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-

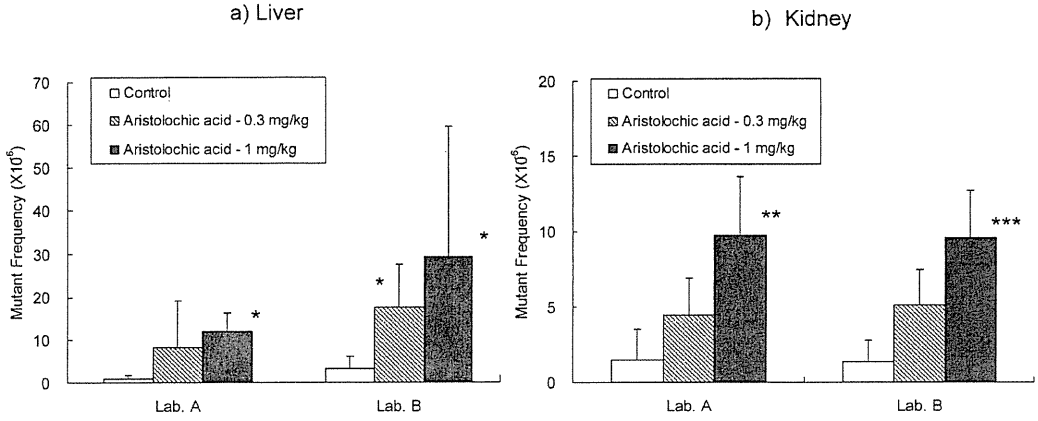

Fig. 1. Comparison between two laboratories in *gpt* mutant frequency of aristolochic acid-treated rats ($n=5$) in a) Liver, b) Kidney. Values represent mean +/−SD. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ (Steel test).

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 ($\times 10^{-6}$)	Mutant frequency Average	Mutant frequency 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).

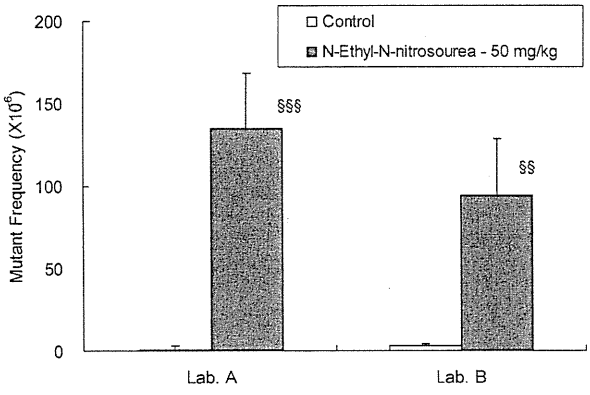

Fig. 2. Comparison between two laboratories in *gpt* mutant frequency of *N*-Ethyl-*N*-nitrosourea-treated rats ($n=5$) in liver. Values represent mean +/−SD. §§$p<0.01$, §§§$p<0.001$ (welch's t-test).

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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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, $1.14\text{–}2.98\times10^{-6}$ (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* ($\times 10^{-6}$)	Total MF* 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.

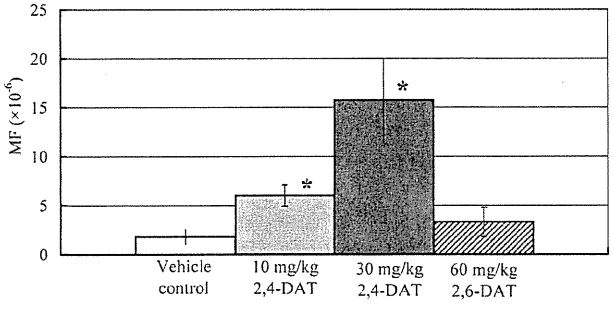

Fig. 1. Total mean MFs from 3 laboratories in the liver of 2,4-DAT or 2,6-DAT-treated rats. *$p<0.05$ Steel's test.

