatment [3,4], or in juvenile rats [5–8]. These studies indicate that the liver MN assay may be useful for the prediction of chemical hepatocarcinogenesis, even using chemicals that are insensitive to rodent bone marrow (BM) MN assays. Hepatocarcinogens are activated in the liver to yield genotoxic metabolites, which often do not reach BM cells due to their short lifetime [9]. In fact, several rodent hepatocarcinogens, including nitro-aromatic amines, alkylnitrosamines, and azo-derived chemicals, give negative results in BM MN assays [1]. Although many carcinogenic chemicals have been identified, tumor induction most frequently occurs in the liver [10]. Thus, it is anticipated that the MN-inducing potential of genotoxic hepatocarcinogens can be detected using liver MN assays [1,11,12].

Recently, participating members of the European Center for the Validation of Alternative Methods (ECVAM) Workshop agreed that the integration of *in vivo* genotoxicity tests, such as the BM MN assay, into repeated-dose toxicity studies presents one approach for reducing the number of animals used in experimentation [13]. Furthermore, to reduce the number of experimental animals, the International Conference on Harmonization (ICH) guidance on genotoxicity testing has recently been revised to recommend integration of *in vivo* genotoxicity studies, such as the BM MN assay, into general toxicity studies routinely conducted at the pre-clinical

* Corresponding author at: Safety Assessment Department, Nonclinical Research Center, Drug Development Service Segment, Mitsubishi Chemical Medience Corporation, 14-1 Sunayama, Kamisu-shi, Ibaraki 314-0255, Japan. Tel.: +81 479 46 7552; fax: +81 479 46 7505.
E-mail address: Takasawa.Hironao@mg.medience.co.jp (H. Takasawa).

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stage of pharmaceutical development [14]. However, the liver MN assays are typically performed using *in situ* liver perfusion *via* the portal vein or vena cava to isolate hepatocytes. Liver perfusion precludes conducting other tests that are commonly performed as part of repeat-dose general toxicology studies, such as histopathological examination.

To overcome the above-described limitations of *in situ* liver perfusion, we have recently established a remarkably practical and simple method to isolate hepatocytes from animals for the evaluation of liver MN inducibility [15]. Using this method, we reported that the rodent hepatocarcinogens diethylnitrosamine (DEN) and 2,4-diaminotoluene (2,4-DAT) significantly induced MN in the rat liver with repeated treatment regimens at least 14 days after dosing [15]. The establishment of this hepatocyte isolation method, which does not require perfusion of the entire liver, is expected to facilitate integration of the liver MN assay into general toxicity studies.

In the present study, we investigated the effectiveness of the liver MN assay conducted as part of a repeated-dose treatment protocol in order to confirm if this assay could be integrated into general toxicity studies. A genotoxic rodent hepatocarcinogen, 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), and a genotoxic noncarcinogen, 2,6-diaminotoluene (2,6-DAT), were repeatedly administered to rats for up to 28 days, and MN induction in the liver and BM were then concurrently evaluated. In addition, samples from the identical animals used in the MN assays were subjected to histopathological examination.

In our previous report [15], DEN and 2,4-DAT were examined using only the liver MN assay; however, BM cells had been also collected from the identical animals. Furthermore, liver tissues other than those used for the liver MN assay had been fixed with formalin for later histopathological examination. Here, to further evaluate liver responses to hepatocarcinogens and noncarcinogens, the results of the MN assays and histopathology were compared between three hepatocarcinogens (1,2-DMH, DEN, and 2,4-DAT) and a noncarcinogen (2,6-DAT). Thus, this is a complementary paper to our previous report [15].

2. Materials and methods

2.1. Animals

Five-week-old male rats [Crl:CD(SD)] were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were housed in an air-conditioned room maintained at 22 ± 3 °C and 55% ± 20% relative humidity under a 12-h light/dark cycle and allowed free access to a commercial pellet diet and tap water. The rats were quarantined and acclimated for a period of one week prior to the start of drug administration. The animal experiments were approved by the Institutional Animal Care and Use Committee of the test facility prior to conducting the experiments.

2.2. Chemicals

1,2-DMH (hydrochloride salt, CAS No. 306-37-6, >98% purity) and DEN (CAS No. 55-18-5, >99% purity) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 2,6-DAT (CAS No. 823-40-5, >98% purity) and 2,4-DAT (CAS No. 95-80-7, >95% purity) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were dissolved in physiological saline at appropriate concentrations to prepare dose formulations for administration to animals.

