reliable and contributes to the predictive accuracy of liver tumor promoters (Ito et al., 2003; Tsuda et al., 2010). However, there have been no reports describing the expression of mutational enzymes corresponding to GST-P in renal cells, and therefore, no medium-term animal models to rapidly predict renal carcinogens have been developed. In addition, alternative *in vivo* studies to long-term bioassays are required to gain information about modes of action underlying carcinogenesis, including the participation of genotoxic mechanisms (Cohen and Arnold, 2011).

*In vivo* mutation assays using reporter gene transgenic rodents can be combined with additional assays to investigate modes of action underlying chemical carcinogenesis, such as the formation of DNA adducts, induction of cell proliferation, and occurrence of oxidative stress (Kuroda et al., 2013; Tasaki et al., 2013; Ishii et al., 2014). In the previous studies, we developed a medium-term *gpt* delta rat model (the GPG model) capable of rapidly detecting *in vivo* mutagenicity and tumor-promoting activity in the liver (Matsushita et al., 2013, 2014). In the GPG model, partial hepatectomy (PH) is performed to collect samples for an *in vivo* mutation assay. Because genotoxic compounds can effectively induce gene mutations under conditions giving rise to cell proliferation (Cohen and Arnold, 2011), treatment with diethylnitrosamine (DEN) for subsequent tumor-promoting analysis is conducted at 18 h after PH (Tsuda et al., 1980; Kobayashi et al., 1997). To apply this concept of the GPG model to a new assay for renal carcinogens (*i.e.*, the GNP model), unilateral nephrectomy (UN) was performed in place of PH. Although UN also induces compensatory cell proliferation in the residual kidney tissue (Mulroney et al., 1996; Mulroney and Pesce, 2000), detailed kinetics of cell proliferation, including sex differences, remain to be fully elucidated. In addition, although expression of specific proteins in renal cells as markers for preneoplastic lesions has not been found, as mentioned above, morphological alterations regarded as preneoplastic lesions, such as atypical tubules (ATs) and atypical tubular hyperplasia (AH), have been identified. Moreover, because about 90% of target sites of environmental renal carcinogens are renal tubules, these lesions could be suitable markers in the GNP model, corresponding to GST-P foci in the liver (Dietrich and Swenberg, 1991a; National Toxicology Program, 2014).

In the present study, the kinetics of cell proliferation in renal tubules of residual kidneys from male and female rats after UN were investigated to determine the optimal timing of DEN treatment. Subsequently, the optimal dose of DEN and optimal duration of exposure to test chemical were determined based on data describing the development incidences and/or multiplicities of AT and/or AH after treatment with DEN followed by trisodium nitrilotriacetic acid monohydrate (NTA-$H_2O$), a potent tumor promoter of renal carcinogenesis. Finally, the GNP model was validated using a genotoxic carcinogen, nongenotoxic carcinogens and a noncarcinogen.

## 2. Materials and methods

### 2.1. Chemicals

DEN was obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and NTA, potassium dibasic phosphate (PDP), and *d*-limonene (DL) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5-Bromo-2′-deoxyuridine (BrdU), aristolochic acid (AA), and phenylbutazone (PBZ) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Experimental animals and housing conditions

The protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five- or nine-week-old specific pathogen-free F344/NSlc rats or five-week-old specific pathogen-free F344/NSlc-Tg (*gpt* delta) rats carrying approximately 5 tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan) and acclimated for 1 week prior to testing. The rats were housed in polycarbonate cages (2–3 rats per cage) with hardwood chips for bedding in a conventional animal facility. Animals were maintained under controlled temperature (23 ± 2 °C), relative humidity (55 ± 5%), air changes (12 times/h), and lighting (12-h light–dark cycle) conditions with free access to a basal diet (CRF-1; Oriental Yeast Co., Ltd, Tokyo, Japan) and tap water. At the end of each experiment, the rats were euthanized by exsanguination *via* transection of the abdominal aorta under deep anesthesia.

### 2.3. Experiment I

To determine the optimal timing of DEN injection as a tumor initiator, the time course for cell proliferation in residual kidney tissues after the UN in male and female rats was investigated.

#### 2.3.1. Animal treatment

Three male and female 10-week-old F344 rats in each group were subjected to UN (left kidney) under deep anesthesia. Male rats were sacrificed at 6, 18, 24, or 48 h after UN, and female rats were sacrificed at 6, 18, 24, 48, or 72 h after UN. All rats were injected intraperitoneally (i.p.) with BrdU (100 mg/kg) 2 h before sacrifice. Residual right kidneys were fixed in ice-cold acetone and processed by embedding in paraffin, sectioning (4-μm), and immunostaining for BrdU after histochemical demonstration of γ-glutamyltranspeptidase (γ-GT) activity.

#### 2.3.2. Immunohistochemical staining for BrdU

For immunohistochemical staining of BrdU, sections were treated sequentially with normal goat serum, monoclonal mouse anti-BrdU (1:100 dilution; Becton Dickinson, Franklin Lakes, NJ, USA), and high polymer stain (HISTOFINE Simple Stain, Nichirei Bioscience Inc., Tokyo, Japan) after denaturation of DNA with 4*N* HCl. Before the denaturation step, sections were processed histochemically for demonstration of γ-GT activity based on previously reported methods (Rutenburg et al., 1969) using L-glutamyl-4-methoxy-β-naphthylamide (Polysciences, Ltd., Warrington, PA, USA) as a substrate in order to distinguish among the three types of tubules, as previously described (Umemura et al., 2004, 2009). The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride. At least 3000 tubule cells of the proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal tubule (DT) were counted for each kidney, and BrdU-labeling indices (LIs) were calculated as the percentages of cells positive for BrdU incorporation.

### 2.4. Experiment II

To determine the optimal dose of DEN and experimental period to detect renal preneoplastic lesions in the residual kidney, rats were treated with two different doses of DEN, followed by treatment with NTA as a tumor promoter.

#### 2.4.1. Animal treatment

The experimental design is described in Fig. 1. A total of 180 female 6-week-old F344 rats were administered NTA solution at a concentration of 0 or 1000 ppm in drinking water for 4 weeks ($n = 90$ per dose), and UN was then performed in all rats under deep anesthesia. The dose of NTA was determined based on a previous 2-year carcinogenicity study (Goyer et al., 1981). The rats treated with 0 ppm NTA were given distilled water (DW). At 48 h after UN, an i.p. injection of DEN was administered at doses of 20 and 40 mg/kg in

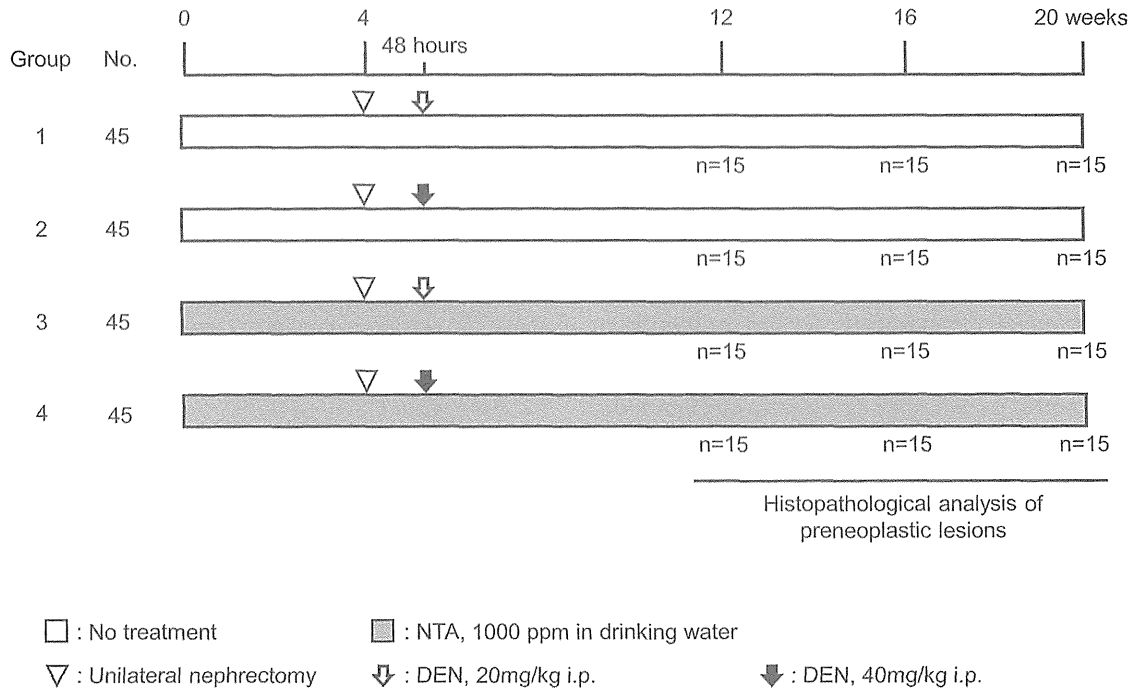


**Fig. 1.** Treatment protocol for experiment II. Animals were 6-week-old female F344 rats. Development of preneoplastic lesions was evaluated histopathologically in residual kidneys at 12, 16, and 20 weeks after the start of the experiment, *i.e.*, at 8, 12, and 16 weeks after the unilateral nephrectomy.

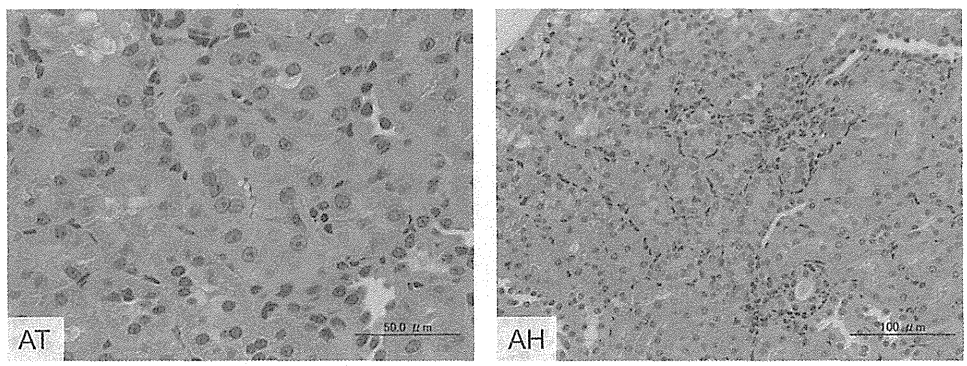


**Fig. 2.** Representative photographs of atypical tubules (ATs) and atypical hyperplasia (AH) in the kidney of rats. HE stain.

rats treated with 0 or 1000 ppm NTA, respectively. The rats continued to consume water containing NTA until they were sacrificed at 8, 12, or 16 weeks after UN ($n=15$ per time point). The residual right kidneys were fixed in 10% neutral-buffered formalin, and four pieces were taken from each kidney, routinely processed by embedding in paraffin, sectioning (4-μm), and H&E staining. Renal tubular lesions of AT and AH (Fig. 2) were diagnosed as preneoplastic lesions according to generally accepted guidelines (Dietrich and Swenberg, 1991a).

### 2.5. *Experiment III*

The animal model was validated using a genotoxic renal carcinogen, two nongenotoxic renal carcinogens, and a noncarcinogen.

#### 2.5.1. *Animal treatment*

Six-week-old F344/NSlc-Tg (*gpt* delta) rats ($n=15$ per dose) were fed 50,000 ppm PDP or 2500 ppm PBZ in their basal diets. The rats treated with AA received 1% sodium bicarbonate at a dose of 0.3 mg/kg body weight by gavage once a day. The rats given DL were administered 600 mg/kg in corn oil by gavage once a day. The doses of PDP and PBZ were determined based on previous reports in which these chemicals exerted tumor-promoting effects in rat kidneys (Hiasa et al., 1992; Maekawa et al., 1987). The dose of AA was selected based on a previous report in which the *gpt* mutant frequencies (MFs) were increased in rats treated with AA for 4 weeks (Kawamura et al., 2012). The dose of DL was based on a previous carcinogenicity test (National Toxicology Program, 1990a). A control group was fed the basal diet without chemical supplementation. After 4 weeks, test chemical treatment was interrupted in all animals. At 6 weeks, an i.p. injection of DEN at a dose of 40 mg/kg was administered, and UN was performed under deep anesthesia 48 h before DEN administration in all rats. The excised left kidney tissues were perfused with saline to remove residual blood and stored at −80 °C for the *gpt* assay. Test chemical exposure resumed at 7 weeks, and animals were sacrificed at 19 weeks. The experimental procedures and sample preparation after the end of the experiment were the same as for experiment II.