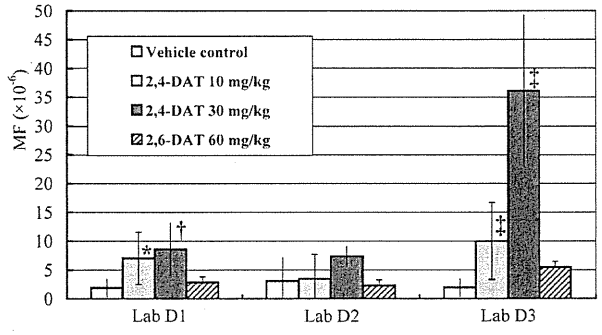

Fig. 2. Individual mean MFs in the liver of 2,4-DAT or 2,6-DAT-treated rats. *$p<0.05$ Dunnett's test; †$p<0.01$ Dunnett's test; ‡$p<0.05$ Steel's test.

mg/kg/day 2,4-DAT-treated groups ($5.07–7.73 \times 10^{-6}$ 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.

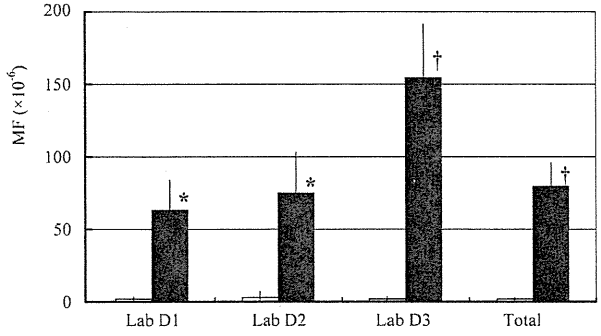

Fig. 3. Individual mean MFs in the liver of vehicle or ENU-treated rats. Mean MFs in the vehicle control for diaminotoluen (□) and 50 mg/kg ENU-treated groups (■) are indicated. *$p<0.01$ Welch's *t*-test; †$p<0.001$ Welch's *t*-test.

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.

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Regular article

Evaluation of the *in vivo* Mutagenicity of Nickel Subsulfide in the Lung of F344 *gpt* delta Transgenic Rats Exposed by Intratracheal Instillation: A Collaborative Study for the *gpt* delta Transgenic Rat Mutation Assay

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This study was conducted to evaluate the effectiveness of a transgenic rat mutation assay using F344 *gpt* delta rats. We investigated the mutagenic potential in the lung of nickel subsulfide (Ni_3S_2), an insoluble fine-crystalline-metallic compound and a carcinogen in the rodent and human lung. Ni_3S_2 carcinogenicity has been proposed to act via both genotoxic and non-genotoxic mechanisms. Ni_3S_2 was intratracheally instilled into male *gpt* delta rats at doses of 0.5 and 1 mg/animal once a week for four weeks; these doses of Ni_3S_2 are high enough to induce inflammation in the lung. Following a period of 28 and 90 days after the first administration, the *gpt* mutant frequencies (MFs) in lung were determined in four independent laboratories, and Spi$^-$ selection for larger deletion mutations was done in one laboratory. The *gpt* MFs of the rats treated with Ni_3S_2 were not increased: all four laboratories obtained similar results with no statistical differences. The Spi$^-$ MFs were also not increased by exposure to Ni_3S_2. These results indicate that intratracheally instilled Ni_3S_2 is non-mutagenic in the lung of *gpt* delta transgenic rats; however, whether Ni_3S_2 is non-mutagenic in the lung or it induces mutations which are not detectable by transgenic rodent mutation assays requires further investigation.

Key words: F344 *gpt* delta transgenic rat, nickel subsulfide, *gpt* assay, Spi$^-$ assay

Introduction

Transgenic animals, such as Big Blue® rats and mice (1,2), Muta™ mice (3) and *gpt* delta rats and mice (4,5), are powerful tools for the detection of *in vivo* mutagenicity. The transgenic rodent mutation assay using *gpt* delta transgenic mice and rats was developed by Nohmi *et al.* (4,5). This assay system is composed of two detection methods, the 6-thioguanine (6-TG) assay for point mutations and Spi$^-$ selection for deletion (6,7). This system is convenient for the identification of gene mutation by DNA sequencing due to small gene size (456 bp). In addition, positive selections are easier to evaluate mutant frequencies (MFs) than conventional color selections. Spontaneous MFs are comparatively lower than that of other transgenic rodent mutation assay system (7). Among the numerous substances which have been evaluated using this system are a variety of chemical compounds (8–10), radiation (11,12), and micro/nanoparticles (13), validating the usefulness of this transgenic rodent mutation assay system (7).

