8-Methoxypsoralen, a potent human CYP2A6 inhibitor, inhibits lung adenocarcinoma development induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in female A/J mice

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Abstract. Previously, we demonstrated that 8-methoxypsoralen (methoxsalen), a potent human cytochrome P450 2A6 (CYP2A6) inhibitor, strongly suppresses lung adenoma induction by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice. In the present study, we examined the inhibitory effects of methoxsalen on the development of lung adenocarcinomas, as well as on adenomas and alveolar hyperplasia. Female A/J mice were treated with methoxsalen at doses of 12.5 or 1.25 mg/kg body weight, administered by stomach tube once daily for 3 days. One hour after the final treatment, NNK was injected i.p. at a dose of 2 mg/mouse. The experiments were terminated 52 weeks after the first methoxsalen treatment, and lung adenomas and adenocarcinomas were analyzed histopathologically. Pretreatment with methoxsalen significantly reduced the incidence of adenocarcinomas from 94.7 to 46.7% (12.5 mg/kg) and 44.4% (1.25 mg/kg), and their tumor multiplicity from 4.68 to 0.87 (12.5 mg/kg) and 0.61 (1.25 mg/kg) tumors/mouse. The tumor multiplicity of adenomas and adenocarcinomas in the methoxsalen-treated groups was significantly reduced from 12.47 to 5.67 (12.5 mg/kg) and 4.28 (1.25 mg/kg) tumors/mouse. Approximately 60% of the adenocarcinomas arose within adenomas. In comparing the methoxsalen + NNK and NNK alone groups, there was no significant difference in the frequency of such compound lesions, indicating that pretreatment with methoxsalen did not suppress the eventual progression of adenomas to adenocarcinomas. These results clearly demonstrate that methoxsalen, a potent human CYP2A6 inhibitor, inhibits not only lung adenoma but also adenocarcinoma development.

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Abbreviations: methoxsalen, 8-methoxypsoralen; CYP2A6, cytochrome P450 2A6; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

Key words: cytochrome P450 2A6, lung carcinogenesis, methoxsalen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Introduction

Cytochrome P450 2A6 (CYP2A6) is an enzyme that plays a major role in the metabolic activation of promutagens, such as tobacco-specific *N*-nitrosamines (1). These include 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which conceivably plays an important role in tobacco-related human lung cancer, given its strong potential to induce lung tumorigenesis in rodents (2). CYP2A6 is known to contribute to coumarin 7-hydroxylation (3). In humans, 70-80% of nicotine is metabolized by CYP2A6 to the inactive metabolite cotinine (4), and then further metabolized to trans-3'-hydroxycotinine (5,6). In our previous study, Japanese male smokers with CYP2A6 gene deletion-type polymorphisms were shown to have a reduced risk of lung cancer in a hospital-based case control study (7). Moreover, CYP2A6 gene deletions have been linked to a decreased risk of tobacco dependence and a decrease in the number of cigarettes smoked (7,8).

Methoxsalen (8-methoxypsoralen) is a potent CYP2A6 inhibitor *in vitro* (9,10), strongly suppressing coumarin and nicotine metabolism *in vivo* in humans (11,12). Furthermore, its treatment *in vivo* increases the routing of NNK to inactive 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)-glucuronide. In mutagenicity testing using *Salmonella typhimurium* YG7108 expressing high levels of CYP2A, it was found that methoxsalen at low concentrations inhibited the mutagenic activity of NNK (13).

If one of the causes of human lung cancer is dependent on the metabolic activation of a tobacco-specific *N*-nitrosamine, inhibition of CYP2A6 by methoxsalen might result in the

