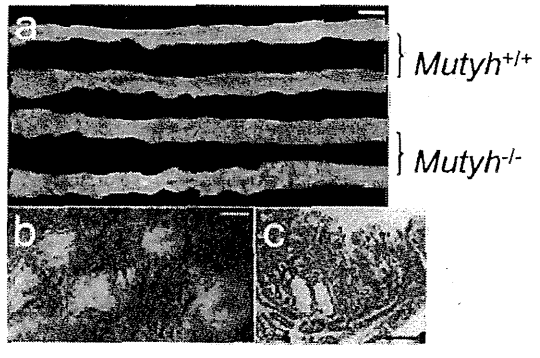
multiple formation of tumor in the small intestines of *Mutyh*-deficient mice provides a suitable model system to investigate the processes of intestinal tumorigenesis.

Xie *et al.* showed that *Mutyh/Ogg1* double-deficient mice predominantly developed lung and ovarian tumors as well as lymphomas. They also showed that 8.6% of *Mutyh/Ogg1* double-deficient mice exhibited adenomas/carcinomas in their gastrointestinal tracts, which were not observed in wild-type mice[7]. We and other groups have previously reported that there was little difference in the number of intestinal tumors in wild-type and Ogg1-null mice, although an Ogg1 deficiency resulted in 8-oxoG buildup in genomic DNA and an elevated mutation frequency in the latter[4,8,9]. Thus, the development of intestinal tumors in *Mutyh/Ogg1* double-deficient mice supports the notion that having a Mutyh deficiency does indeed increase susceptibility to intestinal tumorigenesis regardless of the genetic background or environmental factors.

I. $KBrO_3$-induced intestinal tumors in wild-type and *Mutyh*-deficient mice



II. Frequency of $KBrO_3$-induced intestinal tumors in wild-type and *Mutyh*-deficient mice (tumors/mouse)

Genotype	Sex	Mean ± S.D.
Mutyh $^{+/+}$	♂	0.50 ± 0.55
	♀	1.00 ± 0.71
Mutyh $^{-/-}$	♂	72.75 ± 24.24
	♀	51.00 ± 28.35

Figure 2 $KBrO_3$-induced tumors in the small intestine of *Mutyh*-deficient mice.
I. a, The upper part of the small intestines (duodenum and a part of jejunum) from $KBrO_3$-treated mice are shown. Multiple polyp formations are observed in the $KBrO_3$-treated *Mutyh*-deficient mice (-/-), but not in the treated wild-type mice (+/+). Upper: female, lower: male. **b,** A high-power view of the polyps in the $KBrO_3$-treated *Mutyh*-deficient female mouse. **c,** A section of the $KBrO_3$-induced polyp stained with haematoxylin and eosin. Scale bars; **a:** 1 cm, **b:** 1 mm, **c:** 100 μm. **II.** $KBrO_3$ was administered to wild-type and *Mutyh*-deficient mice in their drinking water for 16 weeks. Body weight and water consumption were monitored weekly. The animals were sacrificed at the age of 20 weeks, and their intestines were removed and fixed in 4% paraformaldehyde fixative. Microscopic inspection for tumor formation was performed (adopted from reference 5).

1) Familial adenomatous polyposis with a recessive trait

- Patients have germ-line mutations in the *MUTYH* gene (About 80% of the mutations were identified as Y165C or G382D in the patients of Europe and USA)
- Onset: 50 years of age (median, range:16~59 years of age) *
- No. of tumors: 40/colon(median, range: 18~100 tumors) *

*United Kingdom patients (156) with multiple (5~100) adenomas

2) Tumors from the patients all showed G:C to T:A mutations in the *APC* gene.

3) Almost all the G:C to T:A mutations occur in the GAA sequence context of the *APC* gene

Figure 3 MutYH-associated polyposis (MAP).

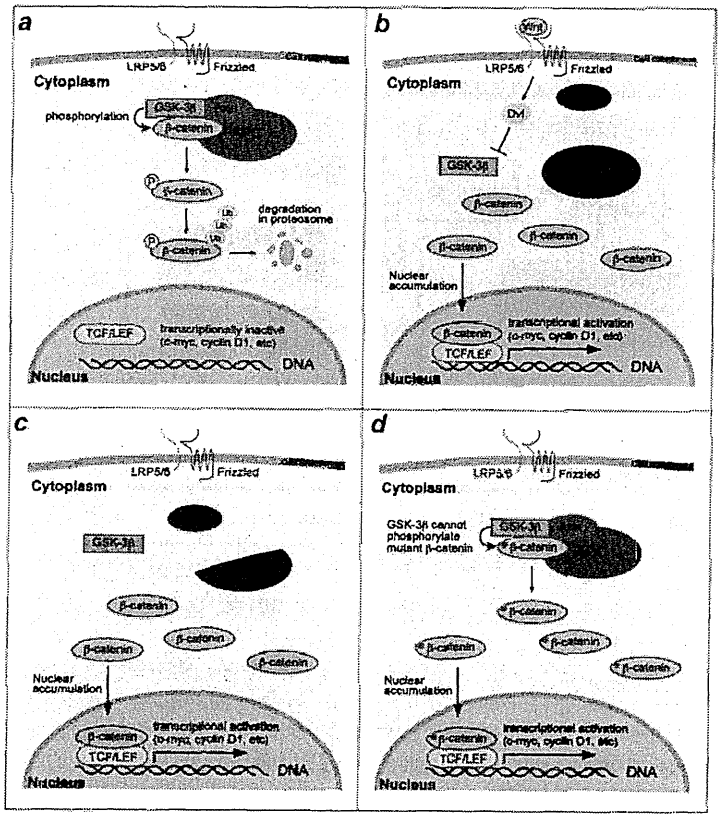


Figure 4 Wnt signaling pathway (canonical).

a) In the absence of Wnt signaling, β-catenin is phosphorylated by GSK3β in a complex with axin and Apc, and is subsequently ubiqutinated and degradated in the proteasome. *b*) In the presence of Wnt signaling, activated Dishevelled (Dvl) inhibits phoshorylation of β-catenin, consquently leading to stabilization of β-catenin. Nuclear accumulated β-catenin activates transcription of the target genes. Mutations in either APC (*c*) or in the phosphorylation sites of β-catenin (*d*) abolish the phoshorylation of β-catenin, which in turn leads to an accumulation of nuclear β-catenin, thereby up-regulating the expression of the target genes without Wnt signaling, and resulting in carcinogenic cell growth.

It is of interest that the deficiency of Mutyh but not Ogg1 makes mice susceptible to intestinal tumorigenesis, although the deficiency of either Mutyh or Ogg1 increases G:C to T:A transversion at almost equal frequency in the small intestines of mice. It is possible that this difference may be attributed to the additional substrate; MUTYH excises 2-hydroxyadenine, an oxidized adenine, paired with guanine, beside adenine paired with 8-oxoguanine, from DNA. However, we recently reported the involvement of Mutyh in cell death caused by oxidative DNA damage[10]. Thus, the defect in Mutyh would simultaneously compromise both DNA repair and cell-death induced by oxidative DNA damage (Figure 5). This may explain why among the factors involved in suppressing oxidative damage-induced mutagenesis, only MUTYH is, so far, identified to be associated with hereditary colorectal cancers in humans.

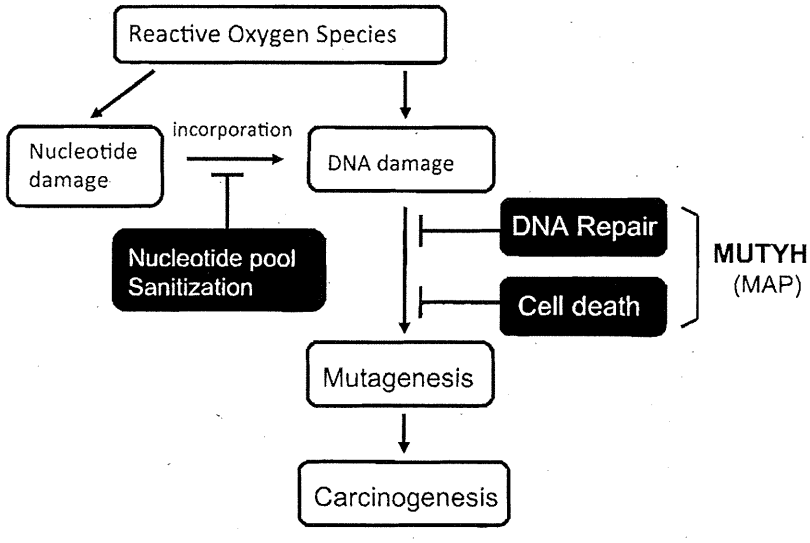


Figure 5 The roles of MUTYH in the avoiding mechanisms for ROS-induced mutagenesis and carcinogenesis.
The defect in Mutyh simultaneously compromise both DNA repair and cell-death induced by oxidative DNA damage. Thus, the defect in Mutyh makes mice highly susceptible to oxidative stress-induced tumorigenesis. This may provide molecular bases for explaining why among the factors involved in suppressing oxidative damage-induced mutagenesis, only MUTYH is, so far, identified to be associated with hereditary colorectal cancers in humans.

