Development of new medium-term animal models for predicting chemical carcinogenicity with underlying modes of action using reporter gene transgenic rat

レポーター遺伝子導入ラットを用いた中期発がん評価系の開発

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Contents

Abbreviations

AA	Aristolochic acid
2-AAF	2-Acetylaminofluorene
AH	Atypical hyperplasia
APAP	Acetaminophen
AT	Atypical tubule
BNF	β-Naphthoflavone
BrdU-LIs	5-Bromo-2'-deoxyuridine-labeling indices
BT	Barbital
CYP	Cytochrome P450
DADS	Diallyl disulfide
DEN	Diethylnitrosamine
DL	*d*-Limonene
DT	Distal tubule
DW	Distilled water
ES	Estragole
GST-P	Glutathione *S*-transferase placental form
ICH	International Conference on Harmonisation

i.p.	intraperitoneal
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinolone
MF	Mutant frequency
NTA	Trisodium nitrilotriacetic acid
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PBO	Piperonyl butoxide
PBZ	Phenylbutazone
PCT	Proximal convoluted tubule
PCNA-LIs	Proliferating cell nuclear antigen-labeling indices
PDP	Potassium dibasic phosphate
PH	Partial hepatectomy
PhB	Phenobarbital
PHE	Phenytoin
PST	Proximal straight tubule
SF	Safrole
6-TG	6-Thioguanine
UN	Unilateral nephrectomy

General introduction

In our daily life, we are regularly exposed to a large number of new and different environmental chemicals, including pharmaceuticals, agrochemicals, and food additives. Currently, several thousand new chemicals are developed or discovered each day through the ongoing efforts of organic chemists in various fields (Binetti et al. 2008; Mahadevan et al., 2011). Environmental chemicals may pose a risk to humans, and therefore, their safety has been evaluated by extensive toxicity studies using animals. In particular, carcinogenicity is a key component of safety assessments, because resulting lesions can be irreversible and often fatal. Additionally, environmental chemical exposure plays an important role in the generation of sporadic neoplasms in humans (Sørensen et al., 1988; Lee et al., 2007; Davis et al., 2013), although inherited genetic factors or infectious diseases also may make a minor contribution (Lichtenstein et al., 2000; Danaei, 2012).

The current gold standard for the evaluation of chemical carcinogenicity is a 2-year lifetime bioassay using rodent species. Although conventional lifetime bioassays can provide information regarding target organs and doses of carcinogens, these assays are associated with high animal burden and a long time frame, often exceeding 3 years (Paules et al., 2011), meaning that only a small fraction of chemicals have been evaluated for their carcinogenicity (Mahadevan et al., 2011). In addition, information regarding modes of action in carcinogenesis

cannot be obtained in lifetime bioassays; thus, additional assays are required to permit extrapolation from rodents to humans (Cohen and Arnold, 2011). The International Conference on Harmonisation (ICH) guidelines recommend the combination of an alternative medium-term *in vivo* study and a lifetime bioassay using rats for assessment of carcinogenicity, instead of lifetime bioassays using 2 species of rodents (ICH, 1997). A medium-term rat liver animal model (e.g., the Ito model), and a 6-month transgenic animal model (e.g., using rasH2 or *p53*-deficient mice) are recommended as alternative *in vivo* studies by ICH guidelines (ICH, 1997). However, neither alternative bioassay proposed by ICH guidelines can provide data regarding mode of action in chemical carcinogenesis (Cohen and Arnold, 2011). Therefore, the development of *in vivo* assays for rapid detection of carcinogens with their carcinogenic modes of action is currently desired.

It is well recognized that chemical-induced carcinogenesis involves a multistep process, with individual steps classically including initiation, promotion, and progression (Barrett, 1993). Initiation is now considered to correspond to an event causing permanent damage to DNA (Cohen and Arnold, 2011). DNA-reactive chemicals, acting either directly or following metabolic activation, can form DNA adducts, ultimately leading to irreversible gene mutations (Garner, 1998; Hemminki et al., 2000); compounds of this type are referred to as genotoxic carcinogens. Although not all DNA adducts have mutagenic potential largely due to

in vivo DNA repair systems, DNA-reactive carcinogens have been assumed to induce a linear, nonthreshold dose-response in terms of extrapolating of risk estimates to humans (Cohen and Arnold, 2011). On the other hand, non-DNA-reactive carcinogens also referred to as tumor-promoters or non-genotoxic carcinogens are thought to induce tumors by increasing cell proliferation activity at the target site. Such effects can be caused either directly (e.g., by serving as hormones or growth factors) or indirectly (e.g. by inducing regeneration following cytotoxicity) (Cohen and Arnold, 2011). In contrast to initiation or gene mutation induced by genotoxic carcinogens, tumor-promotion or cell proliferation induced by non-genotoxic carcinogens can be a reversible event, with a certain amount of chemical exposure required for promotion to occur (Cohen and Arnold, 2011). Thus characterization of promotion can involve an evaluation of the dose-response and identification of a threshold. Therefore, understanding the modes of action of chemical carcinogenicity, especially for genotoxic mechanisms, is essential for assessment of human risk hazards.

A plethora of genotoxicity assays have been developed not only for investigation of carcinogenic modes of action but also for short-term screening assays to predict carcinogenicity (Kirkland et al., 2007). However, standard assays for assessing chemical genotoxicity such as the Ames test, the micronucleus test, and the chromosomal aberration test have been demonstrated to produce high rates of false-positive results in predicting carcinogenicity.

Because almost all of these assays are performed *in vitro*, results can be significantly skewed by cytotoxicity of the compounds under investigation (Kirkland et al., 2007; Cohen and Arnold, 2011). Various *in vivo* genotoxicity assays also have been developed, permitting the evaluation of genotoxicity in the context of *in vivo* metabolic systems and target organs. However, these *in vivo* assays understandably cannot evaluate the tumor-promoting potential of chemicals in terms of predicting carcinogenicity. Given that liver is the most common target organ of chemical carcinogenesis and that almost all known carcinogens exhibit tumor-promotion potential when administered repeatedly, a model was developed for rapid detection of tumor-promoting activity in the liver (Ito et al., 2003; Tsuda et al., 2010). The "Ito model" utilizes a combination of initiation, treatment with a DNA-reactive carcinogen, and subsequent proliferative stimulus by partial hepatectomy (PH). The inclusion of a reliable preneoplastic marker glutathione *S*-transferase placental form (GST-P), an enzyme induced in preneoplastic lesions of hepatocytes, permits rapid prediction of tumor-promotion potential. However, modes of action, including involvement of genotoxicity, cannot be investigated in this model, as mentioned above.

In vivo mutation assays using reporter gene transgenic rodents can be combined with additional assays for further investigation of underlying carcinogenic modes of action because both classes of tests can be conducted under the same conditions as part of a lifetime

carcinogenicity bioassay. In fact, we have demonstrated the usefulness of the combination of *in vivo* mutation assay with evaluation of other parameters related to chemical carcinogenesis, such as enzymatic activity, formation of DNA adducts, cell proliferation, or oxidative stress using *gpt* delta rodents for understanding the modes of action of various carcinogens (Suzuki et al., 2012b; Kuroda et al., 2013; Tasaki et al., 2013; Ishii et al. 2014). Therefore, it was conceivable, based on the concept of two-step carcinogenesis, that tumor-promoting activity could be evaluated in combination with *in vivo* mutagenicity tests and additional assays for elucidation of chemical carcinogenesis using *gpt* delta rats.

In the present study, I attempted to develop new medium-term animal models using the *gpt* delta rat. These models were expected to permit the simultaneous evaluation of *in vivo* mutagenicity and tumor-promoting potential. I present the development of these new models over the course of three chapters. In the first chapter, I evaluate the potential for development of the GPG model, using *gpt* delta rats, that is capable of detecting *in vivo* mutagenicity and tumor-promoting activity in the liver. In the second chapter, I describe the improvement of the GPG model by the inclusion of a test-chemical washout period as part of the protocol, a refinement that avoids possible interactions between the tumor initiator (diethylnitrosamine; DEN) and test chemicals. The applicability of the GPG model is validated using various types of carcinogens. In addition, I demonstrate the availability of additional assays using excised or

residual liver tissue for further analysis of the underlying modes of action of chemical carcinogenesis. In the third chapter, the standard protocol of the GNP model, which is capable of evaluating *in vivo* mutagenicity and tumor-promoting activity in the kidney, is established based on the results of preliminary studies. This final chapter also demonstrates the usefulness of this model by means of validation studies using several types of carcinogens.

Chapter 1

Development of a medium-term animal model using *gpt* delta rats to evaluate chemical carcinogenicity and genotoxicity in the liver

1.1. Introduction

Environmental chemicals, including pharmaceuticals, agrochemicals and food additives, are important in various aspects of daily life. However, these chemicals may pose a risk to humans, and their toxicities have been extensively assessed in animal studies. In particular, carcinogenicity is a key component of safety assessments because the resulting lesions can be irreversible and are often fatal. The current gold standard for assessing the risk of cancer is a lifetime bioassay in rodents, but this method requires over 3 years to complete, including histopathological procedures (Pules et al., 2011). It is estimated that only approximately 1500 chemicals have been tested over the past 30 years despite the addition of nearly 4000 new chemicals in the Chemical Abstracts Service (CAS) Registry database every day (Binetti et al., 2008; Mahadevan et al., 2011). Although conventional lifetime bioassays can provide data regarding the potential carcinogenicity and target organs of various chemicals, these assays do not provide any information about the associated modes of action that influence carcinogenesis (Cohen and Arnold, 2011). The development of bioassays that can rapidly detect chemical carcinogenicity and provide information about the underlying modes of action is currently being pursued.

Thresholds in dose-related chemical carcinogenicity curves depend on the involvement of genotoxic mechanisms (Cohen and Arnold, 2011). Mutagenicity and

carcinogenicity are important factors when determining risk assessments (Kirkland and Speit, 2008). Although *in vitro* genotoxic assays, such as the Ames test, the micronucleus test and the chromosomal aberration test, are considered standard tools for investigating chemical mutagenicity, the results of these methods are not necessarily indicative of carcinogenicity (Kirkland and Speit, 2008). Reporter gene mutation assays are promising genotoxic techniques because *in vivo* metabolic processes can be evaluated at the target organs (World Health Organization, 2006). Comprehensive toxicity studies and the measurement of DNA adducts, oxidative stress and enzymatic activities have been demonstrated in animal models using *gpt* delta rodents (Umemura et al., 2009; Tasaki et al., 2010; Jin et al., 2011; Suzuki et al., 2012a). Using the reliable preneoplastic marker GST-P foci, medium-term rat liver bioassays have been developed to rapidly detect tumor-promoters because the liver is the most common target organ for carcinogenesis (Ito et al., 2003). However, the conventional medium-term bioassays do not provide information regarding the involvement of genotoxic mechanisms in carcinogenesis as a result of exposure to test compounds.

In this study, I evaluated the possibility of developing a new animal model designed to rapidly detect chemical carcinogenicity and underlying molecular mechanisms using a reporter gene mutation assay and a medium-term liver bioassay. The conditions were optimized to establish a tentative experimental protocol, and validation of the model was confirmed using

several carcinogens.

1. 2. Materials and Methods

1. 2. 1. Chemicals

Diethylnitrosamine (DEN) and safrole (SF) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Phenobarbital (PhB), 2-acetylaminofluorene (2-AAF), piperonylbutoxide (PBO), and phenytoin (PHE) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and acetaminophen (APAP) was purchased from MP Biomedicals (Irvine, CA, USA). 2-Amino-3-methylimidazo[4,5-*f*]quinolone (IQ) and aristolochic acid (AA) were obtained from Toronto Research Chemicals (North York, ON, Canada) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

1. 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 five 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 (two or three 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.

1. 2. 3. Animal treatments

1. 2. 3. 1. Experiment I

The effects of a single administration of DEN on the development of GST-P positive foci were evaluated. A PH was performed on ten week-old male F344/NSlc rats (n=5 rats per dose). After 18 h, an intraperitoneal (i.p.) injection of DEN was administered at doses of 0, 10, 50, and 100 mg/kg. Six weeks after the start of the experiment, the rat livers were fixed in 10% neutral-buffered formalin. The fixed tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P positive foci.

1. 2. 3. 2. Experiment II

Changes in the development of GST-P positive foci over time following administration of PhB after a PH and single dose exposure to DEN were examined. Six-week-old male F344/NSlc rats (n=10 rats per dose) were fed PhB at concentrations of 0 and 500 ppm in their

basal diets. This dose was selected based on a previous carcinogenicity test (Bulter, 1978). After 4 weeks, a PH was performed. An i.p. injection of DEN at a dose of 10 mg/kg was administered 18 h after the PH. The rats continued to feed on a diet containing PhB until they were sacrificed at 10, 12, or 14 weeks after the start of the experiment. The livers were fixed in 10% neutral-buffered formalin, and the tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P positive foci.

1. 2. 3. 3. Experiment III

Validation of the animal model was confirmed using genotoxic, non-genotoxic carcinogens and a non-carcinogen. Six-week-old male F344/NSlc-Tg (*gpt* delta) rats (n=15 per dose) were fed 20 ppm 2-AAF, 12000 ppm PBO or 6000 ppm APAP in their basal diets. A control group was fed the basal diet without chemical supplementation. The 2-AAF dose was selected based on a preliminary study in which no toxic effects were observed in rats treated with 20 ppm (data not shown). The doses of PBO and APAP were selected based on previous carcinogenicity tests (National Toxicology Program (NTP), 1993a; Takahashi et al., 1994). A PH was performed on all rats after 4 weeks, and an i.p. injection of DEN at a dose of 10 mg/kg was administered 18 h after the PH. The excised liver tissues were perfused with saline to remove residual blood and stored at –80°C for the *gpt* assay. The rats continued to feed on the basal

diets containing the various chemicals. Ten weeks after the start of the experiment, the livers were fixed in 10% neutral-buffered formalin. The fixed tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P positive foci.

