Mechanisms of chemopreventive effects of 8-methoxypsoralen against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced mouse lung adenomas

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Recently we reported that the occurrence of lung adenoma caused by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was completely prevented by pretreatment of female A/J mice with 8-methoxypsoralen, a potent inhibitor of cytochrome P450 (P450 or CYP) 2A [Takeuchi et al. (2003) Cancer Res., 63, 7581–7583]. Thus, the aim of this study was to confirm that 8-methoxypsoralen exhibits chemopreventive effects by inhibiting CYP2A in the mouse lung. The involvement of CYP2A in the metabolic activation of NNK in the lung was first evidenced by the fact that the mutagenic activation of NNK by mouse lung microsomal enzymes in a genetically engineered Salmonella typhimurium YG7108. The expression of mRNA for CYP2A5, but not for CYP2A4 or CYP2A12, in the mouse lung was proven by reverse transcriptase–polymerase chain reaction, probably indicating that CYP2A5 present in the mouse lung was involved in the metabolic activation of NNK. In accordance with these in vitro data, treatment of gpt delta transgenic mice with 8-methoxypsoralen prior to NNK completely inhibited the mutation of the gpt delta gene. The in vivo chemopreventive effects of 8-methoxypsoralen towards NNK-induced adenoma was seen only when the agent was given to female A/J mice prior to, but not posterior to, NNK, lending support to the idea that NNK is activated by CYP2A5 in the mouse lung as an initial step to cause adenoma. The inhibition by 8-methoxypsoralen of NNK-induced adenoma was seen in a dose-dependent manner; the dose to show apparent 50% suppression was calculated to be 1.0 mg/kg. To our surprise, CYP2A protein(s) was expressed in the lesion of NNK-induced lung adenomas, probably suggesting that 8-methoxypsoralen could inhibit the possible occurrence of further mutation of the adenoma cells induced by NNK. Based on these lines of evidence, we propose that 8-methoxypsoralen inhibits the CYP2A5-mediated metabolic activation of NNK in the mouse lung, leading to the prevention of NNK-induced adenoma.

Introduction

Tobacco smoke contains >4000 components; at least 60 chemicals including nitrosamines are proven to be carcinogenic. Among them, NNK, a tobacco-specific N-nitrosamine, is believed to be one of the most promising candidates of lung carcinogen in humans, since NNK is known to induce lung tumors in laboratory animals such as mice, rats and hamsters (1–4). NNK is known to be metabolically activated to elicit their genotoxicity (1,5). The first activation step of NNK is thought to be the methylene or methyl hydroxylation of the carbon atom located at the α-position of the N-nitroso group primary mediated by P450, leading to the formation of electrophiles which can methylate or pyridyloxobutylate DNA, respectively (1). This initial event has been reported to result in the formation of O^\alpha-methylguanine, an adduct that leads to GC→AT transitional mispairing and the subsequent activation of the K-ras proto-oncogene (6,7), an initiating event in tumor development.

P450 is a heme-containing enzyme responsible for the oxidation of a wide variety of exogenous compounds such as drugs, environmental pollutants and foodstuffs, and endogenous compounds including steroids, fatty acids and prostaglandins (8–10). Some of the oxidative reactions catalyzed by P450 result in the formation of reactive intermediates, which bind to endogenous macromolecules such as DNA, RNA and proteins to induce organ toxicities (11,12). Thus, the catalytic property and the content of P450 can be considered to be the determinants of the formation of reactive metabolites and the subsequent toxicity of chemicals.

CYP2A6 is one of the major members of P450 expressed in human livers (13) and to lesser amounts in the lung (14,15). This cytochrome is involved in the mutagenic activation of a wide variety of promutagens including NNK (5,16,17). The genetic polymorphism of CYP2A6 was originally discovered as one of the causes of inter-individual differences in the metabolism of coumarin (18,19). In further study by us, we first found the novel deletion-type mutants of the CYP2A6 gene (CYP2A6*4A and *4B). Following this, we also found single nucleotide polymorphisms (CYP2A6*7 and *11), reducing CYP2A6 enzymatic activities in a Japanese population (20–23). Combining these two concepts that CYP2A6 is capable of activating NNK and that there are genetic polymorphisms in CYP2A6, we hypothesized that subjects harboring these

Abbreviations: ABC, avidin-biotin complex; CPR, NADPH-cytochrome P450 reductase; GC, guanine–cytosine; N, nicotinamide; ENU, ethylnitrosourea; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; P450, general term for cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcriptase.