References

1. Sekiguchi M, Tsuzuki T. Oxidative nucleotide damage: consequences and prevention. Oncogene, 21: pp.8895-8904, 2002
2. Tsuzuki T, Nakatsu Y, Nakabeppu Y. Significance of error-avoiding mechanisms for oxidative DNA damage in carcinogenesis. Cancer Sci, 98: pp.465-470, 2007
3. Tsuzuki T, Egashira A, Igarashi H, Iwakuma T, Nakatsuru Y, Tominaga Y, Kawate H, Nakao K, Nakamura K, Ide F, Kura S, Nakabeppu Y, Katsuki M, Ishikawa T, Sekiguchi M. Spontaneous tumorigenesis in mice defective in the *MTH1* gene encoding 8-oxo-dGTPase. Proc. Natl. Acad. Sci. USA, 98: pp.11456-11461, 2001
4. Sakumi K, Tominaga Y, Furuichi M, Xu P, Tsuzuki T, Sekiguchi M, Nakabeppu Y, *Ogg1* knockout-associated lung tumorigenesis and its suppression by *Mth1* gene disruption. Cancer Res, 63: pp.902-905, 2003
5. Sakamoto K, Tominaga Y, Yamauchi K, Nakatsu Y, Sakumi K, Yoshiyama K, Egashira A, Kura S, Yao T, Tsuneyoshi M, Maki H, Nakabeppu Y, Tsuzuki, T. MUTYH-null mice are susceptible to spontaneous and oxidative stress-induced intestinal tumorigenesis. Cancer Res., 67: pp.6599-6604, 2007
6. Al-Tassan N, Chmiel N H, Maynard J, Fleming N, Livingston AL, Williams GT, Hodges AK, Davies DR, David SS, Sampson JR, Cheadle JP. Inherited variants of MYH associated with somatic G:C to T:A mutations in colorectal tumors. Nat Genet, 30: pp.227-232, 2002
7. Xie Y, Yang H, Cunanan C, Okamoto K, Shibata D, Pan J, Barnes DE, Lindahl T, McIlhatton M, Fishel R, Miller JH. Deficiencies in mouse *Myh* and *Ogg1* result in tumor predisposition and G to T mutations in codon 12 of the K-ras oncogene in lung tumors. Cancer Res, 64: pp.3096–3092, 2004
8. Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T, Barnes DE. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proc. Natl. Acad. Sci .USA, 96: pp.13300–13305. 1999
9. Minowa O, Arai T, Hirano M, Monden Y, Nakai S, Fukuda M, Itoh M, Takano H, Hippou Y, Aburatani H, Masumura K, Nohmi T, Nishimura S, Noda T. *Mmh/Ogg1* gene inactivation results in accumulation of 8-hydroxyguanine in mice. Proc. Natl Acad. Sci. USA, 97: pp.4156–4161, 2000
10. Oka S, Ohno M, Tsuchimoto D, Sakumi K, Furuichi M, Nakabeppu Y. Two distinct pathways of cell death triggered by oxidative damage to nuclear and mitochondrial DNAs. EMBO J, 27: pp.421-432 2008

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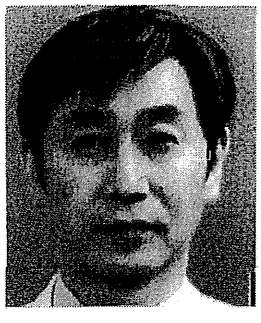

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Specialty and Present Interest:
Oxidative DNA damage, Mutagenesis and Tumorigenesis, Gastrointestinal Tumor

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Regular article

Evaluation of the Genotoxicity of Aristolochic Acid in the Kidney and Liver of F344 *gpt* delta Transgenic Rat Using a 28-Day Repeated-dose Protocol: A Collaborative Study of the *gpt* delta Transgenic Rat Mutation Assay

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Transgenic rat gene-mutation assays can be used to assess genotoxicity of chemicals in target organs for carcinogenicity. Since gene mutations in transgenes are genetically neutral and thus accumulate along with treatment periods, the assays are suitable for genotoxicity risk assessment of chemicals using repeated-dose treatment methodologies. However, few studies have been conducted to examine the suitability of the assays in repeat-dose treatment protocols. In order to prove the utility of the transgenic rat assays, we treated *gpt* delta rats with aristolochic acid at 0.3 and 1 mg/kg by gavage daily for 28 days, and autopsied the rats 3 days after the final treatment, which is a protocol recommended by the International Workshop on Genotoxicity Testing (IWGT). Aristolochic acid exists in herbs and some other plants, and is carcinogenic in the kidney, bladder and stomach in rats. The mutant frequency (MF) in both the kidney and the liver increased significantly in a dose-dependent manner when the rats were treated with aristolochic acid. We concluded that the *gpt* delta rat assay is sensitive enough to detect gene mutations induced by aristolochic acid and also that the 28-day repeated-dose protocol is suitable for assessing genotoxicity of chemicals.

Key words: F344 *gpt* delta transgenic rat, aristolochic acid, 28-day repeated-dose protocol, *gpt* assay

Introduction

Transgenic gene-mutation assays are of a high value for the assessment of *in vivo* genotoxicity (1,2). In this method, mutations in reporter genes integrated in the rodent chromosomes can be identified in any organs/tissues after the reporter genes are recovered from the rodent cells to bacterial cells. Transgenic gene-mutation assays are suitable for the risk assessment of potential genotoxic chemicals dosed via repeated-dose treatment, since mutations can be analyzed in various time points during treatment and sampling periods (3). In addition, mutations in the reporter genes accumulate over time as the treatments progress (4,5). It is, therefore, expected that these assays enableus to assess the genotoxicity of chemicals with various dose levels, dosing periods and target organs.

Present issues to be solved for the use of transgenic gene-mutation assays include how the detection sensitivity can be confirmed and how the dosing periods can be standardized. In a genotoxicity assessment of 90 carcinogens, transgenic gene-mutation models are shown to have a high sensitivity and a good positive predictability (4). However, the majority of the 90 carcinogens assessed in that study are such strong mutagens that they could be used as positive controls in genotoxicity studies, and there are not enough data available on genotoxicants with a lower potency that allow assess-

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ment of the method's sensitivity.

A recent trend regarding the use of experimental animals in toxicological studies focuses on replacement, reduction, and refinement (the '3R' principles), and a movement towards these '3Rs' can be noted in presently reviewed guidelines for the assessment of genotoxicity. In addition to an *in vivo* bone marrow micronucleus test, we may select one more *in vivo* study instead of an *in vitro* study using cultured cells (6), the latter of which shows a comparatively high false-positive rate (7). It is now under discussion and, if conditions permit, we may integrate the *in vivo* genotoxicity assessment into a 28-day repeated-dose toxicity study for example. This approach would contribute to a reduction in the number of animals to be used experimentally. One of the promising candidates for the additional *in vivo* test is a test using a transgenic gene-mutation assay (8). However, nearly 70% of studies with transgenic gene-mutation assays have been conducted using a single dosing or repeated-dosing regimen within a 5-day period (3), and there are not enough data compiled for genotoxicity assessment using repeated treatment. This is contrast to the recommended protocol by the International Workshop on Genotoxicity Testing (IWGT), i.e., autopsy and sample collection on day 3 after the completion of a 28-day repeated treatment (28 + 3 protocol) (9,10,11).

We initiated this study with the aim of testing the adequacy and detection capabilities of the IWGT-recommended general protocol for 28-day repeated-dose studies. For this work, we used F344 *gpt* delta rats, which were developed in Japan (8,12). Aristolochic acid, which exists in herbs and some other plants (13), was used as the test substance, since it is genotoxic *in vitro* and *in vivo* (14,15,16) and carcinogenic in rats (17). In the carcinogenicity in rats, repeated treatment over 6–9 months induced tumors in the kidney, bladder, and stomach (17). In *in vivo* genotoxicity studies in Big Blue transgenic rats (18,19), aristolochic acid was dosed orally for 12 weeks at the same doses used in the carcinogenicity study (17) and the frequency of *cII* mutation in the kidney (18,19), a target organ for carcinogenicity, and the liver, a non-target organ, increased substantially.

In the current study, oral treatments with aristolochic acid increased *gpt* mutant frequency (MF) significantly in the kidney and the liver of F344 *gpt* delta rats in a dose-dependent manner, which suggests that four weeks treatment recommended by IWGT is sensitive enough to detect gene mutations.

Materials and Methods

F344 *gpt* delta rats: All animals were bred at Japan SLC, Inc. (Shizuoka, Japan). The F344 *gpt* delta transgenic rat strain was developed by backcrosses of the original SD *gpt* delta transgenic rat wtih wild-type F344 rats. The *gpt* delta rat contains approximately 5 to 10 copies of the lambda EG10 transgene in chromosome 4 as a heterozygote (12). Male SD *gpt* delta rats were mated with wild-type F344 females to produce heterozygous F1 rats. F1 males (heterozygote for the transgene) were then backcrossed with F344 females. After 15 backcross matings, animals were designated as F344 *gpt* delta rats.

Chemical: Aristolochic acid (CAS#313-67-7, purity 98%, as 8-methoxy-6-nitrophenanthro-(3,4-D)-1,3-dioxolo-5-carboxylic acid, aristolochic acid-I) was purchased from Sigma-Aldrich (Tokyo, Japan). *N*-Ethyl-*N*-nitrosourea (ENU, CAS#759-73-9) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The dosing solution of aristolochic acid was prepared by dissolving the chemical in purified water. The dosing solution of ENU was prepared by dissolving the chemical in saline.

Animals and treatments: The rats were used in the experiment at 7 weeks of age, after a 1-week acclimation period. The rats were housed individually in stainless steel cages, with free access to tap water and a CRF-1 pellet diet (Oriental Yeast Co., Ltd., Tokyo, Japan). The animal room conditions were maintained at a room temperature of 23 ± 2°C, a relative humidity of 55 ± 10%, and a light-dark cycle of 12:12 h. The study protocol was approved by the Animal Care and Utilization Committee of Meiji Seika Pharma Co., Ltd. The treatments were conducted in accordance with the protocol recommended by the IWGT (9,10,11). Five *gpt* delta rats per group were dosed with aristolochic acid at 0, 0.3, or 1 mg/kg by gavage daily for 28 days, and necropsied 3 days after the final treatment for collection of the kidney and liver. The following parameters were monitored: clinical signs, body weight, food intake, hematology, blood chemistry, autopsy findings, organ weights, and histopathology. In addition, a positive control group was given an i.p. injection of 50 mg/kg ENU daily for 5 days, and autopsied 26 days after the final treatment for collection of the liver. The collected organs were immediately frozen in liquid nitrogen and stored at −80°C. The frozen samples were sent to Kirin Group Office Co., Ltd. (Lab. A) and Suntory Business Expert Ltd. (Lab. B) for *gpt* assays.