#### 2.5.2. *In vivo mutation assay*

6-Thioguanine (6-TG) was used according to previously described methods (Nohmi et al., 2000). Briefly, genomic DNA was

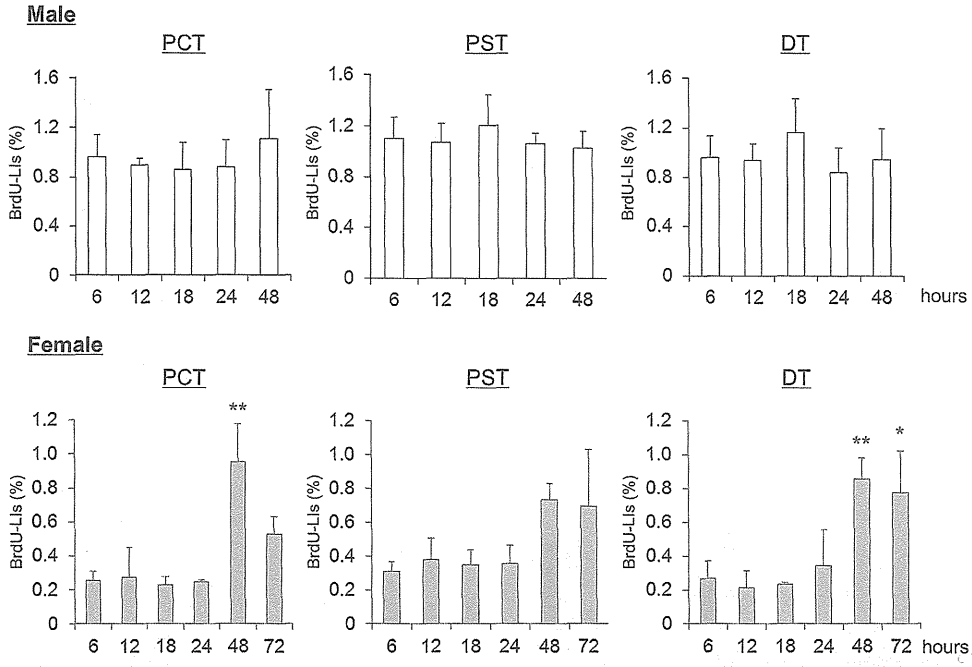


**Fig. 3.** BrdU-LIs in the proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal tubule (DL) of residual kidneys of male (white column) and female (gray column) F344 rats after unilateral nephrectomy ($n = 3$). Values are means ± SDs. *,** Significantly different from the 6-h group at $p < 0.05$ and $p < 0.01$, respectively.

extracted from each kidney, and lambda EG10 DNA (48 kb) was rescued in phages by *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying genes encoding *gpt* and chloramphenicol acetyltransferase. The infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the selection of 6-TG-resistant colonies. Positive colonies were counted on day 3 and collected on day 4. The *gpt* MFs were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

### 2.6. Statistical analysis

The data for BrdU-LIs, multiplicity of preneoplastic lesions in experiment III, and *gpt* MFs were analyzed with analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. The data for multiplicity of preneoplastic lesions in experiment II were analyzed with the Student–Welch test. The incidence of preneoplastic lesions was compared with Fisher's exact probability test.

## 3. Results

### 3.1. Experiment I

Fig. 3 illustrates changes in BrdU-LIs for each tubule type in residual kidney tissues after UN in male and female rats. In female rats, BrdU-LIs in the PCT, PST, and DT were highest at 48 h after UN, with significant changes in the PCT at 48 h and in the DT at 48 and 72 h. However, no obvious changes in BrdU-LIs were observed in all types of tubules in male rats.

### 3.2. Experiment II

Two rats from each of the groups of rats treated with 20 mg/kg DEN followed by 12 weeks of DW treatment or 40 mg/kg DEN followed by 16 weeks of DW treatment and one rat from each of the groups of rats treated with 40 mg/kg DEN followed by 8 weeks of NTA treatment, 20 mg/kg DEN followed by 12 weeks of NTA treatment, 40 mg/kg DEN followed by 12 weeks of NTA treatment, 20 mg/kg DEN followed by 16 weeks of DW treatment, or 20 mg/kg DEN followed by 16 weeks of NTA treatment died due to surgical complications during UN and were eliminated from further evaluation. The incidences and multiplicities of renal preneoplastic lesions in rats exposed to NTA and DEN are given in Fig. 4. DEN induced preneoplastic lesions in the kidneys during each experimental period, and more lesions were observed in rats treated with 40 mg/kg DEN than in rats treated with 20 mg/kg DEN. NTA treatment enhanced the formation of preneoplastic lesions for both doses of DEN in each experimental period. In rats treated with 40 mg/kg DEN followed by NTA treatment, the incidence and multiplicity of preneoplastic lesions at 12 weeks after UN and the multiplicity of preneoplastic lesions at 16 weeks after UN were significantly increased compared to those in rats treated with 40 mg/kg DEN alone.

### 3.3. Experiment III

One rat from each of the AA, PDP, PBZ, and DL treatment groups died due to surgical complications during UN and were eliminated from further evaluation. Table 1 shows the MFs in the excised kidneys of *gpt* delta rats treated with AA, PDP, PBZ, or DL for 4 weeks followed by a 2-week washout period. The MFs in rats exposed to AA were significantly increased compared with that in rats in the control group. There were no significant changes in MFs in the rats treated with PDP, PBZ, or DL. In the *gpt* mutation spectra, AT:TA transversions were significantly increased in rats treated with AA (Table 2). The results of histopathological analyses of preneoplastic lesions are illustrated in Fig. 5. The incidence of preneoplastic lesions was significantly increased in the kidneys of rats treated with AA and PDP, and the multiplicity of preneoplastic lesions was significantly increased in kidneys of rats treated with AA, PDP, and PBZ. No significant changes were observed in the rats exposed to DL. Representative photographs of the kidney of rats treated with PDP and DL were illustrated in Fig. 6. Calcium depositions were

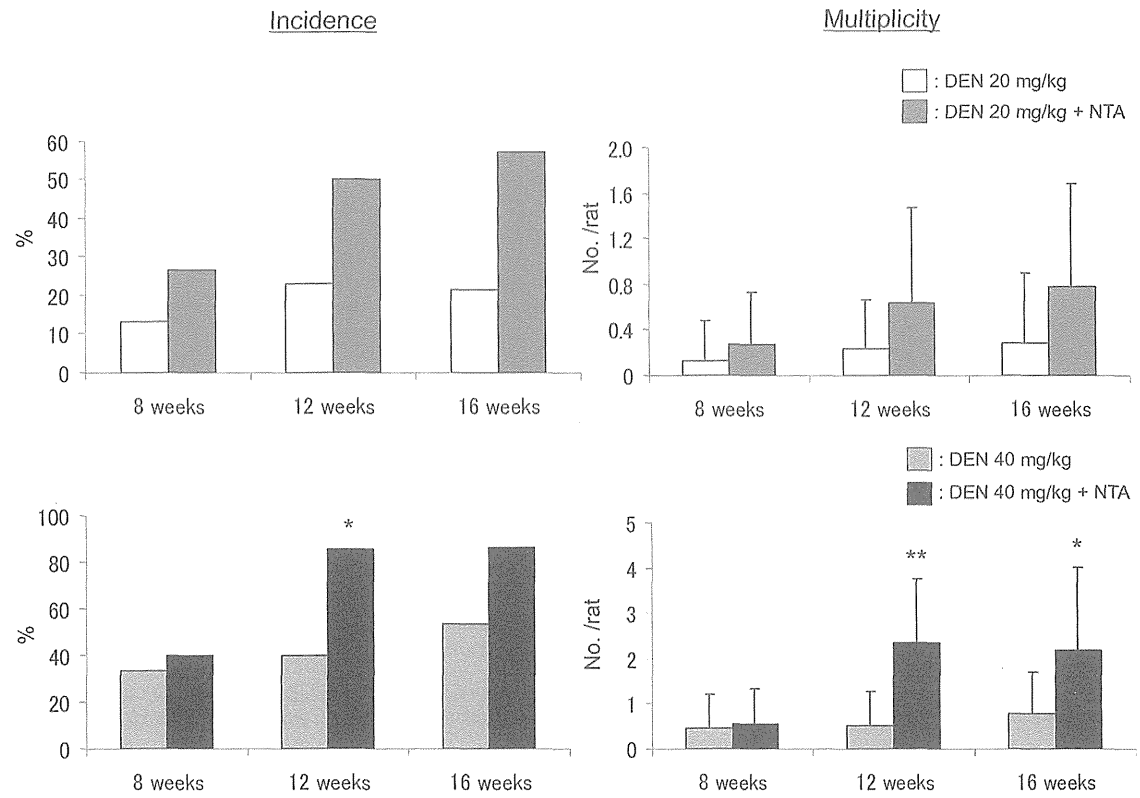


**Fig. 4.** Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with DEN and NTA ($n \approx 13$–15). Values of multiplicity are means ± SDs. The horizontal axes represent the treatment period after unilateral nephrectomy. *,** Significantly different from the DEN 40 mg/kg group at $p < 0.05$ and $p < 0.01$, respectively.

**Table 1**
*gpt* MFs in kidneys of F344 *gpt* delta rats treated with AA, PDP, PBZ and DL.

| Group | Animal no. | $Cm^R$ colonies ($\times 10^5$) | 6-$TG^R$ and $Cm^R$ colonies | MF ($\times 10^{-5}$) | Mean ± SD |
|---|---|---|---|---|---|
| Control | 101 | 3.7 | 2 | 0.54 | 0.43 ± 0.22 |
| | 102 | 10.0 | 3 | 0.30 | |
| | 103 | 8.9 | 2 | 0.22 | |
| | 104 | 9.5 | 3 | 0.32 | |
| | 105 | 7.9 | 6 | 0.76 | |
| AA | 201 | 3.7 | 4 | 1.07 | 1.20 ± 0.42** |
| | 202 | 6.9 | 5 | 0.73 | |
| | 203 | 5.5 | 10 | 1.81 | |
| | 204 | 6.2 | 6 | 0.97 | |
| | 205 | 6.3 | 9 | 1.42 | |
| PDP | 301 | 5.9 | 1 | 0.17 | 0.57 ± 0.32 |
| | 302 | 4.8 | 5 | 1.05 | |
| | 303 | 7.5 | 4 | 0.54 | |
| | 304 | 6.4 | 4 | 0.63 | |
| | 305 | 6.2 | 3 | 0.48 | |
| PBZ | 401 | 5.5 | 3 | 0.55 | 0.48 ± 0.32 |
| | 402 | 5.4 | 1 | 0.19 | |
| | 403 | 7.4 | 2 | 0.27 | |
| | 404 | 7.8 | 7 | 0.90 | |
| | 405 | 3.6 | 0[a] | – | |
| DL | 501 | 5.1 | 4 | 0.78 | 0.47 ± 0.29 |
| | 502 | 4.4 | 1 | 0.23 | |
| | 503 | 6.9 | 5 | 0.72 | |
| | 504 | 7.2 | 1 | 0.14 | |
| | 505 | 6.2 | 3 | 0.48 | |

** Significantly different from the control group at $p < 0.01$.
[a] No mutant colonies were detected on the plate, with this data being excluded from the calculation of MF.