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alleles of the CYP2A6 gene have less risk for tobacco-related lung cancer. Our large-scale epidemiological studies clearly supported this idea (24–26). Based on these results, it was assumed that CYP2A6 determines the cancer risk caused by tobacco-smoking via its ability to activate the nitrosamines. However, it was necessary to further ascertain this idea using experimental animals.

8-Methoxypsoralen is a naturally occurring furanocoumarin derivative found in many plant species such as celery, figs, limes, parsnip and parsley (27). Oral administration of 8-methoxypsoralen, followed by ultraviolet A light, has photosensitizing effects. Thus, this drug has been used clinically for the treatments of psoriasis (28,29). 8-Methoxypsoralen is also known as a potent mechanism-based inhibitor of CYP2A6 and the orthologous forms, mouse CYP2A5 and rat CYP2A3 (30–32). Recently, we demonstrated that lung tumorigenesis caused by NNK was completely suppressed in female A/J mice which had been pretreated with 8-methoxypsoralen (33), probably indicating that 8-methoxypsoralen prevented the occurrence of lung tumorigenesis by inhibiting the metabolic activation of NNK catalyzed by CYP2As present in the lungs of mice. However, we still needed to provide direct evidence as to whether or not 8-methoxypsoralen inhibited the mutagenic activation of NNK.

Thus, in the present study, we first examined the inhibitory effects of 8-methoxypsoralen on the mutagenic activation of NNK by both liver or lung microsomes from mice and the forms of mouse CYP2A (CYP2A4, CYP2A5 or CYP2A12) expressed in the Salmonella typhimurium YG7108. To evaluate the in vivo effects of 8-methoxypsoralen on the mutagenicity and tumorigenicity of NNK in vivo, we carried out two assays using the gpt delta transgenic mice carrying a shuttle vector as a model of mutation and using female A/J mice as an experimental animal model for lung tumorigenesis, respectively. Based on results presented in this paper, we propose that suppression of the initiation step of carcinogenesis by pretreatment with CYP2A inhibitors can result in the chemoprevention of lung cancer caused by tobacco smoking.

Materials and methods

Animals and chemicals

Seven-week-old female C57BL/6J mice, 9-week-old female C57BL/6J transgenic mice homozygous for the gpt delta gene and 5-week-old female A/J mice (Japan SLC, Shizuoka, Japan) were used. NNK (purity ≥ 99%) was purchased from Tokyo Research Chemicals (Toronto, Canada). ENU and 8-methoxypsoralen were obtained from Sigma (St Louis, MO). All other chemicals and solvents were of the highest grade commercially available.

Expression of CYP2A in S. typhimurium YG7108 cells

Original S. typhimurium YG7108 and three strains of the genetically engineered S. typhimurium YG7108 cells, each co-expressing a mouse CYP2A and CYP2B or CYP2F1, were used in our laboratory, were used. The P450 and CPR in the genetically engineered S. typhimurium YG7108 cells were expressed according to the method previously described by Fujita et al. (34). The content of P450 holo-protein in the S. typhimurium cells was determined by Fe2+ -CO difference spectra according to the method of Omura and Sato (35). The expression levels of P450 ranged from 77 nmol/l culture for CYP2A4 to 340 nmol/l culture for CYP2A12. The activity of CPR in sonicated bacterial cells was measured with cytochrome c as an electron acceptor by measuring the absorbance change at 550 nm at 20°C according to the method of Philips and Langdon (36). The unit of CPR was defined as the amount of the enzyme that reduced 1 μmol of cytochrome c/min. The molar ratio of the expressed CPR to P450 varied from 1.0 for CYP2A12 to 1.9 for CYP2A4.