Detection of *gpt* mutation: The *gpt* assays were conducted in accordance with previously published methods in Lab. A and Lab. B separately (1,20). Genomic DNA was extracted from the liver or the kidney using the RecoverEase™ DNA Isolation Kit (Agilent Technologies, Santa Clara, CA) and lambda EG10 phages were recovered with Transpack® Lambda Packaging Extract (Agilent Technologies). *Escherichia coli* YG6020 was infected with the phage, spread onto M9 salt plates containing chloramphenicol (Cm) and 6-thioguanine (6-TG) (21), and then incubated for 72 h at 37°C for selection of the colonies harboring a plasmid

carrying a chloramphenicol acetyltransferase gene and a mutated *gpt* gene. The mutant frequencies (MFs) of the *gpt* gene in the liver and kidney were calculated by dividing the number of confirmed 6-TG resistant colonies by the number of rescued plasmids.

Statistical analysis: The data for MFs were expressed as mean ± SD. Statistically significant differences in MFs between the treated groups and the negative control were analyzed by Dunnett's multiple test or Steel's test. Statistically significant differences in MFs between the positive and negative control groups were analyzed by Welch's t-test. Differences in body weight, food intake, hematology, blood chemistry, and organ weights between the control and treated groups were analyzed by Dunnett's multiple test.

Results

***gpt* Mutations in the liver and kidney induced by aristolochic acid:** In order to estimate the mutagenicity of aristolochic acid, *gpt* delta rats were treated orally for 28 days and mutations in the liver and kidney were analyzed in Lab. A and Lab. B (Fig. 1). Two laborato-

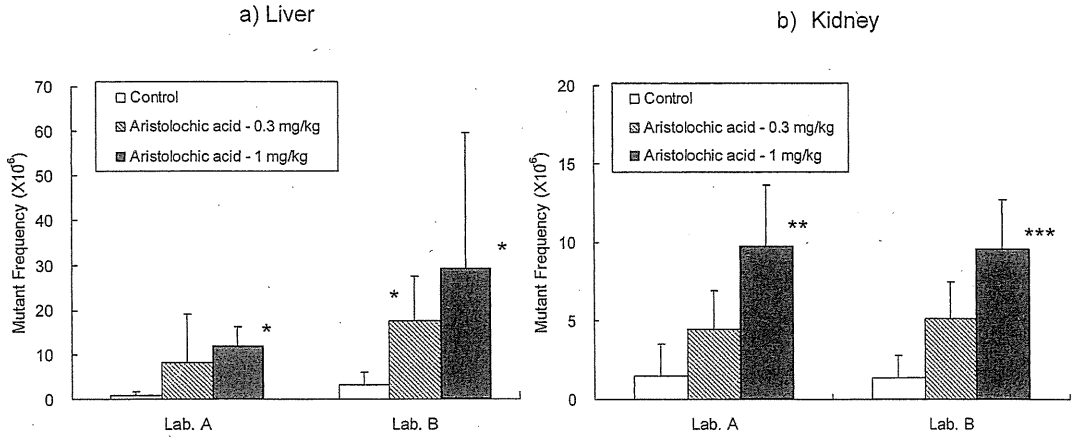

Fig. 1. Comparison between two laboratories in *gpt* mutant frequency of aristolochic acid-treated rats ($n=5$) in a) Liver, b) Kidney. Values represent mean +/−SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (Steel test).

Table 1. *gpt* Mutant frequencies in the liver of *gpt* delta rats treated with aristolochic acid

Treatment	Animal No.	Total population	Number of mutants	Mutant frequency ($\times 10^{-6}$)	Mutant frequency Average	Mutant frequency SD
Control (Purified water)	1	1,755,000	6	3.42	1.92	1.02
	2	1,158,000	1	0.86		
	3	1,527,000	2	1.31		
	4	654,000	1	1.53		
	5	813,000	2	2.46		
Aristolochic acid (0.3 mg/kg)	11	606,000	5	8.25	12.28***	8.05
	12	729,000	3	4.12		
	13	540,000	10	18.52		
	14	798,000	6	7.52		
	15	261,000	6	22.99		
Aristolochic acid (1 mg/kg)	21	1,107,000	28	25.29	15.29***	6.25
	22	1,149,000	14	12.18		
	23	888,000	15	16.89		
	24	1,104,000	10	9.06		
	25	1,227,000	16	13.04		
N-Ethyl-*N*-nitrosourea (50 mg/kg)	51	336,000	46	136.90	110.16§§§	26.03
	52	447,000	44	98.43		
	53	507,000	54	106.51		
	54	417,000	56	134.29		
	55	576,000	43	74.65		

$^{**}p<0.01$, $^{***}p<0.001$ (Dunnett test), $^{§§§}p<0.001$ (welch's t-test).

Table 2. *gpt* Mutant frequencies in the kidney of *gpt* delta rats treated with aristolochic acid

Treatment	Animal No.	Total population	Number of mutants	Mutant frequency ($\times 10^{-6}$)	Average	SD
Control (Purified water)	1	1,020,000	2	1.96	1.69	1.07
	2	921,000	3	3.26		
	3	2,820,000	1	0.35		
	4	1,656,000	2	1.21		
	5	597,000	1	1.68		
Aristolochic acid (0.3 mg/kg)	11	1,254,000	6	4.78	4.82**	1.36
	12	510,000	2	3.92		
	13	669,000	4	5.98		
	14	1,932,000	6	3.11		
	15	474,000	3	6.33		
Aristolochic acid (1 mg/kg)	21	954,000	10	10.48	9.14***	3.60
	22	1,965,000	19	9.67		
	23	1,719,000	9	5.24		
	24	987,000	14	14.18		
	25	1,797,000	11	6.12		

**$p<0.01$, ***$p<0.001$ (Dunnett test).

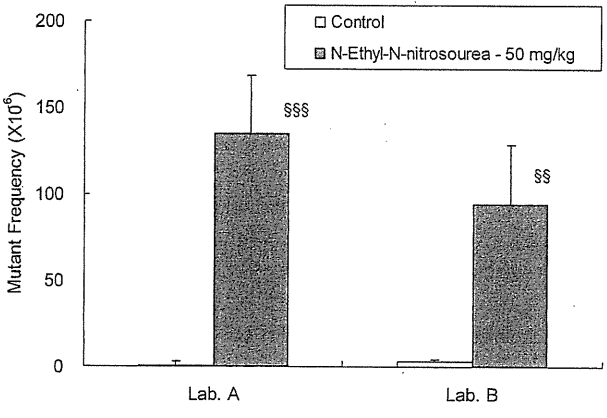

Fig. 2. Comparison between two laboratories in *gpt* mutant frequency of *N*-Ethyl-*N*-nitrosourea-treated rats ($n=5$) in liver. Values represent mean +/−SD. §§$p<0.01$, §§§$p<0.001$ (welch's t-test).

ries generated quite similar results. In the liver, the mean numbers of *gpt* MFs in both Lab. A and Lab. B were 1.92 ± 1.02, 12.28 ± 8.05, and 15.29 ± 6.25 ($\times 10^6$) in the groups treated with 0, 0.3, and 1 mg/kg aristolochic acid, respectively (Table 1). The numbers of *gpt* MFs in the liver in the aristolochic acid treatment groups increased in a dose-dependent manner to approximately 6.4- and 8.0-fold that in the controls, for the 0.3 and 1 mg/kg treatments, respectively. These increases in MFs were statistically significant ($p=0.00054$ and 0.00011, respectively).

In the kidney, the mean numbers of *gpt* MFs in both Lab. A and Lab. B were 1.69 ± 1.07, 4.82 ± 1.36, and 9.14 ± 3.60 ($\times 10^6$) in the groups treated with 0, 0.3, and 1 mg/kg aristolochic acid, respectively (Table 2). The *gpt* MFs in the kidney in the aristolochic acid treatment groups increased in a dose-dependent manner to approximately 2.9- and 5.4-fold that in the controls. These increases in MFs were also statistically significant ($p=0.00843$ and 0.00043, respectively).

In the positive control group treated with 50 mg/kg ENU for 5 days, Lab. A and Lab. B showed very similar *gpt* MF in the liver of rats (Fig. 2). The *gpt* MF in the liver was 110.16 ± 26.03 ($\times 10^6$), which was approximately a 57.4-fold increase compared with the negative control group (Table 1). This increase in MFs was also statistically significant ($p=0.00036$).

Evaluation of the toxicity of aristolochic acid: A summary of the toxicity data generated for aristolochic acid is shown in Table 3. No mortalities occurred at any dose level during the dosing period. In the clinical observation, hematology, autopsy, and measurements of body weights, organ weights, and food intakes, no significant changes related to treatment with aristolochic acid were found at any dose. In the blood chemistry, the ALT value increased very slightly in the 1 mg/kg group. In the histopathology, very slight mononuclear infiltrations of the liver and very slight basophilic tubules in the kidney were observed in both of the 0.3 mg/kg and 1 mg/kg groups.