These chemicals were selected for reasons as follows: DEN and 2,4-DAT were used in our previous report and evaluated for their ability to induce MN in the liver of adult rats [15]; 1,2-DMH was expected to be positive in the liver MN assay in this study according to the previous report using young rats [8] although this chemical was reported to be negative in the BM MN assay in the 28-day repeated dose study [16]; and 2,6-DAT is structural isomer of 2,4-DAT and is classified as noncarcinogen [17], and furthermore this chemical was expected to be negative in the liver MN assay in this study.

2.3. Dose levels and treatment

Two dose levels were used for the administration of 1,2-DMH to be consistent with our previous study that used two dose levels of DEN and 2,4-DAT [15]. As 10 mg/kg/day was reported to be the maximum tolerated dose (MTD) of 1,2-DMH during a 28-day experiment [16], the high-dose level was set at 10 mg/kg/day, and the low-dose level was set at 5 mg/kg/day. The high dose level was expected to be positive in the present study based on the results of a previous report on MN induction in juvenile rats [8]. 2,6-DAT is known to be noncarcinogenic in rodents [17], and this chemical was expected to give a negative response in this investigation. Therefore, three dose levels in total were used for 2,6-DAT. The high-dose level was set at 100 mg/kg/day, which was equivalent to 40% of the level used in a previous report [18] in which rats were given a single oral gavage, and this dose level was anticipated to be the MTD for 28 days. The two lower doses were set at 50% and 25% of the high-dose level. For DEN and 2,4-DAT, the high dose levels were set at 12.5 and 50 mg/kg/day, respectively, as these levels were used in our previous investigation [15], and the low dose levels were set at 50% of the high dose level. Five animals were randomly assigned to each treatment group except for the 5- and 14-day experiments of 1,2-DMH, in which 3 animals were allocated to each group. The high dose level of each test chemical was administered once daily for 5, 14 or 28 days by oral gavage, and the lower dose level(s) of each chemical was administered up to 28 days. As the negative control, physiological saline used as the vehicle for preparation of the test formulations was administered in the same way as the test chemicals. All dose volumes were 10 mL/kg/day.

2.4. Liver MN assay

On the day after the last administration of the test chemicals for each time point (5, 14, and 28 days), the animals were euthanized by exsanguination under deep anesthesia without overnight fasting. The livers were excised, and the lateral left lobe of the liver was then removed. An approximately 1 g portion of the lobe was sliced into several pieces of 0.5- to 1-mm thicknesses. The sliced tissues were washed with cold Hank's balanced salt solution (HBSS) and treated with a digestion solution containing collagenase (Wako Pure Chemical Industries, Ltd., Osaka Japan) to isolate hepatocytes (HEPs) using the methods established by Narumi et al. [15]. Isolated HEPs were fixed with 10% neutral buffered formalin, and the resultant suspensions of HEPs were stored at room temperature until needed for microscopic observation.

Immediately prior to microscopic observation, each HEPs suspension was mixed and stained with an equal volume of staining solution consisting of acridine orange (AO, 500 μg/mL) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μg/mL). The resultant mixture was dropped onto a clean glass slide and covered with a cover slip (24 mm × 50 mm) to prepare slide specimens. The specimens were observed under a fluorescent microscope at 400× magnification with U-excitation (Ultraviolet rays excitation, wave length: 330–385 nm), and the number of micronucleated HEPs (MNHEPs) per 2000 parenchymal HEPs was counted for each animal according to the previous reports [5–8].

2.5. BM MN assay

After the removal of livers as described in the liver MN assay, femurs were removed from the same animals. BM cells were collected from the femurs according to a method reported by Kawabata et al. [19], and the resultant BM cell suspensions were stored at room temperature until used for microscopic observation.

Immediately prior to microscopic observation, each BM-sample was mixed and stained with an equal volume of an AO (500 μg/mL) solution. The resultant mixture was dropped onto a clean glass slide and covered with a cover slip (24 mm × 50 mm) to prepare slide specimens. The specimens were observed under a fluorescent microscope at 600× magnification with B-excitation (Blue light excitation, wave length: 420–490 nm), and the number of micronucleated immature erythrocytes (MNIMEs) per 2000 erythrocytes was counted for each animal. As a parameter of hematopoietic function in BM, the proportion of immature erythrocytes (IMEs) among 1000 erythrocytes was also calculated for each animal.

2.6. Histopathological examination

Upon euthanasia, the residual liver tissue of the left lateral lobe after the isolation of HEPs was fixed with 10% phosphate buffered formalin, embedded in paraffin, and then stained with hematoxylin and eosin (H.E.) according to the standard method. Histopathological examination was performed under a light microscope.