1. 2. 3. 4. Experiment IV

The animal model was further validated using genotoxic and non-genotoxic carcinogens and a genotoxic non-hepatocarcinogen. Six-week-old male F344/NSlc-Tg (*gpt* delta) rats (n=15 per dose) were fed 20 ppm IQ, 5000 ppm SF or 2400 ppm PHE in their basal diets. The rats treated with AA received 0.3 mg/kg body weight in 1% sodium bicarbonate by gavage once a day. A control group was fed the basal diet without chemical supplementation. The IQ dose was selected based on a preliminary study in which no toxic effects were observed in rats treated with 20 ppm (data not shown). The doses of SF and PHE were selected based on previous carcinogenicity tests (Wislocki et al., 1977; NTP, 1993b), and the dose of AA was determined 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). A PH was performed on all rats after 4 weeks, and an i.p. injection of DEN at a dose of 10 mg/kg was administered 18 h after the PH. The excised liver tissues were perfused with saline to remove residual blood and

stored at –80°C for the *gpt* assay. The rats continued to feed on the basal diets containing the various chemicals. Ten weeks after the start of the experiment, the livers were fixed in 10% neutral-buffered formalin. The fixed tissues were embedded in paraffin, sectioned and evaluated using immunohistochemistry for the quantitative analysis of GST-P positive foci.

1. 2. 4. *In vivo* mutation assays

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* MFs were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

1. 2. 5. Immunohistochemical staining for GST-P

Immunohistochemical staining was performed using polyclonal antibodies against GST-P (1:1000 dilution; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). The number and area of GST-P positive foci consisting of 5 or more nucleated hepatocytes in a cross-section were evaluated using an image analyzer (IPAP, Sumika Technoservice, Hyogo, Japan) (Watanabe et al., 1994).

1. 2. 6. Statistics

The number and area of GST-P positive foci in experiment I were analyzed using ANOVA followed by Dunnett's multiple comparison test. The number and area of GST-P positive foci in experiments II, III and IV and the *gpt* MFs in experiments III and IV were analyzed by assessing the variance for homogeneity using the F-test. The Student's t-test and Welch's t-test were used for homogeneous and heterogeneous data, respectively. The *gpt* MFs in the rats treated with SF in experiment IV were analyzed using the Mann-Whitney U test.

1. 3. Results

1. 3. 1. Experiment I

Two of the rats in the control group died due to surgical complications of the PH and were eliminated from further evaluation. Treatment with DEN increased the number and area of GST-P positive foci in a dose-dependent manner compared with the control group (Table 1), although the differences were not significant in the rats that were treated with 10 mg/kg and 50 mg/kg.

1. 3. 2. Experiment II

Two rats from the 14-week control group, one rat from the 10-week PhB group and one rat from the 12-week PhB group died due to surgical complications of the PH and were eliminated from further evaluation. The number and area of GST-P positive foci were significantly increased in the rats treated with PhB in each experimental time period (Table 1).

1. 3. 3. Experiment III

Three rats in the control group, one rat in the group treated with 2-AAF, five rats in the group treated with PBO and one rat in the group treated with APAP died due to surgical complications of the PH and were eliminated from further evaluation. Table 2 shows the MFs in

the excised livers of *gpt* delta rats that were treated with 2-AAF, PBO or APAP for 4 weeks. The MFs in the rats treated with 2-AAF were significantly increased compared with the rats in the control group. No significant changes were observed in the rats treated with PBO or APAP. In the *gpt* mutation spectra, GC:TA and GC:CG transversions and single base pair deletions were significantly increased in the rats treated with 2-AAF (Table 3). The number and area of GST-P positive foci were significantly increased in livers of the rats treated with 2-AAF or PBO and significantly decreased in the livers of the rats treated with APAP (Table 1).

1. 3. 4. Experiment IV

One rat in the control group, four rats in the group treated with IQ, eight rats in the group treated with SF, three rats in the group treated with PHE and two rats in the group treated with AA died due to surgical complications of the PH and were eliminated from further evaluation. Table 4 shows the MFs in the excised livers of *gpt* delta rats that were treated with IQ, SF, PHE or AA for 4 weeks. The MFs in the rats treated with IQ, SF and AA were significantly increased compared with the rats in the control group. In the *gpt* mutation spectra, GC:TA transversions, GC:AT transitions and single base pair deletions were significantly increased in the rats treated with IQ, and AT:TA transversions were significantly increased in the rats treated with AA (Table 5). No significant changes were observed in the rats treated with SF.

The number and area of GST-P positive foci were significantly increased in the livers of the rats treated with IQ, SF and PHE (Table 1).

1. 4. Discussion

Chemical carcinogenesis involves multiple gene alterations, which can be divided into initiation and promotion phases. A medium-term rat liver bioassay involving the quantitative analysis of GST-P positive foci following cell proliferative stimuli via PH was established to detect the tumor-promoting activities of various chemicals. Reporter gene mutation assays using transgenic animals have been developed to detect *in vivo* mutagenicity. Because this assay can be performed under conditions that are similar to the conventional long-term bioassay, the results may represent the tumor initiation phase of chemical carcinogenesis. GST-P positive foci have been analyzed in *gpt* delta rats (Kanki et al., 2005; Toyoda-Hokaiwado, 2010; Jin et al., 2011). The GPG animal model described in this study can detect the *in vivo* mutagenicity and tumor-promoting activities of various chemicals by combining the reporter gene mutation assay and the medium-term liver bioassay.

In this animal model, *gpt* delta rats were exposed to chemicals, and a PH was performed to collect liver samples for an in vivo mutation assay. The rats were subsequently administered a single i.p. injection of DEN, and the tumor-promoting activity of the chemical was evaluated based on the development of GST-P positive foci. The Organisation for Economic Co-operation and Development (OECD) guidelines state that 4 weeks of exposure is sufficient for detecting mutations in the reporter gene (OECD, 2011), which is supported by

additional data (Hibi et al., 2011; Suzuki et al., 2012a). Therefore, the period of exposure prior to PH in this study was determined to be 4 weeks. Initial exposure to a potent genotoxic carcinogen is necessary to detect tumor-promoting activities over a short period of time. In this model, DEN was selected because correlations between the administration of DEN and the induction of GST-P foci in the rat liver have been extensively reported (Ogiso et al., 1985; Kushida et al., 2005; Nagahara et al., 2010; Kakehashi et al., 2011). However, the dose of DEN should be as low as possible to avoid any effects on the metabolism of the test chemical because DEN has been shown to influence various parameters, including the induction of cytochrome P450 (CYP) and glutathione *S*-transferase (Basak et al., 2000; Aibu et al., 2011). I took advantage of the rapid induction of cell proliferation following PH because genotoxic compounds can effectively induce gene mutations under conditions of high cell proliferation (Cohen and Arnold, 2011). Tsuda et al. (1980) reported that the initiator should optimally be administered 18 h after PH to effectively enhance initiation. Based on these data, appropriate dosages of DEN were investigated in a dose-response study consisting of single i.p. injections of DEN 18 h after PH at doses of 10 mg/kg and higher. The optimal dosage of DEN was established as 10 mg/kg based on the quantitative analysis of GST-P positive foci. PhB, a liver tumor-promoter in rodents (Fukushima et al., 2005), was used to determine the optimal duration of exposure following a PH in experiment II. The results of this study demonstrate that

treatment with PhB at 500 ppm in the diet for 6 weeks is effective in detecting the effects of tumor-promotion. The tentative protocol for the GPG animal model is shown in Fig. 1.

The animal model was validated using several carcinogens. 2-AAF, IQ and SF are genotoxic murine liver carcinogens that produce deoxyguanine adducts via metabolic activation and play a key role in liver carcinogenesis (Heflich and Neft, 1994; Schut and Snyderwine, 1999; Bagnyukova et al., 2008; Shen et al., 2012). A significant increase in the MFs of the *gpt* genes in the rats treated with 2-AAF, IQ and SF was shown using the GPG model. Spectrum analysis in the *gpt* mutant colonies revealed that guanine-related mutations and single base pair deletions were induced by 2-AAF and IQ, but not SF, which is in agreement with previous reports (Schaaper et al., 1990; Ross and Leavitt, 1998; Xie et al., 2012). In the conventional medium-term bioassay, 2-AAF, IQ and SF exposure induced a marked increase in the development of GST-P positive foci (Ito et al., 1988), implying that these chemicals also exert a strong tumor-promoting action. The GPG animal model showed that the development of GST-P positive foci at 10 weeks was markedly increased in the livers of rats treated with these carcinogens. PBO and PHE were reported to act as hepatocarcinogens in F344 rats fed a diet containing 12000 ppm and 2400 ppm for 2 years, respectively (NTP, 1993b; Takahashi et al., 1994). These compounds are classified as non-genotoxic carcinogens based on the results of various genotoxicity studies (NTP, 1993b; Beamand et al., 1996). An increase in the

development of GST-P positive foci was observed in rats treated with PBO or PHE in a conventional medium-term bioassay (Ito et al., 1988; Muguruma et al., 2009). Treatment with PBO and PHE at the carcinogenic dose in the GPG animal model did not increase the *gpt* MF, although the development of GST-P positive foci was significantly increased. APAP was not reported to be hepatocarcinogenic in F344 rats fed a diet containing 6000 ppm for 2 years (NTP, 1993a). In the present study, treatment with APAP in the GPG model at a dose of 6000 ppm did not increase the *gpt* MF and inhibited the development of GST-P positive foci. Ito et al. (1988) showed that APAP had an inhibitory effect on the development of GST-P positive foci in a conventional medium-term bioassay. AA has been reported to be carcinogenic in the kidney and the stomach of rodents (Mengs et al., 1982). In an *in vivo* genotoxicity study in Big Blue transgenic rats, AA exposure elevated *cII* MFs and produced AA-specific deoxyadenine and deoxyguanine adducts in the kidney and the liver (Mei et al., 2006). A significant increase in *gpt* MFs in rats treated with AA was observed in the GPG model, and AT:TA transversions were the predominant mutation in the mutation spectra analysis, which is similar to a previous report (Mei et al., 2006). AA did not have an enhancing effect on the development of GST-P positive foci, which may reflect the fact that AA exerts initiation activity, but not carcinogenicity, in the liver.

Overall, the validation results show the possibility of developing a new animal model

using *gpt* delta rats. However, a possible limitation of the tentative protocol is that the test chemicals are co-administered simultaneously with DEN. Although there did not appear to be any mutual effects between DEN and the test chemicals, this treatment regimen may modify the detoxification or metabolic activation of DEN. Several isoforms of CYP have been reported to participate in the metabolic activation of DEN, with CYP2E1 in particular playing an essential role (Kang et al., 2007). Because many liver tumor-promoters in rodents can induce several types of CYPs and/or modify the expression of phase II enzymes, I worked toward improving the timing of the regimen to avoid the possibility of mutual effects. Validation studies of the modified protocol based on changes in the timing of chemical administration have been performed in the next study.

In this chapter, I have demonstrated the potential for develop a GPG medium-term animal model to evaluate *in vivo* mutagenicity and tumor-promoting activities of test chemicals in the liver concurrently. Given that a limitation of the original protocol is the potential interaction between the test chemical and DEN, the next study establishes a modified protocol that includes a test chemical washout period.

1. 5. Abstract

In this study, the potential for development of an animal model (GPG) capable of rapidly detecting chemical carcinogenicity and the underlying modes of action were examined in *gpt* delta rats using a reporter gene assay to detect mutations and a medium-term rat liver bioassay to detect tumor-promotion. The tentative protocol for the GPG model was developed based on the results of dose-response exposure to diethylnitrosamine (DEN) and treatment with phenobarbital over time following DEN administration. Briefly, *gpt* delta rats were exposed to various chemicals for 4 weeks, followed by a partial hepatectomy (PH) to collect samples for an in vivo mutation assay. The mutant frequencies (MFs) of the reporter genes were examined as an indication of tumor initiation. A single intraperitoneal (i.p.) injection of 10 mg/kg DEN was administered to rats 18 h after the PH to initiate hepatocytes. Tumor-promoting activity was evaluated based on the development of glutathione *S*-transferase placental form (GST-P) positive foci at week 10. The genotoxic hepatocarcinogens 2-acetylaminofluorene (2-AAF), 2-amino-3-methylimidazo[4,5-*f*]quinolone (IQ) and safrole (SF), the non-genotoxic hepatocarcinogens piperonyl butoxide (PBO) and phenytoin (PHE), the non-carcinogen acetaminophen (APAP) and the genotoxic non-hepatocarcinogen aristolochic acid (AA) were tested to validate the GPG model. The results of validation study demonstrated that potential for development of new medium-term animal model for predicting chemicals with carcinogenicity

and *in vivo* mutagenicity in the liver.

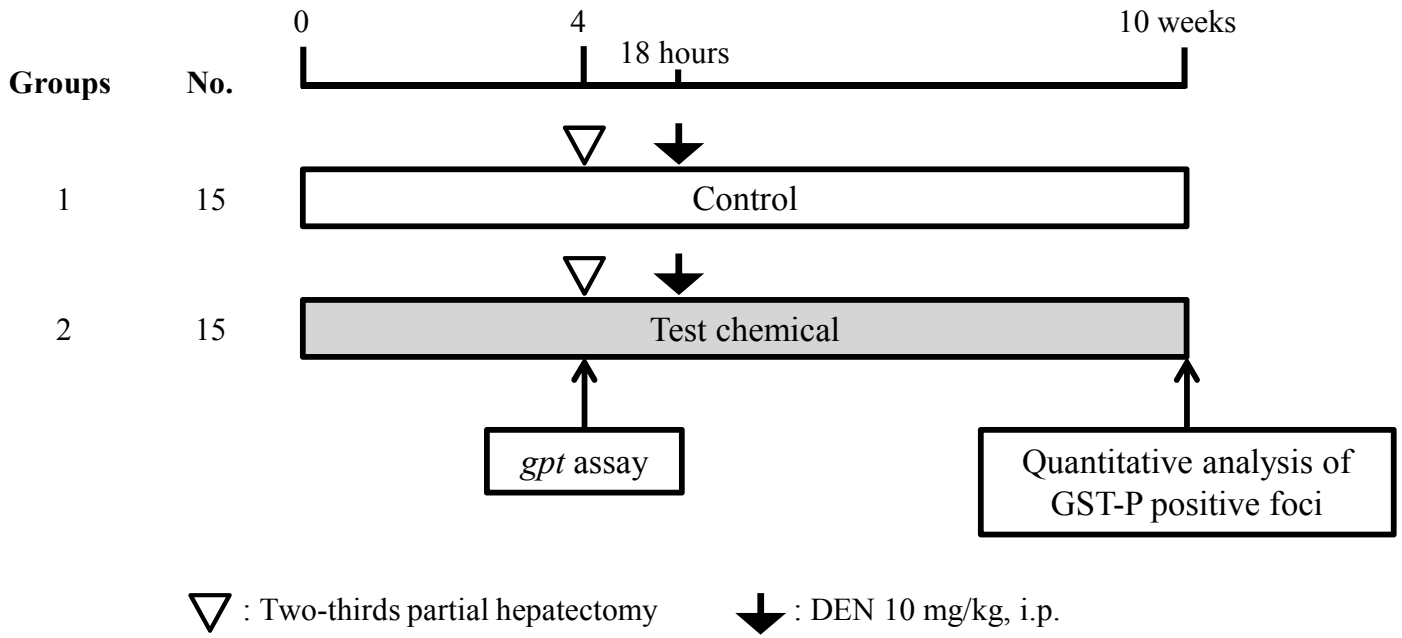

Fig. 1. Tentative protocol for the GPG model. Six-week-old male F344 *gpt* delta rats were exposed to test chemicals for 10 weeks. A partial hepatectomy (PH) was performed at week 4, and the rats were administered a single i.p. injection of 10 mg/kg diethylnitrosamine (DEN) 18 h after PH. The *gpt* assay, which is an indicator of *in vivo* mutagenicity, was performed using the liver samples excised via PH at week 4. Tumor-promoting activities were evaluated based on the development of GST-P positive foci induced by DEN at week 10.