**Table 2**
Mutation spectra of *gpt* mutant colonies in kidneys of F344 *gpt* delta rats treated with AA, PDP, PBZ and DL.

| | Control | | AA | | PDP | | PBZ | | DL | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) |
| Transversions | | | | | | | | | | |
| GC-TA | 1[a] (6.3) | 0.02 ± 0.05[b] | 3(8.8) | 0.11 ± 0.12 | 5(29.4) | 0.17 ± 0.14 | 2(11.8) | 0.06 ± 0.10 | 3(21.4) | 0.11 ± 0.17 |
| GC-CG | 2(12.5) | 0.05 ± 0.11 | 1(2.9) | 0.03 ± 0.07 | 2(11.8) | 0.08 ± 0.18 | 0 | 0 | 0 | 0 |
| AT-TA | 0 | 0 | 12(35.3) | 0.43 ± 0.39* | 0 | 0 | 1(5.9) | 0.02 ± 0.04 | 0 | 0 |
| AT-CG | 0 | 0 | 2(5.9) | 0.06 ± 0.14 | 0 | 0 | 0 | 0 | 2(14.3) | 0.07 ± 0.10 |
| Transitions | | | | | | | | | | |
| GC-AT | 11(68.8) | 0.31 ± 0.17 | 8(23.5) | 0.28 ± 0.08 | 6(35.3) | 0.18 ± 0.15 | 10(58.8) | 0.28 ± 0.13 | 5(35.7) | 0.17 ± 0.11 |
| AT-GC | 1(6.3) | 0.03 ± 0.06 | 4(11.8) | 0.13 ± 0.14 | 1(5.9) | 0.04 ± 0.08 | 1(5.9) | 0.02 ± 0.04 | 1(7.1) | 0.03 ± 0.07 |
| Deletion | | | | | | | | | | |
| Single bp | 1(6.3) | 0.02 ± 0.05 | 3(8.8) | 0.10 ± 0.14 | 3(17.6) | 0.09 ± 0.09 | 3(17.6) | 0.10 ± 0.10 | 3(21.4) | 0.09 ± 0.14 |
| Over 2 bp | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Insertion | 0 | 0 | 1(2.9) | 0.05 ± 0.12 | 0 | 0 | 0 | 0 | 0 | 0 |
| Complex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Number of colonies with independent mutations.
[b] Mean ± SDs.
* Significantly different from the control group at $p < 0.05$.

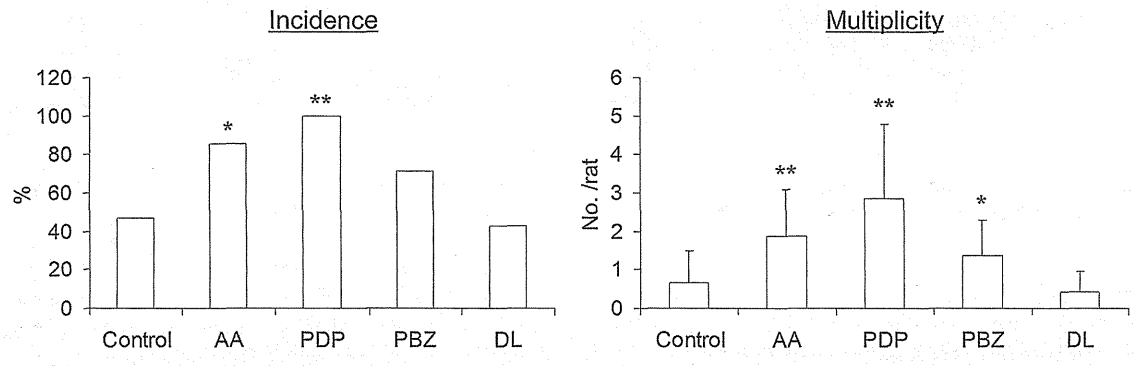


**Fig. 5.** Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with AA, PDP, PBZ, and DL ($n = 14–15$). Values of multiplicity are means ± SDs. *,**Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

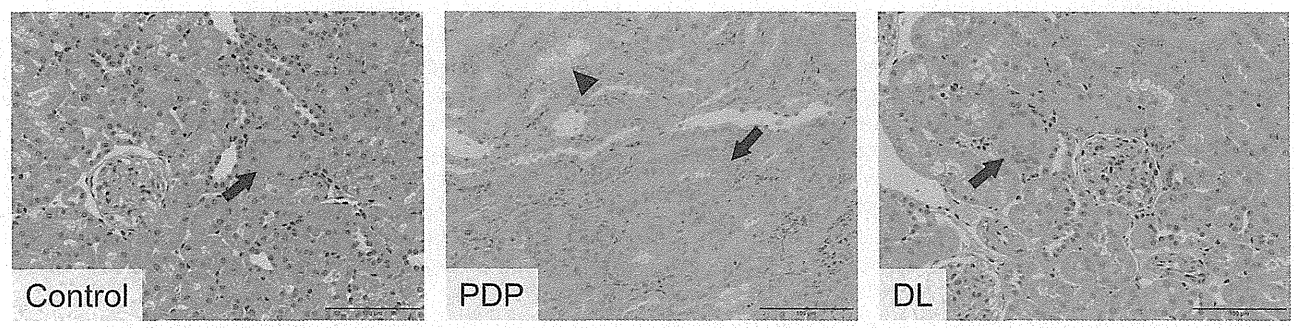


**Fig. 6.** Representative photographs of the residual kidneys of female F344 *gpt* delta rats treated with PDP and DL in the validation study. Calcium depositions were observed in the proximal tubules of rats treated with PDP (arrowhead). There were no hyaline droplets indicating accumulation of $\alpha_{2u}$-globulin in the proximal tubular epithelium in DL-treated rats. Arrows represent preneoplastic lesions in renal tubules. HE stain.

observed in the proximal tubules of rats treated with PDP. There were no hyaline droplets in the proximal tubular epithelium of rats treated with DL.

## 4. Discussion

Although the kidney is a major organ targeted by chemical carcinogenesis, reliable medium-term animal models for identification of renal carcinogens have not been established. In this study, we applied the concepts of the GPG model (Matsushita et al., 2013, 2014) to develop the GNP model, which was able to detect the *in vivo* mutagenicity and tumor-promoting activity of renal carcinogens. In the GPG model, *gpt* delta rats are subjected to PH to harvest samples for an *in vivo* mutation assay. Subsequently, DEN is administered at 18 h after PH to effectively initiate hepatocyte carcinogenesis for subsequent analysis of tumor-promoting effects by taking advantage of the induction of compensatory cell proliferation after PH. In the GNP model, UN is performed in place of PH. However, since the detailed kinetics of cell proliferation in the residual kidney tissue after UN remained unclear, clarifying the time to reach peak cell proliferation after UN was needed to determine the optimal time for initiation of renal cell carcinogenesis.

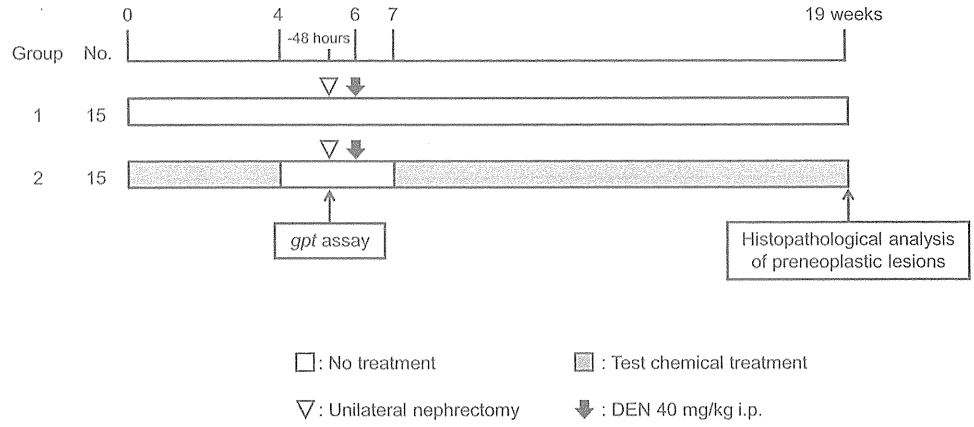


**Fig. 7.** Standard protocol for the GNP model. Animals were 6-week-old female F344 *gpt* delta rats. The *gpt* assay was performed in excised kidney samples as an indicator of *in vivo* mutagenicity. Tumor-promoting activities were evaluated based on the enhancement of preneoplastic lesions induced by DEN in residual kidney samples.

Thereafter, BrdU-LIs in three types of tubules located at the cortex and outer stripe of outer medulla in the residual kidney were examined following UN in male and female rats. Our data demonstrated that BrdU-LIs increased, reaching a peak at 48 h after UN in female rats, although there were no remarkable changes in male rats. Compensatory mechanisms in the residual kidney tissue following UN have been reported to involve sex differences, *i.e.*, hypertrophic responses are observed in male rats, while hyperplastic responses are observed in female rats (Mulroney et al., 1996; Mulroney and Pesce, 2000). Thus, our cell kinetic study revealed that female rats were suitable for use in the GNP model. Additionally, the use of female rats is expected to yield an additional advantage, *i.e.*, it will be possible to eliminate the interference of male rat-specific renal carcinogens, so-called $\alpha_{2u}$-globulin-mediated carcinogens, in terms of the risk of human cancer. Because DEN is capable of inducing renal tubular tumors as well as hepatocyte tumors (Nogueira, 1987; Athar and Iqbal, 1998; Umemura et al., 2000), we performed a study to investigate optimal timing of DEN administration to induce renal tumors. We found that female *gpt* delta rats should be administered DEN at 48 h after UN in the GNP model. No reports have identified a reliable marker for renal preneoplastic lesions, such as specific enzymes corresponding to GST-P in the liver. However, characteristic tubular lesions, such as ATs and AH, are known to appear early, prior to tumor formation. ATs are normal in size, but contain epithelial cells showing atypia. In contrast, AH consists of aggregations of proliferating atypical cells that are single- or multilayered. These lesions are believed to represent preneoplastic lesions of renal tubular cell tumor (Dietrich and Swenberg, 1991a).

For the second half of the GNP model protocol, we determined the optimal dose of DEN and duration of treatment with the renal cell tumor promoter. Two doses of DEN (20 and 40 mg/kg) were applied using the tentative standard protocol described above, and rats were then treated with NTA, a typical promoter of renal tubular cell tumors, for 8, 12, or 16 weeks in order to select optimal conditions for achieving the tumor-promoting effects of NTA. Since development of preneoplastic lesions was significantly enhanced in rats treated with 40 mg/kg DEN followed by NTA treatment for 12 and 16 weeks as compared to rats treated with DEN alone, the dose of DEN was chosen as 40 mg/kg, and the duration of test chemical treatment was chosen as 12 weeks. In addition, as in the GPG model (Matsushita et al., 2014), we added 2- and 1-week washout periods before and after DEN injection, respectively, to avoid interaction between DEN and the test chemical. From these studies, we developed the standard protocol for the GNP model as follows. Female *gpt* delta rats were treated with the test chemical for 4 weeks, followed by a 2-week washout period, and i.p. injection of DEN was subsequently performed at a dose of 40 mg/kg. UN was carried out 48 h before DEN administration, and the *gpt* assay was performed using excised kidney samples. At 1 week after DEN administration, test chemical treatment was resumed. The incidences and/or multiplicities of preneoplastic lesions were evaluated in residual kidney samples at 12 weeks after resuming test chemical treatment (Fig. 7).

To validate the GNP model established above, we applied 1 genotoxic renal carcinogen, 2 nongenotoxic renal carcinogens, and 1 noncarcinogen to the model. The genotoxic renal carcinogen AA was reported to produce AA-specific DNA adducts, and 7-(deoxyadenosine-$N^6$-yl) aristolactum I (AAI-dA) was found to induce the most persistent DNA adducts *in vivo* (Mengs et al., 1982; Mei et al., 2006). In the present study, we observed a significant increase in the MFs of *gpt* in rats treated with AA, and frequencies of AT:TA transversion mutations were predominantly elevated in spectrum analysis. Accordingly, it is highly probable that AA-specific deoxyadenine adducts may be responsible for AA-induced gene mutations (Mei et al., 2006; Xing et al., 2012). Ishii et al. (2014) demonstrated that *gpt* delta rats could be powerful tools not only for examination of *in vivo* genotoxicity but also for investigation of the relationship between DNA base modifications and gene mutations. Thus, in the GNP model, it is possible to measure chemical-specific DNA modifications using excised or residual kidney samples, which could be helpful for further understanding the causes of chemical-induced gene mutations. In addition, the GNP model showed that AA was capable of exerting tumor-promoting effects on the kidney. This was inconsistent with the negative results of AA in the liver using the GPG model (Matsushita et al., 2014), and this difference may be explained by the observation that the carcinogenic effects of AA target the kidney (Mengs et al., 1982).