Mutation assay

The mutation assay with the parental YG7108 cells was performed as described by Maron and Ames (37), with modifications. Briefly, when needed, NNK was metabolically activated outside the S. typhimurium YG7108 cells. The effects of the specific inhibitor of P450 forms on the activation of NNK by liver or lung microsomes from mice were examined and lung microsomes (70 pmol of total P450/reaction) from 7-week-old female C57BL/6J mice were added, which had been suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 10% (v/v) glycerol as described previously (38). The parental S. typhimurium YG7108 cells were pre-exposed to 100 or 1000 μM of NNK in the presence or absence of various concentrations of specific inhibitors, substrates or antibodies of P450 at 37°C for 20 min before plating. The modifiers employed were α-naphthoflavone (CYP1A), coumarin (CYP2A), 8-methoxypsoralen (CYP2A), chloramphenicol (CYP2B), tolbutamide (CYP2C), quinidine (CYP2D), chlorozoxanole (CYP2E1) and ketoconazole (CYP3A). All inhibitors were dissolved in dimethylsulfoxide to give a final concentration of the organic solvent in the incubation mixtures lower than 1%. Antibodies against rat CYP2A1 were raised in rabbits to be added to the reaction mixtures up to 50 μg/ml protein. The mixtures were preincubated at 25°C for 30 min, followed by the addition of NNK as previously described. An NADPH-generating system was added to the reaction mixtures to start incubation.

When the genetically engineered S. typhimurium YG7108 was used, NNK was metabolically activated inside the bacterial cells without addition of any other enzymes. In this experiment, the mutagen-producing activity of a form of mouse CYP2As in the activation of NNK was expected to be clarified. The Salmonella cells were first preincubated to 0.1–250 μM of NNK for 20 min before plating. The NADPH-generating system was not added to the reaction mixtures since the mutagenic activation of promutagens was not affected by the addition of NADPH, probably because NADPH present in the bacterial cells was utilized as an electron donor. The plates were incubated at 37°C for 2 days. Assays were carried out at least twice in duplicate. When the variability of the values of duplicate determinations was within ±20%, the results were adopted. Induced revertants/pmole of NNK were calculated as the number of induced colonies per pmol of NNK content expressed in the tester strain. The spontaneous revertant number per plate ranged from 20 to 40. Apparent kinetic analysis for the activation of NNK was performed using a computer program (Microgen, Microlab, Software, Northampton, MA) designed for non-linear regression analysis. The concentration of 8-methoxypsoralen which decreased the number of revertants to 50% of control (in the absence of 8-methoxypsoralen) was defined as IC50.

Detection of CYP2As in the mouse lung

Total RNAs were prepared from the lung or liver of the 9-week-old female C57BL/6J mouse using RNAasy Midi kit (QIAGEN, Hilden, Germany). To examine the expression levels of CYP2A mRNA in the mouse tissues, reverse transcriptase–polymerase chain reaction (RT–PCR) was carried out. Total RNA (1 μg) and oligodeoxothyimidyl acid primer (0.5 μg) were mixed, incubated at 70°C for 10 min and then cooled on ice. Subsequently, moloney murine leukemia virus reverse transcriptase (20 units) (Toyobo, Tokyo, Japan), RNase inhibitor (20 U) (TaKaRa, Tokyo, Japan), and 0.5 mM of each deoxynucleoside triphosphates, were added to the RNA-primer mixtures and then incubated at 42°C for 50 min. PCR was performed in a solution containing cDNA synthesized in the above reaction mixtures (1 μM), 1.5 mM MgCl2, 0.2 mM each of four deoxynucleoside triphosphates, each primer (1 μM), AmpliTaq Gold polymerase (2.5 U) (PerkinElmer Life Sciences) and 10× AmpliTaq reaction buffer (5 μl) (PerkinElmer Life Sciences). PCR was carried out under the following conditions: 30 cycles of reactions composed of cycle denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The PCR products (96 bp for CYP2A4 and CYP2A25 and 143 bp for CYP2A12) were subjected to a 2% agarose gel and then visualized by ethidium bromide staining. The sequences of primers used are as follows: CYP2A4, 5’-GCCGACTCTATGAGTTGTTT-3’ and 5’-TTATAAGTCC-CTCCAGGCT-3’; CYP2A5, 5’-GCCAAGCTCTATGAGTTGTTT-3’ and 5’-TTATAAGTCCCTCTGCCAGGGCC-3’; CYP2A12, 5’-TTAGTCCATTGTCTCGGC-3’ and 5’-GGTACTCTATACTGAGTTG-3’; β-actin, 5’-ATTGGTCTGACAGGATCAGAAG-3’ and 5’-GCTCAGAGAGGACACTGAT-3’.
volume of saline as a vehicle control. Two weeks after the final treatment, mice were killed under ether anesthesia. They were quickly frozen in liquid nitrogen and stored at −80°C until analysis. The aminopterin mutagenesis assay was performed as previously described (39).