Table 1. Quantitative analysis of GST-P positive foci in chapter 1.

Groups	No. of rats	No. of foci (No./cm^2)	Area of foci (mm^2/cm^2)
Experiment I			
Control	3	0.21 ± 0.36[a]	0.002 ± 0.003
DEN 10 mg/kg	5	7.65 ± 3.42	0.072 ± 0.034
DEN 50 mg/kg	5	20.06 ± 3.60	0.326 ± 0.103
DEN 100 mg/kg	5	28.31 ± 5.78**	1.042 ± 0.297**
Experiment II			
10 weeks			
Control	10	5.72 ± 2.47	0.038 ± 0.019
PhB	9	19.81 ± 4.08**	0.153 ± 0.035**
12 weeks			
Control	10	8.59 ± 4.33	0.053 ± 0.028
PhB	9	22.36 ± 4.89**	0.171 ± 0.043**
14 weeks			
Control	8	7.39 ± 2.60	0.053 ± 0.019
PhB	10	26.53 ± 4.41**	0.243 ± 0.048**
Experiment III			
Control	12	4.70 ± 1.53	0.027 ± 0.011
2-AAF	14	24.79 ± 6.15**	0.630 ± 0.315**
PBO	10	7.94 ± 2.22**	0.054 ± 0.015**
APAP	14	0.98 ± 0.42**	0.005 ± 0.002**
Experiment IV			
Control	14	4.40 ± 1.59	0.025 ± 0.01
IQ	11	7.83 ± 3.33**	0.046 ± 0.019**
SF	7	37.02 ± 10.03**	0.586 ± 0.293**
PHE	12	17.29 ± 5.55**	0.113 ± 0.040**
AA	13	4.70 ± 1.86	0.029 ± 0.015

Note. DEN, diethylnitrosamine; PhB, phenobarbital; 2-AAF, 2-acetylaminofluorene; PBO, piperonyl butoxide; APAP, acetaminophen; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; SF, safrole; PHE, phenytoin; AA, aristolochic acid; GST-P, glutathione *S*-transferase placental form.

[a] Mean ± SD.

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

Table 2. *gpt* MFs in livers of F344 *gpt* delta rats treated with 2-AAF, PBO and APAP

Group	Animal no.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies	MF ($\times 10^{-5}$)	Mean ±SD
Control	101	11.75	5	0.43	0.44 ± 0.10
	102	22.46	6	0.27	
	103	11.07	6	0.54	
	104	8.46	4	0.47	
	105	10.62	5	0.47	
2-AAF	201	8.33	12	1.44	2.07 ± 0.85**
	202	12.20	14	1.15	
	203	7.79	15	1.93	
	204	8.15	21	2.58	
	205	8.96	29	3.24	
PBO	301	7.70	1	0.13	0.49 ± 0.27
	302	8.42	7	0.83	
	303	7.65	5	0.65	
	304	15.03	5	0.33	
	305	8.10	4	0.49	
APAP	401	18.77	4	0.21	0.40 ± 0.14
	402	18.68	7	0.37	
	403	11.39	7	0.61	
	404	15.53	6	0.39	
	405	14.45	6	0.42	

Note. 2-AAF, 2-acetylaminofluorene; PBO, piperonyl butoxide; APAP, acetaminophen; MF, mutant frequency.

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

Table 3. Mutation spectra of *gpt* mutant colonies in livers of F344 *gpt* delta rats treated with 2-AAF, PBO and APAP

	Control		2-AAF		PBO		APAP	
	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})	Number (%)	Mutation frequency (10^{-5})
Transversions								
GC-TA	6 [a] (23.1)	0.11 ± 0.08[b]	32 (35.2)	0.72 ± 0.27**	5 (22.7)	0.13 ± 0.16	7 (23.3)	0.01 ± 0.09
GC-CG	1 (3.8)	0.01 ± 0.02	9 (9.9)	0.20 ± 0.17*	1 (4.5)	0.02 ± 0.05	3 (10.0)	0.03 ± 0.05
AT-TA	1 (3.8)	0.02 ± 0.04	8 (8.8)	0.17 ± 0.21	2 (9.1)	0.03 ± 0.06	3 (10.0)	0.04 ± 0.05
AT-CG	1 (3.8)	0.11 ± 0.02	3 (3.3)	0.07 ± 0.15	1 (4.5)	0.02 ± 0.06	1 (3.3)	0.02 ± 0.04
Transitions								
GC-AT	15 (57.7)	0.26 ± 0.08	19 (20.9)	0.39 ± 0.35	9 (40.9)	0.20 ± 0.14	14 (46.7)	0.19 ± 0.09
AT-GC	0	0	4 (4.4)	0.10 ± 0.11	1 (4.5)	0.02 ± 0.05	0	0
Deletion								
Single bp	1 (3.8)	0.02 ± 0.04	12 (13.2)	0.28 ± 0.21*	2 (9.1)	0.04 ± 0.06	2 (6.7)	0.03 ± 0.04
Over 2bp	0	0	1 (1.1)	0.02 ± 0.05	1 (4.5)	0.02 ± 0.05	0	0
Insertion	1 (3.8)	0.02 ± 0.04	3 (3.3)	0.07 ± 0.07	0	0	0	0
Complex	0	0	0	0	0	0	0	0

Note. 2-AAF, 2-acetylaminofluorene; PBO, piperonyl butoxide; APAP, acetaminophen.

[a] Number of colonies with independent mutations. [b] Mean ± SD.

*,** Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

Table 4. *gpt* MFs in livers of F344 *gpt* delta rats treated with IQ, SF, PHE and AA

Group	Animal no.	Cm^R colonies ($\times 10^5$)	$6\text{-}TG^R$ and Cm^R Colonies	MF ($\times 10^{-5}$)	Mean ± SD
Control	101	15.1	3	0.20	0.38 ± 0.19
	102	6.8	4	0.59	
	103	15.9	7	0.44	
	104	12.2	2	0.16	
	105	8.1	4	0.50	
IQ	201	8.9	18	2.03	3.35 ± 1.22**
	202	7.2	34	4.69	
	203	6.1	18	2.94	
	204	10.4	26	2.49	
	205	4.4	20	4.58	
SF	301	10.0	8	0.80	1.18 ± 0.74**
	302	5.0	5	1.00	
	303	5.6	14	2.49	
	304	10.1	7	0.69	
	305	5.4	5	0.92	
PHE	401	7.9	3	0.38	0.36 ± 0.26
	402	4.5	1	0.22	
	403	11.4	1	0.09	
	404	5.9	2	0.34	
	405	7.7	6	0.78	
AA	501	8.6	13	1.50	1.18 ± 0.41**
	502	9.8	17	1.73	
	503	12.9	12	0.93	
	504	11.3	9	0.79	
	505	9.5	9	0.95	

Note. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; SF, safrole; PHE, phenytoin; AA, aristolochic acid; MF, mutant frequency.

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

Table 5. Mutation spectra of *gpt* mutant colonies in livers of F344 *gpt* delta rats treated with IQ, SF, PHE and AA

	Control		IQ		SF		PHE		AA	
	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	5 [a] (25.0)	0.11 ± 0.09[b]	50 (43.1)	1.40 ± 0.41**	13 (33.3)	0.41 ± 0.38	4 (30.8)	0.10 ± 0.17	11 (18.3)	0.21 ± 0.09
GC-CG	1 (5.0)	0.01 ± 0.03	4 (3.5)	0.11 ± 0.25	6 (15.4)	0.17 ± 0.13	1 (7.7)	0.03 ± 0.06	1 (1.7)	0.02 ± 0.05
AT-TA	0	0	6 (5.2)	0.20 ± 0.18	3 (7.7)	0.09 ± 0.09	0	0	29 (48.3)	0.55 ± 0.30**
AT-CG	0	0	1 (0.9)	0.03 ± 0.06	2 (5.1)	0.06 ± 0.08	0	0	0	0
Transisions										
GC-AT	8 (40.0)	0.14 ± 0.11	14 (12.1)	0.40 ± 0.16*	6 (15.4)	0.17 ± 0.14	6 (46.2)	0.16 ± 0.15	7 (11.7)	0.15 ± 0.13
AT-GC	3 (15.0)	0.07 ± 0.13	0	0	4 (10.3)	0.13 ± 0.15	1 (7.7)	0.03 ± 0.08	2 (3.3)	0.04 ± 0.09
Deletion										
Single bp	3 (15.0)	0.04 ± 0.04	39 (33.6)	1.17 ± 0.58*	3 (7.7)	0.10 ± 0.17	1 (7.7)	0.03 ± 0.08	8 (13.3)	0.16 ± 0.16
Over 2bp	0	0	1 (0.9)	0.02 ± 0.04	0	0	0	0	0	0
Insertion	0	0	1 (0.9)	0.02 ± 0.05	2 (5.1)	0.06 ± 0.08	0	0	2 (3.3)	0.04 ± 0.06
Complex	0	0	0	0	0	0	0	0	0	0

Note. IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; SF, safrole; PHE, phenytoin; AA, aristolochic acid.

[a] Number of colonies with independent mutations. [b] Mean ± SD.

*,** Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

Chapter 2

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

2. 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 ICH guideline recommends lifetime bioassays using rats and an additional medium-term *in vivo* study in place of the lifetime bioassay using 2 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 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.

I 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 (Kuroda et al., 2013; Tasaki et al., 2013; Ishii et al., 2014). I then attempted to develop a new medium-term animal model using *gpt* delta rats capable of rapidly detecting chemical carcinogenicity, *in vivo* mutagenicity, and the underlying modes of action. In chapter 1, I confirmed the potential for

development of a new animal model, in which 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 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 2 compounds should be avoided. In fact, several isoforms of 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 (Muguruma et al., 2007; Graham and Lake, 2008; 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), PBO or 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 new protocol.

2. 2. Materials and Methods

2. 2. 1. Chemicals

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 AA were from Sigma–Aldrich (St. Louis, MO, USA).

2. 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 344/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 (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. 2. 3. Animal treatments

2. 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. 2). 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 (NTP, 1993b; Takahashi et al., 1994; Le Bon et al., 2003). 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. Test chemical exposure resumed at 7 weeks, and at 13weeks, 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. 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 (Shimada et al., 2010; Kawamura et al., 2012; Suzuki et al., 2012a). 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. At13 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. 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. 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 at530 nm and emission at 585 nm. CYP2B1 activity was measured by 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. 2. 6. *In vivo* mutation assay

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* MFs were calculated by dividing the number of *gpt* mutants by the number of rescued phages.

2. 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 mono-clonal 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 Technoser-vice, 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 percentagesof cells staining positive for PCNA.

2. 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 Biosystems7900HT 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. 2. 9. Statistics

The data for the number and area of GST-P positive foci, CYP 2E1enzymatic 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.

2. 3. Results

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

2. 3. 2. Enzymatic assay

Fig. 3 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.

2. 3. 3. GST-P analysis

Table 6, Figs. 4 and 5 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.

2. 3. 4. *In vivo* mutation assay

Table 7 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 8).

2. 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. 6A and 7). As shown in Fig. 6B, 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.

2. 4. Discussion

In the previous study, I attempted to develop a new medium-term animal model, GPG, capable of detecting *in vivo* mutagenicity and tumor-promoting activity. 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

this 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 (Ito et al., 1988; Nims et al., 1994; Muguruma et al., 2007; Beltrán-Ramírez et al., 2008; Tasaki et al., 2010). The present data clearly 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 preneoplastic lesion induced by DEN. From the overall data, I established a modified protocol using the GPG

model as follows (Fig. 8): *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 CYPs 1A 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, I 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, I 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., 2012a). It is likely that these 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 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 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.

I have established a new medium-term animal model; I termed this model the GPG model. A summary of the GPG model validation study is shown in Table 9. The validation results indicate that the GPG model could be a powerful tool in understanding chemical carcinogenesis and provide valuable information regarding human risk hazards.

2. 5. Abstract

I 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 new 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 new protocol has been established with 2- and 1- week washout periods before and after DEN injection, respectively. The applicability of the new protocol was demonstrated using the genoxotic 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.