PDP has been reported to exert its tumor-promoting effects in the kidneys of rats (Hiasa et al., 1992; Konishi et al., 1995). Additionally, PBZ has carcinogenic effects on the rat kidney and is classified as a nongenotoxic carcinogen based on negative results in various *in vivo* genotoxicity tests (National Toxicology Program, 1990b; Kari et al., 1995; Machemer and Hess, 1971; Müller and Strasser, 1971; Rathenberg and Müller, 1972; Gebhart and Wissmüller, 1973; Charles and Leonard, 1978). In the GNP model, while exposure to neither PDP nor PBZ elevated the MFs of *gpt* delta rats, significant enhancements in the frequencies of preneoplastic lesions were observed in both treatment groups. In addition, calcium deposition was detected in the proximal tubules of rats treated with PDP, and this was considered the mechanism through which PDP exerted its tumor-promoting effects. DL has been reported to act as a renal carcinogen in male rats only through a mechanism mediated by $\alpha_{2u}$-globulin (Dietrich and Swenberg, 1991b). Treatment

**Table 3**
Summary: GNP model validation study.

| Test chemical | *gpt* assay | Histopathological analysis of preneoplastic lesions | Classification |
|---|---|---|---|
| AA | + | + | Genotoxic carcinogen |
| PDP | – | + | Nongenotoxic |
| PBZ | – | + | carcinogen |
| DL | – | – | Noncarcinogen |

with DL did not increase the MFs of *gpt* delta rats and did not affect the development of preneoplastic lesions in the kidney, in line with the observation that DL is not carcinogenic in female rats. Moreover, there were no hyaline droplets indicating accumulation of $\alpha_{2u}$-globulin in the proximal tubular epithelium. $\alpha_{2u}$-Globulin-mediated renal carcinogenesis is not thought to be relevant in humans (Hard, 1998; Doi et al., 2007). However, these false-positive results in terms of human risk assessment can be avoided in the GNP model using female rats. Overall, our validation study demonstrated that the GNP model could be a valid tool to detect renal carcinogens and provide a variety of results and insights regarding the mechanisms underlying carcinogenesis (Table 3).

In conclusion, we have established a new medium-term *gpt* delta rat model for predicting chemicals with renal carcinogenicity; we termed this model the GNP model. Based on the results of our validation studies, we propose that the GNP model may represent a reliable system for analysis of chemical renal carcinogenicity and the underlying mode of action.

## Conflict of interest

The authors declare that there are no conflicts of interest. K. M. is an employee of Otsuka Pharmaceutical Co., Ltd.

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# Improvement and validation of a medium-term *gpt* delta rat model for predicting chemical carcinogenicity and underlying mode of action

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ABSTRACT

We have developed a new medium-term animal model, "GPG", in which an *in vivo* mutation assay in partially hepatectomized tissue and a tumor-promoting assay were performed. The tumor-promoting assay measures glutathione *S*-transferase placental form positive foci induced by diethylnitrosamine (DEN) in the residual tissue. Given that a limitation of the original protocol is the potential interaction between the test chemical and DEN, the present study establishes a modified protocol that includes a test chemical washout period. Using CYP2E1 inhibitor and CYP1A or CYP2B inducers, a period of 2 weeks after cessation of exposure to the chemicals was confirmed to be sufficient to return their enzymatic activities to normal levels. Additionally, to avoid the effects of DEN on the pharmacokinetics of the test chemical, re-exposure to the test chemical started 1 week after DEN injection, in which tumor-promoting activities were clearly detected. Consequently, a modified protocol has been established with 2- and 1-week washout periods before and after DEN injection, respectively. The applicability of the modified protocol was demonstrated using the genotoxic hepatocarcinogen, estragole (ES), the genotoxic renal carcinogen, aristolochic acid (AA), and the non-genotoxic hepatocarcinogens, β-naphthoflavone and barbital. Furthermore, the increase of cell cycle-related parameters in ES-treated livers, but not in AA-treated livers, may indicate that the liver is not the carcinogenic target site of AA despite its genotoxic role. Thus, since various parameters related to carcinogenesis can be evaluated concurrently, the GPG model could be a rapid and reliable assay for the assessment of human cancer hazards.



## 1. Introduction

A key consideration in terms of safety assessments for environmental chemicals is to detect their carcinogenicity. Lifetime bioassays in rodents have been conducted to assess chemical carcinogenicity, but this method requires long time periods and a large number of animals. The International Conference on Harmonization (ICH) guideline recommends lifetime bioassays using rats and an additional medium-term *in vivo* study in place of the lifetime bioassay using two species of rodents requested by earlier guidelines (ICH, 1997). In fact, as alternative *in vivo* carcinogenicity studies, the rat medium-term animal model, *i.e.*, the Ito model, or 6-month carcinogenicity models using transgenic mice such as rasH2 and *p53*-deficient mice are proposed (ICH, 1997). In particular, Ito model using the preneoplastic marker glutathione *S*-transferase placental form (GST-P) foci is highly reliable *in vivo* assay to predict liver carcinogen (Ito et al., 2003; Tsuda et al., 2010). However, neither bioassay provides information regarding the involvement of genotoxic mechanisms in carcinogenesis.

We have noted that *in vivo* mutation assays using reporter gene transgenic rodents can be combined with additional assays to investigate modes of action underlying carcinogenesis, such as measurements of DNA adducts, oxidative stress and cell proliferative activities (Ishii et al., 2014; Tasaki et al., 2013; Kuroda et al., 2013). We then attempted to develop a new medium-term animal model using *gpt* delta rats capable of rapidly detecting chemical

*Abbreviations:* PH, partial hepatectomy; GST-P, glutathione *S*-transferase placental form; CYP, cytochrome P450; i.p., intraperitoneal; DEN, diethylnitrosamine; DADS, diallyl disulfide; PBO, piperonyl butoxide; PHE, phenytoin; ES, estragole; AA, aristolochic acid; BNF, β-naphthoflavone; BT, barbital.

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carcinogenicity, *in vivo* mutagenicity, and the underlying modes of action. In our previous study (Matsushita et al., 2013), we confirmed the potential for development of a new animal model, in which partial hepatectomy (PH) was performed in *gpt* delta rats followed by a *gpt* assay using the excised liver samples for evaluation of *in vivo* mutagenicity. Quantitative analysis of GST-P positive foci was examined following diethylnitrosamine (DEN) treatment using residual liver samples for evaluation of tumor-promoting activity. The positive results of *gpt* assay in rats exposed to several genotoxic carcinogens indicated that the excised liver sample from *gpt* delta rats treated with the test chemicals for 4 weeks is able to be used for *in vivo* mutation assay. In addition, the positive results of GST-P quantitative analysis in *gpt* delta rats treated with several tumor-promoters implied that the residual liver sample after PH is able to be used for GST-P quantitative analysis. However, since the test chemical and DEN are simultaneously administrated in the original protocol, the interaction of the two compounds should be avoided. In fact, several isoforms of cytochrome P450 (CYP) affect metabolic activation of DEN (Verna et al., 1996), and many liver tumor-promoters in rodents were reported to induce several types of CYPs and/or modify the expression of phase II enzymes (Graham and Lake, 2008; Muguruma et al., 2007; Wieneke et al., 2009).

In the present study, a washout period for the test chemical was added to the original protocol. To confirm elimination of the effects of test chemical, the relevant CYPs activities induced by diallyl disulfide (DADS), piperonyl butoxide (PBO) or phenytoin (PHE) were measured with or without the washout period. In addition, the effect of setup of the washout period on the ability to detect tumor promotion activity was verified in the modified protocol. Then, a genotoxic hepatocarcinogen, a genotoxic non-hepatocarcinogen as well as non-genotoxic hepatocarcinogens having inducible potency for CYPs were applied to validate the modified protocol.

## 2. Materials and methods

### 2.1. Reagents

DEN, estragole (ES) and β-naphthoflavone (BNF) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). PBO, PHE and barbital (BT) were obtained from Wako Pure Chemical Industry (Osaka, Japan), and DADS and aristolochic acid (AA) were from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Experimental animals and housing conditions

The protocol was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Five-week-old specific pathogen-free F344/NSlc rats or F344/NSlc-Tg (*gpt* delta) rats carrying approximately 5 tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan) and acclimated for 1 week prior to testing. Animals were maintained under controlled temperature (23 ± 2 °C), relative humidity (55 ± 5%), air changes (12 times/h), and lighting (12 h light–dark cycle) conditions with free access to a basal diet (Charles River Formula (CRF)-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. At the end of each experiment, the rats were euthanized by exsanguination *via* transection of the abdominal aorta under deep anesthesia.

### 2.3. Animal treatments

#### 2.3.1. Experiment I

Elimination of the test chemical effects on the metabolic parameters and sufficiency of sensitivity for detecting tumor-promoting activity were confirmed (Fig. 1). Six-week-old male F344/NSlc rats ($n = 24$) were treated with DADS at a dose of 50 mg/kg body weight in corn oil by gavage once a day. The rats ($n = 12$ rats per dose) were fed 12,000 ppm PBO or 2400 ppm PHE in their basal diets. A control group did not receive the test chemical treatment ($n = 12$). The doses were selected based on a previous report (Le Bon et al., 2003; Takahashi et al., 1994; National Toxicology Program, 1993). After 4 weeks, test chemical treatment was interrupted in the PBO or PHE treated group, and half the number of rats was treated with DADS ($n = 12$). The other half of rats given DADS ($n = 12$) had been treated with the test chemical throughout the experiment. At 6 weeks, an i.p. injection of DEN at a dose of 10 mg/kg was administered, and PH was performed at 18 h before DEN administration in all rats. The excised liver samples were perfused with saline to remove residual blood and stored at −80 °C for the measurement of enzymatic activity of CYP2E1, CYP1A2 and CYP2B1 in rats given DADS, PBO and PHE, respectively. CYP1A2 or CYP2B1 activities were also evaluated in rats given PBO or PHE in the original protocol by using excised liver samples obtained from previous study (Matsushita et al., 2013). Test chemical exposure resumed at 7 weeks, and at 13 weeks, animals were sacrificed and the residual liver samples fixed in 10% neutral-buffered formalin. The fixed tissues were evaluated using immunohistochemistry for the detection of GST-P.

#### 2.3.2. Experiment II

The modified protocol was validated. Six-week-old male F344/NSlc-Tg (*gpt* delta) rats ($n = 15$ per dose) were fed 5000 ppm BNF, or 2500 ppm BT in their basal diets. The rats treated with ES received 150 mg/kg body weight in corn oil by gavage once a day. The rats treated with AA received 0.3 mg/kg body weight in 1% sodium bicarbonate by gavage once a day. A control group did not receive the test chemical treatment. The doses of ES, AA and BNF were based upon previous reports (Suzuki et al., 2012; Shimada et al., 2010; Kawamura et al., 2012). The BT dose was based on a preliminary study in which no toxic effects were observed (data not shown). After 4 weeks, test chemical treatment was interrupted in all animals. At 6 weeks, an i.p. injection of DEN at a dose of 10 mg/kg was administered, and PH was performed at 18 h before DEN administration in all rats. The excised liver tissues were perfused with saline to remove residual blood and stored at −80 °C for the *gpt* assay. Test chemical exposure resumed at 7 weeks. At 13 weeks, animals were sacrificed and a portion of the residual liver samples was fixed in 10% neutral-buffered formalin. The fixed tissues were evaluated using immunohistochemistry for the detection of GST-P in all animals and proliferating cell nuclear antigen (PCNA) in rats given ES or AA. The remaining residual liver samples of rats treated with ES or AA were stored at −80 °C for quantitative PCR.

### 2.4. Preparation of microsomes

Livers were homogenized with a Teflon homogenizer and the resulting homogenate was centrifuged for 10 min at 10,000 × *g*, 4 °C. The supernatant was re-centrifuged at 105,000 × *g*, 4 °C for 1 h to obtain microsomal fractions. Protein concentrations were determined with the Advance Protein Assay Reagent (Cytoskelton Ltd., Denver, CO, USA).