**Tamorigenicity test**

Female A/J mice were maintained in the Animal Facility of the Faculty of Medicine, Kagawa University, according to the institutional animal care guidelines. The animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, Oriental MF (Oriental Yeast), under controlled conditions of humidity at 60% ± 10%, lighting with 12 h light/dark cycle and temperature at 24 ± 2°C.

The animals were pretreated with 8-methoxypsoralen at a dose of 0.125, 1.25 or 12.5 mg/kg body wt in 0.2 ml corn oil or an equal volume of corn oil as a vehicle control via stomach tube, daily for 3 days. One hour after the last pretreatment, animals were given a single dose of NNK at a dose of 100 mg/kg body wt in 0.1 ml saline via p.o. or an equal volume of saline as a vehicle control. The experiment was terminated at 16 weeks after the first 8-methoxypsoralen pretreatment. In separate experiments, three daily doses of 8-methoxypsoralen (12.5 mg/kg) were given to mice 1, 3 and 7 days after a single dose of NNK injection. At autopsy, their lungs were excised and weighed, infused with 10% neutral buffered formalin and carefully inspected grossly. All of the macroscopically detected lung nodules were counted, and adenomas were diagnosed according to the criteria of ‘Tumors of the Mouse’ (40), and the number of hyperplasias and adenomas was counted under a microscope.

**Immunohistochemistry**

Lungs were immunostained for CYP2A by the ABC method, all staining processes from deparaffinization to counterstaining with hematoxylin being performed automatically using the Ventana Discovery™ staining system (Ventana Medical Systems, AZ, USA). Anti rabbit CYP2A1 polyclonal antibodies were used at 1:50 dilution.

**Statistical analysis**

The incidence of lung proliferative lesions were analyzed by the Fisher’s exact probability test and data for multiplicity by Student’s t-test.

**Results**

**Effects of CYP2A inhibitors and anti-CYP2A antibodies on the mutagenic activation of NNK catalyzed by liver and lung microsomes from mice**

To estimate a possible P450 form(s) responsible for the mutagenic activation of NNK in mice, effects of the representative inhibitors of P450s and anti-P450 antibodies on the genotoxic activation of NNK catalyzed by liver and lung microsomes from mice were examined. Among the inhibitors tested, coumarin and 8-methoxypsoralen, the inhibitor of CYP2A, efficiently inhibited the mutagenic activation of NNK catalyzed by liver microsomes (Figure 1A). Anti-CYP2A1 antibodies efficiently inhibited the activation of NNK by mouse liver microsomes (Figure 1B).

As was seen with liver microsomes, 8-methoxypsoralen and anti-CYP2A1 antibodies inhibited the mutagenic activation of NNK catalyzed by lung microsomes in a dose-dependent manner (Figure 2).

**Metabolic activation of NNK by mouse CYP2As expressed in S typhimurium YG7108**

To determine which one of the mouse CYP2A forms is responsible for the mutagenic activation of NNK, we
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Fig. 3. Mutagenic activation of NNK by CYP2A4, CYP2A5 and CYP2A12 expressed in S. typhimurium YG7108 cells. NNK (0.1–250 nM) was exposed to S. typhimurium YG7108 cells expressing a mouse CYP2A. Closed diamond, closed circle and open circle represent the results with S. typhimurium YG7108 cells expressing CYP2A4, CYP2A5 and CYP2A12, respectively. Each value and bar indicates the mean and SD (range) of duplicate determinations, respectively.