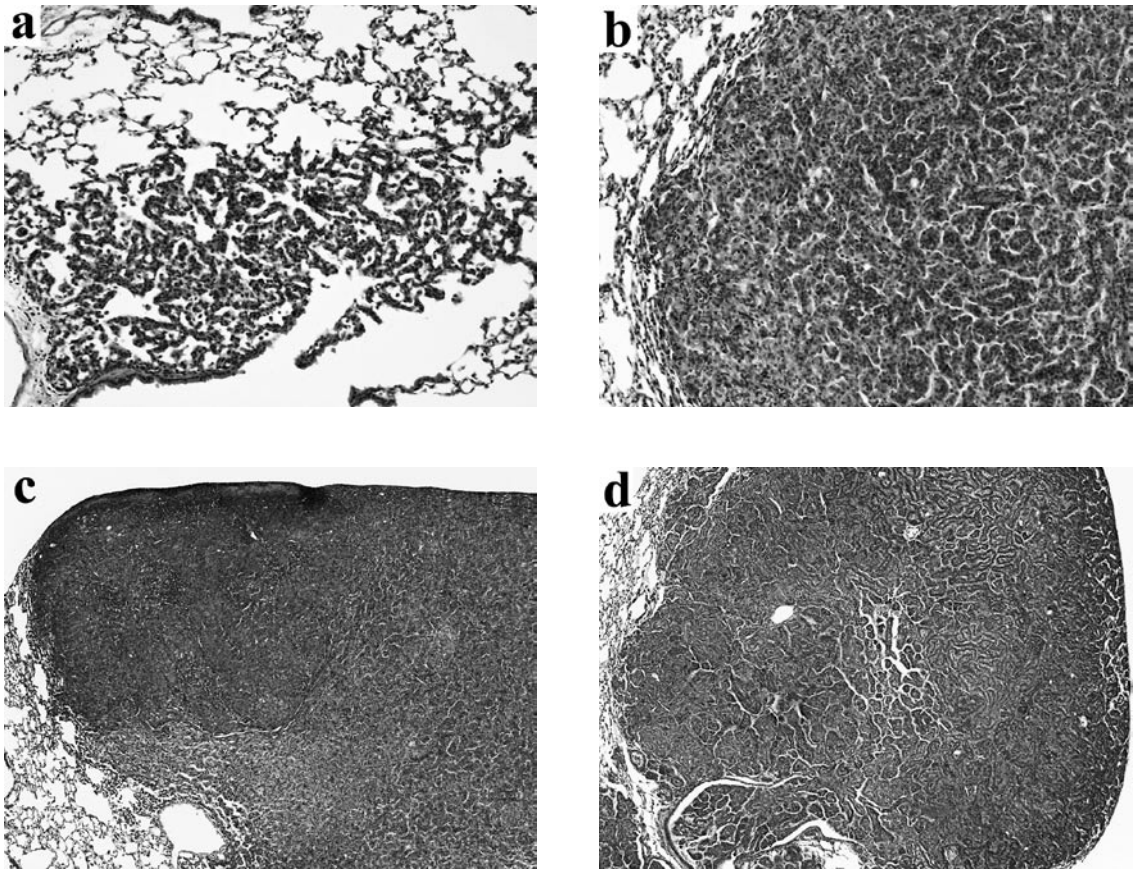

Figure 1. Histopathology of proliferative lung lesions induced by NNK (Group 4). (a) Hyperplasia, (b) adenoma, (c) adenocarcinoma within adenoma and (d) adenocarcinoma. Hematoxylin and eosin staining. Original magnification: a and b, x10; c and d, x4.

chemoprevention of tobacco-related lung cancer. Previously, we demonstrated that it indeed strongly inhibits lung tumorigenesis in terms of adenoma development induced by NNK in female A/J mice in a medium-term study (13-15). In the present investigation, the inhibitory effects of methoxsalen on adenocarcinoma development were assessed in a 52-week model.

Materials and methods

Chemicals. Methoxsalen was purchased from Sigma (St. Louis, MO, USA) and NNK from Toronto Research Chemicals (Toronto, Canada).

Animals. Female A/J mice (5 weeks of age) purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were maintained at the Kagawa University Animal Facility according to their 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 consisting of Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan) under controlled humidity (60±10%), lighting (12-h light/dark cycle) and temperature (24±2˚C) conditions.