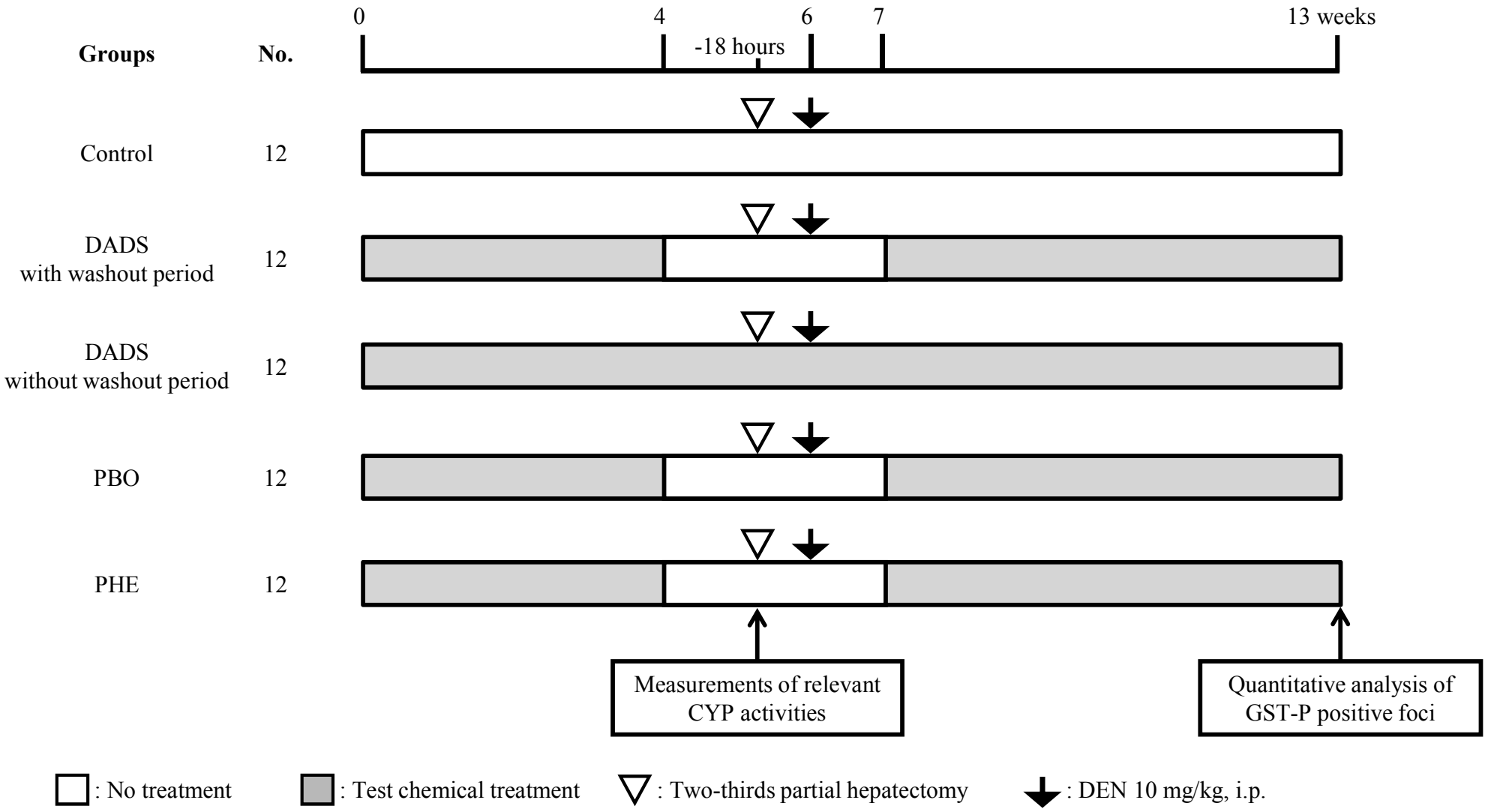

Fig. 2. Treatment protocol for experiment I in chapter 2. Animals were 6-week-old male F344 rats.
Diallyl disulfide (DADS): 50 mg/kg body weight by gavage once a day. Piperonylbutoxide (PBO): 12000 ppm in diet. Phenytoin (PHE): 2400 ppm in diet.
CYP2E1, CYP1A2 and CYP2B1 activities were evaluated in 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.

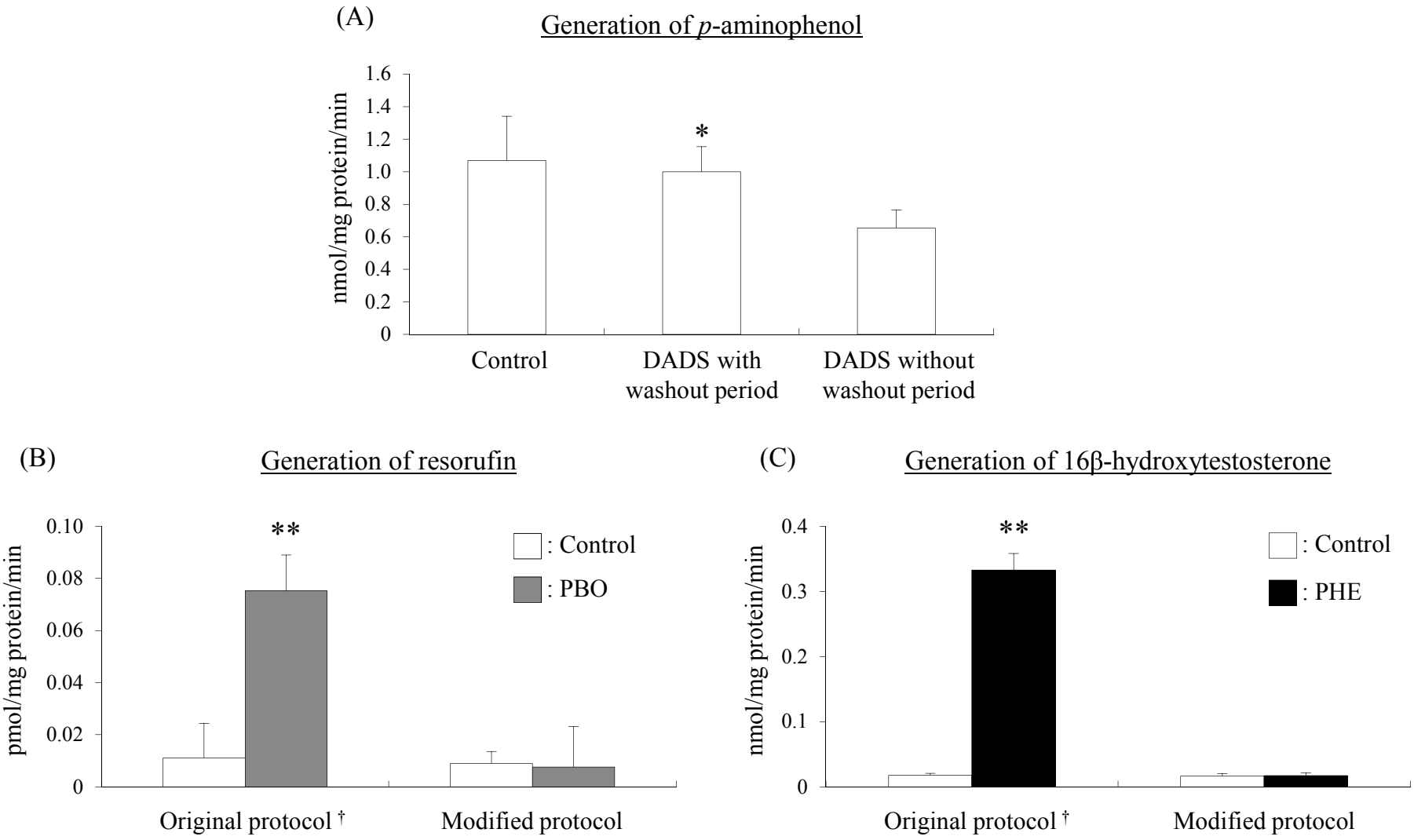

Fig. 3. Changes in CYP2E1 activity in excised livers of rats given diallyl disulfide (DADS) with or without a washout period (A). Changes of CYP1A2 (B) and CYP2B1 (C) activities in excised livers of rats given piperonylbutoxide (PBO) and phenytoin (PHE), respectively, in original and modified protocol. The values are means ± SD of data for 5 rats. †Samples were obtained from validation study of tentative protocol of GPG model. *, **Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

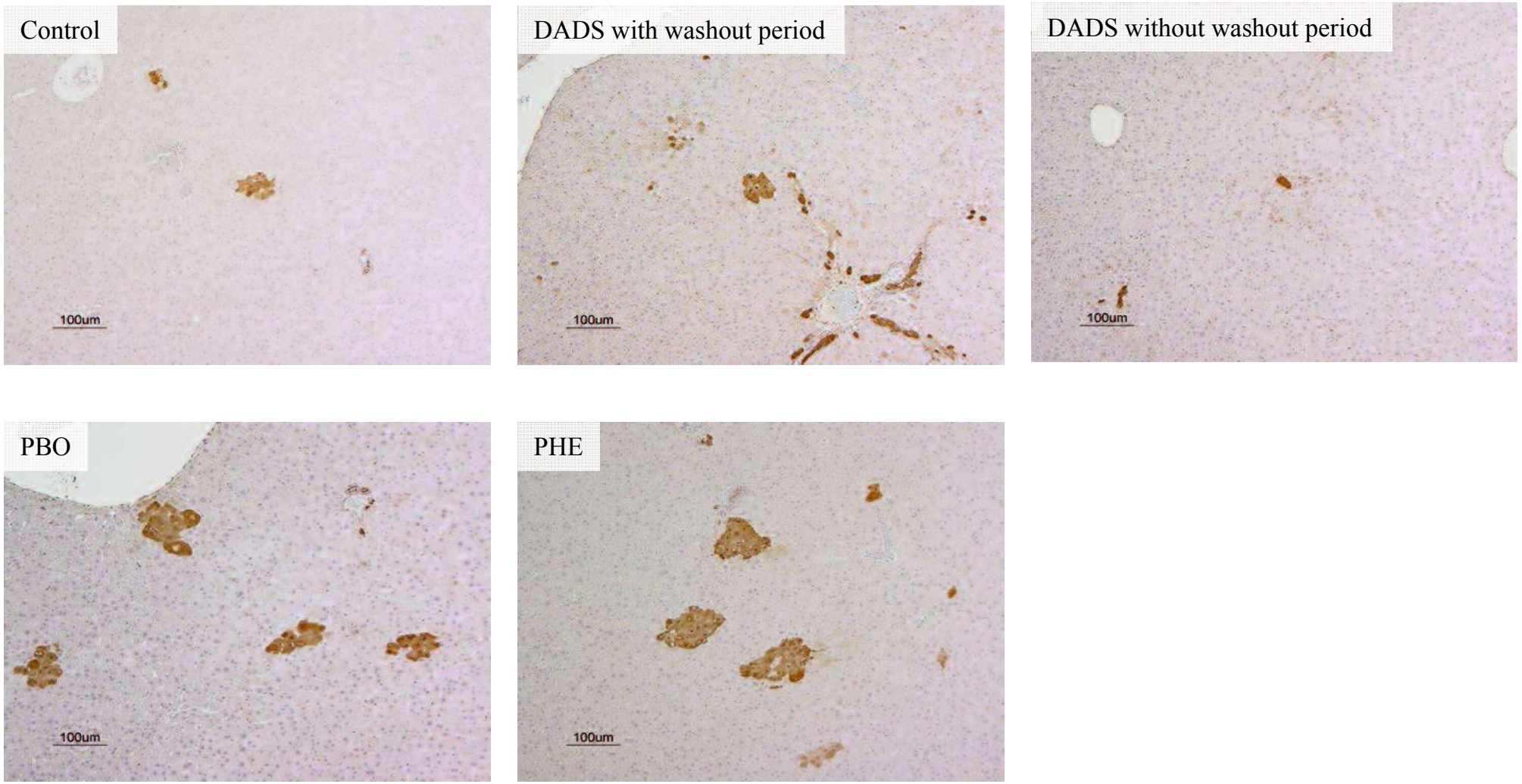

Fig. 4. Representative photographs of glutathione *S*-transferase (GST-P) immunohistochemistry in the residual livers of rats treated with diallyl disulfide (DADS) with or without a washout period, piperonyl butoxide (PBO) and phenytoin (PHE) in experiment I in chapter 2.

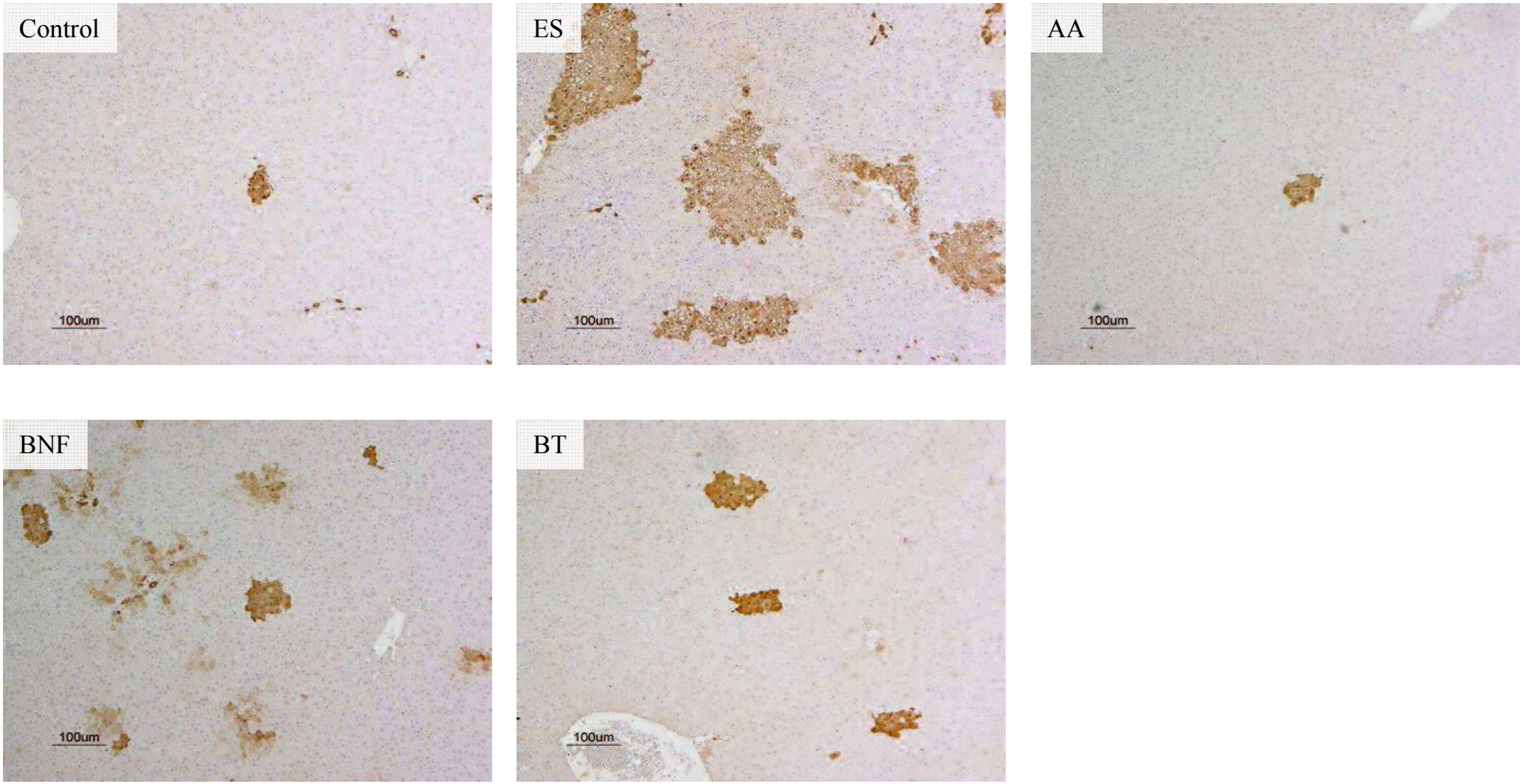

Fig. 5. Representative photographs of glutathione *S*-transferase (GST-P) immunohistochemistry in the residual livers of rats treated with estragole (ES), aristolochic acid (AA), β-naphthoflavone (BNF) and barbital (BT) in validation study of modified GPG model.