Discussion

The aim of the present study was to assess the utility of *gpt* delta transgenic rats and the adequacy of the IWGT-recommended general protocol (9) through a genotoxicity risk assessment of aristolochic acid in the kidney and liver of rats. Aristolochic acid was administered orally to *gpt* delta rats at doses of 0.3 and 1 mg/kg for 28 days, and the animals were autopsied 3 days after the last treatment so that the liver and kidney

Table 3. Summary of toxicity data in *gpt* delta rats treated with aristolochic acid

Animal species, age, sex	F344 *gpt* delta rat, 8 weeks old, male		
Dosing method	Aristolochic acid was dissolved in water and administerd by oral gavage once a day for 4 weeks. Dosing volume: 10 mL/kg		
Dosing volume	Vehicle control (Purified water)	Aristolochic acid (mg/kg/day) 0.3	1
Number of animals	5	5	5
Number of deaths	0	0	0
Clinical signs	No abnormal signs	No abnormal signs	No abnormal signs
Body weight		—	—
Food consumption		—	—
Hematology		—	—
Blood biochemistry		—	Increase in ALT value* (1.4 fold)
Autopsy	No remarkable changes	No remarkable changes	No remarkable changes
Organ weight		—	—
Histopathology	No remarkable changes	Mononuclear cell infiltration in the liver (±: 2/5 rats) Basophilic change in the renal tubules (±: 4/5 rats)	Mononuclear cell infiltration in the liver (±: 3/5 rats) Basophilic change in the renal tubules (±: 3/5 rats, +: 1/5 rat)

—: No significant differneces compared with vehicle control. *$p<0.05$ (Dunnett test). Grade in histopathology: ±: Very slight, +: Slight.

could be collected for the detection of mutations with the *gpt* assay. A significant and dose-dependent increase in the MF was noted in the kidney, a carcinogenicity target organ, in the groups treated with aristolochic acid compared with the MF in the negative control group (Table 2). In a previous genotoxicity study using Big Blue transgenic rats (18,19), aristolochic acid was administered repeatedly for 12 weeks at 0.1 and 1 mg/kg and the frequency of *cII* mutations in the kidney increased approximately 3- and 8-fold compared with the control group, respectively. The increases in *gpt* MF in the present study were approximately 3- and 5-fold, at the dose levels of 0.3 and 1 mg/kg, respectively, indicating an almost equivalent ability to detect mutations as in the assessment with Big Blue rats treated for 12 weeks, and also demonstrating that a 28-day dosing period is sufficient for detection.

The rat carcinogenicity study was conducted at dose levels of 0.1, 1.0, and 10 mg/kg (17). That study revealed adenoma in the kidney, and hyperplasia and carcinoma in the renal pelvis after 6 months of treatment at 10 mg/kg, adenoma in the kidney and hyperplasia in the renal pelvis after 9 months of treatment at 1 mg/kg, and hyperplasia in the renal pelvis after 12 months of treatment at 0.1 mg/kg. An increased MF in the kidney was observed in *gpt* delta rats treated at 1 mg/kg (Table 2), which strongly suggested that the carcinogenicity observed in the kidney was related to the genotoxicity. However, *gpt* MF was also increased in the liver, a non-target organ of carcinogenicity (Table 1). Similar results are reported with Big Blue rat (19). Accordingly, aristolochic acid was judged to have genotoxicity in the liver. In the rat carcinogenicity study with aristolochic acid, the maximum duration of administration was 9, 9 and 16 months in the groups treated with 0.1, 1, and 10 mg/kg aristolochic acid, respectively (17). That study duration of 9–16 months was rather short compared with the 2 year duration that is typical of carcinogenicity studies. This might be a reason why no induction of liver cancer was detected. Alternatively, other factors such as accelerating cell proliferation may be required for the induction of cancer in the liver. Further work is needed to discuss the relationship between genotoxicity and carcinogenicity in the liver of rats treated with aristolochic acid.

In the present study, we also carried out hematology, blood chemistry, autopsy, and organ weight measurements to examine the general toxicity of aristolochic acid using the same animals. These analyses did not identify any abnormalities except a slight increase in ALT that might be effects of aristolochic acid administration. The histopathology revealed basophilic changes in the kidney and mononuclear infiltration in the liver. However, these effects were very slight, and thus we judged that aristolochic acid did not induce significant tissue damage in the study. In a 28-day repeated-dose toxicity study using *gpt* delta rats, it would be possible to assess not only genotoxicity but also general toxicity.

This is another merit of the IWGT-recommended protocol and *gpt* delta rats. However, in ordinary repeated-dose toxicity studies, autopsy is conducted one day after the final treatment (28 + 1 protocol). In transgenic gene-mutation assays, in contrast, sampling time of about 3 days after the final treatment is set as a period in which DNA adducts are converted to mutations. The difference of the sampling time might be a roadblock to integrate transgenic rat assays into 28-day repeat dose toxicity assays. However, if we sample the organs one day after the final treatment (28 + 1 protocol), it will be almost equivalent to 26 days administration time plus 3 days sampling time (26 + 3 protocol). Because the administration periods of 26 days and 28 days are not substantially different in terms of total dose, we expect that the results from sampling one day after the last treatment (28 + 1 protocol) will be very similar to those from the assays conducted with the protocol recommended by IWGT (28 + 3 protocol). In addition, if we set the dosing period for general toxicity studies to be 1 month (30 + 1 protocol) instead of 4 weeks (28 + 3 protocol), we would expect to have similar results to those generated if sampling was conducted 3 days after the 28 day of treatment. On the other hand, the length of the recovery period after the final treatment is very important in general toxicity evaluation, because the result may be different depending on the length. Since the basophilic changes in the kidney, which were noted 3 days after the final treatment with aristolochic acid, were regenerative, acute tissue injury might be observed in the case where necropsy was conducted 1 day after the final treatment. Therefore, we suggest that 28 + 3 protocol recommended by IWGT should not be rigid and also that the protocols can be flexibly adapted to repeat dose toxicity protocols such as 28 + 1 or 30 + 1.

For studies using transgenic rat gene-mutation assays, we need to further promote the standardization of experimental procedures. There have not been any reports comparing the results for the same chemical evaluated at different laboratories. In the present study, the *gpt* assay on organs originating from the same animal was conducted in two different laboratories and the results were combined for assessment (Figs. 1 and 2). Figure 1 shows the results of the *gpt* assay of aristolochic acid in each laboratory. The results of the analyses on the mutations in the kidney in the two laboratories were similar to each other and almost comparable. As for the liver, some differences were noted in the statistical analyses in the low dose groups, but otherwise we noted similar tendencies. Figure 2 shows the *gpt* assay results of the positive control (ENU) in each laboratory. The results of the analyses on mutations in the positive control in the two laboratories were highly comparable. In transgenic gene-mutation assays, the recovery of the reporter genes and the method for identifaication of mutated-colonies are influential factors on the results of the study. It is, therefore, expected that international validation of standardized technical procedures among laboratories will proceed in the future.

In conclusion, the genotoxicity of aristolochic acid was sensitively detected in the kidney and the liver in the 28-day repeated treatment study using *gpt* delta rats, and thus the adequacy of the IWGT-recommended protocol (28 + 3) was confirmed.

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References

1 Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191–215.
2 Nohmi T, Masumura K. Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens. Environ Mol Mutagen. 2005; 45: 150–61.
3 Lambert IB, Singer TM, Boucher SE, Douglas GR. Detailed review of transgenic rodent mutation assays. Mutat Res. 2005; 590: 1–280.
4 Suzuki T, Hayashi M, Ochiai M, Wakabayashi K, Ushijima T, Sugimura T, Nagao M, Sofuni T. Organ variation in the mutagenicity of MeIQ in Big Blue lacI transgenic mice. Mutat Res. 1996; 369: 45–9.
5 de Vries A, van Oostrom CT, Dortant PM, Beems RB, van Kreijl CF, Capel PJ, van Steeg H. Spontaneous liver tumors and benzo[a]pyrene-induced lymphomas in XPA-deficient mice. Mol Carcinog. 1997; 19: 46–53.
6 Hayashi M. Update on the maintenance of the ICH S2 genetic toxicology. Pharm Regul Sci. 2008; 39: 515–21.
7 Kirkland D, Aardema M, Henderson L, Müller L. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. Mutat Res. 2005; 584(1–2): 1–256.
8 Toyoda-Hokaiwado N, Inoue T, Masumura K, Hayashi H, Kawamura Y, Kurata Y, Takamune M, Yamada M, Sanada H, Umemura T, Nishikawa A, Nohmi T. Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. Toxicol Sci. 2010; 114: 71–8.
9 Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus HJ, Mirsalis JC, Suzuki T, Yajima N. *In vivo* transgenic mutation assays. Mutat Res. 2003; 540: 141–51.
10 Heddle JA, Dean S, Nohmi T, Boerrigter M, Casciano D, Douglas GR, Glickman BW, Gorelick NJ, Mirsalis JC, Martus HJ, Skopek TR, Thybaud V, Tindall KR, Yajima

N. *In vivo* transgenic mutation assays. Environ Mol Mutagen. 2000; 35: 253–9.
11 International Program on Chemical Safety (2006): Transgenic animal mutagenicity assays. Environmental Health Criteria 233, World Health Organization (WHO), Geneva.
12 Hayashi H, Kondo H, Masumura K, Shindo Y, Nohmi T. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi- selection. Environ Mol Mutagen. 2003; 41: 253–9.
13 Kite GC, Yule MA, Leon C, Simmonds MS. Detecting aristolochic acids in herbal remedies by liquid chromatography/serial mass spectrometry. Rapid Commun Mass Spectrom. 2002; 16: 585–90.
14 Arlt VM, Stiborova M, Schmeiser HH. Aristolochic acid as a probable human cancer hazard in herbal remedies: a review. Mutagenesis. 2002; 17: 265–77.
15 Schmeiser HH, Stiborova M, Arlt VM. Chemical and molecular basis of the carcinogenicity of Aristolochia plants. Curr Opin Drug Discov Devel. 2009; 12: 141–8.
16 Zhang H, Cifone MA, Murli H, Erexson GL, Mecchi MS, Lawlor TE. Application of simplified *in vitro* screening tests to detect genotoxicity of aristolochic acid. Food Chem Toxicol. 2004; 42: 2021–8.
17 Mengs U, Lang Wm Poch J-A, The carcinogenic action of aristolochic acid in rats. Arch Toxicol 1982; 51: 107–19.
18 Chen L, Mei N, Yao L, Chen T. Mutations induced by carcinogenic doses of aristolochic acid in kidney of Big Blue transgenic rats. Toxicol Lett. 2006; 165: 250–6.
19 Mei N, Arlt VM, Phillips DH, Heflich RH, Chen T. DNA adduct formation and mutation induction by aristolochic acid in rat kidney and liver. Mutat Res. 2006; 602: 83–91.
20 Nohmi T, Katoh M, Suzuki H, Matsui M, Yamada M, Watanabe M, Suzuki M, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T. A new transgenic mouse mutagenesis test system using Spi- and 6-thioguanine selections. Environ Mol Mutagen. 1996; 28: 465–70.
21 Seidman MM, Dixon K, Razzaque A, Zagursky RJ, Berman ML. A shuttle vector plasmid for studying carcinogen-induced point mutations in mammalian cells. Gene. 1985; 38: 233–7.