Fig. 4. Expression levels of mRNAs for CYP2A4, CYP2A5 and CYP2A12 in mouse lungs. Total RNA was prepared from lungs and livers of female mice. The expression of mRNAs for CYP2A4, CYP2A5 and CYP2A12 in the mouse lung or liver was determined by RT–PCR as described in Materials and methods. The sequence of specific primers for the mouse CYP2As is also described under Materials and methods. One tenth of each PCR solution was applied and separated in a 2% agarose ethidium bromide-stained gel. Each lane represents three individual samples.

performed the mutation assays using the genetically engineered S. typhimurium YG7108 each expressing CYP2A4, CYP2A5 or CYP2A12 together with CPR. As shown in Figure 3, CYP2A4 and CYP2A5, but not CYP2A12, was involved in the mutagenic activation of NNK. Apparent kinetic parameters for the mutagenic activation of NNK by CYP2A4 and CYP2A5 were calculated from the standard Michaelis–Menten velocity equation. Apparent $K_m$ and $V_{max}$ values for the activation by CYP2A4 and CYP2A5 were estimated to be 0.17 ± 0.03 µM and 770 induced revertants/pmol CYP2A4/µM and 0.18 ± 0.04 µM and 750 induced revertants/pmol CYP2A5/µM, respectively. Thus, both CYP2A forms showed similar apparent $V_{max}/K_m$ values (4500 for CYP2A4 and 4200 for CYP2A5).

However, examination for the expression of CYP2As in mouse lung by RT–PCR indicated that mRNA for CYP2A5, but not for CYP2A4, was expressed, as shown in Figure 4. Taken together, these results suggested that CYP2A5 was a principal enzyme responsible for the mutagenic activation of NNK in the mouse lung.

Effects of 8-methoxypsoralen on in vivo mutation caused by NNK in the gpt delta transgenic mice

To elucidate the role of mouse CYP2A in NNK-induced in vivo mutagenesis, we assessed the effects of 8-methoxypsoralen against NNK-induced mutation by using the transgenic mice carrying the gpt delta gene as a monitor of mutation. Oral feeding of 8-methoxypsoralen did not result in any apparent toxic effects even at a high concentration of 8-methoxypsoralen (100 mg/kg). No significant change was seen in food and water consumption and body wt (data not shown). As shown in Figure 5, the frequency of spontaneous mutation in the gpt delta gene in the lung was 6.4 × 10⁻⁶. A single i.p. injection of NNK resulted in a 10-fold higher frequency of mutation (62.2 × 10⁻⁶). The NNK-induced mutations in the lung were reduced by 89 and 94% when mice were pretreated with 8-methoxypsoralen at a dose of 12.5 or 50 mg/kg, respectively, prior to NNK. On the other hand, pretreatment of mice with 8-methoxypsoralen did not reduce ENU (a direct mutagen)-induced mutation whereas treatment of mice with ENU resulted in a 9-fold higher frequency of mutation (55.0 × 10⁻⁶) in the gpt gene of the lung.

Effects of 8-methoxypsoralen on the NNK-induced lung tumors in A/J mice

As described previously, our previous study had demonstrated that pretreatment of female A/J mice with 8-methoxypsoralen strongly inhibited NNK-induced lung tumorigenesis (33). Here, we further investigated the inhibitory effects of 8-methoxypsoralen on the NNK-induced lung tumorigenesis. Lung whitish nodules were readily detected in the group of mice treated with NNK alone macroscopically but were rare in the group of mice treated with 8-methoxypsoralen alone and the group of mice pretreated with 12.5 mg/kg of 8-methoxypsoralen + NNK (Table I). Lung carcinoma could not be seen in any of the animals. Numbers and/or incidence of macroscopically observed lung nodules induced by NNK were reduced by 8-methoxypsoralen in a dose-dependent manner ($P < 0.01$). The numbers of tumors/mouse were reduced from
21.04 (NNK alone) to 17.05, 7.89 and 1.25 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxypsoralen, respectively, with the inhibitions by the latter two doses being statistically significant ($P < 0.01$).