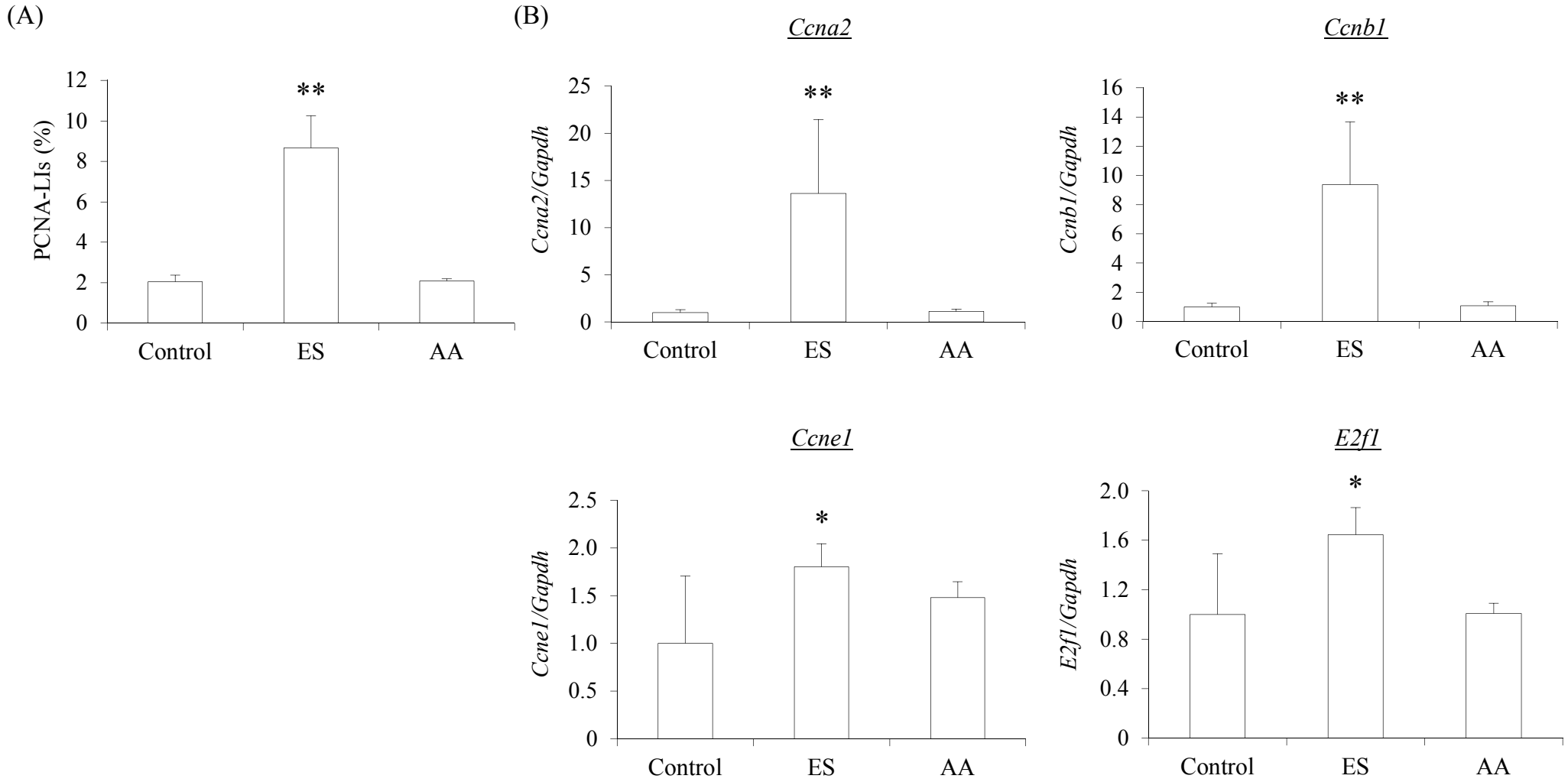

Fig. 6. Proliferating cell nuclear antigen-labeling indices (PCNA-LIs) for hepatocytes (A) and changes in mRNA levels of cell-cycle related factors (B) in the residual livers of *gpt* delta rats treated with estragole (ES) or aristolochic acid (AA). Values are means ± SD of data for 5 rats.
*, **Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

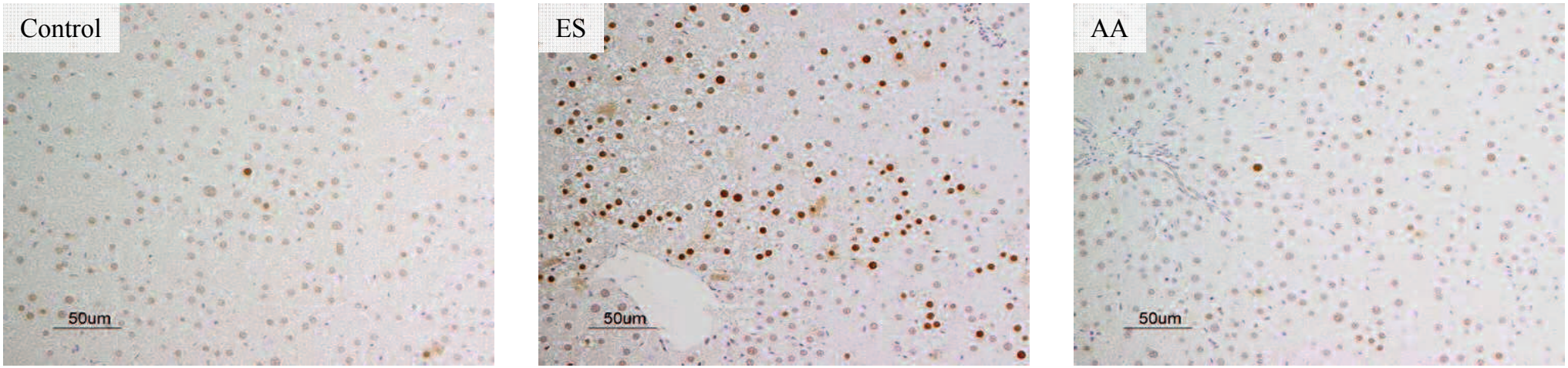

Fig. 7. Representative photographs of proliferating cell nuclear antigen (PCNA) immunohistochemistry in the residual livers of *gpt* delta rats treated with estragole (ES) or aristolochic acid (AA).

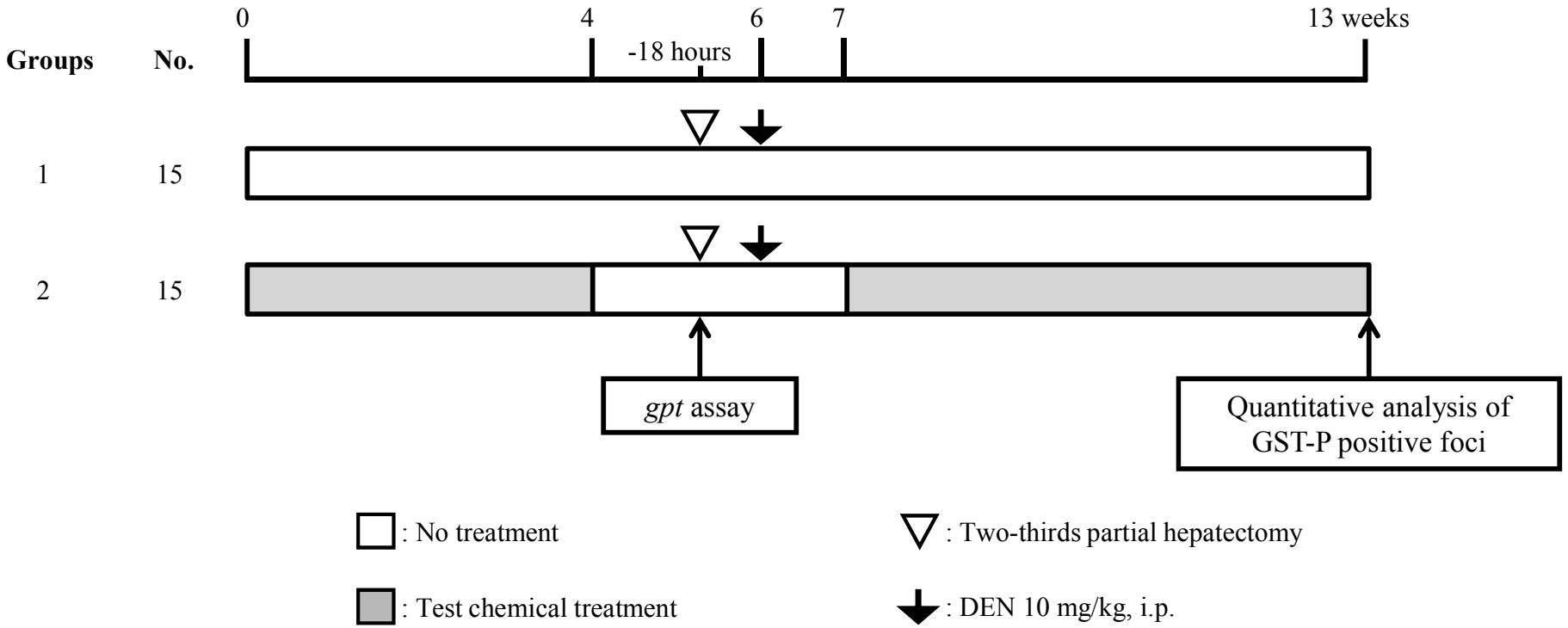

Fig. 8. Standard protocol for the GPG model. Animals were 6-week-old male F344 *gpt* delta rats. The *gpt* assay is performed in excised liver samples as indicator of *in vivo* mutagenicity. Tumor-promoting activities are evaluated based on the enhancement of glutathione *S*-transferase placental form (GST-P) positive foci induced by diethylnitrosamine (DEN) in residual liver samples.

Table 6. Quantitative analysis of GST-P positive foci in in chapter 2.

	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

Note. DADS, diallyl disulfide; PBO, piperonyl butoxide; PHE, phenytoin; ES, estragole; AA, aristolochic acid; BNF, β-naphthoflavone; BT, barbital; GST-P, glutathione *S*-transferase placental form.

[a] Mean ± SD.

*, ** Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

Table 7. *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	

Note. ES, estragole; AA, aristolochic acid; BNF, β-naphthoflavone; BT, barbital; MF, mutant frequency.

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

Table 8. 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 ± 0.32**	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

Note. ES, estragole; AA, aristolochic acid; BNF, β-naphthoflavone; BT, barbital.

[a] Number of colonies with independent mutations. [b] Mean ± SD.

*, ** Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

Table 9. Sammary: GPG model validation study

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

Note. ES, estragole; 2-AAF, 2-acetylaminofluorene; SF, safrole, IQ, 2-amino-3-methylimidazo[4,5-*f*]quinolone; BNF, β-naphthoflavone; BT, barbital; PBO, piperonyl butoxide; PHE, phenytoin; AA, aristolochic acid; APAP, acetaminophen; GST-P, glutathione *S*-transferase placental form.

[a] These results were obtained in the validation study of tentative protocol.

Chapter 3

A medium-term *gpt* delta rat model as an *in vivo* system for analysis of renal carcinogenesis and the underlying mode of action

3. 1. Introduction

Carcinogenicity is a key factor in safety assessments of environmental chemicals because resulting neoplastic lesions can be irreversible and often fatal. The kidney receives an abundant supply of blood in order to perform its vital roles in metabolism and excretion of xenobiotics, which may increase the risk of carcinogen exposure (Radford et al., 2013). In addition, as the kidney possesses phase I and phase II detoxification mechanisms, it is highly probable that DNA damage can be caused by reactive metabolites or oxidative stress generated during chemical metabolism (Choudohary et al., 2005; Mizerovská et al., 2011; Priestap et al., 2012; Kakehashi et al., 2013). Indeed, NTP background data for lifetime bioassays using rodents demonstrated that the kidney is the second organ targeted by chemical carcinogenesis after the liver (NTP, 2014). However, because tremendous amounts of time and large numbers of animals are required in lifetime bioassay using rodents, the ICH guideline recommends alternative *in vivo* studies, including medium-term rat liver animal models, e.g., the Ito model, or 6-month carcinogenicity models using transgenic mice, such as rasH2 and *p53*-deficient mice (ICH, 1997). In particular, quantitative analysis of GST-P foci, which is used as a preneoplastic hepatocyte marker in the Ito model, is very 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 chapters 1 and 2, I 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. In the GPG model, 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 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; NTP, 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.

3. 2. Materials and Methods

3. 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), AA, and phenylbutazone (PBZ) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3. 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.