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Regular article

Evaluation of *In Vivo* Mutagenicity by 2,4-Diaminotoluene and 2,6-Diaminotoluene in Liver of F344 *gpt* delta Transgenic Rat Dosed for 28 Days: A Collaborative Study of the *gpt* delta Transgenic Rat Mutation Assay

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The transgenic rodent (TGR) assay has been widely used to study *in vivo* gene mutations by chemicals or radiation; however, an optimal protocol has not yet been established to assess unknown genotoxic potential. The International Workshop on Genotoxicity Testing (IWGT) strongly recommends a repeated-dose regimen for the TGR assay protocol for regulatory safety assessment as follows: a treatment period of 28 days and a sampling time of 3 days following the final treatment. In this study, TGR assays using F344 *gpt* delta transgenic rats were conducted at three laboratories to evaluate the validity of the IWGT protocol, as part of a collaborative study of the transgenic rat mutation assay. Male F344 *gpt* delta transgenic rats were orally treated with 2,4-diaminotoluene (2,4-DAT; hepatic carcinogen in rodents; 10 and 30 mg/kg/day) or 2,6-diaminotoluene (2,6-DAT; non-carcinogen in rodents; 60 mg/kg/day) once daily for 28 days. Rats were euthanized 3 days after the last dosing, and then mutant frequencies (MFs) of the *gpt* gene in the livers were studied. As a result, a significant increase in the MF was observed at 30 mg/kg in the 2,4-DAT-treated group, but not in the 2,6-DAT-treated group. These results were commonly observed among the three laboratories. In addition, the overall results from the three laboratories were in general agreement. These results indicate that 2,4-DAT induces gene mutation in the liver of *gpt* delta rats, but 2,6-DAT does not. These results also indicate that the F344 *gpt* delta transgenic rat mutation assay can distinguish differences in the *in vivo* mutagenic potential between a hepatic carcinogen and a non-carcinogen. Results from one laboratory showed more variability than those from the other two laboratories, and this appearance was due to the smaller number of colonies scored. Thus, these results demonstrate that the IWGT protocol for the TGR assays is valid, and show that consistent results are obtained among different laboratories.

Key words: F344 *gpt* delta transgenic rat, diaminotoluenes, 28 consecutive daily treatment, *gpt* assay

Introduction

Transgenic rodent (TGR) assays have been widely used to study *in vivo* gene mutations by chemicals or radiation; however, an optimal protocol has not yet been established to assess unknown genotoxic potential. The International Workshop on Genotoxicity Testing (IWGT) strongly recommends a repeated-dose regimen for the TGR assay protocol for regulatory safety assessment as follows: a treatment period of 28 days and a sampling time of 3 days following the final treatment (i.e., IWGT protocol) (1,2). The monograph criteria for TGR mutagenicity assays published by the World Health Organization (WHO) are consistent with the IWGT protocol (3); however, little TGR assay data have been obtained using the IWGT protocol.

In this study, we conducted the TGR assays at three different laboratories using F344 *gpt* delta transgenic rats to evaluate the validity of the IWGT protocol, as a part of a collaborative study of the transgenic rat mutation assay. We used F344 *gpt* delta transgenic rats because of its useful features (see below).

For *gpt* delta transgenic rodents, transgenic mice were first developed as a new model in 1996 (4). Subsequent-

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ly, Sprague-Dawley (SD) *gpt* delta transgenic rats (5) and F344 *gpt* delta transgenic rats (6) were developed in 2003 and 2010, respectively. The TGR assay using *gpt* delta transgenic rodents has two distinct selections for detecting different types of mutations: 6-thioguanine (6-TG) selection (i.e., *gpt* assay) for point mutations such as base substitutions and frameshifts, and Spi$^-$ selection for deletion mutations (4). F344 *gpt* delta transgenic rats also have the advantage that its background strain (i.e., F344 strain) is frequently used for the 2-year cancer bioassay (6).

In this study, we used 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT). 2,4-DAT is carcinogenic in rodents, inducing liver, mammary gland and subcutaneous tumors in rats, and liver tumors and lymphomas in female mice (7), while 2,6-DAT does not induce tumors in rats or mice (8).

Diaminotoluenes have been extensively studied in genotoxicity assays. For *in vitro* genotoxicity studies, both 2,4-DAT and 2,6-DAT are mutagenic in the Ames test (9–12). 2,4-DAT induces DNA damage, DNA repair and micronucleus formation in the metabolically competent HepG2 cell line (13). Using rat and human hepatocytes *in vitro*, both 2,4-DAT and 2,6-DAT induce DNA repair (14,15), but 2,4-DAT is inactive by an alkaline elution assay for DNA strand breaks (16).

For *in vivo* genotoxicity studies, 2,4-DAT induces unscheduled DNA synthesis (UDS) in the rat liver, while 2,6-DAT does not (17). 2,4-DAT induces DNA damage (comets) in the mouse liver, while 2,6-DAT does not (18). 2,4-DAT induces *LacI*, *LacZ* and *gpt* mutations in the liver of Big Blue™ mice (19,20), Muta™Mouse transgenic mice (21) and F344 *gpt* delta transgenic rats (6), respectively, while 2,6-DAT does not (6,20,21). Both 2,4-DAT and 2,6-DAT weakly induce micronuclei in rat bone marrow (22), while neither 2,4-DAT nor 2,6-DAT in peripheral blood of F344 *gpt* delta transgenic rat (6).

Thus, *in vivo* genotoxicity studies in the liver are able to discriminate between the carcinogen 2,4-DAT and the non-carcinogen 2,6-DAT. In particular, the results of TGR assays using the liver are correlated with those of the bioassays for carcinogenicity of 2,4-DAT and 2,6-DAT; however, the MF is not increased when the treatment period (19) and the sampling time (20) are not appropriate. Thus, the treatment period and the sampling time are important factors in the TGR assay protocol. In this collaborative study, we conducted TGR assays by the IWGT protocol with 2,4-DAT and 2,6-DAT to evaluate the validity of the IWGT protocol.

Materials and Methods

Participating laboratories: Laboratories that participated in this study are shown in Table 1.

Animal housing, treatment of animals and tissue collection for the ENU-treated group were conducted at Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd. (Kanagawa, Japan), and those for all other treated groups were conducted at Lab D3. In this collaborative study, the *gpt* mutation assays were performed at three laboratories (i.e., Labs D1-D3) after preliminary technical training.

Table 1. Participants in the collaborative study

Lab No.	Investigators
D1	A. Akahori, K. Suzuki, M. Nakajima: Biosafety Research Center, Foods, Drugs and Pesticides
D2	T. Shiragiku, Y. Ohara: Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd.
D3	H. Sui, K. Kawakami, N. Sakurai, T. Toyoizumi, H. Okutomi, R. Ohta, T. Nagata, M. Furuya, H. Inada: Hatano Research Institute, Food and Drug Safety Center
*	Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd.

*Animal housing, treatments of animals and tissue collection for ENU-treated group were conducted by this laboratory. The *gpt* MFs from rats in all treated groups were studied by Labs D1–D3.

Test chemicals: 2,4-Diaminotoluene (2,4-DAT; CAS no. 95–80–7, 99.9% pure), 2,6-diaminotoluene (2,6-DAT; CAS no. 823–40–5, 99.5% pure) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). *N*-Ethyl-*N*-nitrosourea (ENU; CAS no. 759–73–9) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Distilled water and physiological saline were purchased from Hikari Pharmaceutical Co., Ltd. (Tokyo, Japan) and Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan), respectively. 2,4-DAT was dissolved in distilled water (2 and 6 mg/mL). 2,6-DAT was suspended in distilled water (12 mg/mL). ENU was dissolved in physiological saline (5 mg/mL). All test chemical solutions and suspensions were stored in a refrigerator under shaded conditions until use, and used for treatment within five days after preparation.

Animals and treatment: Male 6-week-old F344/NSlc-Tg (*gpt* delta) rats (i.e., F344 *gpt* delta transgenic rats) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were observed daily for their general conditions for at least seven days (i.e., quarantine period). The 7-week-old healthy rats during the quarantine period were randomly assigned to the control and treated groups, and then used for the study. The animals were individually housed in TPX cages (CLEA Japan, Inc., Tokyo, Japan) on paper-based bedding, Paper-clean (Japan SLC, Inc.), with pellet chow and tap water *ad libitum* in an air-conditioned room (12 h light/12 h dark cycle; 21–25°C; 40–75% humidity).

The animals (6 rats/group) were treated by oral gavage with distilled water (i.e., vehicle control), 2,4-DAT (10 and 30 mg/kg/day) or 2,6-DAT (60 mg/kg/day) at a dosing volume of 5 mL/kg once a day for 28 consecutive days (7,8). For the positive control

group, five rats were treated intraperitoneally with ENU (50 mg/kg/day; 10 mL/kg) once a day for five consecutive days. Changes in the general condition and body weight of treated animals were monitored regularly until the dissection day. The animal experiments were conducted in accordance with the guideline in each laboratory.

Tissue collection and delivery: All treated animals except for the ENU-treated group were euthanized 3 days after the last treatment by exsanguination under deep anesthesia with sodium pentobarbital. The livers (i.e., target organ for carcinogenesis) and kidneys (i.e., non-target organ) were collected and weighed. The liver was divided into four aliquots, quickly frozen in liquid nitrogen, and stored below −70°C until delivery to the participants. For the ENU-treated group, five treated animals were euthanized 26 days after the last treatment. The liver was isolated and cut into slices, quickly frozen in liquid nitrogen, and stored below −70°C until delivery to the participants. Liver samples from rats in the ENU-treated group were delivered by the Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd. (Kanagawa, Japan), and those in all other treated groups were delivered by Lab D3 to the participating laboratories. The liver samples were stored below −70°C until used for genomic DNA isolation in each laboratory.