2.7. Statistical analysis

Differences in the incidences of MNHEPs and MNIMEs between the test and the vehicle control groups were analyzed using the conditional binomial test reported by Kastenbaum and Bowman [20]. To analyze the percentage of IME ratios between groups, Dunnett's multiple comparison test was applied for the 28-day experiment since two or three groups were treated with each test chemical. For the 5- and 14-day experiments, Student's *t*-test was applied since there was only one group of each test chemical.

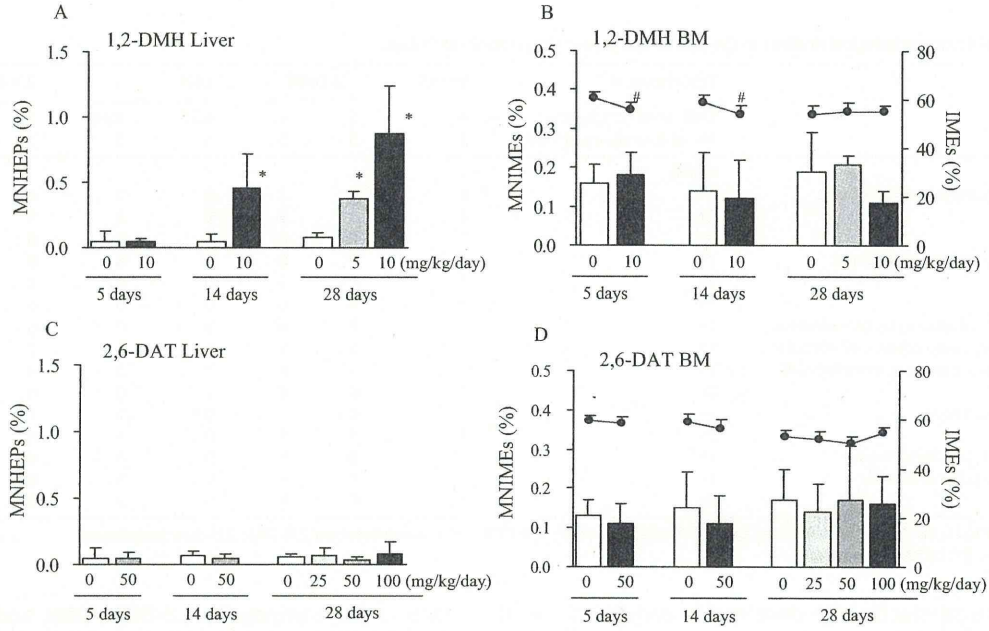

Fig. 1. Proportion of MNHEPs (%) in rat livers (bar graphs, left) and ratio of MNIMEs (bar graphs, right) and IMEs-ratio (line graphs, right) in rat BM treated for 5, 14, or 28 days with 1,2-DMH (A, B) or 2,6-DAT (C, D). Values are presented as the mean and SD ($n=5$ or 3). $^{*}p<0.01$, Kastenbaum and Bowman test, $^{\#}p<0.05$, Student's t-test.

3. Results

3.1. Liver and BM MN assays

Fig. 1 shows the results of the liver and BM MN assays in rats treated with 1,2-DMH and 2,6-DAT, and Fig. 2 shows the results of the BM MN assay with DEN and 2,4-DAT administered to rats used in the previously reported liver MN assay [15]. In the liver MN assay, the mean MNHEPs % in the vehicle control groups were $0.05\pm0.07\%$, $0.05\pm0.05\%$, and $0.08\pm0.03\%$ for 5, 14 and 28 days in 1,2-DMH experiment, respectively. For 2,6-DAT in the same way, the mean MNHEPs % in the vehicle control groups were $0.05\pm0.07\%$, $0.07\pm0.03\%$, and $0.06\pm0.02\%$ for 5, 14 and 28 days, respectively. 1,2-DMH significantly increased the proportion of MNHEPs after repeated dosing for 14 days or longer (mean MNHEPs %: 0.45% at 12.5 mg/kg/day for 14 days, 0.37% at 6.25 mg/kg/day for 28 days and 0.87% at 12.5 mg/kg/day for 28 days), but had no effect in the 5-day experiment (mean MNHEPs %: 0.05% at 6.25 mg/kg/day, Fig. 1A). In the 28-day experiment, a dose-dependent increase in the incidence of MNHEPs was observed. Additionally, the incidence of MNHEPs tended to display a duration-dependent increase. In contrast to 1,2-DMH, treatment with 2,6-DAT did not result in significant increases in the incidence of MNHEPs in the liver at any examined time point; the mean MNHEPs % ranged from 0.03% to 0.08% (Fig. 1C).