Recently, a protocol or guideline for the use of transgenic rodent gene mutation assays was discussed and proposed by the International Workshop on Genotoxicity Testing (IWGT) (14–16). It recommends a repeat-dose regimen with daily treatments for a period of 28 days and sampling time for 3 days following the final treatment. This protocol is being developed into an OECD Test Guideline (17). IWGT also suggests alternative treatment regimens; for example, weekly dose administration may be appropriate for some evaluations and a longer sampling time may be more appropriate if slowly proliferating tissues are of interest (16). We adopted the fundamental protocol of a collaborative

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study, which doses were a negative control group and a minimum of two dose revels (17).

The purpose of our study was to evaluate *in vivo* mutagenicity of nickel subsulfide (Ni_3S_2) by transgenic gene mutation assays in the lung as the target organ using F344 *gpt* delta rat. Ni_3S_2 is an insoluble fine-crystalline-metallic compound and a representative rodent and human lung carcinogenic metal.

Epidemiological studies of nickel refinery workers have demonstrated increased mortality from lung and nasal cancer, and it is apparent that the cancer risks have been associated with exposure to insoluble nickel compounds such as Ni_3S_2 and nickel oxide (18,19). Ni_3S_2 clearly has carcinogenic activity in the lungs of male and female F344/N rats exposed by inhalation, but it is not carcinogenic in male or female B6C3F1 mice exposed by inhalation (20). Injection of Ni_3S_2 has been shown to cause numerous carcinogenic lesions in rodents depending on the site of injection: lesions include sarcomas, rhabdomyosarcomas, fibrosarcomas, fibrous histiocytomas, mesotheliomas, renal-cell neoplasms, retinoblastomas, melanomas, and gliomas (19,21-27).

A variety of nickel compounds, including Ni_3S_2, are able to interact with molecular oxygen and generate reactive oxygen species (ROS) (27); however, like most carcinogenic metal ions, nickel compounds have weak or no mutagenic activity in bacteria (27-33), except when present at high toxic concentrations (34). In mammalian cells, carcinogenic nickel compounds are also generally poor mutagens (27,29,30,32,33,35). These compounds are, however, clastogenic in mammalian cells, inducing DNA strand breaks and accompanying chromosomal aberrations, sister-chromatid exchanges and the formation of micronuclei (29,30,32,36-39), and these clastogenicities are thought to be the mechanism by which Ni_3S_2 transforms mammalian cells *in vitro* (27,35,37,39).

Kawanishi *et al.* showed that Ni_3S_2 induced the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) DNA adducts and DNA strand breaks, both *in vitro* and after intratracheal instillation into the lungs of Wistar rats (40). In these studies, intratracheal instillation of Ni_3S_2 also caused inflammation in the lungs of the rats. In contrast, Mayer *et al.* reported that Ni_3S_2 has no mutagenic activity in *lacZ* transgenic mice and *lacI* transgenic rats treated by single inhalation exposure for 2 h at a dose close to the maximum tolerated dose (MTD) of Ni_3S_2, even though it was mutagenic in *lacI* transgenic cells *in vitro* (41).

We assessed the *in vivo* mutagenicity of Ni_3S_2 administered into the lung by intratracheal instillation using *gpt* delta transgenic rats. The administration was limited to once a week for 4 weeks and lung sampling was at 7 days after fourth treatment due to the reduction of body weight over a few days by treatment under anesthesia, instead of 28 consecutive daily treatments and sampling time in IWGT recommendation. To manifest maximum mutation frequency, the rats were sacrificed 28 or 90 days after the first administration. The period of 90 days between the initial administration of Ni_3S_2 and sacrifice (a little less than 10 weeks after the final administration of Ni_3S_2) was chosen to allow the lung to recover from Ni_3S_2 induced inflammation.

Materials and Methods

Four laboratories (Table 1) participated in a collaborative study to evaluate the transgenic rodent gene mutation assay using *gpt* delta rats recommended by IWGT. This study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were cared for in accordance with the Guideline for the care and use of laboratory animals in JBRC.