For all treated animals except for the ENU-treated group, histopathological and biochemical examinations were also performed to examine the general toxicities by 2,4-DAT and 2,6-DAT. For histopathological examination, a portion of each tissue (i.e., liver and kidney) from each animal was fixed in 10% buffered formalin solution, and routinely processed into paraffin blocks. Hematoxylin and eosin-stained tissue preparations cut from the blocks were examined by light microscopy. For biochemical examination, the blood was collected from the abdominal caval vein of rats using heparinized plastic syringes, and routinely processed into plasma samples, which were analyzed with an automated clinical biochemistry analyzer (JCA-BM6010; Japan Electron Optics Laboratory Ltd., Tokyo).

Extraction of genomic DNA: High molecular weight total genomic DNA was extracted from the liver by the standard phenol/chloroform method (23) at Lab D1, and using the RecoverEase™ DNA Isolation kit (Agilent Technologies, Santa Clara, CA) at Labs D2 and D3. The genomic DNA was stored at 4°C until used for *in vitro* packaging.

***In vitro* packaging:** The λ phage vectors (i.e., λ EG10) carrying *gpt* genes were recovered from genomic DNA by *in vitro* packaging reactions, which were carried out using Transpack® Lambda Packaging Extract (Agilent Technologies) according to the manufacturer's instructions. The phage solutions were used for *gpt* mutation assay immediately after preparation or stored at 4°C and then used within four days after preparation.

***gpt* Mutation assay:** Five animals (at Labs D1 and D3) or six animals (at Lab D2) per group were analyzed by the *gpt* mutation assay, which was performed according to the previously published methods (4,24). The *gpt* mutant frequency (MF) was calculated according to:

$$MF = \frac{\left[\begin{array}{c}\text{Total number of confirmed mutant}\\ \text{colonies on 6-thioguanine plates}\end{array}\right]}{[\text{Total number of colonies on titer plates}]} \times \text{dilution factor}$$

The bacterial strains (i.e., *Escherichia coli* C and YG6020) were delivered from the Division of Genetics and Mutagenesis, National Institute of Health Sciences (Tokyo, Japan) to each participating laboratory. These strains were stored below −70°C until use.

Statistical analysis: The MFs were statistically analyzed using total MFs which were calculated after summation of total colonies and mutant colonies, respectively, from three laboratories, and individual MFs which were calculated by the values of each laboratory. The total MFs were of five animals per group (i.e., data of sixth animal in each group assayed only at Lab D2 was omitted). Variance was analyzed by Bartlett's test ($p<0.05$) between the MF in each 2,4-DAT-and 2,6-DAT-treated group and the vehicle control group. Subsequently, multiple comparisons of the treated groups with the vehicle control group were performed using either Dunnett's test (parametric, one-tailed) (25,26) or Steel's test (one-tailed) (27). For MF in the ENU-treated group, variance was analyzed by the F test ($p<0.05$). Subsequently, pairwise comparison of the ENU-treated group with the vehicle control group was performed using either Student's *t*-test (one-tailed) or Welch's *t*-test (one-tailed). For body weight, hematology, blood chemistry and organ weights, variance was analyzed by the F test ($p<0.05$) between the values in each 2,4-DAT-or 2,6-DAT-treated group and the vehicle control group. Subsequently, pairwise comparison of each treated group with the vehicle control group was performed using either Student's *t*-test (two-tailed) or Welch's *t*-test (two-tailed).

Results

gpt Mutant frequency in liver: Summary data of total MF from three laboratories are shown in Table 2, and depicted in Figs. 1 and 2. The numbers of colonies scored per animal were not less than 900,000 (Table 2). Total MF in the vehicle control group was similar between animals, $1.14–2.98\times10^{-6}$ (mean value: 1.80×10^{-6}), which are slightly lower than in the previous report (i.e., 4.4×10^{-6}) (28).

All total MFs from each animal in 10 and 30

Table 2. The total MFs* in the liver of 2,4-DAT or 2,6-DAT-treated rats

Group	Animal No.	Number of colonies*	Number of 6TG^r mutants	Total MF*	
				($\times 10^{-6}$)	Mean ± SD ($\times 10^{-6}$)
Vehicle control	M01001	2,868,000	6	2.09	
(Distilled water)	M01002	2,454,000	3	1.22	
10 mL/kg × 28	M01003	2,016,000	6	2.98	
	M01004	3,858,000	6	1.56	
	M01005	3,495,000	4	1.14	1.80 ± 0.76
2,4-DAT 10 mg/kg × 28	M02001	1,464,000	8	5.46	
	M02002	1,092,000	7	6.41	
	M02003	2,718,000	21	7.73	
	M02004	1,122,000	6	5.35	
	M02005	1,380,000	7	5.07	6.00 ± 1.09†
2,4-DAT 30 mg/kg × 28	M03001	1,014,000	13	12.82	
	M03002	969,000	21	21.67	
	M03003	1,074,000	19	17.69	
	M03004	1,326,000	21	15.84	
	M03005	1,032,000	11	10.66	15.74 ± 4.28†
2,6-DAT 60 mg/kg × 28	M04001	1,236,000	4	3.24	
	M04002	1,539,000	9	5.85	
	M04003	2,064,000	6	2.91	
	M04004	3,273,000	9	2.75	
	M04005	3,948,000	7	1.77	3.30 ± 1.52
ENU 50 mg/kg × 5	51	1,191,000	109	91.52	
	52	1,134,000	99	87.30	
	53	1,746,000	93	53.26	
	54	1,188,000	88	74.07	
	55	1,044,000	95	91.00	79.43 ± 16.24‡

*Sum of data from three laboratories. †$p<0.05$ Steel's test; ‡$p<0.001$ Welch's t-test.

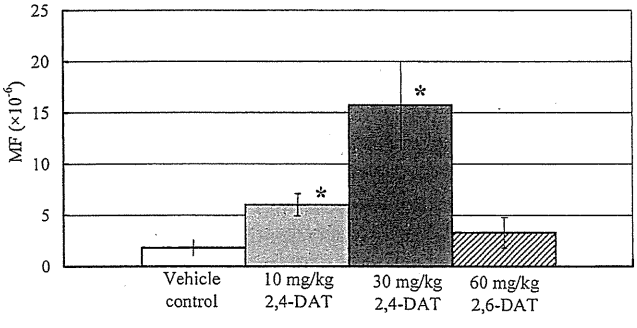

Fig. 1. Total mean MFs from 3 laboratories in the liver of 2,4-DAT or 2,6-DAT-treated rats. *$p<0.05$ Steel's test.

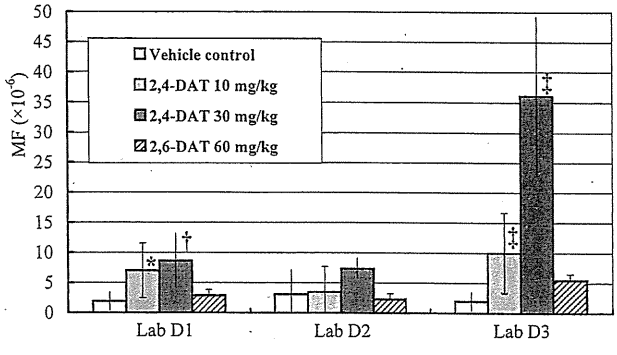

Fig. 2. Individual mean MFs in the liver of 2,4-DAT or 2,6-DAT-treated rats. *$p<0.05$ Dunnett's test; †$p<0.01$ Dunnett's test; ‡$p<0.05$ Steel's test.

mg/kg/day 2,4-DAT-treated groups (5.07–7.73×10^{-6} and 10.66–21.67×10^{-6}, respectively) were higher than in the vehicle control group (approximately 3.3 and 8.7 times, respectively), with significance (both $p<0.05$; Steel's test) and increased in a dose-related manner (Table 2 and Fig. 1). In contrast, almost all total MFs from each animal in the 2,6-DAT-treated group (i.e., 1.77–5.85×10^{-6}; mean value: 3.30×10^{-6}) were similar to the vehicle control group, and were not significantly increased (Table 2 and Fig. 1). The total MF from one animal (i.e., 5.85×10^{-6}) in the 2,6-DAT-treated group was similar to that in the 10 mg/kg/day 2,4-DAT-treated group (Table 2).

All total MFs from each animal in ENU-treated group (53.26–91.52×10^{-6}; mean value: 79.43×10^{-6}) were apparently higher than the vehicle control group (approximately 44.1 times), and were significantly increased ($p<0.001$; Welch's t-test) (Table 2 and Fig. 3).

The individual mean MFs are shown in Table 3 and Figs. 2 and 3. Detailed data from each laboratory are presented in online supplemental Appendices 1–3 at http://www.j-ems.org/ journal/. The individual mean MFs in the vehicle control group (i.e., 1.88, 3.06 and 1.93×10^{-6}, respectively) were similar among the three

laboratories (Table 3).

In the 10 and 30 mg/kg/day 2,4-DAT-treated groups, the MFs from Labs D1 and D3 increased in a dose-related manner, and a significant difference was observed compared with the vehicle control (Table 3 and Fig. 2). Meanwhile, there was no significant increase in the MF from Lab D2 in any 2,4-DAT-treated group; although, the mean MF in 30 mg/kg/day 2,4-DAT-treated group (i.e., 7.34×10^{-6}) was approximately 2.4 times higher than that in the vehicle control (i.e., 3.06×10^{-6}) (Table 3 and Fig. 2). Thus, increase in the MF by 2,4-DAT treatment was observed in all laboratories, which agreed with the results obtained for the total MF from the three laboratories.

In the 2,6-DAT-treated group, no significant increase was observed in the MF from any laboratory, which agreed with the results obtained for total MF (Table 3 and Fig. 2).

In the ENU-treated group (i.e., positive control group), all individual mean MFs apparently increased (mean values: 63.02, 74.82 and 154.44×10^{-6}, respectively) significantly when compared with the vehicle control (Table 3 and Fig. 3).