### 2.5. Enzyme assays

CYP2E1 activity was measured by aniline hydroxylase activity assay based on modification of the method described by Imai et al. (1966), which detects the formation of *p*-aminophenol by colorimetric assay at 630 nm. Methoxyresofurin-O-dealkylase activity was assessed as CYP1A2 activity according to the method previously described (Umemura et al., 2006). The formation of resorufine was measured fluorometrically using excitation at 530 nm and emission at 585 nm. CYP2B1 activity was measured by

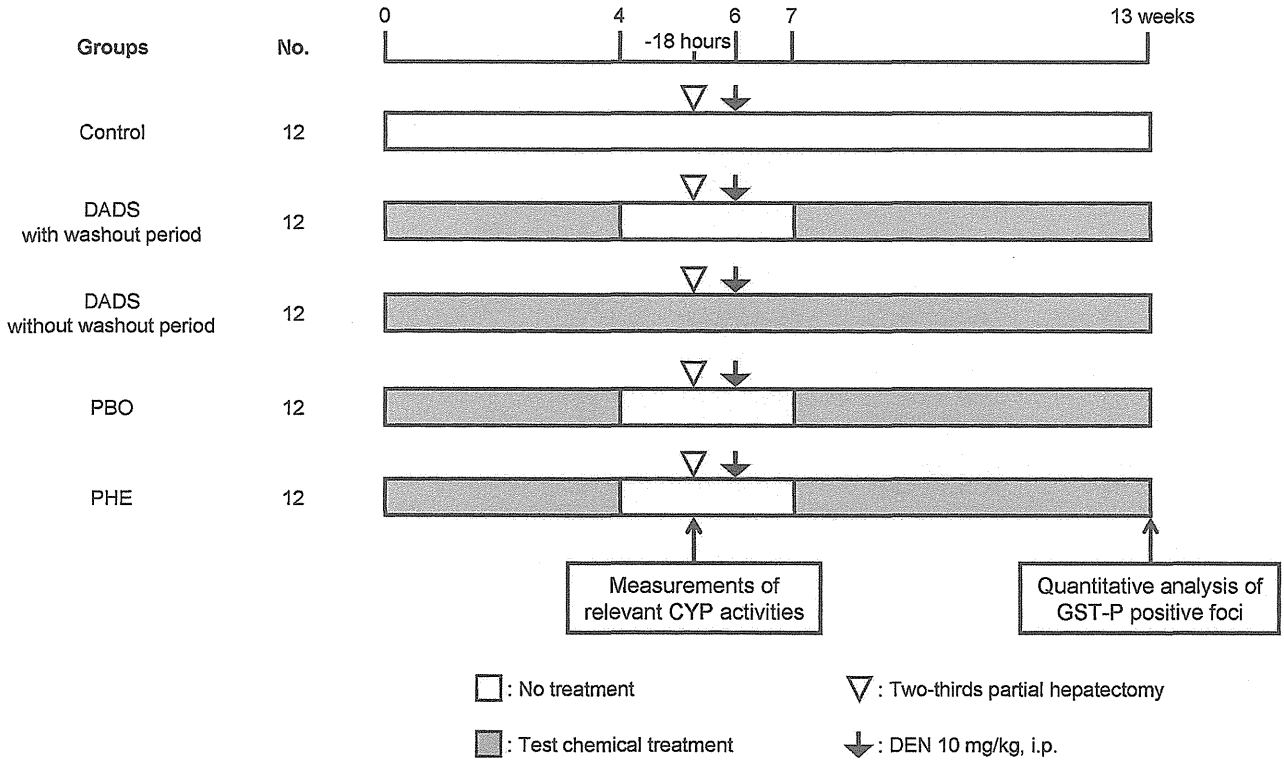


**Fig. 1.** Treatment protocol for experiment I. Animals were 6-week-old male F344 rats. Diallyl disulfide (DADS): 50 mg/kg body weight by gavage once a day. Piperonylbutoxide (PBO): 12,000 ppm in diet. Phenytoin (PHE): 2400 ppm in diet. CYP2E1, CYP1A2 and CYP2B1 activities were evaluated in excised livers of rats treated with DADS, PBO and PHE, respectively. Development of glutathione *S*-transferase placental form (GST-P) positive foci was evaluated in residual livers of all rats at week 13.

testosterone 16β-hydroxylation activity assay according to modification of the method described by Imaoka et al. (1989). The formation of 16β-hydroxytestosterone was analyzed by high performance liquid chromatography at 240 nm.

### 2.6. *In vivo mutation assay*

6-Thioguanine (6-TG) was used according to the method described in Nohmi et al. (2000). Briefly, genomic DNA was extracted from each liver, and the lambda EG10 DNA (48 kb) was rescued in phages by *in vitro* packaging. For 6-TG selection, the packaged phages were incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to plasmids carrying genes encoding *gpt* and chloramphenicol acetyltransferase. The infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. To determine the total number of rescued plasmids, the infected cells were poured on plates containing chloramphenicol without 6-TG. The plates were incubated at 37 °C for the selection of 6-TG-resistant colonies. Positive colonies were counted on day 3 and collected on day 4. The *gpt* mutant frequencies (MFs) were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

### 2.7. *Immunohistochemical staining for GST-P and PCNA*

Immunohistochemical staining was performed using rabbit polyclonal antibodies against GST-P (1:1000 dilution; Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) and mouse monoclonal antibodies against PCNA (PC10, 1:100; Dako Denmark A/S, Glostrup, Denmark). The number and area of GST-P positive foci consisting of five or more nucleated hepatocytes in a cross-section were evaluated using an image analyzer (IPAP, Sumika Technoservice, Hyogo, Japan) (Watanabe et al., 1994). At least 2000 intact hepatocytes in the liver per animal treated with ES or AA were counted; labeling indices (LIs) were calculated as the percentages of cells staining positive for PCNA.

### 2.8. *Quantitative real-time PCR for mRNA expression*

Total RNA was extracted from residual liver samples using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. cDNA copies of total RNA were obtained using a High Capacity cDNA Reverse Transcription kit (Life Technologies). All PCR reactions were performed with primers for rat *Ccna2, Ccnb1, Ccne1, E2f1* and TaqMan® Rodent GAPDH Control Reagents as an endogenous reference in the Applied Biosystems 7900HT FAST Real-Time PCR Systems. TaqMan® Fast Universal PCR Master Mix and TaqMan® Gene Expression Assays (Life technologies) were used. The expression levels of the target gene were calculated using the relative standard curve method and were determined as ratios to GADPH levels.

### 2.9. *Statistics*

The data for the number and area of GST-P positive foci, CYP 2E1 enzymatic activity, *gpt* MFs, PCNA-LIs and mRNA expression were analyzed with ANOVA, followed by Dunnett's multiple comparison test. Data for CYP1A2 and CYP2B1 enzymatic activity were analyzed by assessing the variance for homogeneity using the *F*-test. Student's *t*-test and Welch's *t*-test were used for the homogeneous and heterogeneous data, respectively.

## 3. Results

### 3.1. *Survival condition of animals*

Three rats given DADS without a washout period in experiment I, three rats given ES, and one rat given AA, BNF and BT in

**Table 1**
Quantitative analysis of GST-P positive foci.

| | No. of rats | No. of foci (No./cm$^2$) | Area of foci (mm$^2$/cm$^2$) |
|---|---|---|---|
| Experiment I | | | |
| Control | 12 | 15.27 ± 3.36[a] | 0.166 ± 0.043 |
| DADS with washout period | 12 | 12.58 ± 1.72 | 0.118 ± 0.021 |
| DADS without washout period | 9 | 2.75 ± 1.91** | 0.025 ± 0.017** |
| PBO | 12 | 24.94 ± 7.23** | 0.356 ± 0.133** |
| PHE | 12 | 38.15 ± 6.96** | 0.515 ± 0.113** |
| Experiment II | | | |
| Control | 15 | 24.96 ± 8.74 | 0.461 ± 0.248 |
| ES | 12 | 103.62 ± 20.79** | 16.310 ± 8.391** |
| AA | 14 | 23.33 ± 7.37 | 0.395 ± 0.190 |
| BNF | 14 | 40.83 ± 13.30* | 0.922 ± 0.422 |
| BT | 14 | 51.20 ± 15.09** | 1.227 ± 0.484 |

[a] Mean ± SD.
*,** Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

experiment II died due to surgical complications of PH and were eliminated from further evaluation.

### 3.2. Enzymatic assay

Fig. 2 illustrates changes in CYP2E1, CYP1A2 and CYP2B1 activities in rats given DADS, PBO and PHE, respectively. Whereas significant depression of CYP2E1 activity was observed in rats given DADS without a washout period, there were no significant changes in rats given DADS with a washout period. In the original protocol, significant elevation of CYP1A2 and CYP2B1 activity was observed in rats given PBO and PHE, respectively. On the other hand, there were no significant changes in the experimental results when applying the modified protocol.

### 3.3. GST-P analysis

Table 1, Figs. 3 and 4 show the results of the quantitative analysis of GST-P positive foci. In experiment I, whereas the number and area of GST-P positive foci were significantly decreased in rats given DADS without a washout period, no significant changes were observed in rats given DADS with a washout period. The number and area of GST-P positive foci were significantly increased in the rats treated with PBO and PHE. In experiment II, the number and area of GST-P positive foci were significantly increased in rats given ES, and the number of GST-P positive foci was increased significantly upon treatment with BNF or BT. Although there were no statistically significant differences, the area of GST-P positive foci in the rats treated with BNF or BT were clearly increased in comparison to control rats. There were no remarkable changes in the rats treated with AA.

### 3.4. In vivo mutation assay

Table 2 presents the MFs in the excised livers of *gpt* delta rats treated with ES, AA, BNF or BT. The MFs in the rats given ES or AA were significantly elevated and no significant changes were observed in BNF or BT treatment groups. In the *gpt* mutation spectra, AT:CG transversions and AT:GC transitions increased significantly in the rats treated with ES, and AT:TA transversions increased significantly in the rats treated with AA (Table 3).

**Table 2**
*gpt* MFs in livers of F344 *gpt* delta rats treated with ES, BNF, BT and AA.

| Group | Animal no. | Cm$^R$ colonies ($\times 10^5$) | 6-TG$^R$ and Cm$^R$ colonies | MF ($\times 10^{-5}$) | Mean ± SD |
|---|---|---|---|---|---|
| Control | 101 | 5.0 | 1 | 0.20 | 0.71 ± 0.37 |
| | 102 | 4.1 | 3 | 0.73 | |
| | 103 | 3.7 | 4 | 1.07 | |
| | 104 | 7.9 | 4 | 0.51 | |
| | 105 | 4.8 | 5 | 1.05 | |
| ES | 201 | 4.4 | 11 | 2.52 | 3.04 ± 1.00** |
| | 202 | 4.1 | 17 | 4.15 | |
| | 203 | 4.8 | 18 | 3.77 | |
| | 204 | 4.5 | 14 | 3.14 | |
| | 205 | 3.1 | 5 | 1.63 | |
| AA | 301 | 4.2 | 7 | 1.65 | 2.34 ± 0.65** |
| | 302 | 3.3 | 6 | 1.83 | |
| | 303 | 7.7 | 17 | 2.21 | |
| | 304 | 4.5 | 14 | 3.14 | |
| | 305 | 4.6 | 13 | 2.86 | |
| BNF | 401 | 6.4 | 3 | 0.47 | 0.75 ± 0.53 |
| | 402 | 5.4 | 2 | 0.37 | |
| | 403 | 3.2 | 5 | 1.59 | |
| | 404 | 5.2 | 5 | 0.97 | |
| | 405 | 5.3 | 2 | 0.38 | |
| BT | 501 | 4.4 | 9 | 2.06 | 1.22 ± 0.65 |
| | 502 | 4.4 | 2 | 0.46 | |
| | 503 | 5.8 | 4 | 0.69 | |
| | 504 | 4.5 | 7 | 1.56 | |
| | 505 | 3.8 | 5 | 1.32 | |

** Significantly different from the control group at $P < 0.01$.

### 3.5. PCNA analysis and quantitative real time RT-PCR

Whereas the PCNA-LIs were significantly increased in rats treated with ES, no remarkable changes were observed in rats treated with AA (Figs. 5A and 6). As shown in Fig. 5B, expression levels of *Ccna2*, *Ccnb1*, *Ccne1* and *E2f1* mRNA increased significantly in rats given ES. In comparison, expression levels of these genes did not change in the AA group.

## 4. Discussion

In the previous study, we attempted to develop a new medium-term animal model, GPG, capable of detecting *in vivo* mutagenicity and tumor-promoting activity (Matsushita et al., 2013). In the original protocol, administration of DEN is performed in the course of treatment with the test chemical, which may result in their interaction. With test chemicals having the potential to affect DEN metabolism, an incorrect conclusion about the property of the chemical could be reached. Since induction of drug metabolic enzymes by xenobiotics is an adaptive response, it is generally considered to be reversible (Maronpot et al., 2010). Therefore, it is highly probable that introduction of the optimal washout period into the protocol is effective for avoiding the interaction. In addition, given that gene mutation induced by exposure to genotoxic carcinogen is irreversible event (Cohen and Arnold, 2011), the effects of washout period on the outcome in the following *in vivo* mutation assay are probably negligible. As a matter of fact, assessment of the mutagenic potential of an environmental chemical was performed using the sample collected 2 weeks after the last treatment (Wu et al., 2012).