Chemicals: Nickel subsulfide (Ni_3S_2) (CAS.No. 12035-72-2) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The purity of the Ni_3S_2 particles (150-mesh) was 99.7%. The particle characterization of the Ni_3S_2 particle was determined using scanning electron microscopy (SU-8000, Hitachi Ltd., Tokyo Japan). The mean size was $2.9 \pm 1.74\ \mu m$ in diameter and more than 90% of the Ni_3S_2 particles had diameters less than 5 μm (Fig. 1). Perfluorocarbon (PF-5060) (CAS.No. 96508-42-1) was obtained from 3M (St. Paul, MN, USA).

Animals and treatment: Five-week-old male *gpt* delta rats [F344/NSlc-Tg(*gpt* delta)] were obtained from Japan SLC (Shizuoka, Japan). The inbred F344/NSlc-Tg (*gpt* delta) rat was established by backcrossing outbred *gpt* delta SD males with inbred F344 females. This strain has been backcrossed more than 15 times and is genetically homogeneous (42). The *gpt* delta transgenic rat contains approximately 5 to 10 copies of the lambda EG10 transgene in chromosome 4 and is maintained as a heterozygote (6). The animals were quarantined for one week and acclimated for 6 weeks to allow growth to a

Table 1. Participants in the collaborative study

Lab	Investigators
A	T. Kamigaito, T. Noguchi: Japan Bioassay Research Center
B	K. Narumi, R. Takashima, S. Hamada: Mitsubishi Chemical Medience Corporation
C	H. Sanada: Central Research Laboratories, Kaken Pharmaceutical Co., Ltd.
D	K. Masumura, M. Hasuko, T. Nohmi: National Institute of Health Sciences

The *gpt* MFs in all treated groups were studied by Labs A-D.
The Spi$^-$ MFs in all treated groups were studied by Lab D.
*Animal housing, treatments to animals and tissue collection for Ni_3S_2- and ENU-treated groups were conducted by Japan Bioassay Research Center and Pharmaceutical Research Center, Meiji Seika Pharma Co., Ltd. respectively.

body weight of about 250 g. The animals were housed individually in stainless steel wire mash cages under barrier system room controlled environmental conditions (temperature of 24 ± 3°C, and relative humidity of 55 ± 15%). Fluorescent lighting was controlled automatically to provide a 12-h light-dark cycle. All animals were given basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum*. Twelve-week-old rats were divided by stratified randomization into 5-body weight matched groups.

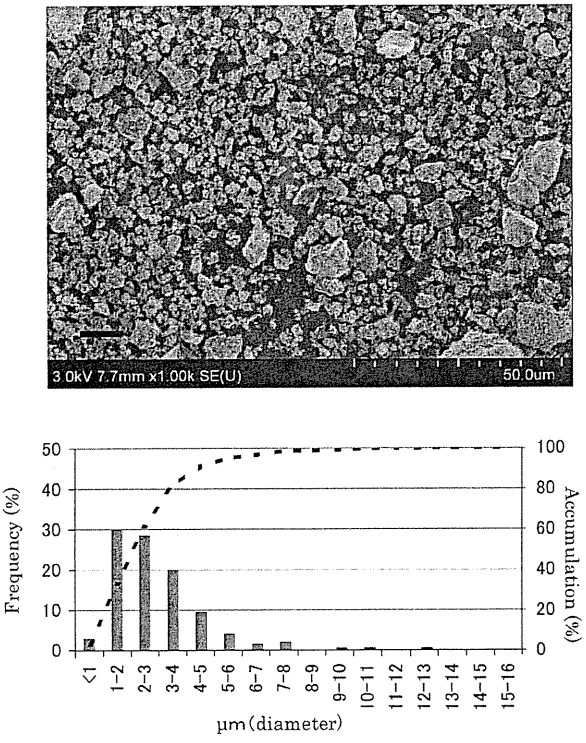

Fig. 1. Characteristics of nickel subsulfide particles in test substance. Upper panel: Scanning electron micrograph of Ni_3S_2 particles. Bar shows 10 μm. Lower panel: Frequency distribution (bar) and cumulative distribution (dotted line) in Ni_3S_2 particl.