3. 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.

3. 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 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.

3. 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; Umemura et al., 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.

3. 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 2 different doses of DEN, followed by treatment with NTA as a tumor-promoter.

3. 2. 4. 1. Animal treatment

The experimental design is described in Fig. 9. 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 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 4 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. 10) were diagnosed as preneoplastic lesions according to generally accepted guidelines (Dietrich and Swenberg, 1991a).

3. 2. 5. Experiment III

The animal model was validated using a genotoxic renal carcinogen, two non-genotoxic renal carcinogens, and a non-carcinogen.

3. 2. 5. 1. Animal treatment

Six-week-old F344/NSlc-Tg (*gpt* delta) rats (n = 15 per dose) were fed 50000 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 (Maekawa et al., 1987; Hiasa et al., 1992). The dose of AA was selected based on a previous report in which the *gpt* 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 (NTP, 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.

3. 2. 5. 2. *In vivo* mutation assay

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

3. 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. 3. Results

3. 3. 1. Experiment I

Fig. 11 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. 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. 12. 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. 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 10 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 11). The results of histopathological analyses of preneoplastic lesions are illustrated in Fig. 13. 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. 14. Calcium depositions were 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.

3. 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, I applied the concepts of the GPG model 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. 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 α_{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 (Nogueria 1987; Athar and Iqbal, 1998; Umemura et al., 2000), I performed a study to investigate optimal timing of DEN administration to induce renal tumors. I 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, I 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, I 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, I 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. 15).

To validate the GNP model established above, I applied 1 genotoxic renal carcinogen, 2 non-genotoxic renal carcinogens, and 1 non-carcinogen 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, I 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, 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 non-genotoxic carcinogen based on negative results in various *in vivo* genotoxicity tests (Machemer and Hess, 1971; Müller and Strasser, 1971; Rathenberg and Müllar, 1972; Gebhart and Wissmüller, 1973; Charles and Leonard; 1978; NTP, 1990b; Kari et al., 1995). 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 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 α_{2u}-globulin (Dietrich and Swenberg, 1991b). Treatment 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 α_{2u}-globulin in the proximal tubular epithelium. α_{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 12).

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

modes of action.

3. 5. Abstract

The kidney is a major target site of chemical carcinogenesis. However, a reliable *in vivo* assay for rapid identification of renal carcinogens has not been established. The purpose of this study was to develop a new medium-term *gpt* delta rat model (the GNP model) to facilitate identification of renal carcinogens. In this model, I carried out an *in vivo* mutation assay using unilaterally nephrectomized kidney tissue and a tumor-promoting assay using residual kidney tissue, with diethylnitrosamine (DEN) as the renal tumor initiator. To clarify the optimal time of DEN injection after nephrectomy, time-dependent changes in bromodeoxyuridine-labeling indices in the tubular epithelium of nephrectomized rats were examined. The optimal dose of DEN injection and sufficient duration of subsequent nitrilotriacetic acid treatment were determined for detection of renal preneoplastic lesions. The standard protocol for the GNP model was determined as follows. Six-week-old female *gpt* delta rats were treated with test chemicals for 4 weeks, followed by a 2-week washout period, and 40 mg/kg DEN was administered intraperitoneally to initiate renal carcinogenesis. Unilateral nephrectomy was performed 48 h before DEN injection, followed by *gpt* assays using excised kidney tissues. One week after DEN injection, rats were further exposed to test chemicals for 12 weeks, and histopathological analysis of renal preneoplastic lesions was performed as an indicator of tumor-promoting activity in residual kidney tissue. Validation studies using aristolochic acid,

potassium dibasic phosphate, phenylbutazone, and *d*-limonene indicated the reliability of the GNP model for predicting renal carcinogens and the underlying mode of action.

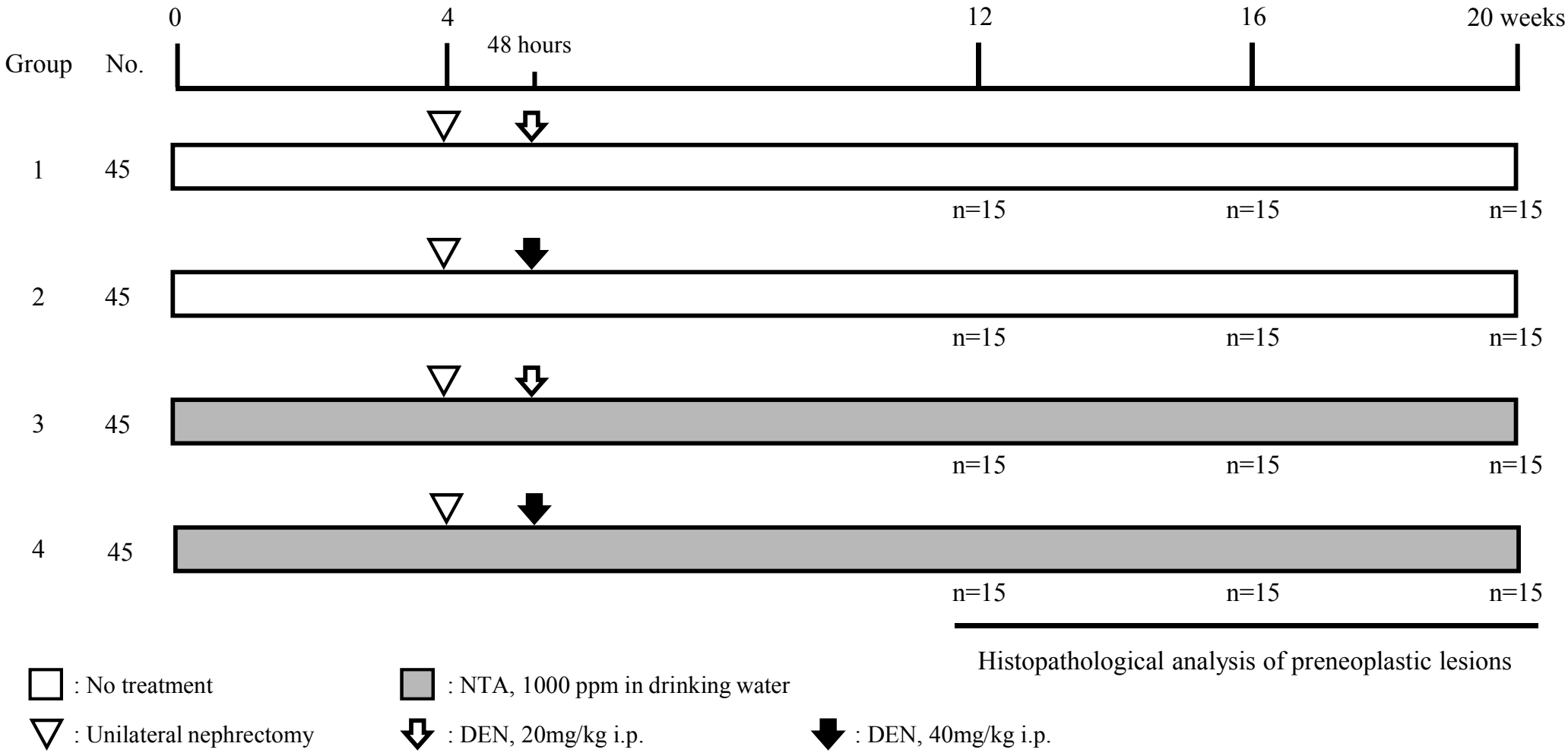

Fig. 9. Treatment protocol for experiment II in chapter 3. Animals were 6-week-old female F344 rats.
Development of preneoplastic lesions was evaluated histopathologically in residual kidneys at 12, 16, and 20 weeks after the start of the experiment, i.e., at 8, 12, and 16 weeks after the unilateral nephrectomy.

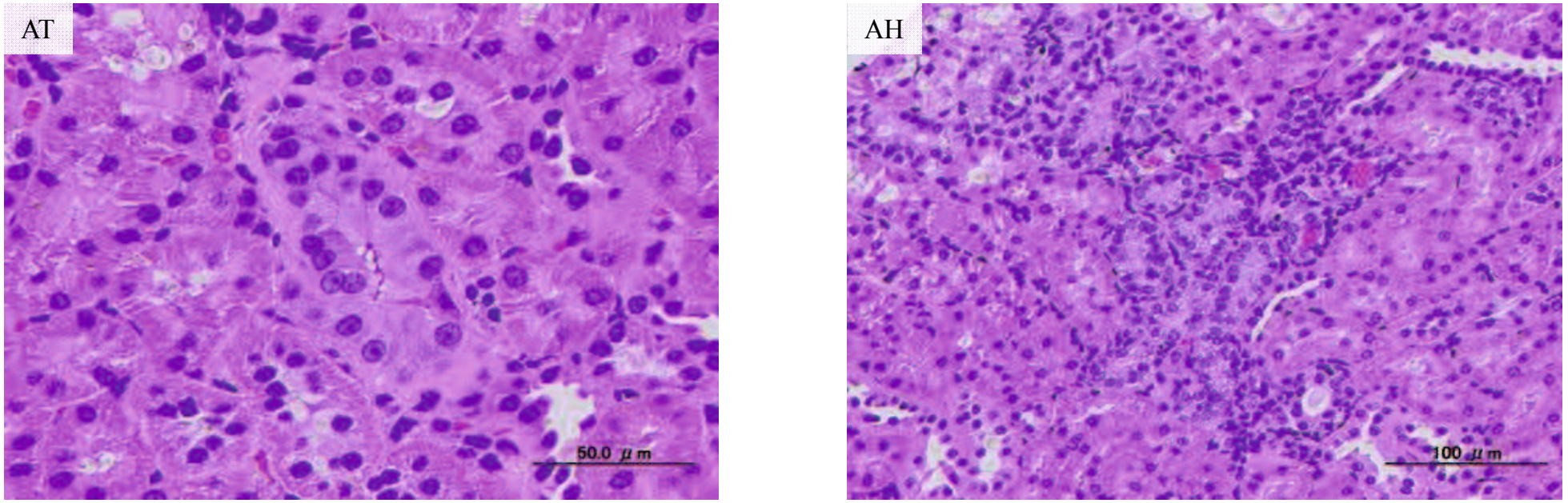

Fig. 10. Representative photographs of atypical tubules (ATs) and atypical hyperplasia (AH) in the kidney of rats. HE stain.

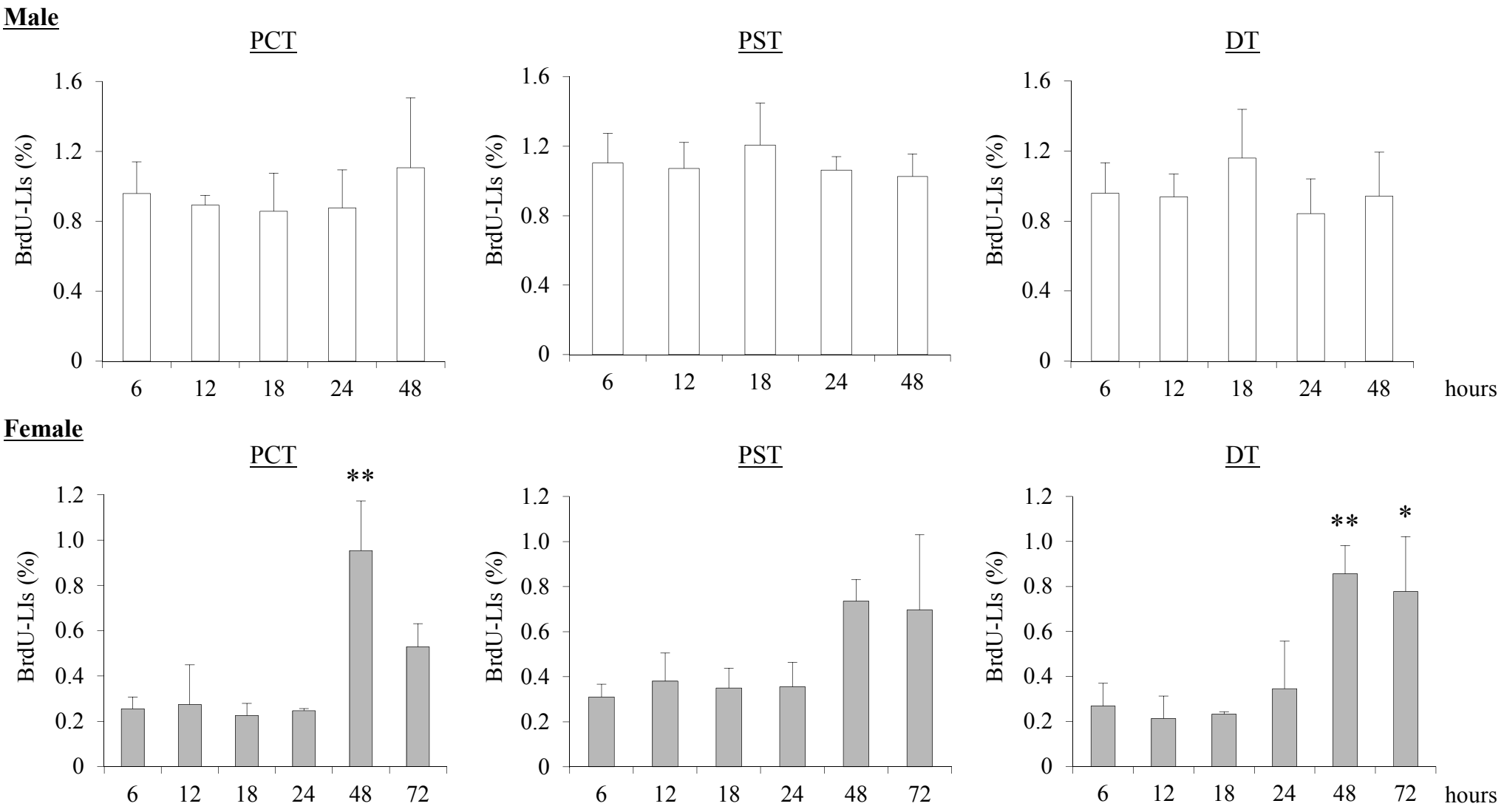

Fig. 11. 5-Bromo-2′-deoxyuridine-labeling indices (BrdU-LIs) in the proximal convoluted tubule (PCT), proximal straight tubule (PST), and distal tubule (DL) of residual kidneys of male (white column) and female (gray column) F344 rats after unilateral nephrectomy (n = 3). Values are means ± SD.
*,**Significantly different from the 6 h group at $P < 0.05$ and 0.01, respectively.