From these results, 2,4-DAT and ENU increased *gpt* MF in the liver of male F344 *gpt* delta transgenic rats in this study, but 2,6-DAT did not.

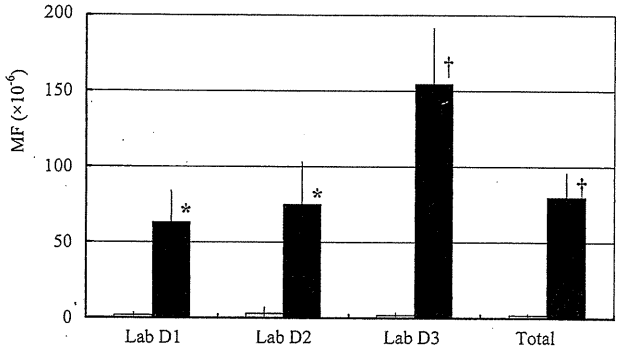

Fig. 3. Individual mean MFs in the liver of vehicle or ENU-treated rats. Mean MFs in the vehicle control for diaminotoluen (□) and 50 mg/kg ENU-treated groups (■) are indicated. *$p<0.01$ Welch's *t*-test; †$p<0.001$ Welch's *t*-test.

General toxicity evaluation: The summary of general toxicity data is shown in Table 4. Changes in the general condition of animals were observed only in the 2,6-DAT-treated group. There were no deaths in any treated group. Body weights of animals were significantly decreased in 30 mg/kg/day 2,4-DAT-and 2,6-DAT-treated groups. Significant changes in the hematological and blood biochemical findings were observed in both 2,4-DAT-and 2,6-DAT-treated groups. Weights of the liver and kidneys were significantly decreased only in the 2,6-DAT-treated group. Histological findings of the kidneys in both 2,4-DAT-and 2,6-DAT-treated groups were similar to the vehicle control group; however, for the liver, proliferation of bile duct and single cell necrosis of hepatocytes, which were very slight or slight, were observed in all animals in the 30 mg/kg/day 2,4-DAT-treated group. These histological findings are presented in online supplemental Appendix 4 at http://www.j-ems.org/journal/. Very slight single cell necrosis of hepatocytes was observed in one animal in the vehicle control group. Very slight hypertrophy of hepatocytes was observed in three animals each in the 30 mg/kg/day 2,4-DAT-treated and 2,6-DAT-treated groups. No histological changes of the liver were observed in the 10 mg/kg/day 2,4-DAT-treated group.

Discussion

In this collaborative study of the transgenic rat mutation assay, three participating laboratories conducted the *gpt* mutation assay using the liver of F344 *gpt* delta transgenic rats treated with 2,4-DAT or 2,6-DAT to evaluate the validity of the IWGT protocol (i.e., treatment period of 28 days and sampling time of 3 days following the final treatment).

In this collaborative study, the individual mean MFs in the vehicle control group (i.e., 1.88×10^{-6} at Lab D1; 3.06×10^{-6} at Lab D2; 1.93×10^{-6} at Lab D3) were similar between laboratories (Table 3 and Fig. 2). Although the data of MF in the liver of F344 *gpt* delta transgenic rats are limited, they were previously reported as follows: $6.02 \pm 2.38 \times 10^{-6}$ (6) and 4.4×10^{-6} (28) in the untreated group. Thus, the individual mean MFs in the vehicle control group of this study were likely values;

Table 3. Interlaboratory comparison of MFs in the liver of 2,4-DAT or 2,6-DAT-treated rats

Group	Individual mean MF ± SD ($\times 10^{-6}$)			Total mean MF ± SD ($\times 10^{-6}$)
	Lab 1	Lab 2	Lab 3	Lab 1-3
Distilled water 10 mL/kg × 28	1.88 ± 1.51	3.06 ± 4.01	1.93 ± 1.53	1.80 ± 0.76
2,4-DAT 10 mg/kg × 28	7.00 ± 4.54*	3.45 ± 4.23	9.97 ± 6.68‡	6.00 ± 1.09‡
2,4-DAT 30 mg/kg × 28	8.61 ± 4.49†	7.34 ± 1.68	36.07 ± 13.17‡	15.74 ± 4.28‡
2,6-DAT 60 mg/kg × 28	2.85 ± 2.19	2.26 ± 3.50	5.48 ± 5.06	3.30 ± 1.52
ENU 50 mg/kg × 5	63.02 ± 20.87§	74.82 ± 28.34§	154.44 ± 37.16‖	79.43 ± 16.24‖

*$p<0.05$ Dunnett's test; †$p<0.01$ Dunnett's test; ‡$p<0.05$ Steel's test (vs vehicle); §$p<0.01$ Welch's *t*-test; ‖$p<0.001$ Welch's *t*-test.

Table 4. General toxicity of 2,4-DAT and 2,6-DAT in male F344 *gpt* delta transgenic rats

Group (mg/kg/day)			Distilled water: vehicle control (0)	2,4-DAT (10)	2,4-DAT (30)	2,6-DAT (60)
Number of animals			6	6	6	6
General conditions		Decrease in locomotor activity	0	0	0	6
		Eyelid closure	0	0	0	6
		Salivation	0	0	0	2
Body weights				—	▼	▼
Hematological findings		RBC		▽	▽	—
		MCV		—	▲	—
		MCH		▲	▲	—
		Neutrophil		—	—	▲
		Platelet		▲	▲	▼
Blood biochemical findings		TP		—	▽	▽
		Total cholesterol		▲	▲	▽
		Triglyceride		▽	—	▼
		Phospholipid		▲	▲	▼
		Ca		—	—	▽
		Total bilirubin		—	△	—
		Inorganic phosphorus		—	△	—
Organ weight (absolute)		Liver		—	—	▼
		Kidneys		—	—	▼
Organ weight (relative)		Liver		▲	▲	—
		Kidneys		—	▲	▲
Histological findings	Liver	Hypertrophy, hepatocyte	0	0	(±)3	(±)3
		Degeneration, hepatocyte, periportal	0	0	(±)3	0
		Proliferation, Kupffer cell	0	0	0	(±)1
		Proliferation, bile duct	0	0	(±)5; (+)1	0
		Single cell necrosis, hepatocyte	(±)1	0	(±)5; (+)1	(±)2
	Kidneys	Eosinophilic body, proximal tubule epithelium	(±)6	(±)6	(±)6	(±)6
		Hyaline droplet, proximal tubule	(±)6	(±)6	(±)5	(±)5; (+)1
		Basophilic tubular epithelium	(±)1	(±)1	0	0
		Dilatation, lumen, distal tubule	0	(±)1	0	0

—, No change; (±), very slight; (+), slight; ▽△, $p<0.05$; ▼▲, $p<0.01$.

however, they were slightly lower than reported previously.

In this collaborative study, liver samples from ENU-treated animals were used as a positive control. ENU increased the total MF (79.43×10^{-6}) which was approximately 44.1 times higher than in the vehicle control group (Table 2 and Fig. 3). Clear increases in MF (i.e., approximately 33.5 times at Lab D1; approximately 24.5 times at Lab D2; approximately 80.0 times at Lab D3) were also observed in each mean MF from three laboratories (Table 3 and Fig. 3). These positive results coincide with previous reports using *gpt* delta transgenic mice (29). Thus, the results of both (i.e., vehicle and positive) control groups indicate that the total data from the three laboratories in this study are reliable.

In previous reports, 2,4-DAT, a liver carcinogen in rats and mice, gave negative and positive results in the TGR mutagenicity assays as follows: increase in *LacI* MF of liver samples from male and female Big Blue™ transgenic mice with a treatment period of 10 days (80 mg/kg/day by oral gavage) and sampling time of 10 days (approximately 1.7 times only in female) and 28 days (approximately 2 times in male and 1.9 times in female) following the final treatment, but not in males with a sampling time of 10 days (19); approximately 2.1 times increase in *LacI* MF of liver samples from male Big Blue™ transgenic mice with a treatment period of 90 days (1000 ppm in the diet), but not with 30 days (20); 4.5 and 1.9 times increase in *LacZ* MF of liver and kidney samples from male Muta™Mouse transgenic mice with a treatment period of 28 days (200 mg/kg/day by topical application) and sampling time of 7 days following the final treatment, respectively, but not in skin samples (21); approximately 2.2–7.1 times increase in *gpt* MF and 1.9–3.6 times increase in Spi⁻ MF of liver samples from male F344 *gpt* delta transgenic

rats with a treatment period of 13 weeks (125, 250 and 500 ppm in the diet) (6). These data indicate that adequate dosing (i.e., treatment period) and sampling (i.e., sampling time and sampling organ) regimens may be necessary to observe the *in vivo* gene mutations induced by 2,4-DAT treatment.

In this collaborative study, total MFs from three laboratories in 10 and 30 mg/kg/day 2,4-DAT-treated groups were 6.00×10^{-6} and 15.74×10^{-6}, approximately 3.3 and 8.7 times higher than in the vehicle control group, respectively (Table 2 and Fig. 1). Thus, clear increases in total MF were observed by 2,4-DAT treatment, dose-dependently.

Similar increases in MF at two doses were also observed in the individual mean MF from two laboratories, Lab D1 and Lab D3, that is, significant increases in mean MF in 10 and 30 mg/kg/day 2,4-DAT-treated groups (i.e., 3.7 and 4.6 times at Lab D1; 5.2 and 18.7 times at Lab D3) were observed (Table 3 and Fig. 2). At Lab D2, there was no significant increase in MF in any 2,4-DAT-treated group; however, the mean MF in the 30 mg/kg/day 2,4-DAT-treated group was 2.4 times higher than in the vehicle control. We speculate that the results of the 2,4-DAT treated group at Lab D2 may have been caused as follows: zero MF, which was frequently observed in the vehicle control and 10 mg/kg/day 2,4-DAT-treated group, and high MF (i.e., 10.58×10^{-6}) from one animal in the vehicle control group (Appendix 2). Especially, this high MF was calculated by a small number of colonies scored, and it was considered that this MF influenced the variability of MF in the vehicle control group. In fact, this single MF in the vehicle control group was determined as an outlier by a box-and-whisker plot (30). Thus, this outlier value was excluded and then the statistical re-analysis was conducted for the results of Lab D2. As a result, the statistical re-analysis showed that a significant increase ($p < 0.01$) in MF in 30 mg/kg/day 2,4-DAT-treated group (data not shown).