Perfluorocarbon is a volatile organic compound (bp 56°C) which does not affect respiratory function in rodents and is an efficient vehicle for Ni_3S_2 particle delivery to pulmonary alveoli by intratracheal instillation (43). Ni_3S_2 particles homogenously suspended in perfluorocarbon were administrated into the endotrachea of the rats by intratracheal instillation, using a microspray cannula (Series 1A-1B Intratracheal Aerosolizer, PennCentury, Inc., PA, USA). Ni_3S_2 was administered at doses of 0 (vehicle control), 0.5, or 1.0 mg/animal in 0.3 mL perfluorocarbon once a week for four weeks. Each dose was administered to five rats under isoflurane anesthesia. Administration was limited to one time a week due to the harmful effects of the anesthesia. The dose of administered Ni_3S_2 was determined so as not to exceed the highest exposure dose produced only minimal interference with lung defense mechanisms based on particle clearance (40,44,45). Animals were sacrificed 28 days or 90 days after the first administration. The lung tissue was removed, weighed and a piece of the organ was fixed in 10% neutral buffered formalin and prepared and stained with hematoxylin and eosin. The remaining tissue was frozen in liquid nitrogen. Frozen lung tissue was broken, divided, packed with dry ice and sent to each of the collaborating laboratories for DNA extraction and mutation analysis (Table 1).

As a positive control, the livers of *N*-ethyl-*N*-nitrosourea (ENU) treated transgenic rats were analyzed. This treatment was conducted by Pharmaceutical

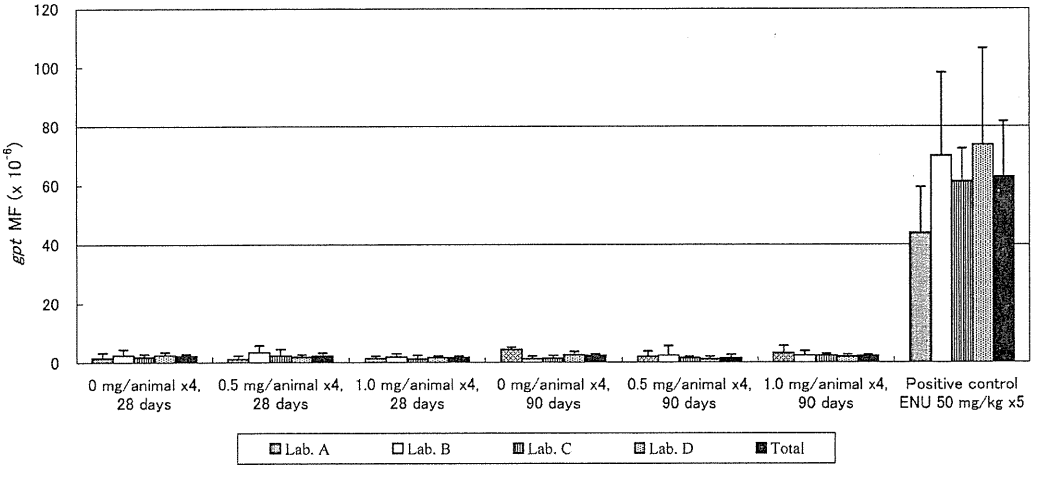

Fig. 2. *gpt* Mutant frequencies of each laboratoriy in the lung of male F344 *gpt* delta rats treated with Ni_3S_2 by intratracheal instillation and liver treated with ENU (positive control).

Research Center of Meiji Seika Pharma Co., Ltd. (Kanagawa, Japan). ENU was intraperitoneally administered to the *gpt* delta F344 male rats at a dose of 50 mg/kg once a day for five consecutive days. Twenty-six days after the final treatment the rats were killed and the livers were removed, frozen and distributed to the collaborating research laboratories (46).

DNA isolation and mutation assay: The *gpt* mutation assay was conducted at four collaborating laboratories and the Spi$^-$ assay was at a single laboratory using previously reported methods (4,6). A RecoverEase™ DNA Isolation Kit (Agilent Technologies, Santa Clara, CA) was used to extract genomic DNA according to the manufacturer's instructions. The transgene was rescued from rat genomic DNA by *in vitro* lambda phage packaging using Transpack™ Packaging Extract (Agilent Technologies) according to the manufacturer's instructions.

For the *gpt* assay, the *E.coli* YG6020 strain which expresses Cre recombinase was infected with the rescued lambda phage. Total colonies and 6-TG resistant mutant colonies confirmed by replating were counted to calculate the frequencies of point mutations (*gpt* assay).