In the BM MN assay, no significant increases in the proportion of MNIMEs were detected during the 28 days of treatment with either 1,2-DMH or 2,6-DAT (Fig. 1B and D, respectively). In the 28-day experiment with 2,4-DAT, one animal each was found dead on day 15 and 24 after the initial administration at 50 mg/kg/day, while no mortality was observed in the other groups administered 2,4-DAT. All surviving animals were used for the evaluation of the BM MN assay for 2,4-DAT.

For both 2,4-DAT and DEN, no significant increases in the proportion of MNIMEs in the BM MN assay were observed at any time point up to 28 days (Fig. 2). In the 5-day experiment with DEN (Fig. 2A) and the 5- and 14-day experiments with 1,2-DMH (Fig. 1B), significant decreases in the IMEs ratio were detected. However, no marked differences were observed in the IMEs ratios for the 28-day experiments for any test chemical compared to the vehicle control group.

3.2. Histopathology

Table 1 presents the test chemical-related histopathological findings in the liver and Fig. 3 shows the typical hepatotoxicity

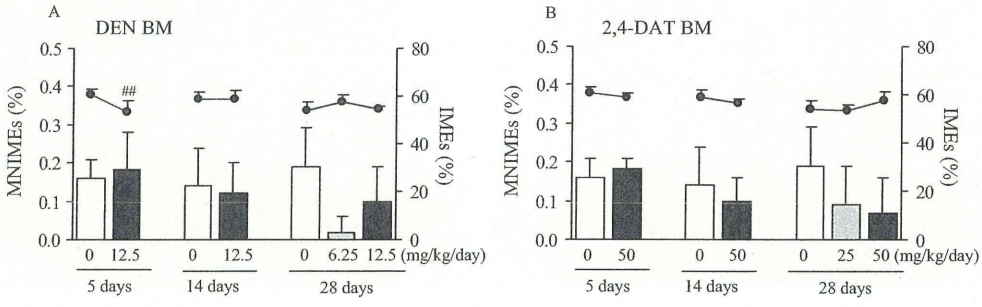

Fig. 2. Proportion of MNIMEs (bar graphs) and IMEs ratios (line graphs) in rat BM treated for 5, 14, or 28 days with DEN (A) or 2,4-DAT (B). Values are presented as the mean and SD ($n=5$). $^{\#\#}p<0.01$, Student's t-test.

Table 1
Test chemical-related histopathological findings in the liver of rats repeatedly treated for 28 days.

Finding	Test chemical	Vehicle	1,2-DMH		DEN		2,4-DAT		2,6-DAT
	Dose level (mg/kg/day)	0	5	10	6.25	12.5	25	50	100
	No. of animals examined	5	5	5	5	5	5	3	5
	Grade								
Hypertrophy, hepatocyte, centrilobular	1+	0	0	2	0	0	4	1	0
	2+	0	0	3	0	2	1	1	0
	3+	0	0	0	0	3	0	1	0
Hypertrophy, hepatocyte, perilobular	1+	0	0	0	0	0	0	1	0
	2+	0	0	0	0	0	1	1	0
	3+	0	0	0	0	0	0	1	0
Acidophilic change, hepatocyte, centrilobular	1+	0	5	0	2	0	0	0	0
Single cell necrosis, hepatocyte, centrilobular	1+	0	5	5	4	5	3	3	0
Cell infiltration, inflammatory, centrilobular	1+	0	0	0	2	2	4	0	0
	2+	0	0	0	1	0	0	0	0
Anisokaryosis, hepatocyte	1+	0	0	3	0	0	1	3	0
	2+	0	0	0	0	5	0	0	0
Increase, mitotic figure, hepatocyte	1+	0	0	0	0	4	0	0	0
Proliferation, oval cell, perilobular	1+	0	0	0	0	3	0	1	0
	2+	0	0	0	0	2	0	0	0

1,2-DMH: 1,2-dimethyl hydrazine hydrochloride; DEN: diethylnitrosamine; 2,4-DAT: 2,4-diaminotoluene; 2,6-DAT: 2,6-diaminotoluene.
Grade—1+: minimal; 2+: mild; 3+: moderate.

observed in rats treated at the high-dose level for each test chemical in the 28-day experiments.