The multiplicities and incidence of lung hyperplasias and adenomas are also summarized in Table I. Numbers and/or incidence of microscopically observed adenoma and hyperplasia induced by NNK were also reduced by 8-methoxypsoralen in a dose-dependent manner. Numbers of adenoma and hyperplasia were reduced from 15.61 (NNK alone) to 11.90, 5.44 and 1.15 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxypsoralen, respectively (Table I). Similarly, adenoma numbers were reduced from 13.44 to 10.25, 4.44 and 0.85 by 0.125, 1.25 and 12.5 mg/kg body wt of 8-methoxypsoralen, respectively, with the inhibitions by the latter two doses being statistically significant ($P < 0.01$).

To examine the mechanism(s) for the suppression of the NNK-induced lung tumorigenesis by 8-methoxypsoralen, we determined whether or not treatment of mice with 8-methoxypsoralen after the administration of NNK also inhibited the NNK-induced lung tumorigenesis. As shown in Table II, pretreatment of mice with 8-methoxypsoralen inhibited the incidence and multiplicities of macroscopically and microscopically examined lung lesions. However, treatment with 8-methoxypsoralen on days 1, 3 and 7 after NNK administration did not affect the incidence and multiplicities of examined lung lesions, suggesting that 8-methoxypsoralen abolished NNK-induced lung tumorigenesis via the inhibition of initiation event in carcinogenesis but not subsequent events including promotion and progression in carcinogenesis.

Expression of CYP2A in mouse lung adenomas induced by NNK

It was of interest to know if CYP2A2s were expressed in the region of NNK-induced lung lesions. Thus, we performed immunohistochemical examination for the expression of CYP2A using rabbit polyclonal anti-rat CYP2A1 antiserum in paraffin-embedded tumors containing NNK-induced lung adenomas. As shown in Figure 6, the expression of a protein(s) cross-reactive to antibodies to CYP2A1 was clearly seen in the region of NNK-induced lung tumor. Together with the data shown in Figure 3, it seemed possible to assume that CYP2A1 protein was expressed in the region of NNK-induced lung adenomas.

Discussion

The lung is one of the major target organs for NNK-induced tumor formation in laboratory animals including mice, rats and hamsters, regardless of the route of administration (1). A number of reports have appeared to date indicating that P450 is responsible for the metabolic activation of NNK (1,33,41–44). P450 forms belonging to the CYP2A subfamily have been studied in rats (CYP2A1, CYP2A2 and CYP2A3), mice (CYP2A4 and CYP2A5) and hamsters (CYP2A8, CYP2A9 and CYP2A16). CYP2As from these laboratory animals differ markedly in catalytic specificity despite similarity in their amino acid sequences (~70–90%) (45–50). In the present study, among mouse CYP2As, recombinant CYP2A4 and CYP2A5 activated NNK efficiently at nM concentrations of NNK in the mutation assays using the *S.typhimurium* YG7108, each expressing a form of the mouse CYP2A along with CPR (Figure 3). To our knowledge, the mutagenicity of N-nitrosoamines including NNK at the nM level could not be detected in other studies reported so far. Furthermore, in our preliminary study, rat CYP2A3 and hamster CYP2A16 also activated NNK at nM levels of NNK using our genetically engineered *S.typhimurium* YG7108 expressing a CYP2A. Collectively, these data may suggest that CYP2A subfamily member(s) play an important role in the mutagenic activation of NNK.