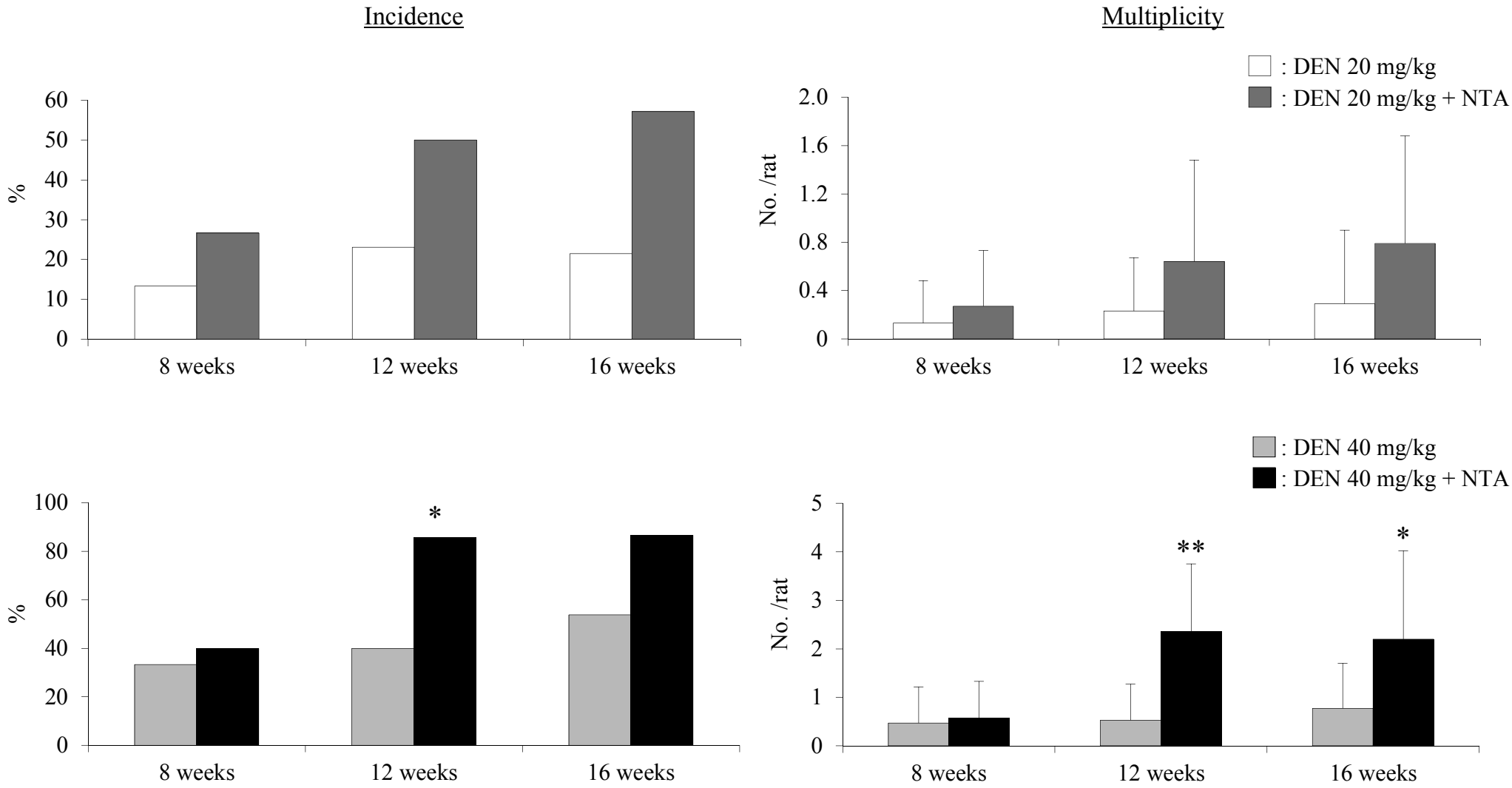

Fig. 12. Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with diethylnitrosamine (DEN) followed by trisodium nitrilotriacetic acid (NTA) treatment (n = 13–15). Values of multiplicity are means ± SDs. The horizontal axes represent the treatment period after unilateral nephrectomy. *,**Significantly different from the DEN 40 mg/kg group at $P < 0.05$ and 0.01, respectively.

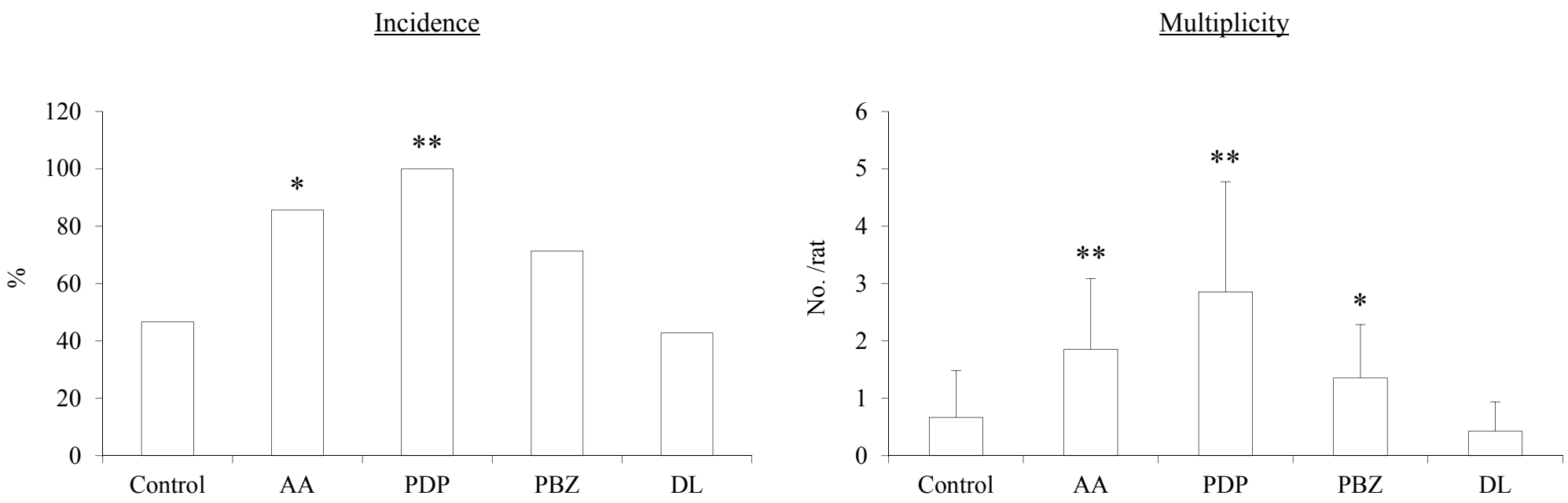

Fig. 13. Incidence and multiplicity of renal preneoplastic lesions in the residual kidneys of female F344 rats treated with aristolochic acid (AA), potassium dibasic phosphate (PDP), phenylbutazone (PBZ), and *d*-limonene (DL) in the validation study of GNP model (n = 14–15). Values of multiplicity are means ± SD. *,**Significantly different from the control group at $P < 0.05$ and 0.01, respectively.

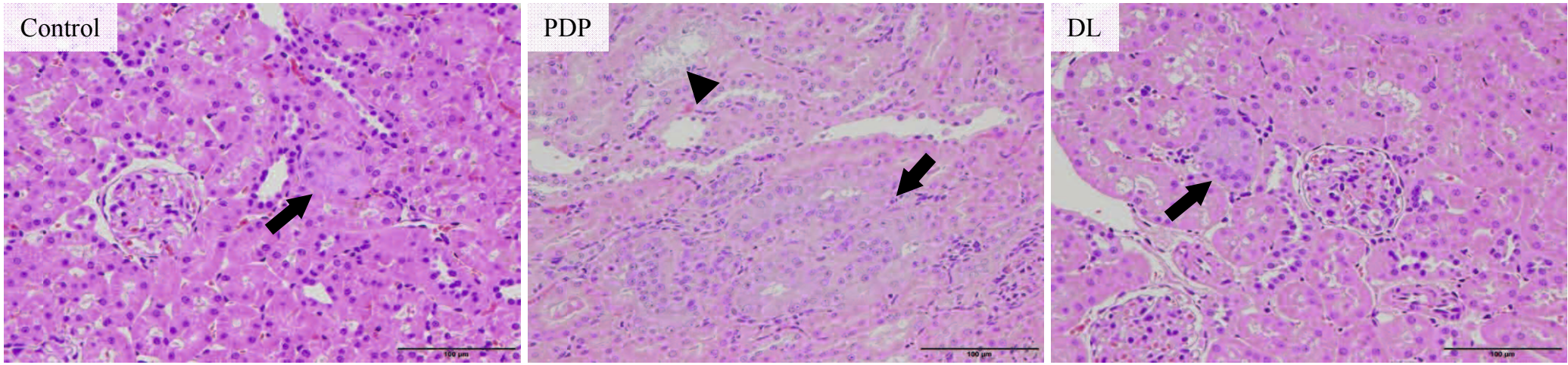

Fig. 14. Representative photographs of the residual kidneys of female F344 *gpt* delta rats treated with potassium dibasic phosphate (PDP) and *d*-limonene (DL) in the validation study of GNP model. Calcium depositions were observed in the proximal tubules of rats treated with PDP (arrowhead). There were no hyaline droplets indicating accumulation of α_{2u}-globulin in the proximal tubular epithelium in DL-treated rats. Arrows represent preneoplastic lesions in renal tubules. HE stain.

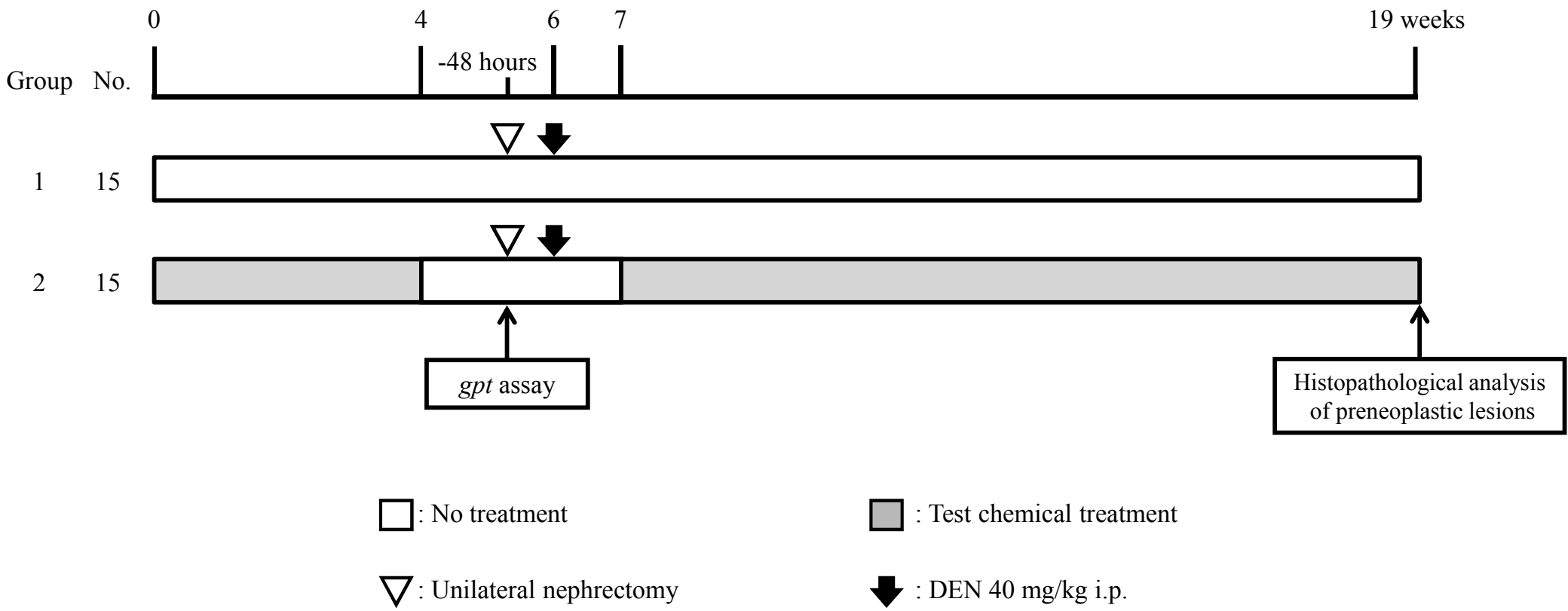

Fig. 15. Standard protocol for the GNP model. Animals were 6-week-old female F344 *gpt* delta rats. The *gpt* assay was performed in excised kidney samples as an indicator of *in vivo* mutagenicity. Tumor-promoting activities are evaluated based on the enhancement of preneoplastic lesions induced by diethylnitrosamine (DEN) in residual kidney samples.

Table 10. *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	

Note. AA, aristolochic acid; PDP, potassium dibasic phosphate; PBZ, phenylbutazone; DL, *d*-limonene; MF, mutant frequency.

** 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 11. 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 2bp	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

Note. AA, aristolochic acid; PDP, potassium dibasic phosphate; PBZ, phenylbutazone; DL, *d*-limonene.

[a] Number of colonies with independent mutations. [b] Mean ± SD.

* Significantly different from the control group at $P < 0.05$.

Table 12. Sammary: GNP model validation study

Test chemical	*gpt* assay	Histopathological analysis of preneoplastic lesions	Classification
AA	+	+	Genotoxic carcinogen
PDP	-	+	Non-genotoxic carcinogen
PBZ	-	+	
DL	-	-	Non-carcinogen

Note. AA, aristolochic acid; PDP, potassium dibasic phosphate; PBZ, phenylbutazone; DL, *d*-limonene.