For the Spi$^-$ assay, the *E.coli* XL1-Blue MRA strain and the XL1-Blue MRA(P2) strain were infected with the rescued lambda phage. Total recued plaques formed in *E.coli* XL1-Blue plates (representing total rescued lambda phage) and mutant plaques formed in the XL1-Blue MRA(P2) plates and confirmed by replating in *E.coli* WL95(P2) (representing mutant phages with deletion mutations) were counted to assess the frequencies of deletion mutations.

Statistical analysis: Bartlett's test was preliminarily used to determine whether the variances of data among control and treated groups were different or not. Body weight, organ weight and mutant frequency were analyzed by Dunnett's' multiple test because the variances of the data was not statistically different between each group. Incidences of histopathological lesions were analyzed by Fisher exact test. The level of significance was set at a p value of less than 0.05 (two-sided analysis with).

Results

Rats were exposed by intratracheal instillation to 0, 0.5 and 1.0 mg Ni_3S_2/animal once a week for four weeks. The total doses of each treated group were estimated to be 7 and 14 mg/kg, respectively. There was no difference in body weight gain between the Ni_3S_2-treated rats and the controls at 28 or 90 days after the first administration of Ni_3S_2. However, the absolute lung weight and the relative lung weight were increased in the 1.0 mg/animal group at 28 days compared to the untreated control group. No significant difference was observed at 90 days (Table 2).

Mutant frequency (MF): A summary of the *gpt* MFs determined in the four participating laboratories is shown in Table 3 and Fig. 2. There were no significant differences between the *gpt* MFs of the treated rats and the vehicle controls, at 28 days or 90 days. Under the same experimental conditions for mutation assay, ENU showed a clear positive result. The MFs obtained by the four laboratories were similar with no statistical differences between them as shown in appendix A-D (available at http:www.j-ems.org/journal/). The *gpt* MFs of the vehicle control groups in the four laboratories were 1.63 ± 1.73, 2.54 ± 1.95, 1.87 ± 1.13 and $2.60 \pm 0.98 \times 10^{-6}$ at 28 days and 4.40 ± 0.88, 1.28 ± 0.94, 1.44 ± 1.00 and $2.70 \pm 1.08 \times 10^{-6}$ at 90 days, respectively.

The *gpt* MF in the livers of *gpt* delta rats treated with ENU was $62.88 \pm 18.92 \times 10^{-6}$, dramatically increased compared to the *gpt* MFs in the lung of the vehicle control (2.28 ± 0.66 or $2.50 \pm 0.46 \times 10^{-6}$), and the spontaneous *gpt* MF in the liver of F344 *gpt* delta rat previously reported (4.4×10^{-6}) (7). Again, there was no statistical difference in the *gpt* MFs reported by the four different laboratories: 43.90 ± 15.51, 70.05 ± 28.31, 61.30 ± 11.27 and $73.74 \pm 32.81 \times 10^{-6}$.

The Spi$^-$ MFs were determined in only one of the participating laboratories (Table 4). Similar to the *gpt* MF results, there were no significant differences between the Spi$^-$ MFs of the treated rats and the vehicle controls.

Table 2. Body weight and lung weight of male F344 *gpt* delta rats treated with Ni_3S_2

Group	Sampling time (days)	No. of animals	Body weight		Lung weight	
			at first treatment (g)	at dissection (g)	Absolute (g)	Relative (%)
0 mg/animal × 4	28	5	289 ± 8	286 ± 11	1.302 ± 0.052	0.46 ± 0.02
0.5 mg/animal × 4	28	5	291 ± 11	291 ± 12	1.327 ± 0.067	0.46 ± 0.03
1.0 mg/animal × 4	28	5	288 ± 11	287 ± 7	1.420 ± 0.076*	0.49 ± 0.02*
0 mg/animal × 4	90	5	283 ± 8	372 ± 5	1.247 ± 0.063	0.34 ± 0.02
0.5 mg/animal × 4	90	5	284 ± 11	372 ± 8	1.277 ± 0.081	0.34 ± 0.02
1.0 mg/aniaml × 4	90	5	288 ± 7	374 ± 6	1.309 ± 0.042	0.35 ± 0.01

*Significant difference compared with control group (Dunnett's test, $p < 0.05$).