Centrilobular hypertrophy of hepatocytes was observed in all of the surviving animals treated at the high dose levels of the three hepatocarcinogens, 1,2-DMH, DEN, and 2,4-DAT. Notably, centrilobular hypertrophy was also detected at the low dose level of 2,4-DAT, which additionally induced perilobular hypertrophy of hepatocytes. For all chemicals, the observed hepatocellular

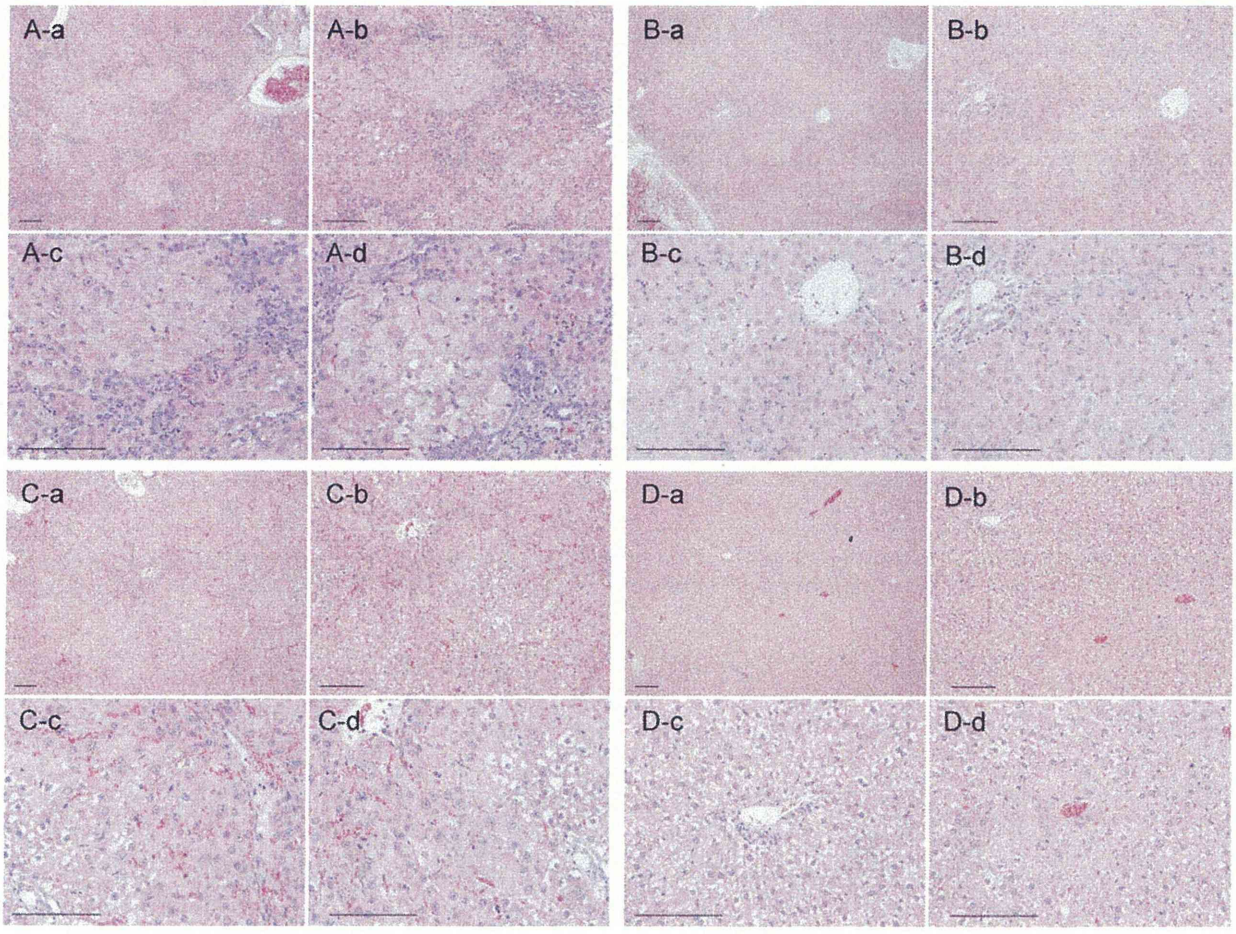

Fig. 3. H.E. stained histopathological images of rat livers treated for 28 days with 12.5 mg/kg/day of DEN (A), 50 mg/kg/day of 2,4-DAT (B), 10 mg/kg/day of 1,2-DMH (C), or 100 mg/kg/day of 2,6-DAT (D). Original magnifications 10× (a), 20× (b), and 40× (c, centrilobular area; d, perilobular area). Hepatotoxicity was observed in the livers of rats administered DEN, 2,4-DAT, and 1,2-DMH. Bar = 100 μm.

Table 2
Mean body weight changes of rats repeatedly treated with 1,2-DMH and 2,6-DAT for 28 days.