General discussion

To establish the protocol of the GPG model, detailed conditions were determined based on earlier guidelines, previous reports, and the results of preliminary studies. In the GPG model, PH is performed to harvest samples for *gpt* assay to detect *in vivo* mutagenicity. Tumor-promoting potential then is evaluated by immunohistochemical analysis of GST-P positive foci in the residual liver tissue following induction by DEN. A test chemical treatment period of 4 weeks prior to the sampling by PH for *gpt* assay was selected based on the OECD guidelines (OECD, 2011). Notably, these guidelines state that 4 weeks of exposure is sufficient for the detection of gene mutations in the reporter gene in both rapidly and slowly proliferating tissues. Based on the concept of the initiation-promotion model, tumor-promoting potential is expected to be rapidly detectable by test chemical treatment following exposure to genotoxic carcinogens (Ito et al., 2003). DEN was selected as the tumor initiator in this model because DEN has been used for this purpose in conventional medium-term animal models; various reports show the correlation between administration of DEN and formation of GST-P positive foci in the rat liver (Ogiso et al., 1985; Ito et al., 2003; Kushida et al., 2005; Nagahara et al., 2010; Kakehashi et al., 2011). Given that genotoxic carcinogens were reported to induce gene mutations most effectively when administered 18 h after PH (Tsuda et al., 1980), we treated animals with DEN at 18 h after PH in this model. The results of a preliminary dose-response

study demonstrated that 10 mg/kg DEN was sufficient to induce GST-P positive foci in rat liver. The results of the preliminary study in which animals were exposed to PhB for different intervals following DEN administration revealed that 6 week test chemical treatment after PH was sufficient to detect tumor-promoting activity. Based on these overall considerations, I established the tentative GPG protocol (Fig. 1).

To validate the model, the assays, *gpt* assay using excised liver tissue; quantitative analysis of GST-P positive foci using residual liver tissue, were tested using a total of 7 different compounds. The genotoxic hepatocarcinogens and the genotoxic renal carcinogen yielded positive results when tested by *gpt* assay of the tissue obtained by PH. In the *gpt* mutation spectrum analysis, the majority of lesions induced in rat liver by the genotoxic hepatocarcinogens (2-AAF, IQ, and SF) were guanine-related mutations. This pattern was consistent with the mutagenicity of these compounds, all of which are known to induce formation of deoxyguanine DNA adducts (Schaaper et al., 1990; Ress and Leavitt, 1998; Schen et al., 2012). On the other hand, genotoxic renal carcinogen AA yielded a significant elevation in the number of AT:TA transversions in rat liver. This pattern was consistent with the known ability of this compound to generate reactive metabolites that produce deoxyadenine DNA adducts (Mei et al., 2006; Xie et al., 2012). These results indicated that genotoxic carcinogens and their underlying molecular mechanisms can be detected accurately with the GPG model.

Genotoxic or non-genotoxic hepatocarcinogens showed positive results in quantitative analysis of GST-P positive foci using residual liver tissues, which indicated that the tumor-promoting activities were sufficiently evaluated in this model. A non-carcinogen, APAP, yielded negative results in both *gpt* assay and GST-P analysis. Overall, data from our validation study demonstrated the potential development of new medium-term animal model in which *in vivo* mutagenicity and carcinogenicity can be evaluated in a single study.

However, a possible limitation of the tentative protocol is that DEN is administered in the course of test chemical treatment, which may lead to their interaction. Since induction of drug metabolic enzymes by xenobiotics is an adaptive response and generally is considered to be reversible (Maronpot et al., 2010), optimizing the length of the washout period in the protocol may preclude the interaction. Therefore, I designed a modified GPG protocol that included 2- and 1-week washout periods before and after DEN, respectively. Because CYP2E1, CYP1A, and CYP2B are considered to contribute to metabolic activation of DEN (Nims et al., 1994; Verna et al., 1996; Beltrán-Ramírez et al., 2008), elimination of the interaction of DEN and test chemical in the modified protocol were confirmed using CYP2E1 inhibitor DADS, CYP1A inducer PBO, and CYP2B inducer PHE. It has been reported that simultaneous administration of DEN and DADS significantly inhibited the formation of GST-P positive foci induced by DEN alone (Haber-Mignard et al., 1996). On the other hand, Fukushima et al.

(1997) showed that DADS exposure in the promotion phase did not affect the formation of GST-P positive foci. Therefore, the inhibitory effect of DADS on the formation of DEN-induced GST-P foci was presumed to reflect inactivation of CYP2E1 enzyme, which would in turn result in inhibition of metabolic activation of DEN. In the present study, normal levels of CYP2E1 activity were seen in PH tissue in rats treated with DADS with a washout period, whereas levels of CYP2E1 activity in rats continuously exposed to DADS (i.e., without a washout) were significantly decreased. In accord with these results, formation of GST-P positive foci was significantly inhibited in rats treated with DEN in the course of DADS treatment compared to that in rats treated with DEN alone. Although CYP1A or CYP2B activities in excised liver samples from rats treated with PBO or PHE in the original protocol were obviously increased, the respective values were unchanged compared to control group in livers excised from rats treated by the modified protocol with washout periods. On the other hand, DEN administered intraperitoneally to rats is essentially completely eliminated from the body within 1 week of cessation of dosing (Phillips et al., 1975). Therefore, a 1-week washout period after DEN administration was included in the modified protocol. The positive results of quantitative analysis of GST-P positive foci in rats treated with PBO and PHE demonstrated that tumor-promoting activity can be detected without any problem in the modified protocol. Based on the overall data, I established a modified protocol using the GPG model as described in Fig.

8.

The modified protocol of the GPG model was validated using several types of carcinogens, including a genotoxic hepatocarcinogen (ES), a genotoxic renal carcinogen (AA), and two non-genotoxic hepatocarcinogens (BNF and BT). Although AA was used in the validation study of the tentative protocol, AA was re-tested in the present study to demonstrate clearly that the GPG model can suggest the classification of novel potential carcinogens. *gpt* MFs were significantly increased in rats treated with ES and AA. Spectrum analysis in *gpt* mutant colonies showed that the majority of lesions induced by ES or AA treatment were adenine-related mutations. These results were consistent with previous reports showing that formation of deoxyadenine DNA adducts plays a main role in the genotoxicity of these carcinogens (Mei et al., 2006; Ishii et al., 2011). The formation of GST-P positive foci was significantly enhanced in rats treated with ES, BNF, or BT, demonstrating that tumor-promoting activity was sufficiently detected in this model.

In the validation study, the elevation of *gpt* MFs in rats treated with AA or ES was particularly striking. In terms of genotoxicity, there were no obvious differences between ES and AA, including both *gpt* MFs and mutation spectra. This observation implied that factors other than DNA damage and gene mutation could be involved. For further understanding of this outcome, cell proliferation-related parameters were investigated. We noted that PCNA-LIs and

mRNA expression of cell-cycle-related genes in residual liver tissue were significantly increased only in the ES-treated group. AA also showed no toxic effect in the liver in the validation study (data not shown). On the other hand, a single injection of AA has been reported to initiate hepatocarcinogenesis in a two-step carcinogenicity model (Rossiello et al., 1993). Therefore, lack of carcinogenicity of AA in the liver may indicate that AA does not exert cytotoxicity and therefore does not induce compensatory proliferation of hepatocytes. Furthermore, our results demonstrated that excised or residual liver tissues are useful for further investigation of underlying modes of action, and that the GPG model may suggest the existence of a new class of carcinogen, one that exerts genotoxicity but not carcinogenicity.

Overall, I have established the GPG modified protocol, in which a suitable washout period was added to avoid mutual effects of DEN and the test chemical. Elimination of the interaction was confirmed using compounds that influence several types of CYP activity. The reliability of the modified GPG model was validated by testing with several types of known carcinogens.

On the other hand, although kidney is a major target organ of chemical carcinogenesis, there are no reliable *in vivo* assays capable of rapidly predicting renal carcinogenic potential. This fact is largely due to the lack of useful preneoplastic markers in renal cells, a situation that contrasts with that in hepatocytes. Therefore, I attempted to develop a new medium-term animal

model (the “GNP” model), one that can detect renal carcinogens by *in vivo* mutagenicity, by modifying the concept of the GPG model. In the GPG model, PH is performed to harvest samples for use in the *gpt* assay, and DEN is administered to the animals, at 18 h after PH, to induce gene mutation, taking advantage of the compensatory cell proliferation that occurs after PH. In the GNP model, UN is performed in place of the PH performed in the GPG model. In parallel with the GPG model, a test chemical treatment period of 4 weeks prior to the *gpt* assay was selected for use in the GNP model. Detailed kinetics of compensatory cell proliferation of residual kidney after UN, including sex differences, have not been reported. To determine the suitable timing of DEN treatment for testing of the tumor-promoting assay, a time course of cell proliferative activities of residual kidney tissues (tissues remaining after UN) in male and female rats had to be investigated. We observed that cell proliferative activities in the renal cells, as demonstrated by BrdU-LIs, rose following UN; values peaked at 48 h after UN in all types of renal cells examined in female rats, whereas no obvious changes were observed in male rats. It has been reported that UN induces hyperplastic mechanisms in female rats and hypertrophic mechanisms in male rats (Mulrony et al., 1996; Mulrony and Pesce, 2000). Since hyperplastic mechanisms also have been observed in young male rats following UN (Okada et al., 2010), sexual hormones could play a key role in these sex differences. Therefore, we selected female rats for use in the GNP model, and opted to perform DEN injection at 48 h after UN. In addition,

the use of female rats was expected to eliminate the interference in terms of human risk assessment represented by α_{2u}-globulin-mediated carcinogen, a male rat-specific class of carcinogens. In the GNP model, DEN was selected as the tumor initiator based on the existence of various reports showing a relationship between DEN administration and renal tumor formation (Noguera, 1987; Athar and Iqbal, 1998; Umeura et al., 2000). Although there have been no reports of specific preneoplastic enzymes for renal cells that would serve a role equivalent to that of GST-P in hepatocytes, AT and AH which can easily be distinguished from normal renal cells have been demonstrated to possess predictive value for the assessment of renal carcinogenicity (Dietrich and Swenberg, 1991a). Therefore, formation of AT and AH induced by DEN were selected for use as endpoints in determining tumor-promoting activity in the GNP model.

In order to determine the optimal dose of DEN and optimal duration of test chemical exposure to detect tumor-promoting activity of test chemicals, the tentative protocol for the GNP model consisted of administering DEN at 20 and 40 mg/kg, followed by NTA treatment for 8, 12, or 16 weeks after UN. Formation of preneoplastic lesions was significantly enhanced in rats treated with 40 mg/kg of DEN followed by NTA treatment for 12 or 16 weeks after UN compared to lesion formation in rats that received DEN alone. Therefore, a dose of 40 mg/kg DEN and a duration of test chemical exposure of 12 weeks after UN were selected for use in the

formal GNP model. In addition, since the efficacy of a washout period for avoiding possible interaction of DEN and test chemical were demonstrated in the GPG model, 2- and 1-week washout periods before and after DEN administration, respectively, also were incorporated into the GNP model. The resulting standard protocol for my GNP model is described in Fig. 15.

The GNP model established above was validated using several types of renal carcinogens. AA was reported to induce renal cell tumors in rats by means of genotoxic mechanisms, with AA-specific deoxyadenine DNA adducts playing a main role in the induction of gene mutation by this compound (Mengs et al., 1982; Mei et al., 2006). In the present work, *gpt* MFs in UNed kidney tissue were significantly increased by AA treatment. In terms of mutation spectrum analysis, the largest increases were observed in the number of AT:TA transversions, a pattern that could be responsible for the formation of AA-specific deoxyadenine DNA adducts. Previous work demonstrated that *gpt* delta rats are useful for investigating the relationship between production of chemical-specific DNA adducts and gene mutations (Ishii et al., 2014). Therefore, I propose that further investigation of detailed modes of action, including measurement of DNA modification, could be conducted using excised or residual kidney samples in the GNP model. Specifically, it may be possible to measure using LS-MS/MS the formation of chemical-specific DNA adducts in the GNP model. These additional investigations may be helpful for understanding the relationship between formation of DNA adducts and gene

mutations. Additionally, note that development of preneoplastic renal lesions was significantly enhanced in rats treated with AA, indicating that AA has tumor-promoting potential in the kidney. In the GPG model, AA showed negative results in a tumor-promotion assay, which is consistent with the observation that the carcinogenic target site of AA is kidney and not liver (Mengs et al., 1982).

In the GNP model, non-genotoxic carcinogens (PDP and PBZ) showed negative results by the *gpt* assay and positive results by analysis of preneoplastic lesions, implying that the GNP model correctly identifies renal tumor-promoter compounds. In addition, calcium deposition in proximal tubules was observed in the residual kidney of rats treated with PDP; this deposition is considered to be a key factor in the tumor-promoting activity of PDP. DL-induced accumulation of α_{2u}-globulin in the proximal tubule has been shown to enhance cell proliferative activity, thereby ultimately leading to the formation of renal cell tumors (Dietrich and Swenberg, 1991b). α_{2u}-Globulin is produced in the liver and excreted through the kidney in male rats, but homologous proteins have not been observed in other animals, including female rats and humans (Hard, 1998; Doi et al., 2007). Therefore, enhancement of renal cell tumor formation mediated by α_{2u}-globulin could be a false-positive in terms of human risk assessment. As expected, DL yielded negative results by both *gpt* assay and analysis of preneoplastic lesions in the GNP model using female rats. In addition, hyaline droplets in proximal tubules, a marker

for accumulation of α_{2u}-globulin, were not observed in residual kidney tissue. These results suggested that false-positive results involving accumulation of α_{2u}-globulin can be avoided in the GNP model by using female rats. Overall, data from my validation study demonstrated the usefulness of the GNP model for predicting renal carcinogens along with underlying modes of action.

Conclusion

In the present studies, I have established new medium-term *gpt* delta rat models for predicting chemical carcinogenicity and the underlying modes of action in the liver and the kidney. I designated these models as GPG and GNP models, respectively. In these models, *in vivo* mutagenicity and tumor-promoting activity can be evaluated simultaneously. In addition, further analysis to investigate underlying modes of action can be incorporated into these models. Because multiple parameters involved in chemical carcinogenesis can be evaluated concurrently, GPG and GNP models can be used in accordance with the 3Rs principle of animal testing, providing valuable information regarding human risk assessment and improving understanding of chemical carcinogenesis.

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