DADS, a naturally occurring organosulfur compound, is well known as an inhibitor of CYP2E1 (Siess et al., 1997), which activates DEN to generate its electrophilic form (Verna et al., 1996). In fact, it has been reported that co-administration of DADS and DEN significantly reduced formation of GST-P foci induced by DEN (Haber-Mignard et al., 1996). Likewise, in the present

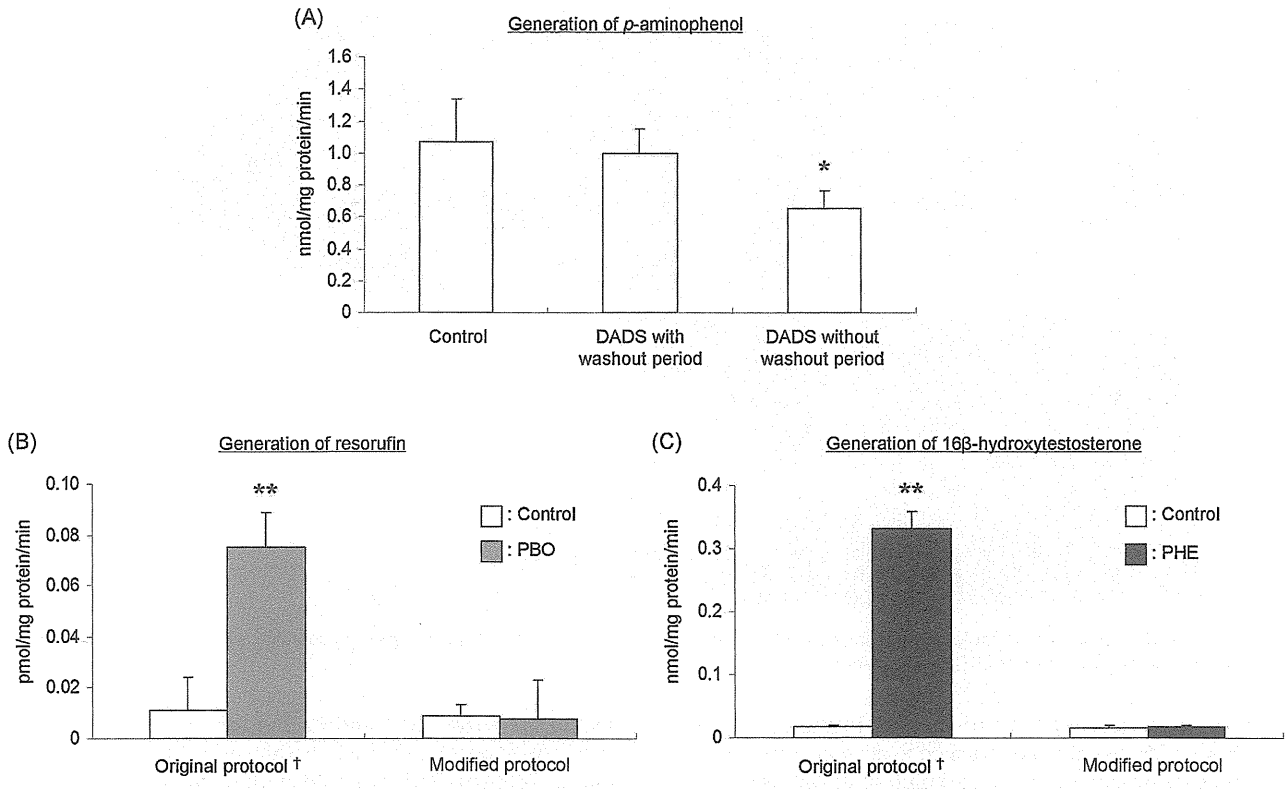


**Fig. 2.** Changes in CYP2E1 activity in excised livers of rats given diallyl disulfide (DADS) with or without a washout period (A). Changes of CYP1A2 (B) and CYP2B1 (C) activities in excised livers of rats given piperonylbutoxide (PBO) and phenytoin (PHE), respectively, in original and modified protocol. The values are means ± SD of data for five rats. †Samples were obtained from a previous validation study (Matsushita et al., 2013). *, **Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

study, treatment with DADS concurrently with DEN administration significantly diminished the number and area of GST-P foci induced by DEN. However, as mentioned above, the decreased level of CYP2E1 following DADS treatment returns to normal within a certain period of time. The present data demonstrate that CYP2E1 activity impaired by 4-week exposure to DADS was almost recovered about 2 weeks after treatment cessation. In line with this result, quantitative data on formation of GST-P foci in rats given DEN and DADS with discontinuous administration was almost identical to that in rats given DEN alone. This is also consistent with the previous report that DADS did not promote formation of GST-P foci in Ito's model (Fukushima et al., 1997). PBO and PHE are liver tumor promoters capable of inducing CYP1A2 and 2B1, respectively, both of which also contribute to metabolic activation of DEN (Tasaki et al., 2010; Muguruma et al., 2007; Nims et al., 1994; Ito et al., 1988; Beltrán-Ramírez et al., 2008). The present data clearly

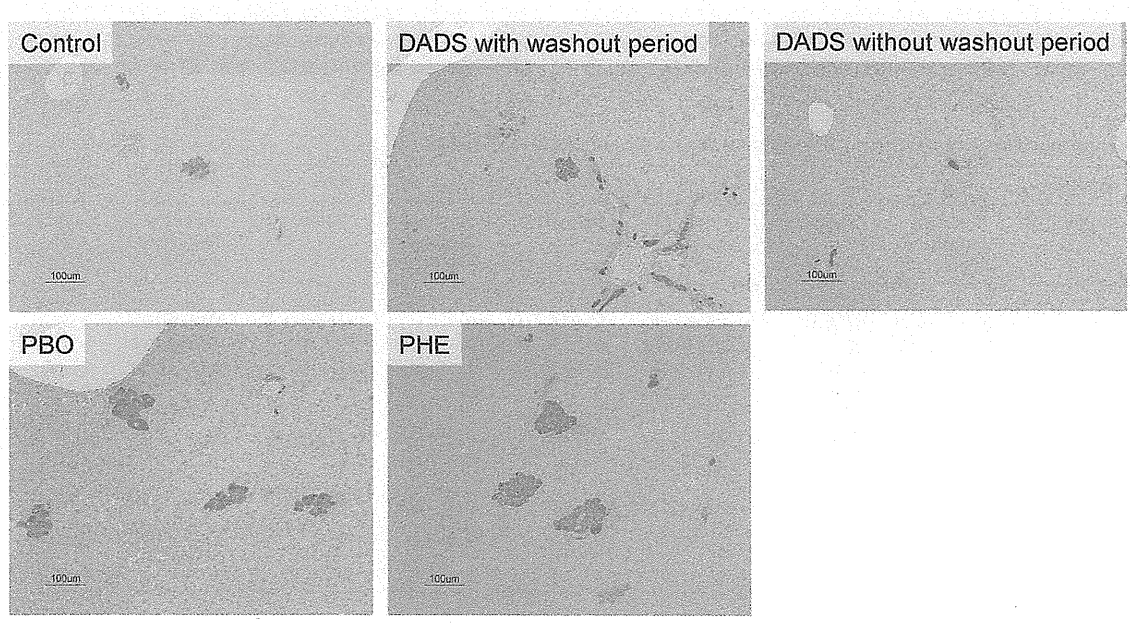


**Fig. 3.** Representative photographs of glutathione *S*-transferase (GST-P) immunohistochemistry in the residual livers of rats treated with diallyl disulfide (DADS) with or without a washout period, piperonyl butoxide (PBO) and phenytoin (PHE) in experiment I.

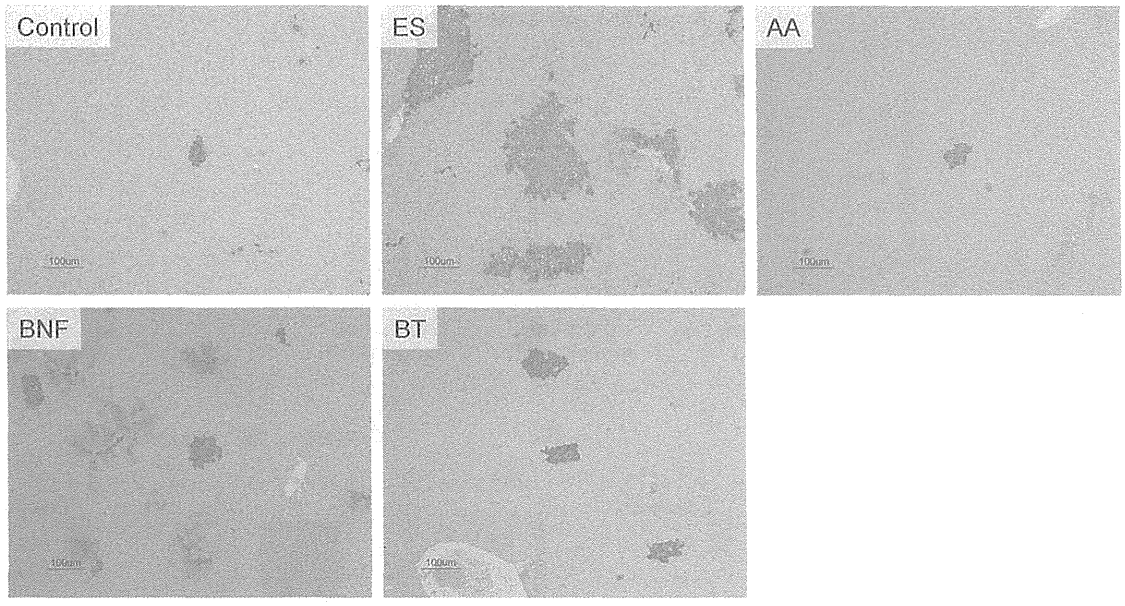


**Fig. 4.** Representative photographs of glutathione *S*-transferase (GST-P) immunohistochemistry in the residual livers of rats treated with estragole (ES), aristolochic acid (AA), β-naphthoflavone (BNF) and barbital (BT) in experiment II.

showed that the two kinds of CYP activity were increased after PBO or PHE exposure for 4 weeks and returned to normal levels 2 weeks after stopping treatment. On the other hand, since DEN is reported to disappear from the body 1 week after a single i.p. administration in rats (Phillips et al., 1975), a 1-week washout period after DEN administration was determined to be enough time to clear the effects of DEN. In fact, re-administration of either chemical 1 week after DEN treatment promoted the pre-neoplastic lesion induced by DEN. From the overall data, we established a modified protocol using the GPG model as follows (Fig. 7): *gpt* delta rats are treated with the test chemical for 4 weeks followed by a 2-week washout period, and DEN is subsequently administered. PH is performed 18 h before DEN administration, and the *gpt* assay is performed using excised liver samples. At 1 week after DEN administration, chemical treatment is resumed. The development of GST-P positive foci is evaluated in residual liver samples at week 13.