Table 3. Summary of *gpt* mutant frequencies in the lung of *gpt* delta rats treated with Ni_3S_2 by intratracheal administration and liver treated with ENU (positive control)

Treatment	Smpling time	Animal No.	Organ	Number of packagings	Total Population	Number of mutants	Mutant frequency ($\times 10^{-6}$)	
								Mean ± SD
0 mg/animal × 4	28 days	1001	lung	17	1,704,000	3	1.76	
		1002	lung	14	2,907,000	8	2.75	
		1003	lung	14	4,026,000	10	2.48	
		1004	lung	14	3,693,000	11	2.98	
		1005	lung	14	4,200,000	6	1.43	2.28 ± 0.66
0.5 mg/animal × 4	28 days	1101	lung	14	2,669,500	6	2.25	
		1102	lung	14	3,577,500	5	1.40	
		1103	lung	13	2,625,000	11	4.19	
		1104	lung	18	3,445,500	5	1.45	
		1105	lung	10	2,851,500	6	2.10	2.28 ± 1.13
1 mg/animal × 4	28 days	1201	lung	13	3,142,500	8	2.55	
		1202	lung	15	3,687,000	4	1.08	
		1203	lung	13	3,823,500	7	1.83	
		1204	lung	15	4,333,500	6	1.38	
		1205	lung	15	6,930,000	13	1.88	1.74 ± 0.55
0 mg/animal × 4	90 days	1011	lung	11	4,149,000	11	2.65	
		1012	lung	11	3,348,000	8	2.39	
		1013	lung	11	5,779,500	17	2.94	
		1014	lung	9	4,371,000	12	2.75	
		1015	lung	13	5,701,500	10	1.75	2.50 ± 0.46
0.5 mg/animal × 4	90 days	1111	lung	10	3,832,500	6	1.57	
		1112	lung	11	2,611,500	3	1.15	
		1113	lung	10	5,424,000	6	1.11	
		1114	lung	13	2,565,000	9	3.51	
		1115	lung	12	7,806,000	5	0.64	1.59 ± 1.12
1 mg/animal × 4	90 days	1211	lung	7	3,405,000	8	2.35	
		1212	lung	7	4,500,000	9	2.00	
		1213	lung	8	3,385,500	10	2.95	
		1214	lung	5	3,940,500	9	2.28	
		1215	lung	7	4,006,500	7	1.75	2.27 ± 0.45
ENU	31 days	51	liver	13	3,336,450	282	84.52	
50 mg/kg × 5		52	liver	11	3,780,300	152	40.21	
		53	liver	11	3,252,900	179	55.03	
		54	liver	11	3,676,500	296	80.51	
		55	liver	11	3,140,550	170	54.13	62.88 ± 18.92†

†The *gpt* MF in the livers of *gpt* delta rats treated with ENU was markedly increased compared to the spontaneous *gpt* MF in F344 *gpt* delta rat livers of 4.4×10^{-6} previously reported (7).

The Spi^- MF for vehicle controls ($6.49 \pm 3.93 \times 10^{-6}$) was higher than the spontaneous Spi^- MF in the lung reported for the *gpt* delta mouse (2.8×10^{-6}) (7). The Spi^- MF in the livers of rats treated with ENU was $16.74 \pm 9.10 \times 10^{-6}$, in the present study, increased compared to the Spi^- MF in the lung of vehicle control and compared to the spontaneous Spi^- MF in F344 *gpt* delta rat liver of 2.8×10^{-6} previously reported (7).

Histopathology: For all the groups, histopathological changes due to the intratracheal instillation of vehicle and Ni_3S_2 were observed in the lung. Table 5 summarizes the histopathological findings of rats and their severity scores at each time point. At 28 days, a slight infiltration of inflammatory cells into the lung was observed in all animals including the negative control group. Eosinophils were found in the perivascular regions. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group and this incidence was significantly greater than that of the control group. Furthermore, focal fibrosis was observed in two animals in the 0.5 mg/animal treatment group and two animals in the 1.0 mg/animal treatment group. Slight to moderate infiltration of alveolar macrophages was found in three animals of 1.0 mg/animal treatment group. At 90 days, focal fibrosis was observed in only two animals, one in the 0.5 mg/animal group and the other in the 1.0 mg/animal group, and mild infiltration of inflammatory cells was seen in only a few rats in the 0.5 mg/animal and control groups. Overall, inflammation in the lung at 90 days was milder than at 28 days, indicating recovery from the intratracheal instillation procedure.