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Lung Tumors in Strain A Mice: Application for Studies in Cancer Chemoprevention

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Abstract Strain A mice develop a high incidence of spontaneous lung tumors during their lifetime. These tumors may be found in some animals as early as 3 to 4 weeks of age, increasing to nearly 100% by 24 months of age. The strain A mouse is also highly susceptible to the induction of lung tumors by several classes of chemical carcinogens and has been used extensively as a mouse lung tumor bioassay for assessing the carcinogenic activity of a variety of chemicals.

In addition to its use in carcinogen detection, the strain A mouse lung tumor model has been employed extensively for the identification of inhibitors of chemical carcinogenesis. A number of chemopreventive agents including β-naphthoflavone, butylated hydroxyanisole, ellagic acid, phenethyl isothiocyanate, phenylpropyl isothiocyanate, phenylbutyl isothiocyanate, phenylhexyl isothiocyanate, indole-3-carbinol, *etc.*, have been shown to inhibit chemically induced lung tumors in strain A mice. In most instances, inhibition of lung tumorigenesis has been correlated with effects of the chemopreventive agent on the metabolic activation and/or detoxification of carcinogens. To date, no chemopreventive agent has been shown to inhibit lung tumorigenesis in strain A mice when administered after the carcinogen, *i.e.*, during the promotion/progression stages of tumor development. Efforts should be made to develop a standardized protocol in strain A mice for evaluating chemopreventive agents as inhibitors of both the initiation and progression stages of lung tumor development.

Key words: Mouse, lung tumors, isothiocyanates, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, benzo(*a*)pyrene, chemoprevention, NNK, strain A, *ras*

Strain A mice develop a high incidence of spontaneous lung tumors during their lifetime. These tumors may be found in some animals as early as 3 to 4 weeks of age with a steady increase to nearly 100% by 24 months of age [1]. The strain A mouse is also highly susceptible to the induction of lung tumors by several classes of chemical carcinogens compared to other inbred mouse strains, and has been used extensively as a mouse lung tumor bioassay to assess the carcinogenic activity of a variety of chemicals [2,3].

The histomorphology [1,2,4] and ultrastructure [5] of spontaneous pulmonary neoplasms in strain A mice have been described. However, in these preliminary papers, much emphasis was placed upon the morphological characteristics of the benign tumor (adenoma), with minor reference to the histomorphologic features of the malignant counterpart. In recent articles, Dixon *et al.* [6] described the morphological features of malignant spontaneous neoplasms of strain A mouse lung and classified these as either papillary carcinoma or mixed carcinoma. Foley *et al.* [7] found that chemically induced tumors in

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strain A mouse lung arise in hyperplasias and progress to adenoma, carcinoma within adenoma, and ultimately, to carcinomas. Although a matter of some controversy, it would appear that most lung tumors in mice arise from type II cells of the alveolar epithelium [8], with a minor component of papillary tumors arising either from Clara cells of the terminal bronchioles or highly undifferentiated type II cells [9].

The biochemical, growth and transplantation characteristics of mouse lung tumors were summarized in detail by Shimkin and Stoner [1]. A recent study has shown that strain A lung tumors stain intensely for lactate dehydrogenase, NADPH diaphorase, and glyceraldehyde-3-phosphate dehydrogenase activities [10]. Phosphatidylglycerol, an important component of pulmonary surfactant, is present in lower quantities in lung tumors than in normal lung tissue [11]. The proliferative rate of type II alveolar epithelial cells in untreated and chemically treated strain A mice is significantly higher than in mouse strains resistant to lung tumorigenesis [12]. Lung tumor susceptibility in strain A mice also appears to be influenced by the H-2 major histocompatibility complex [13].

The development of spontaneous and chemically induced lung tumors in strain A mice is associated with mutational activation of the K-*ras* oncogene [14,15]. This appears to be an early event in lung tumorigenesis. In spontaneous tumors, mutations in codons 12, 13 or 61 of the gene appear to occur randomly, whereas carcinogens induce mutations predominately in specific nucleotides of these codons [14,16]. Interestingly, lung tumor susceptibility in strain A mice is correlated with a 37 base pair deletion in the second intron of the K-*ras* gene [17]. The mechanism(s) by which this deletion influences lung tumor susceptibility is unclear. Other genetic alterations in strain A mouse lung tumors include reductions in the expression of the retinoblastoma (Rb) gene and the growth arrest-specific (*gas*-3) gene [18]. Preliminary results suggest that the p53 suppressor gene is mutated only in some carcinomas and not in adenomas of strain A mouse lung (Ming You, personal communication).