Experimental design. At 7 weeks of age, the mice were pretreated with methoxsalen (12.5 or 1.25 mg/kg body weight in 0.2 ml corn oil, administered by stomach tube) or an equal volume of corn oil (vehicle control) once daily for 3 days. One hour after the final treatment, each group was administered a single dose of NNK (2 mg/0.1 ml saline/mouse) i.p., or an equal volume of saline (vehicle control). They were then maintained without further treatment. The experiment was terminated 52 weeks after the first methoxsalen administration, when all surviving mice were sacrificed under ether anesthesia. At autopsy, the mouse lungs were excised and weighed, and then infused with 10% neutral buffered formalin and carefully inspected grossly. All macroscopically detected lung nodules were counted under a stereomicroscope, and each lung lobe was examined histopathologically.

Statistical analysis. The incidence of proliferative lung lesions was analyzed by Fisher's exact probability test, and data regarding multiplicity by the Student's t-test.

Results

The initial number of mice in each group was 20, but 5 mice in Group 1, 2 each in Groups 2, 3 and 5, and one in Group 4 died during the experimental period and were not included in the effective numbers. There were no inter-group differences in terms of final body or relative organ (lung, liver and kidney) weight (data not shown). Whitish lung nodules were macroscopically prevalent in the groups treated with NNK and were considerably larger than in the previous 16-week experiment (15). Proliferative lung lesions, hyperplasias (Fig. 1a), adenomas (Fig. 1b) and adenocarcinomas (Fig. 1d) were diagnosed according to criteria described in the International Classification

Table I. Incidence and multiplicity of NNK-induced proliferative lung lesions in A/J mice treated with methoxsalen.

Group	Treatment	No.[a]	Hyperplasia		Adenomas		Adenocarcinomas	
			Incidence (%)[b]	Tumors/ mouse[c]	Incidence (%)	Tumors/ mouse	Incidence (%)	Tumors/ mouse
1	Methoxsalen 12.5 mg/kg + NNK	15	11/15 (73.3)	1.27±1.03[d]	15/15 (100)	4.80±3.34[e]	7/15 (46.7)[d]	0.87±1.36[f]
2	Methoxsalen 1.25 mg/kg + NNK	18	12/18 (66.7)[e]	1.33±1.24[d]	18/18 (100)	3.67±3.03[g]	8/18 (44.4)[d]	0.61±0.98[h]
3	Methoxsalen 12.5 mg/kg + saline	18	5/18 (27.8)	0.33±0.59	5/18 (27.8)	0.28±0.46	1/18 (5.6)	0.06±0.24
4	NNK alone	19	18/19 (94.7)	3.47±2.25	19/19 (100)	7.79±3.44	18/19 (94.7)	4.68±3.42
5	Corn oil + saline	18	9/18 (50.0)	0.56±0.62	4/18 (22.2)	0.22±0.43	3/18 (16.7)	0.17±0.38

[a]Number of mice examined. [b]Number of mice observed with each lesion (%). [c]Mean ± SD. Significantly different from Group 4: [d]P<0.005, [e]P<0.05, [f]P<0.0005, [g]P<0.001 and [h]P<0.0001.

Table II. Incidence and multiplicity of NNK-induced proliferative lung lesions in A/J mice treated with methoxsalen.

Group	Treatment	No.[b]	Adenomas + adenocarcinomas		Proliferative lung lesions[a]	
			Incidence (%)[c]	Tumors/mouse[d]	Incidence (%)	Tumors/mouse
1	Methoxsalen 12.5 mg/kg + NNK	15	15/15 (100)	5.67±3.52e	15/15 (100)	6.93±3.75[e]
2	Methoxsalen 1.25 mg/kg + NNK	18	18/18 (100)	4.28±3.86[e]	18/18 (100)	5.61±4.67[e]
3	Methoxsalen 12.5 mg/kg + saline	18	6/18 (33.3)	0.33±0.49	11/18 (61.1)	0.67±0.59
4	NNK alone	19	19/19 (100)	12.47±4.72	19/19 (100)	15.95±5.91
5	Corn oil + saline	18	7/18 (38.9)	0.39±0.50	14/18 (77.8)	0.94±0.64

[a]Hyperplasia + adenomas + adenocarcinomas. [b]Number of mice examined. [c]Number of mice with each lesion (%). [d]Mean ± SD. [e]Significantly different from Group 4 (P<0.0001).