Test chemical (dose, mg/kg/day)	Day 1		Day 6		Day 15		Day 29	
	Mean ± SD	Ratio[a]	Mean ± SD	Ratio[a]	Mean ± SD	Ratio[a]	Mean ± SD	Ratio[a]
Vehicle	229.4 ± 10.1	1.00	271.8 ± 13.8	1.00	343.6 ± 22.2	1.00	431.2 ± 33.5	1.00
1,2-DMH (5)	232.0 ± 13.3	1.01	264.6 ± 13.0	0.97	330.0 ± 15.2	0.96	395.8 ± 30.2	0.92
1,2-DMH (10)	229.6 ± 8.2	1.00	241.0 ± 11.2	0.89	284.9 ± 24.6	0.83	341.0 ± 20.2	0.79
Vehicle	213.3 ± 7.0	1.00	261.1 ± 8.2	1.00	333.8 ± 13.5	1.00	426.8 ± 21.1	1.00
2,6-DAT (25)	213.0 ± 10.0	1.00	243.4 ± 11.7	0.93	307.2 ± 17.7	0.92	374.8 ± 30.4	0.88
2,6-DAT (50)	214.1 ± 7.1	1.00	243.5 ± 9.5	0.93	308.0 ± 15.2	0.92	382.0 ± 25.0	0.90
2,6-DAT (100)	215.8 ± 11.1	1.01	234.2 ± 18.9	0.90	292.6 ± 28.5	0.88	359.2 ± 36.3	0.84

[a] Ratio to the vehicle control.

hypertrophies involved acidophilic changes. At the low dose levels of 1,2-DMH and DEN, centrilobular acidophilic changes were induced without hepatocellular hypertrophy. Centrilobular single cell necrosis of hepatocytes was observed at all dose levels of the three hepatocarcinogens, and hepatocellular anisokaryosis was detected in the high dose groups at a minimum. Additionally, increased mitotic figures and perilobular oval cell proliferation were observed in animals administered the high dose of DEN. Oval cell proliferation was also found in one animal at the high dose level of 2,4-DAT. In contrast, no abnormal findings were observed in animals treated with 2,6-DAT or vehicle control.

4. Discussion

The present study was conducted to further investigate the effectiveness of the liver MN assay for the detection of genotoxic hepatocarcinogens. Moreover, one of the aims of our present and previous studies [15] was to demonstrate that the liver MN assay is suitable for integration into general toxicity studies. Here, the effects of the genotoxic hepatocarcinogens, DEN, 2,4-DAT, and 1,2-DMH, and the noncarcinogen 2,6-DAT administered to rats by repeated oral treatment for up to 28 days were examined in a liver MN assay that incorporated an established hepatocyte isolation method [15] as an alternative to *in situ* liver perfusion.

In the liver MN assay, 1,2-DMH gave a positive response that exhibited a dose- and duration-dependency (Fig. 1A) which was similar to that observed in our previous report examining DEN and 2,4-DAT [15]. However, the noncarcinogen 2,6-DAT did not increase the incidence of MN in the liver MN assay (Fig. 1C). Together, our present and previous [15] results for these four chemicals suggest that the liver MN assay for samples obtained from animals administered a repeated dose regimen is an effective method to detect genotoxic rodent hepatocarcinogens.

For the BM MN assay, a single dosing of 1,2-DMH at 200 mg/kg gave a positive result [21], whereas a repeated dose regimen for 28 days was reported to give a negative result [16]. It was also found that short-term DEN and 2,4-DAT treatments were associated with negative or weak responses in BM MN assays for rats and mice [9,22]. The results of these previous reports [9,16,21] are consistent with the present BM MN assay results for these three hepatocarcinogens (Figs. 1B and 2A, B). Together, the results of our present and previous [15] studies suggest that genotoxic hepatocarcinogenicity, which is typically undetectable by the standard BM MN assay, can be assessed by the liver MN assay. In addition, as the liver is the main target organ for the carcinogenicity of DEN and 2,4-DAT [23,24], whereas 1,2-DMH targets multiple organs in addition to the liver in rats and mice [25], the liver MN assay may be highly organ specific for the prediction of hepatocarcinogenic potential of test chemicals.