Lung tumors in mice have been used extensively for the detection of inhibitors of chemical carcinogenesis. A number of chemopreventive agents including β-naphthoflavone [19,20], butylated hydroxyanisole [21], ethoxyquin [22], quercetin [19], pentamethylether [19], sodium cyanate [23], benzyl isothiocyanate [24], phenethyl isothiocyanate [25,26], phenylpropyl isothiocyanate [25,26], phenylbutyl isothiocyanate [25,26], phenylhexyl isothiocyanate [26], ellagic acid [27], tannic acid [28], indole-3-carbinol [29], D-limonene [30], sulindac [31], green tea and black tea [32], and biochanin A [33] have been shown to inhibit chemically induced lung tumors in mice (Table I). Most of these studies have employed strain A mice, although other mouse strains with high to intermediate susceptibility to lung tumorigenesis have also been used. Several protocols have been employed to evaluate the ability of putative chemopreventive agents to inhibit lung tumorigenesis in strain A mice; however, a thorough discussion of these is beyond the scope of this paper. Instead, we present a protocol used in our laboratories, and in the laboratories of our collaborators, Stephen S. Hecht and Fung-Lung Chung at the American Health Foundation, Valhalla, New York, to test chemopreventive agents in the strain A mouse lung tumor model.

MATERIALS AND METHODS

Animals. Four to six week-old male and female A/J mice are purchased from Jackson Laboratories, Bar Harbor, Maine. Male or female mice can be used in chemoprevention protocols since the lung tumor response in A/J mice to carcinogens is similar in both sexes [1]. The mice are maintained in quarantine on a regular laboratory chow diet for two weeks before use in the bioassays. All mice are housed in Bioclean® laminar flow rooms in groups of four in solid bottom and side polycarbonate cages. Tap water is available *ad libitum*. The mice are maintained under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12 hour cycle of light and darkness). Hygienic conditions are maintained by twice-weekly changes of the cages and water bottles, and the cages are sanitized routinely.

Chemicals. All chemicals tested for inhibitory activity in the lung tumor bioassay are stored as recommended by the manufacturer. Carcinogens are usually obtained from the National Cancer Institute's Chemical Carcinogen Repository at the Midwest Research Institute, Kansas City,

TABLE I. Inhibitors of Carcinogen-Induced Lung Tumors in Mice

Carcinogen	Inhibitor	Reference
Benzo(*a*)pyrene	β-naphthoflavone	[19]
Benzo(*a*)pyrene	BHA[a], ethoxyquin	[21]
7,12-DMBA[a]	BHA	[21]
7-Hydroxymethyl-12-methylbenz(*a*)anthracene	BHA	[21]
Dibenz(*a,h*)anthracene	BHA	[21]
Diethylnitrosamine	BHA, ethoxyquin	[21]
4-NQO[a]	BHA, ethoxyquin	[21]
Uracil mustard	BHA	[21]
Urethane	BHA	[21]
3-Methylcholanthrene	β-naphthoflavone	[20]
Benzo(*a*)pyrene	sodium cyanate	[23]
Benzo(*a*)pyrene	ellagic acid	[27]
Benzo(*a*)pyrene	benzyl isothiocyanate	[24]
NNK[a]	phenethyl isothiocyanate, 3-phenylpropyl isothiocyanate, 4-phenylbutyl isothiocyanate	[25]
Benzo(*a*)pyrene	tannic acid	[28]
NNK[a]	indole-3-carbinol	[29]
NNK[a]	phenethyl isothiocyanate, 3-phenylpropyl isothiocyanate, 4-phenylbutyl isothiocyanate, 5-phenylpentyl isothiocyanate, 6-phenylhexyl isothiocyanate	[26]
NNK[a]	D-limonene, orange oil, lemon oil	[30]
NNK	sulindac	[31]
NNK	green tea, black tea	[32]
Benzo(*a*)pyrene	biochanin A	[33]

[a]7,12-DMBA = 7,12-dimethylbenz(*a*)anthracene; BHA = butylated hydroxyanisole; 4-NQO = 4-nitroquinoline-1-oxide; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

MO, and stored at 4°C in the dark. Chemopreventive agents and carcinogens are tested for purity by high-performance liquid chromatography (HPLC).

Diet. AIN-76A modified diet containing 20% casein, 0.3% DL-methionine, 52% cornstarch, 13% dextrose, 5% corn oil, 5% Alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate is used for routine bioassays of chemopreventive agents in the A/J mouse lung tumor model. Fresh batches of diet are obtained at monthly intervals, and the diet is stored routinely at 4°C in the dark.