The modified GPG model was validated by various types of carcinogens, including the genotoxic hepatocarcinogen ES, the genotoxic renal carcinogen AA, and the non-genotoxic hepatocarcinogens BNF and BT, inducing CYPs1A and 2B, respectively. As expected, ES and AA showed positive in the *gpt* assay, and ES, BNF and BT revealed significant increases in the number of GST-P positive foci. Among these data, we note particularly that AA induced a significant increase in the MF of *gpt* even though it is known that the liver is not a target site of AA. In an attempt to understand this outcome, we compared the data in the GPG model between ES and AA. The present spectrum analysis for *gpt* mutants induced by ES demonstrated that incidences of AT:CG transversion and AT:GC transition increased significantly, in line with the previous report (Suzuki et al., 2012). It is likely that this results from the predominance of the ES-specific adenine adduct (Ishii et al., 2011). Likewise, in concert with the majority of AA-specific adenine adduct (Mei et al., 2006), AT:TA transversions were predominantly found in *gpt* mutants induced by AA in the present study. This phenomenon was also observed in the AA-treated kidney, its carcinogenic target site (Mei et al., 2006). Thus, in terms of the mechanism underlying the genotoxicity, there were no differences between the livers treated with ES or AA, and the kidney treated

**Table 3**
Mutation spectra of *gpt* mutant colonies in livers of F344 *gpt* delta rats treated with ES, BNF, BT and AA.

| | Control | | ES | | AA | | BNF | | BT | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) | Number (%) | Mutation frequency ($10^{-5}$) |
| Transversions | | | | | | | | | | |
| GC-TA | 8[a] (47.1) | 0.33 ± 0.24[b] | 13 (20.0) | 0.60 ± 0.33 | 6 (10.5) | 0.23 ± 0.28 | 4 (23.5) | 0.15 ± 0.21 | 9 (33.3) | 0.40 ± 0.19 |
| GC-CG | 1 (5.9) | 0.05 ± 0.12 | 5 (7.7) | 0.23 ± 0.23 | 0 | 0 | 1 (5.9) | 0.06 ± 0.14 | 2 (7.4) | 0.09 ± 0.12 |
| AT-TA | 0 | 0 | 4 (6.2) | 0.18 ± 0.19 | 34 (59.7) | 1.39 ± 0.13** | 1 (5.9) | 0.04 ± 0.09 | 1 (3.7) | 0.05 ± 0.10 |
| AT-CG | 0 | 0 | 3 (4.6) | 0.16 ± 0.15* | 0 | 0 | 0 | 0 | 0 | 0 |
| Transitions | | | | | | | | | | |
| GC-AT | 6 (35.3) | 0.26 ± 0.16 | 17 (26.2) | 0.80 ± 0.42 | 7 (12.3) | 0.29 ± 0.24 | 6 (35.3) | 0.29 ± 0.39 | 10 (37.0) | 0.46 ± 0.30 |
| AT-GC | 0 | 0 | 20 (30.8) | 0.93 ± 0.39** | 2 (3.5) | 0.11 ± 0.15 | 0 | 0 | 1 (3.7) | 0.03 ± 0.08 |
| Deletion | | | | | | | | | | |
| Single bp | 2 (11.8) | 0.07 ± 0.10 | 3 (4.6) | 0.14 ± 0.22 | 8 (14.0) | 0.32 ± 0.57 | 2 (11.8) | 0.07 ± 0.10 | 4 (14.8) | 0.19 ± 0.19 |
| Over 2bp | 0 | 0 | 0 | 0 | 0 | 0 | 1 (5.9) | 0.04 ± 0.08 | 0 | 0 |
| Insertion | 0 | 0 | 0 | 0 | 0 | 0 | 2 (11.8) | 0.10 ± 0.15 | 0 | 0 |
| Complex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Number of colonies with independent mutations.
[b] Mean ± SD.
* Significantly different from the control group at $P < 0.05$.
** Significantly different from the control group at $P < 0.01$.

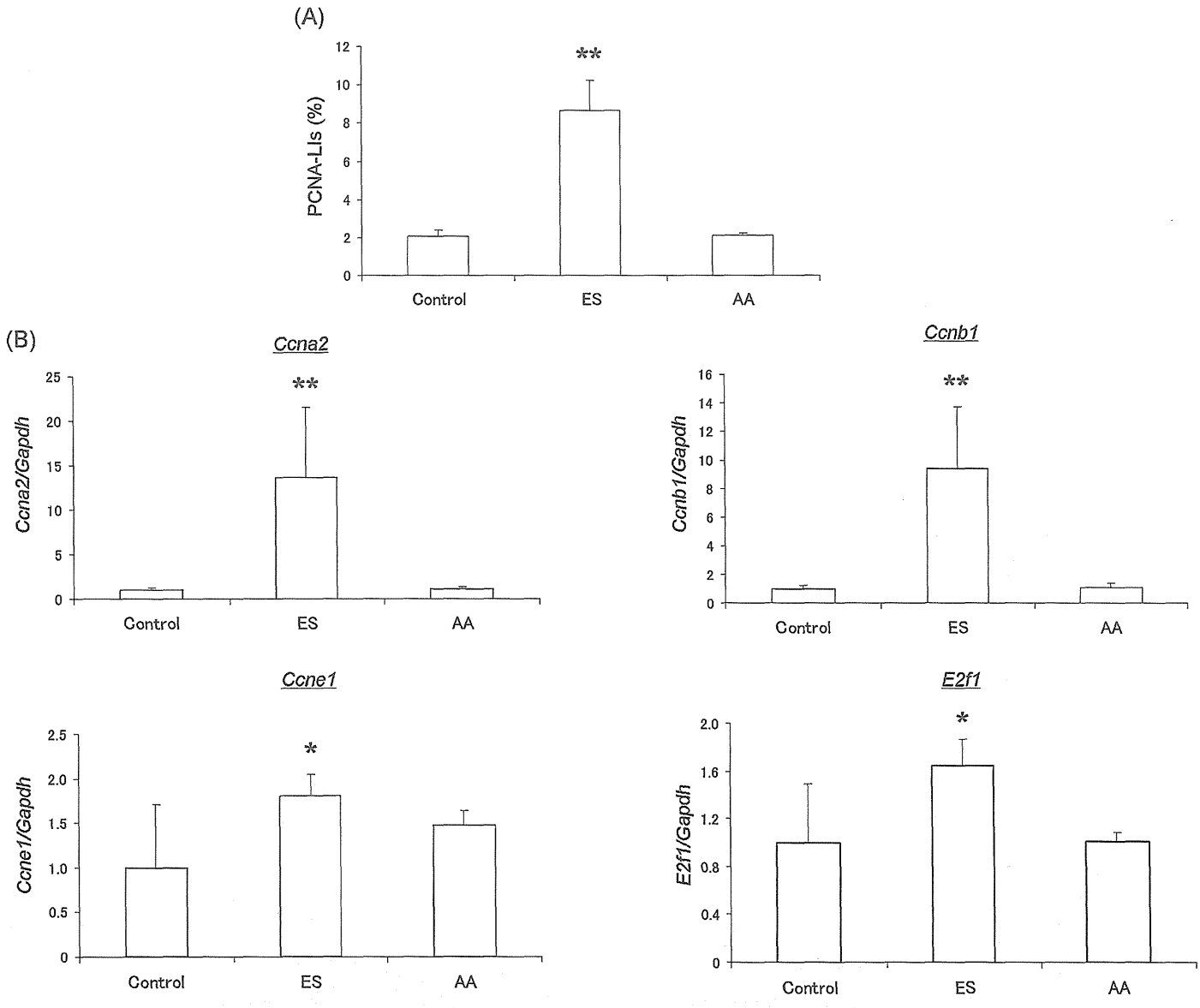


**Fig. 5.** Proliferating cell nuclear antigen-labeling indices (PCNA-LIs) for hepatocytes (A) and changes in mRNA levels of cell-cycle related factors (B) in the residual livers of *gpt* delta rats treated with estragole (ES) or aristolochic acid (AA). Values are means ± SD of data for five rats. *,**Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

with AA. In light of the global gene analysis data showing that the expression levels of cell cycle-related genes in the kidney of rats treated with AA was higher than that in the liver (Chen et al., 2006), mRNA expression levels of more concrete genes, such as *Ccna1*, *Ccnb1*, *Ccne1* and its transcriptional factor *E2f1*, were measured in the residual livers of rats treated with ES or AA. The results show that the mRNA levels of these genes do not increase in the liver of rats given AA and there is no increase in PCNA-positive hepatocytes, in contrast to results for ES-treated liver. It has been thought that cell proliferation may be a prerequisite to transform cells with mutation to tumor cells (Cohen and Arnold, 2011). In fact, exposure to AA followed by a regimen of a liver tumor promoter in the liver

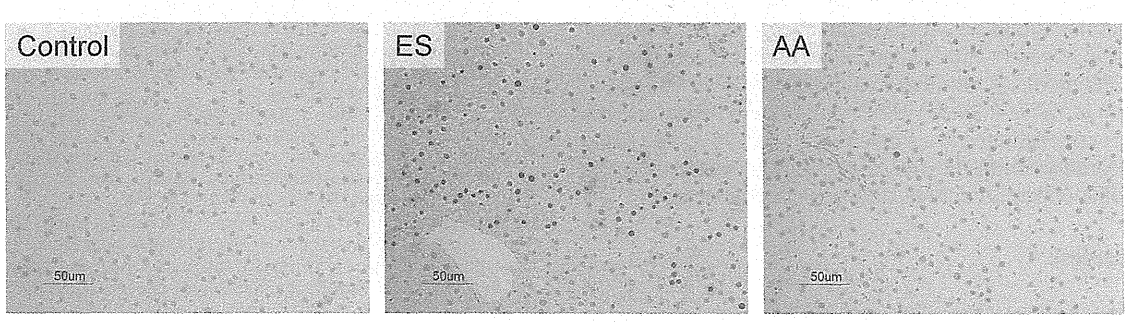


**Fig. 6.** Representative photographs of proliferating cell nuclear antigen (PCNA) immunohistochemistry in the residual livers of *gpt* delta rats treated with estragole (ES) or aristolochic acid (AA).

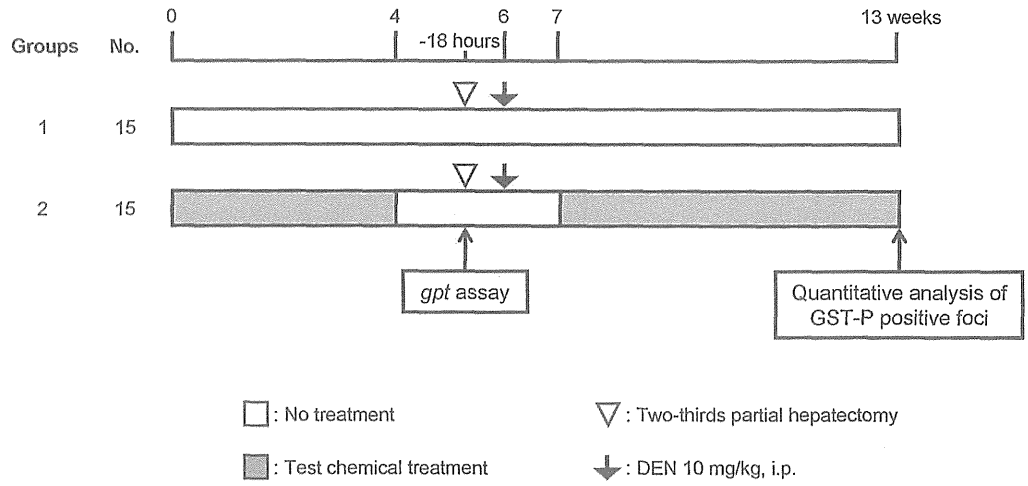


**Fig. 7.** Standard protocol for the GPG model. Animals were 6-week-old male F344 *gpt* delta rats. The *gpt* assay is performed in excised liver samples as indicator of *in vivo* mutagenicity. Tumor-promoting activities are evaluated based on the enhancement of glutathione *S*-transferase placental form (GST-P) positive foci induced by diethylnitrosamine (DEN) in residual liver samples.

**Table 4**
Summary: GPG model validation study.

| Test chemical | *gpt* assay | Quantitative analysis of GST-P positive foci | Classification |
|---|---|---|---|
| ES | + | + | Genotoxic carcinogen |
| 2-AAF[a] | + | + | |
| IQ[a] | + | + | |
| SF[a] | + | + | |
| BNF | – | + | Non-genotoxic carcinogen |
| BT | – | + | |
| PBO[a] | – | + | |
| PHE[a] | – | + | |
| AA | + | – | Potential carcinogen |
| APAP[a] | – | – | Non-carcinogen |

2-AAF: 2-acetylaminofluorene, IQ: 2-amino-3-methylimidazo[4,5-*f*] quinolone, SF: safrole, APAP: acetaminophen.

[a] These results were obtained in the previous validation study (Matsushita et al., 2013).

of rats resulted in significant elevation of GST-P foci (Rossiello et al., 1993). Lack of tumor-promoting activity of AA in the liver was reflected in the results of quantitative analysis for GST-P foci in the GPG model. The overall data indicate that the excised and residual liver samples in the GPG model are useful for investigation of the modes of action underlying carcinogenesis as well as for analysis of reporter gene mutations and GST-P positive foci. Consequently, as in the case of AA, analysis using the GPG model may contribute to the new classification of environmental chemical carcinogens.