For 2,6-DAT, the high dose level (100 mg/kg/day) used in the present study is equivalent to 40–67% of the levels used in previous short-term assays [18,26]. In addition, the mean body weight of animals administered this dose was 16% lower than the vehicle control group at the termination of the 28-day experiment (Table 2). Integration of the liver MN assay into the general toxicity study is one of the aims of this investigation. According to our plan, the pattern of body weight changes in rats treated with 2,6-DAT was comparable to those produced by 1,2-DMH (Table 2) and other two hepatocarcinogens [15], although no statistical analyses were conducted in this investigation. Thus, the high dose level selected for 2,6-DAT was considered to be appropriate for a 28-day repeated dose general toxicity study. However, the top dose of 2,6-DAT may not have reached the MTD for the genotoxicity study because there were no histopathological findings (Table 1 and Fig. 3D), and the effect on the body weight gain was not severe (Table 2). We supposed that the doses at 200–250 mg/kg of 2,6-DAT could not be used for the 28-day treatment since these dose levels are around a half of LD_{50} [18]. Thus, we did not conduct the repeated administration of 2,6-DAT at around these doses. Dose selection may be a problem which confronts us in the future in order to truly integrate a liver MN assay into a repeated dose general toxicity study. In the present study, we did not analyze the parameters of either hematology or blood chemistry. These data also should be analyzed to determine the MTD for the integration of the assay into the general toxicity study. Furthermore, in the 5- and 14-day experiments for 2,6-DAT, a dose of 50 mg/kg/day was selected to allow comparison with the identical dose used for 2,4-DAT, its structural isomer. It may have been possible to administer rats a higher dose of 2,6-DAT, at least 100 mg/kg/day in the 5- and 14-day experiments. However, considering the integration of the liver MN assay into a general toxicity studies at the drug developmental stage, a repeated dose regimen for 28 days or longer would be preferable.

The aromatic amines 2,4-DAT and 2,6-DAT are structural isomers and are often compared with regard to their carcinogenic potential. Both 2,4-DAT and 2,6-DAT are mutagenic in *Salmonella typhimurium* strains [27] and *in vitro* chromosome aberration assays [22,28], and give negative or weakly positive results in the BM MN assay [22]. Despite the fact these chemicals exhibited the same results in these genotoxicity studies, only 2,4-DAT is carcinogenic in rats and mice [24,29]. Therefore, the carcinogenic potency of these two isomers cannot be distinguished by the genotoxicity studies commonly required by regulatory paradigms. In contrast, several *in vivo* assays other than the BM MN assay are reported to give positive responses for only 2,4-DAT [17,26,27,30,31]. For example, these two isomers were examined using short-term assays involving the unscheduled DNA synthesis test [26] and *lacZ* transgenic Muta^{TM}Mouse [31], with the results being compared to those of the ^{32}P-postlabelling technique [17], gene expression profiling technology [30], and *gpt* delta transgenic rats [27] following repeated dosing for approximately one month or longer. Although the results of these previous studies and those for 2,4-DAT [15] and 2,6-DAT in this study (Fig. 1C, D) are all consistent with the reported carcinogenicity of these isomers in rats and mice, our liver MN assay is considered to be easier and less expensive to conduct than these other short-term assays and methods of similar duration.

Table 3
Summary results of our investigations on the liver and bone marrow micronucleus assay.

Test chemical	Liver MN			BM MN			Histopathological findings[a]	Carcinogenic target organ in rats		
Sampling time (day)	5	14	28	5	14	28		Liver	Others	Reference
1,2-DMH	–	+	+	–	–	–	+	Positive	Positive multi-organs	[25]
2,6-DAT	–	–	–	–	–	–	–	Negative	Negative	[29]
DEN	(+)	(+)	(+)	–	–	–	+	Positive	Positive multi-organs	[23]
2,4-DAT	(–)	(+)	(+)	–	–	–	+	Positive	Positive mammary gland	[24]

+: increased or observed in our investigation, –: not increased or not observed in our investigation.
Parentheses indicate the result of the previous study by Narumi et al. [15].
[a] Findings related to hepatocyte proliferation.

The hepatotoxicity of the test chemicals was evaluated in the present study by histopathological examination (Table 1 and Fig. 3), which is commonly conducted in general toxicity studies and which we were able to perform here in conjunction with the liver MN assay, as *in situ* liver perfusion is not necessary for our liver MN assay methods. The three examined hepatocarcinogens displayed hepatocellular hypertrophy, single cell necrosis, and/or acidophilic changes, which represent early stage histopathological findings of hepatotoxicity, while the observed inflammatory cell infiltration, anisokaryosis, increase of hepatocellular mitotic figures, and/or proliferation of oval cells are speculated to have occurred in response to the early stage changes (Table 1 and Fig. 3). It is commonly known that hepatocyte hypertrophy is found in the liver of rodents treated with a hepatocarcinogen. It is considered that single cell necrosis is followed by cell proliferation, and hepatocellular anisokaryosis and an increase in mitotic figures indicate elevated proliferative activity of hepatocytes. Whereas liver oval cells are considered to proliferate and differentiate only under conditions in which hepatocyte proliferation is blocked or delayed [32], the oval cells are expanded by administration of 2-acetylaminofluorene plus PH model [33]. It is considered that the histopathological findings observed in rats treated with these three carcinogens are closely related to hepatocyte proliferation. In contrast to these carcinogens, 2,6-DAT gave no detectable findings in the histopathological examination of liver tissue (Table 1 and Fig. 3). Table 3 shows summary results of our present and previous [15] investigation of the liver and BM MN assays and histopathological examination. The observed histopathological changes correlate with the results of the liver MN assay in our present and previous [15] investigations.