Administration of Chemopreventive Agents. Test compounds are administered ig (by gavage). A major advantage of the gavage route is

that it allows accurate quantitation of dose. However, repeated administration of test compounds by gavage is labor intensive and can lead to significant mortality in treated animals. An alternative method was suggested in the studies of Castonguay *et al.* [34] who administered the test compound in the diet and the carcinogen in the drinking water.

Bioassay. Groups of 6 to 7 week-old A/J male or female mice are administered the test compound in corn oil by gavage at 3–5 concentrations for four consecutive days. These concentrations are chosen from preliminary dose range-finding studies based upon the observation that they elicit no clinical or other (*e.g.*, weight loss) signs of toxicity. The carcinogen is dissolved in saline (if soluble) or corn oil and administered in a single intraperitoneal (ip) dose within 2–4 hours after the last dose of test compound. Controls consist of mice treated with four consecutive ig doses of the test compound only at the above concentrations, or a single ip dose of carcinogen only. Vehicle controls consist of mice given four consecutive ig doses of corn oil plus one ip dose of either saline or corn oil. The mice are housed four per cage as described above. AIN-76A diet and tap water are provided *ad libitum*.

The mice are observed daily during the first week of the study, when the treatments are administered, and weekly thereafter. Body weights are measured weekly for the first month and monthly thereafter. All mice are sacrificed and necropsied 16 weeks after administration of the tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or 24 weeks after treatment with benzo(*a*)pyrene [B(*a*)P]. Sacrifice of B(*a*)P-treated animals at the later time point (*i.e.*, 24 weeks) appears to be necessary to permit the tumors to grow large enough to be visible on the lung surface. The lungs are infused with saline and fixed in Tellyesniczky's fixative [1]. Surface tumors are counted following fixation, using a dissecting microscope. Representative tumor samples are embedded in paraffin and stained with hematoxylin and eosin for histopathological examination.

Statistical comparisons of tumor multiplicities among the various groups are performed by analysis of variance (ANOVA) followed by Newman-Keuls' ranges test. Comparisons of the proportions of animals in groups that develop tumors are performed by the Chi-square test. Body weight and weight gain are analyzed using one-way analysis of variance followed by Tukey's test when significant differences are observed.

DNA Adduct Analysis. Investigations with NNK have shown a linear relationship between NNK-induced lung tumorigenesis in A/J mice and the formation of O^6-methylguanine (O^6-MeGua) adducts in lung DNA [35]. Therefore, studies have been conducted in our laboratories to determine the relationship between inhibition by chemopreventive agents (isothiocyanates and indole-3-carbinol) of NNK-induced lung tumors in A/J mice and reduction in the formation of O^6-MeGua in lung DNA.

Groups of A/J mice are administered corn oil or chemopreventive agents by gavage for four consecutive days. On the fourth day, NNK is administered ip 2 hours after the final gavage. All treatments are identical to those used in the bioassay. Groups of 5 animals are sacrificed at 2 hours or 6 hours following NNK dosing. DNA is isolated from excised lungs by a modification of the method of Marmur [36] and purified by the method of Sebti *et al.* [37]. The DNA is hydrolyzed in 0.1N HCl for 30 minutes at 70°C to release O^6-MeGua and guanine. 7-MeGua, O^6-MeGua, and guanine are quantitated by strong cation exchange HPLC coupled with fluorescence detection. The identities of 7-MeGua, O^6-MeGua, and guanine are confirmed by coelution with authentic standards.

RESULTS AND DISCUSSION

Examples of data obtained from bioassays of chemopreventive agents in the A/J mouse lung tumor model are shown in Tables II, III, and IV. In Table II, a series of arylalkyl isothiocyanate compounds of increasing chain length were evaluated by Morse *et al.* [25,26] for their ability to inhibit lung tumorigenesis by the tobacco-specific carcinogen, NNK. Female A/J mice were administered either phenyl isothiocyanate (PITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), 3-phenylpropyl isothiocyanate (PPITC), 4-phenylbutyl isothiocyanate (PBITC), 5-phenylpentyl isothiocyanate (PPeITC), or 6-phenylhexyl isothiocyanate

TABLE II. Effects of Isothiocyanates on NNK-induced Lung Tumors in Strain A/J Mice[a]