The mutagenic activation of NNK by mouse liver and lung microsomes was also inhibited by anti-CYP2A1 antibodies (Figures 1B and 2B). Since 8-methoxypsoralen is reported to inhibit CYP2A5 and human CYP2A6-mediated coumarin 7-hydroxylation, it has been recognized as a representative inhibitor of CYP2A5 and CYP2A6 (31,51,52). It has also been shown that 8-methoxypsoralen is a substrate for CYP2A6 and that the enzyme inhibition is due to competitive interaction (53,54). The metabolism-dependent inactivation of CYP2A6 by 8-methoxypsoralen has been shown to occur at low concentrations and at high rates (54). Using the *S.typhimurium* YG7108 expressing high levels of mouse or human CYP2A, 8-methoxypsoralen inhibited the metabolic activation of NNK (IC$_{50}$ values were 0.039–0.66 μM (unpublished data). The results of the present study provided strong evidence that the suppression by 8-methoxypsoralen, a typical CYP2A inhibitor, of NNK-induced lung tumorigenesis in mice can be explained by the inhibitory effects of 8-methoxypsoralen on the mutagenic activation of NNK catalyzed by CYP2A5 in the target organ in the initiation events of carcinogenesis. von Pressentin et al. (55) have also reported that the administration of NNK in drinking water resulted in an increased mutation frequency in the organ including the lung of the lac$^{-}$ mice (Muta$^TM$Mouse). In the present study, NNK-induced mutations in the lung of gpt$^-$ delta transgenic mouse were almost completely abolished when mice were pretreated with 8-methoxypsoralen at a dose of 12.5 mg/kg. On the other hand, pretreatment of mice with 8-methoxypsoralen did not reduce the ENU (a direct mutagen)-induced mutation, suggesting that 8-methoxypsoralen might reduce the NNK-induced gene mutation via the inhibition of metabolic activation of NNK. Our preliminary DNA microarray analyses using the lungs from 8-methoxypsoralen and/or NNK-treated mice have shown that treatment with 8-methoxypsoralen and/or NNK did not alter the expression levels of P450s, phase II enzymes and DNA repair enzymes at 1 day after the treatment (unpublished data), when the metabolic activation of NNK had been reported to be almost completely finished (56). The expression of mRNA for CYP2A5, but not for CYP2A4, in the lung from untreated mice (Figure 4) and CYP2A proteins cross-reactive to anti-CYP2A1 antibodies seen in mouse lung adenoma induced by NNK (Figure 6) probably suggested that 8-methoxypsoralen inhibited further occurrence of mutation in the adenomas induced by NNK.

Treatment of mice with 8-methoxypsoralen after NNK administration did not affect the incidence and multiplicities of lung lesions (Table II), probably suggesting that anti-mutagenic activity of 8-methoxypsoralen against NNK-induced mutation in an initiation event in carcinogenesis is a key step in the chemoprevention of this compound against NNK-induced lung tumorigenesis in mice. We also reported that CYP2A6 was capable of activating betel quid-specific N-nitrosamines (57) and that the CYP2A6 gene deletion reduced oral cancer risk in betel quid chewers in Sri Lanka (58). Taken together with the data of this study, it is reasonable
**Table I.** Effects of treatment of female A/J mice with 8-methoxypsoralen prior to NNK on NNK-induced lung tumorigenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>NNK</th>
<th>8-Methoxypsoralen (mg/kg)</th>
<th>No.</th>
<th>Macroscopical lung lesion</th>
<th>Hyperplasia</th>
<th>Adenoma</th>
<th>Hyperplasia and adenoma</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Incidence (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tumors/mouse&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Incidence (%)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–</td>
<td>12.5</td>
<td>20</td>
<td>5/20 (25)</td>
<td>0.25 ± 0.44</td>
<td>1/20 (5)</td>
<td>0.05 ± 0.22</td>
</tr>
<tr>
<td>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>–</td>
<td>23</td>
<td>23/23 (100)</td>
<td>21.04 ± 12.56</td>
<td>18/23 (78)</td>
<td>2.17 ± 1.95</td>
</tr>
<tr>
<td>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>0.125</td>
<td>20</td>
<td>20/20 (100)</td>
<td>17.05 ± 15.24</td>
<td>14/20 (70)</td>
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</tr>
<tr>
<td>&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+</td>
<td>1.25</td>
<td>18</td>
<td>18/18 (100)</td>
<td>7.39 ± 2.63&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11/18 (61)</td>
<td>1.00 ± 0.97</td>
</tr>
<tr>
<td>&lt;sup&gt;5&lt;/sup&gt;</td>
<td>+</td>
<td>12.5</td>
<td>20</td>
<td>12/20 (60)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.25 ± 1.29&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6/20 (30)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.30 ± 0.47</td>
</tr>
</tbody>
</table>

NNK (100 mg/kg) was injected i.p. to mice as described in Materials and methods.