In conclusion, we have established a new medium-term animal model, the GPG model. A summary of the GPG model validation study is shown in Table 4. Since various parameters related to carcinogenesis can be evaluated in a single study, the GPG model could be used in accordance with the 3Rs principle of animal testing and provide valuable information regarding human risk hazards.

## Conflict of Interest

The authors declare that there are no conflicts of interest, although K. Matsushita is an employee of Otsuka Pharmaceutical Co. Ltd.

## Acknowledgments

We appreciate the expert technical assistance of Ms. Ayako Kaneko and Ms. Yoshimi Komatsu. This work was supported by a Grand-in-Aid for Research on Food Sanitation from the Ministry of Health, Labor and Welfare of Japan (H-24-shokuhin-ippan-012).

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# 安全性に関するトピックの動向
# S10：光安全性の評価[*2]

中江 大[*1]

## 1. ICH S10 専門家作業部会 (EWG) (Table 1)

現在のEWGメンバーには，6団体の他にEFTAとWSMIが入っています．その他に，韓国や中国のオブザーバーも参加しました．

## 2. 厚生労働科学研究班

日本国内では，S10のサポートチームという意味もあり，厚生労働科学研究の一環としての研究班(Table 2)があります．Table 2に示すメンバーのうち，*が付いている方々は，EWGのメンバーでもあります．

なお，本稿のTable及びFigは，研究協力者であるJPMAの細井氏及び岩瀬氏が作成したものを参照あるいは引用していることをお断りしておきます．

## 3. ICH S10 ガイドラインの目的

S10ガイドラインの目的は，光安全性評価の国際標準を推奨し，医薬品の臨床試験及び市販承認のために必要な評価方法の統一を進めることです．

また，光安全性試験の開始に関する判断基準などは，ICH M3 (R2) ガイドライン[1]の第14章「光安全性試験」の記載内容を補完する，あるいは追加すべき事項を内容とします．

更に，どのガイドラインにも当てはまることですが，光安全性評価のための *in vitro* 代替法の使用や臨床情報の利用に配慮し，いわゆる「3R原則」に従って動物使用の削減に寄与するものです．

## 4. 留意すべきターム

ICH S10ガイドラインの策定を始めてから現在に至るまで，2種四つのタームが重要視されています．一つは，「評価 (assessment)」と「試験 (testing)」で，EWGにおいて明確に区別しています．すなわち，評価とは，必ずしも実際の試験を必要としない，包括的な概念であります．

もう一つは，「tiered approach」と「integrated approach (又はweight-of-evidence (WoE) approach)」といった二つの考え方です．これは，段階的にアプローチするものと，総合的な判断をするという別の考え方です (後述)．

## 5. Tiered Approach と Integrated Approach

Tiered approachは，第1段階で光反応性に関する評価を行い，第2段階で光毒性に関する評価を行います．第1段階で陰性であれば，第2段階は実施しなくてもよく，また，2段階のいずれかで陰性であれば，フェーズIIIの臨床試験で光毒性に関する予防措置や製品表示を考慮しなくてよいと予想する考え方です．

当初はtiered approachに沿ってガイドライン文書を作成していましたが，途中から変更し，総合的な考え方をするintegrated approachを採用しました．これは，試験法のオプションについて，それぞれの利点と限界を提示し，必要がない限り，特定のオプションを推奨しない立場を取るものです．したがって，医薬品開発製造者 (スポンサー) が試験法のオプションを自ら選択し，その結果を基に規制当局 (DRA) との折衝により，ケースバイケースの対応をすることとしました．

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[*2] 当財団主催の第28回ICH即時報告会 (平成25年7月26日：東京) における講演による．

Table 1　ICH S10専門家作業部会 (EWG)

| | |
|---|---|
| EU: | Ulla Wändel Liminga |
| EFPIA: | Phil Wilcox, Daniel M. Bauer |
| MHLW: | Dai Nakae, Hiroshi Onodera, Osamu Fueki, Shin-ichi Sekizawa |
| JPMA: | Kazuhiro Hosoi, Kazuichi Nakamura, Yumiko Iwase |
| FDA: | Abigail (Abby) Jacobs, Paul C. Brown |
| PhRMA: | Lewis Kinter, Roderick Todd Bunch |
| EFTA (Observer): | Claudine Faller |
| WSMI (Interested Party): | Olaf Kelber |

Table 2　厚生労働科学研究班

医薬品・医療機器等レギュラトリーサイエンス総合研究事業：医薬品の品質，有効性及び安全性確保のための規制の国際調和の推進に係わる研究（H24-医薬-指定-026）
「光毒性試験に関する研究」分班
- 研究分担者
  中江 大*（東京都健康安全研究センター）
- 研究協力者
  小野寺 博志*（MHLW，PMDA）
  笛木 修*（MHLW，PMDA）
  関澤 信一*（MHLW，PMDA）
  細井 一弘*（JPMA，参天製薬株式会社）
  中村 和市*（JPMA，塩野義製薬株式会社）
  岩瀬 裕美子*（JPMA，田辺三菱製薬株式会社）
  白菊 敏之（JPMA，大塚製薬株式会社）
  小島 肇（国立医薬品食品衛生研究所）
  尾上 誠良（静岡県立大学）
  田中 憲穂（食品薬品安全センター/鳥取大学）

*ICH S10 IWGメンバー

## 6. ICH S10ガイドラインの特徴

現在，ICH S10ガイドラインを作成中です．

特徴として，一つ目は，全身及び経皮適用薬にフォーカスしました．二つ目は，従来型のtiered approachでなく，スポンサーの判断でWoEに基づいて光安全性リスクを評価します．前述したように，assessmentという表現は，必ずしも試験を実施することを意味しません．また，光安全性の評価が必要になった場合は，*in vitro*試験，*in vivo*試験，臨床試験のいずれかで判断します．非臨床試験は，偽陰性が少なく，かつ適切と考えられる試験系を使用します．

最終的には，個々のケースに従って規制当局との間で検討します．つまり，スポンサーは，各試験の重みあるいは申請する地域を考慮してリスクアセスメントを行うことが重要です．以上が，現在作っているガイドラインの特徴です．

Fig. 1に示すように，*in vitro*試験より*in vivo*試験，*in vivo*試験より臨床試験が，当然インパクトが大きくなります．原則いずれかの試験で陰性であれば懸念はありませんが，例えば*in vitro*試験が陽性であっても，*in vivo*や臨床試験が陰性であれば懸念はありません．また，経皮適用薬の場合，米国では非臨床試験の位置づけが極めて低いという事情がありますが，あくまでも総合的な判断で評価を行います．

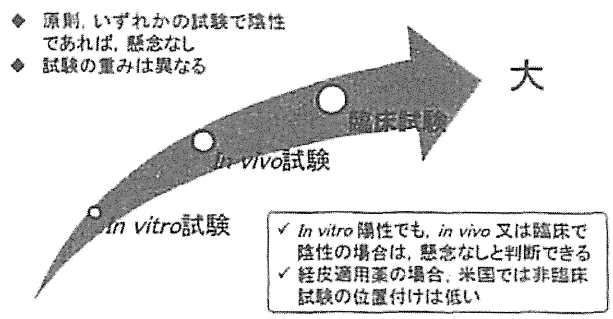


Fig. 1　試験結果のインパクト

## 7. ICHブリュッセル会議

2012年11月に行われたICHサンディエゴ会議で，ICH S10ガイドラインはステップ2に到達し，その後，3極においてパブリックコメントの募集が行われました．

2013年3月末までに3極でのコメント募集が終了し，それらを156件に集約しました．これらのパブリックコメントの対応方針について，先行した打ち合わせも含めてICHブリュッセル会議で議論し，決定しました．すべてのコメントについて対応方針を決定しましたが，それらには，現在あるいは今後のステップ4文書作成時に自動的に対応がなされるものも含まれます．

ICHブリュッセル会議の際に最も問題になった，つまり多くのコメントがあったのは，地域間差，フローチャート，ROSアッセイのバリデーション，眼局所投与医薬品に関するものです．

### 7.1 Step2文書における地域間差 (Table 3)

Step2文書にはいくつかの地域間差がありました．例えばROSアッセイの適用について，日本は非常に推進している立場であり，EUも賛成していますが，アメリカはどちらかというと否定的でした．

また，いわゆる組織分布データの利用について，アメリカでは基準が不明確ですが非常に重視しているのに対し，日本とEUはそれほどでもありません．

*In vitro*の光毒性試験について，日本とアメリカは必ずしも必要ではないとしていますが，EUは必要との態度を表明しました．

経皮適用薬について，臨床の光安全性試験に関して，日

Table 3　Step 2文書における地域間差

| 適用 | 項目 | 日本 | 米 | EU |
|---|---|---|---|---|
| 共通 | ROSアッセイの適用 | 可 | 全身：否定的<br>経皮：不可 | 可（バリデーション結果から最終判断） |
| 全身 | 組織分布データの利用 | 要相談 | 可（基準は不明確） | 要相談 |
| | *In vitro*光毒性試験の先行実施 | 不要 | | 必要 |
| 経皮 | 臨床光安全性評価 | 非臨床で陰性の場合は不要 | 非臨床で陰性の場合でも必要 | 非臨床で陰性の場合は不要 |
| | 光アレルギー性評価（懸念がある場合） | 臨床モニタリング | 臨床モニタリング又は特化した臨床試験 | |
| 眼 | 光毒性評価 | 適切な試験系がないため非臨床試験は不要 | | 何らかのリスクアセスメントは必要 |

本とEUは非臨床で陰性であれば，特に必要としないとの立場であったのに対し，アメリカは必要との立場を崩しませんでした．

懸念がある場合の光アレルギー性評価について，日本は臨床モニタリングで対応することに対し，アメリカとEUは臨床モニタリングでも対応しますが，できれば特化した臨床試験を行うとの方針です．

眼に関して，光毒性試験自体について，日本とアメリカはそもそも適切な試験系がないので非臨床試験が不要で，あまり意味がないとの立場であったのに対し，EUは何らかのリスクアセスメントが必要としています．

以上のような地域間差は，ステップ2文書の段階で明記されていました．

### 7.2　ICHブリュッセル会議における成果（Table 4）

地域間差については，3極ともに多くのコメントが集まりました．それらを検討し，対応した結果，ROSアッセイについて，アメリカは，現在も判断を保留していますが，一応追加データを要求し，その追加データを見て判断するところまで歩み寄っています．

組織分布データの利用に関しては，これを可とし，ケースバイケースで判断するということで妥協されています．

*In vitro*の光毒性試験の先行実施についてはEUの意見を尊重し，動物実験を実施する前に行うことを考慮するという非常に曖昧な表現を用いていますが，実情は玉虫色です．

経皮に関して，光アレルギー性評価については，臨床モニタリングを，上市用の製剤を用いてフェーズIIIの期間中に行うこととしています．

眼に関しては，適切な試験系がないとの理由で，眼局所適用薬をスコープから外すとの結論に達しました．ただし，スコープから外すといっても，一切触れないわけでなく，全身投与薬あるいは経皮適用薬に対して述べている，光安全性評価の一般原則を適用してよいとの表現にしています．

ROSアッセイ等で完全合意には至っていない部分もありますが，地域間差をほぼ解消し，合意されました．

### 7.3　フローチャート

フローチャートについても，非常に多くのコメントが寄せられました．

フローチャートは，図なので直感的な分かりやすさがある反面，もとのフローチャートがtiered approachに基づいたものであり，integrated approachにした時点で誤解を招くようになったとの理由により，ステップ2文書から削除された経緯があります．

しかしながら，多くのコメントがその復活を求めていたため，フローチャート又はその代替物をガイドラインに搭載することを考え，現在その搭載の可否や搭載する場合にどのように搭載するかを検討することとなり，新たなフローチャート又は代替物の草案が提示され議論されています．

Table 4　ICHブリュッセル会議における成果

| 適用 | 項目 | 日本 | 米 | EU |
|---|---|---|---|---|
| 共通 | ROSアッセイの適用 | 可 | 判断保留（20 μMのデータから最終判断） | |
| 全身 | 組織分布データの利用 | 可（ケースバイケース） | | |
| | *In vitro*光毒性試験の先行実施 | （動物実験を実施する前に）考慮 | | |
| 経皮 | 光アレルギー性評価 | 臨床モニタリング（上市用製剤，P3期間中） | | |
| 眼 | 光毒性評価 | 適切な試験系がないため，眼局所適用薬はスコープ外（光安全性評価の一般原則は適用可能） | | |