Table III. Frequency of carcinomas within adenomas in lung carcinoma.

Treatment	Carcinomas within adenomas (%)	Adenocarcinomas alone (%)	Total adenocarcinomas
Methoxsalen 12.5 mg/kg + NNK	9 (69.2)	4 (30.8)	13
Methoxsalen 1.25 mg/kg + NNK	7 (63.6)	4 (36.4)	11
Methoxsalen 12.5 mg/kg + saline	1 (100)	0 (0)	1
NNK alone	55 (61.8)	34 (38.2)	89
Corn oil + saline	2 (66.7)	1 (33.3)	3
Total	74 (63.2)	43 (36.8)	117

of Rodent Tumors: the Mouse (16), and their numbers were counted under a microscope. The incidence and multiplicity of proliferative lung lesions are summarized in Tables I and II. All animals in the NNK-treated groups (Groups 1, 2 and 4) exhibited adenomas, but tumor multiplicity was significantly reduced by methoxsalen treatment. Pretreatment with methoxsalen also significantly reduced the incidence and multiplicity of lung adenocarcinomas (Groups 1 and 2). Without NNK treatment (Groups 3 and 5), there were no significant differences in the incidence or multiplicity of proliferative lung lesions with methoxsalen treatment.

Some lung adenocarcinomas were found within adenomas (Fig. 1c). The frequency of these lesions is summarized in Table III. With NNK treatment alone, 61.8% of the adenocarcinomas were observed to arise within adenomas. In the methoxsalen + NNK cases (Groups 1 and 2), the respective values were 63.6 and 69.2%, with no significant difference compared to Group 3.

Discussion

The present study demonstrated that pretreatment with methoxsalen strongly inhibits NNK-induced lung adenocarci-

noma development. This is in line with our previous finding that pretreatment with 12.5 mg/kg of methoxsalen strongly inhibits NNK-induced lung adenoma development (15,17). The mouse A/J strain is among those most susceptible to lung carcinogenesis; one study demonstrated a 40% incidence and 0.58 tumors/mouse developing spontaneously by 52 weeks of age (18). In the present study, the incidence of macroscopical lung tumors in the vehicle control group (Group 4) was 13/18 (72.2%), while tumor multiplicity was 0.89±0.68 tumors/mouse (data not shown). In the single-dose NNK assay, the incidence of carcinomas was increased in frequency 34 weeks after NNK treatment, and comprised >50% of the pulmonary lesions by 54 weeks (2). The previously reported incidence and multiplicity of carcinomas at 52 weeks after NNK treatment were 73-80% and 2.39 tumors/mouse (19,20). In the present study, the incidence of adenocarcinomas was 94.7%, with a multiplicity of 4.68±3.42 tumors/mouse 52 weeks after NNK treatment. These are relatively high values.

The NNK single-dose assay using A/J mice is recognized as a good animal model of tobacco-related human lung adenocarcinoma. Using this assay, the frequency of hyperplasias decreases progressively over time with an increase in the frequency of adenomas, approximately 50% of which arise within hyperplasias (2). In turn, approximately 30-75% of adenocarcinomas arise within adenomas (2,19). In the present study, the value was 61.8% for Group 3, compared to 63.6 and 69.2% for the methoxsalen and NNK groups, respectively. This suggests that pretreatment with methoxsalen does not suppress the conversion of adenomas to adenocarcinomas, rather that the inhibitory effects of methoxsalen on NNK-induced lung carcinogenesis are due to its impact on the metabolic activation of NNK during the initiation phase.

In conclusion, the results of this study indicate that methoxsalen, a potent human CYP2A6 inhibitor, is a strong chemopreventive agent, not only for NNK-induced lung adenomas, but also for lung adenocarcinoma development. This therefore suggests that CYP2A6 inhibitors may have a chemopreventive effect in tobacco-related lung cancer.

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