When the MN assay is conducted using adult rat liver tissue, measurement of the cell division rate is important, as the liver undergoes low cell division. It is also critical to evaluate whether cell division was suppressed when a negative result is obtained in the liver MN assay. For the BM MN assay results shown in Figs. 1 and 2, the IME-ratio is a useful indicator of cell division suppression. In the liver, internal cell-markers, such as Ki67 and PCNA, or BrdU-incorporation are effective indicators of cell proliferation [34]; however, no appropriate indicators of cell division have been identified, with the exception of counting mitotic figures, as DNA synthesis in the liver occasionally occurs in the absence of cell division [35]. Furthermore, evaluating mitotic figures in the livers of adult rats is considered to be inappropriate because rats over the age of 6 weeks have markedly low cell division activity [15]. Supporting this finding, the percentage of mitotic figures detected in the individual rats examined here ranged from 0.00% to 0.10% for all groups administered 1,2-DMH or 2,6-DAT. Moreover, it is practically and routinely considered that scoring 2000 cells is a marginal number to calculate the incidence of MNHEPs; therefore, the data of mitotic figure counts in hepatocytes were not reported here. In the liver MN assay using PH [36,37], the number of main nuclei in the HPEs (*i.e.* mononucleated, binucleated or multinucleated [3 nuclei or more]) may be useful as a dividing index of HEPs, however, identifying an appropriate indicator of cell division for the liver MN assay remains a future challenge, and so has been for the MN assay in human buccal cells [38]. When a negative result is obtained in the liver MN assay, histopathological examination might provide meaningful information, as was the present case for 2,6-DAT, which did not show any abnormal findings (Fig. 3). We speculate that an absence of abnormal findings in the histopathological examination indicates that the cell division rate is low, but steady, in the adult rat liver, when the liver MN assay also gives a negative result.

It has been speculated that MNHEPs accumulate following repeated dosing with test chemicals due to the long lifespan of HEPs [32]. The proportion of MNHEPs observed for 1,2-DMH (Fig. 1), DEN and 2,4-DAT [15] increased in a dosage- and duration-dependent manner, a finding that supports the speculation that MNHEPs remain in the liver where they accumulate. However, based on the present histopathological examination results, it cannot be excluded that the incidence of MNHEPs was increased by hepatocellular multiplication after treatment with the three test hepatocarcinogens. In the future, it is necessary to determine how this finding relates to the results of the liver MN assay.

Although it has been reported that the rat BM MN assay can be integrated into general toxicity studies [16], a chemical, such as 1,2-DMH, which has to be administered at lower levels during repeated dosing than those used for single or double dosing cannot be detected by the BM MN assay with the repeated dose regimen. To detect MN in the BM according to a previous report [16], a greater number of testing points were necessary in the present study, such as examination of the proportion of MN in peripheral blood and spleen histopathology approximately 4 days after the initial dosing. In the present and previous [15] studies, however, the genotoxic hepatocarcinogens 1,2-DMH, DEN, and 2,4-DAT significantly induced MNHEPs at lower dose levels than those used in the previous reports [5,8,21]. Based on our present findings and the speculation concerning MNHEPs accumulation in the liver as described above, the liver MN assay is considered to be more appropriate for a repeated dose study than the BM MN assay.

An important trend in current toxicology is the 3R-principles, which stands for the replacement, reduction, and refinement of the use of experimental animals. Integration of the liver MN assay into general toxicity studies would help realize one of the 3R-principles, namely a reduction in the number of animals required for the ECVAM [13] and the revised ICH guidance [14]. Furthermore, we expect that the reduction of the number of experimental animals will contribute to a reduction in the cost of the animal experiments; labor, time, animals and other materials used for the experiments. Based on the results of our present and previous studies [15], the liver MN assay can be integrated into 28-day repeated dose toxicity studies that are typically conducted during the toxicological evaluation of pharmaceutical drugs and agrochemicals. Such integration may further facilitate a reduction in the number of experimental animals required for regulatory toxicology.

In conclusion, integrating the liver MN assay into repeated dose toxicity studies is useful and effective for evaluating *in vivo* clastogenicity and predicting the hepatocarcinogenicity of test chemicals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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