Genes and Environment, Vol. 34, No. 1 pp. 25–33 (2012)

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

Hajime Sui[1,6], Ryo Ohta[1], Toshiyuki Shiragiku[2], Ayaka Akahori[3], Kenichiro Suzuki[3], Madoka Nakajima[3], Hiroyuki Hayashi[4], Kenichi Masumura[5] and Takehiko Nohmi[5]

[1]Division of Genetic Toxicology, Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan
[2]Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan
[3]Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan
[4]Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Kanagawa, Japan
[5]Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan

(Received April 13, 2011; Revised June 18, 2011; Accepted July 25, 2011)

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-

[6]Correspondence to: Hajime Sui, Division of Genetic Toxicology, Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Hadano, Kanagawa 257-8523, Japan. Tel: +81-463-82-4751, Fax: +81-463-82-9627, E-mail: sui.h@fdsc.or.jp

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:

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

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. $^\dagger p < 0.05$ Steel's test; $^\ddagger p < 0.001$ Welch's *t*-test.

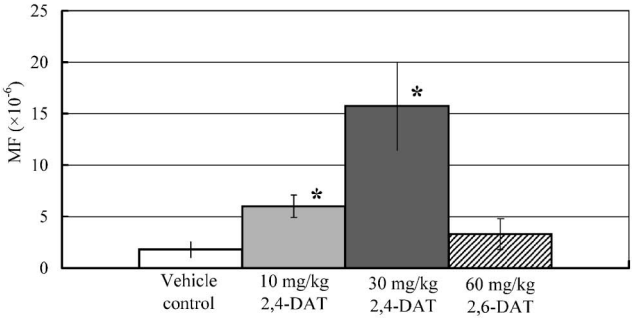

Fig. 1. Total mean MFs from 3 laboratories in the liver of 2,4-DAT or 2,6-DAT-treated rats. $^*p < 0.05$ Steel's test.

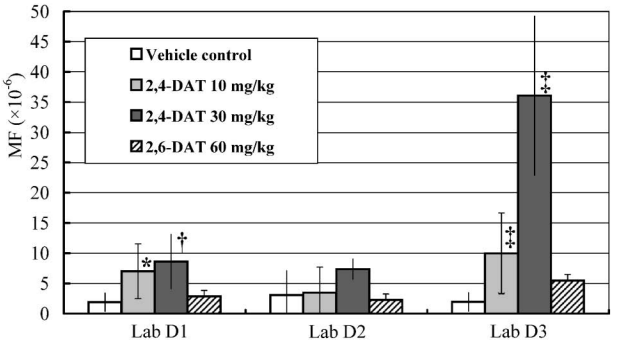

Fig. 2. Individual mean MFs in the liver of 2,4-DAT or 2,6-DAT-treated rats. $^*p < 0.05$ Dunnett's test; $^\dagger p < 0.01$ Dunnett's test; $^\ddagger p < 0.05$ Steel's test.

mg/kg/day 2,4-DAT-treated groups ($5.07–7.73 \times 10^{-6}$ and $10.66–21.67 \times 10^{-6}$, 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., $1.77–5.85 \times 10^{-6}$; 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 ($53.26–91.52 \times 10^{-6}$; 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.

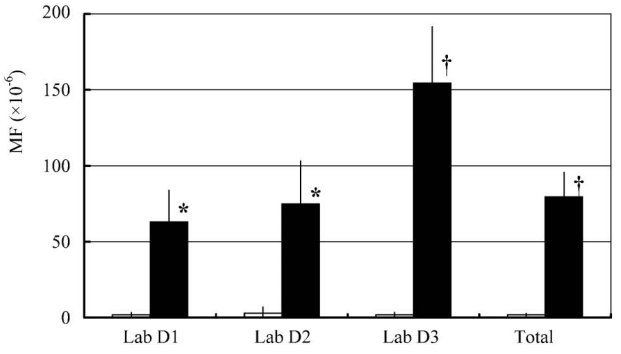

Fig. 3. Individual mean MFs in the liver of vehicle or ENU-treated rats. Mean MFs in the vehicle control for diaminotoluen (□) and 50 mg/kg ENU-treated groups (■) are indicated. *$p<0.01$ Welch's *t*-test; †$p<0.001$ Welch's *t*-test.

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.

Acknowledgments: We thank the organizers of the collaborative study group for the transgenic rat mutation assay for reviewing the manuscript and giving us

helpful suggestions.

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*

delta transgenic mice for quantifying somatic mutations *in vivo*. Environ Mol Mutagen. 2001; 37: 297–303.

30 Yoshimura I. IV 8. Explorative data analysis with diagrammatic representation. In: Yoshimura I, editor. Statistical methods for toxicological and medical data. Tokyo: Scientist Press; 2008. p. 182–6 (in Japanese).

31 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. Environ Mol Mutagen. 2000; 35: 253–9.