<sup>a</sup>Number of mice examined.
<sup>b</sup>Number of mice with lesion (%).
<sup>c</sup>Mean ± SD.
<sup>d</sup>Significantly different from Group 2 by Student’s <i>t</i>-test (<i>P</i> < 0.01).
<sup>e</sup>Significantly different from Group 2 by Fisher’s exact probability test (<i>P</i> < 0.01).

**Table II.** Effects of treatment of female A/J mice with 8-methoxypsoralen before or after the administration of NNK on NNK-induced lung tumorigenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>NNK</th>
<th>8-Methoxypsoralen (mg/kg)</th>
<th>No.</th>
<th>Macroscopical lung lesion</th>
<th>Hyperplasia</th>
<th>Adenoma</th>
<th>Hyperplasia and adenoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Incidence (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tumors/mouse&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Incidence (%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–</td>
<td>Alone</td>
<td>19</td>
<td>3/19 (16)</td>
<td>0.16 ± 0.38</td>
<td>0/19 (0)</td>
<td>0</td>
</tr>
<tr>
<td>&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>Pretreatment</td>
<td>20</td>
<td>19/20 (95)</td>
<td>3.70 ± 3.06</td>
<td>9/20 (45)</td>
<td>0.75 ± 1.07</td>
</tr>
<tr>
<td>&lt;sup&gt;3&lt;/sup&gt;</td>
<td>+</td>
<td>1 day after NNK</td>
<td>19</td>
<td>17/19 (90)</td>
<td>4.26 ± 2.98</td>
<td>11/19 (58)</td>
<td>1.00 ± 1.15</td>
</tr>
<tr>
<td>&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+</td>
<td>3 days after NNK</td>
<td>20</td>
<td>20/20 (100)</td>
<td>3.70 ± 1.84</td>
<td>10/20 (50)</td>
<td>0.50 ± 0.51</td>
</tr>
<tr>
<td>&lt;sup&gt;5&lt;/sup&gt;</td>
<td>+</td>
<td>7 days after NNK</td>
<td>20</td>
<td>19/20 (95)</td>
<td>3.10 ± 1.71</td>
<td>8/20 (40)</td>
<td>0.40 ± 0.50</td>
</tr>
</tbody>
</table>

NNK (100 mg/kg) was injected i.p. into mice.

<sup>a</sup>Number of mice examined.
<sup>b</sup>Number of mice with lesion (%).
<sup>c</sup>Mean ± SD.
<sup>d</sup>Significantly different from Group 2 by Fisher’s exact probability test (<i>P</i> < 0.01).
<sup>e</sup>Significantly different from Group 2 by Student’s <i>t</i>-test (<i>P</i> < 0.005).
to assume that the function of CYP2A6 as an initial event in carcinogenesis plays an important role in lung cancer caused by smoking. In accordance with this idea, we demonstrated in a previous report that the function of CYP2A6 evaluated from CYP2A6 genetic polymorphisms is a key determinant of tobacco-related lung cancer risk in male Japanese smokers (26).

In conclusion, the results of this study indicate for the first time that 8-methoxypsoralen prevents NNK-induced lung mutagenesis and tumorigenesis in mice by inhibition of CYP2A5 in the lung.

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Conflict of Interest Statement: None declared.

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Fig. 6. Immunohistochemical examination for the expression of CYP2A in adenoma induced by NNK in the mouse lung. The boundary of the lesion was usually well demarcated. The paraffin-embedded lung sections were immunostained with rabbit polyclonal anti-rat CYP2A1 antibodies (A–C) or preimmune (D). A, B and D, and C display lung adenomas at 40×, 100× and 200× magnifications, respectively.
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