On the other hand, 2,6-DAT, a non-carcinogen in rodents, did not significantly increase either total MF from the three laboratories or individual MFs from each laboratory in this study (Table 3). Thus, the results obtained in this study agreed with the previous reports as follows: 2,6-DAT give only negative results in TGR mutagenicity assays using Big Blue™, Muta™Mouse transgenic mice (20,21) and F344 *gpt* delta transgenic rats (6). Approximately 1.8 times increase was observed in the total MF in 2,6-DAT-treated group in this study; however, we speculate that this slight increase in total MF in the 2,6-DAT-treated group may have been caused by the MF (i.e., 12.82×10^{-6}) from one animal at Lab D3, which had a low colony count (i.e., 156,000) (Appendix 3).

In this study, we identified the significant decrease of body weight in the 30 mg/kg/day 2,4-DAT-and 60 mg/kg/day 2,6-DAT-treated groups. The decrease in the 2,6-DAT-treated group was most notable, followed by 30 mg/kg/day and 10 mg/kg/day 2,4-DAT-treated groups. In these groups, the histological changes in the liver (i.e., hypertrophy and single cell necrosis of hepatocyte etc.) were also observed in many rats, and these types and the number of corresponding rats were different from that observed in the vehicle control group (Table 4 and Appendix 4). In the 60 mg/kg/day 2,6-DAT-treated group, the significant decrease of liver weight was also observed. Thus, these general toxicity findings indicate that the livers of F344 *gpt* delta rats were exposed to the test chemicals (i.e., 2,4-DAT and 2,6-DAT), and the doses used in this study were sufficient for evaluation of the TGR mutagenicity assays.

In the present study, 2,6-DAT was negative in genotoxicity. This negative result was consistent with the results of carcinogenicity study (8) and the other *in vivo* genotoxicity assays (6,17–21). However, weakly positive result of 2,6-DAT in the rat bone marrow micronucleus study is present (22). This point remains to be clarified.

In the present study, the standard deviations were larger than the mean value in the individual mean MF of all experimental groups except for the higher dose of 2,4-DAT and the positive control groups, obtained at Lab 2 (Appendix 2). In these experimental groups, zero $6TG^r$ mutant was also observed frequently. We suggest that TGR mutagenicity assays are repeatedly conducted on different experimental dates for all animals to confirm the reproducibility of the MF obtained from each assay and to accumulate a larger total population. Actually, total population analyzed at Lab D2 was the smallest among three laboratories. In the previous report, for phage-based assays, a minimum of 125,000 to 300,000 colony (or plaque) per animal is required if spontaneous mutant frequency is in the order of $\sim 3 \times 10^{-5}$ mutants and five to 10 animals per group are analyzed (31).

In conclusion, all three laboratories could distinguish the differences in the *in vivo* mutagenic potential between a hepatic carcinogen (i.e., 2,4-DAT) and a non-carcinogen (i.e., 2,6-DAT) by the F344 *gpt* delta transgenic rat mutation assay using the IWGT protocol. Consequently, the validity of the IWGT protocol for the TGR assays was confirmed in this collaborative study. Because there are still limited data available using the IWGT protocol, further studies using other compounds are needed to validate the TGR assay conducted by the IWGT protocol.

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References

1 Heddle JA, Dean S, Nohmi T, Boerrigter M, Casciano D, Douglas GR, Glickman BW, Gorelick NJ, Mirsalis JC, Martus HJ, Skopek TR, Thybaud V, Tindall KR, Yajima N. *In vivo* transgenic mutation assays. Environ Mol Mutagen. 2000; 35: 253–9.

2 Thybaud V, Dean S, Nohmi T, de Boer J, Douglas GR, Glickman BW, Gorelick NJ, Heddle JA, Heflich RH, Lambert I, Martus HJ, Mirsalis JC, Suzuki T, Yajima N. *In vivo* transgenic mutation assays. Mutat Res. 2003; 540: 141–51.

3 International Programme on Chemical Safety: Transgenic animal mutagenicity assays. Environmental Health Criteria 233, World Health Organization (WHO), Geneva: 2006.

4 Nohmi T, Katoh M, Suzuki H, Matsui M, Yamada M, Watanabe M, Suzuki M, Horiya N, Ueda O, Shibuya T, Ikeda H, Sofuni T. A new transgenic mouse mutagenesis test system using Spi$^-$ and 6-thioguanine selections. Environ Mol Mutagen. 1996; 28: 465–70.

5 Hayashi H, Kondo H, Masumura K, Shindo Y, Nohmi T. Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi$^-$ selection. Environ Mol Mutagen. 2003; 41: 253–9.

6 Toyoda-Hokaiwado N, Inoue T, Masumura K, Hayashi H, Kawamura Y, Kurata Y, Takamune M, Yamada M, Sanada H, Umemura T, Nishikawa A, Nohmi T. Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *In vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. Toxicol Sci. 2010; 114: 71–8.

7 National Toxicology Program. Bioassay of 2,4-diaminotoluene for possible carcinogenicity. Natl Cancer Inst Carcinog Tech Rep Ser. 1979; 162: 1–139.

8 National Toxicology Program. Bioassay of 2,6-toluenediamine dihydrochloride for possible carcinogenicity. Natl Cancer Inst Carcinog Tech Rep Ser. 1980; 200: 1–123.

9 Haworth S, Lawlor T, Mortelmans K, Speck W, Zeiger E. *Salmonella* mutagenicity test results for 250 chemicals. Environ Mutagen. 1983; 1 Suppl 1: 3–142.

10 Cunningham ML, Burka LT, Matthews HB. Metabolism, disposition, and mutagenicity of 2,6-diaminotoluene, a mutagenic noncarcinogen. Drug Metab Dispos. 1989; 17: 612–6.

11 Furlong BB, Weaver RP, Goldstein JA. Covalent binding to DNA and mutagenicity of 2,4-diaminotoluene metabolites produced by isolated hepatocytes and 9000 *g* supernatant from Fischer 344 rats. Carcinogenesis. 1987; 8: 247–51.

12 Cunningham ML, Matthews HB. Evidence for an acetoxyarylamine as the ultimate mutagenic reactive intermediate of the carcinogenic aromatic amine 2,4-diaminotoluene. Mutat Res. 1990; 242: 101–10.

13 Séverin I, Jondeau A, Dahbi L, Chagnon MC. 2,4-Diaminotoluene (2,4-DAT)-induced DNA damage, DNA repair and micronucleus formation in the human hepatoma cell line HepG2. Toxicology. 2005; 213: 138–46.

14 Bermudez E, Tillery D, Butterworth BE. The effect of 2,4-diaminotoluene and isomers of dinitrotoluene on unscheduled DNA synthesis in primary rat hepatocytes. Environ Mutagen. 1979; 1: 391–8.

15 Butterworth BE, Earle LL, Strom S, Jirtle R, Michalopoulos G. Measurement of chemically induced DNA repair in human hepatocytes. Proc Am Assoc Cancer Res. 1983; 24: 69.

16 Sina JF, Bean CL, Dysart GR, Taylor VI, Bradley MO. Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. Mutat Res. 1983; 113: 357–91.

17 Mirsalis JC, Tyson CK, Butterworth BE. Detection of genotoxic carcinogens in the *in vivo-in vitro* hepatocyte DNA repair assay. Environ Mutagen. 1982; 4: 553–62

18 Sasaki YF, Fujikawa K, Ishida K, Kawamura N, Nishikawa Y, Ohta S, Satoh M, Madarame H, Ueno S, Susa N, Matsusaka N, Tsuda S. The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP. Mutat Res. 1999; 440: 1–18.

19 Suter W, Ahiabor R, Blanco B, Locher F, Mantovani F, Robinson M, Sreenan G, Staedtler F, Swingler T, Vignutelli A, Perentes E. Evaluation of the in vivo genotoxic potential of three carcinogenic aromatic amines using the Big Blue™ transgenic mouse mutation assay. Environ Mol Mutagen. 1996; 28: 354–62.

20 Cunningham ML, Hayward JJ, Shane BS, Tindall KR. Distinction of mutagenic carcinogens from a mutagenic noncarcinogen in the big blue transgenic mouse. Environ Health Perspect. 1996; 104 Suppl 3: 683–6.

21 Kirkland D, Beevers C. Induction of *LacZ* mutations in Muta™Mouse can distinguish carcinogenic from non-carcinogenic analogues of diaminotoluenes and nitronaphthalenes. Mutat Res. 2006; 608: 88–96.

22 George E, Westmoreland C. Evaluation of the *in vivo* genotoxicity of the structural analogues 2,6-diaminotoluene and 2,4-diaminotoluene using the rat micronucleus test and rat liver UDS assay. Carcinogenesis. 1991; 12: 2233–7.

23 Sambrook J, Russell DW. Molecular cloning, a laboratory manual. 3rd ed. Vol. 1. New York: Cold Spring Harbor Laboratory Press; 1989.

24 Nohmi T, Suzuki T, Masumura K. Recent advances in the protocols of transgenic mouse mutation assays. Mutat Res. 2000; 455: 191–215.

25 Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc. 1955; 50: 1096–121.

26 Dunnett CW. New tables for multiple comparisons with a control. Biometrics. 1964; 20: 482–91.

27 Steel RGD. A multiple comparison rank sum test: Treatments versus control. Biometrics. 1959; 15: 560–72.

28 Masumura K. Spontaneous and induced *gpt* and Spi$^-$ mutant frequencies in *gpt* delta transgenic rodent. Genes Environ. 2009; 31: 105–18.

29 Swiger RR, Cosentino L, Masumura KI, Nohmi T, Heddle JA. Further characterization and validation of *gpt*