Treatment[b]	No. of mice	Tumors/mouse[c,d]	% of Mice with tumors[e]
Corn oil + NNK	39	9.2 ± 0.5^{1}	100^{1}
PITC + NNK	30	9.8 ± 0.9^{1}	100^{1}
BITC + NNK	29	10.4 ± 0.7^{1}	100^{1}
PEITC + NNK	28	3.3 ± 0.4^{2}	93^{1}
PPITC + NNK	30	0.4 ± 0.1^{3}	37^{2}
PBITC + NNK	28	0.4 ± 0.1^{3}	32^{2}
Corn oil + NNK	60	7.9 ± 0.4^{1}	100^{1}
PEITC + NNK	20	4.1 ± 0.8^{2}	93^{2}
PPITC + NNK	19	0.2 ± 0.1^{4}	11^{3}
PBITC + NNK	19	0.2 ± 0.1^{4}	11^{3}
PPeITC + NNK	20	0.3 ± 0.1^{4}	25^{3}
PHITC + NNK	20	0.1 ± 0.1^{4}	5^{3}

[a]Data of Morse *et al.* [43]: Groups of 20–40 strain A/J mice were administered corn oil or isothiocyanates (5 μmol/mouse/day) by gavage daily for four consecutive days. Two hours after the final dose of corn oil or test compounds, a single dose of saline or NNK (10 μmol/mouse) in saline was administered ip. Sixteen weeks after NNK administration, mice were killed and pulmonary tumors counted.

[b]PITC = phenyl isothiocyanate; BITC = benzyl isothiocyanate; PEITC = phenethyl isothiocyanate; PPITC = phenylpropyl isothiocyanate; PBITC = phenylbutyl isothiocyanate; PPeITC = phenylpentyl isothiocyanate; PHITC = phenylhexyl isothiocyanate.

[c]Mean ± S.E.

[d]Means in this column that bear different superscripts are significantly different ($p < 0.05$) from one another.

[e]Percentages in this column that bear different superscripts are significantly different ($p < 0.0001$) from one another.

(PHITC) by gavage for 4 consecutive days at doses of 5, 1, and 0.2 μmol/day prior to administration of 10 μmol of NNK by ip injection. Mice were sacrificed 16 weeks after NNK administration and lung tumors were quantitated. When compared to vehicle (corn oil + saline) controls, none of the isothiocyanate compounds were shown to influence the occurrence of spontaneous lung tumors in A/J mice (Table II). Animals treated with NNK only had an average of 9.2 ± 0.5 (mean ± S.E.) lung tumors. PITC and BITC were ineffective as inhibitors of NNK lung tumorigenesis. PEITC effectively inhibited NNK-induced lung tumors at a dose of 5 μmol/day but was not inhibitory at doses of 1 or 0.2 μmol/day. PPITC, PBITC, PPeITC, and PHITC were all more potent inhibitors of NNK lung tumorigenesis than PEITC. PHITC appeared to be the most potent tumor inhibitor of all of the isothiocyanates. At a dose of 0.2 μmol/day, PHITC treatment reduced the tumor multiplicity by 85% (data not shown). These results support the hypothesis that increased alkyl chain length enhances the inhibitory activity of an arylalkyl isothiocyanate towards NNK lung tumorigenesis.

DNA adduct studies provided data in support of the lung tumor results. Groups of A/J mice

TABLE III. Effects of Isothiocyanates on NNK-induced DNA Methylation in Lungs of Strain A/J Mice[a]

Treatment	O^6-MeGua (μmol/mol guanine)[b]
Corn oil	17.6 ± 0.9[1]
PEITC	17.2 ± 0.2[1]
PPITC	13.8 ± 0.4[2]
PBITC	13.4 ± 0.5[2]
PPeITC	11.1 ± 0.2[3]
PHITC	9.9 ± 0.2[3]

[a]Data of Morse *et al.* [26]. Groups of 15 strain A/J mice were administered corn oil or isothiocyanates (1 μmol in 0.1 ml corn oil) by gavage for four consecutive days. At 2 hours after the final treatment, mice were given 10 μmol NNK ip. Mice were killed 6 hours after NNK administration and their lungs excised. Following DNA isolation and purification, O^6MeGua was analyzed as described in **MATERIALS AND METHODS**. Values within the same column that bear different superscripts are statistically different from one another ($p < 0.05$).

[b]Mean ± S.E.

TABLE IV. Effects of Phenethyl Isothiocyanate (PEITC) on B(*a*)P-induced Lung Tumors in Strain A/J Mice[a]

Treatment	Dose (μmol)	No. of mice	Tumors/mouse[b]	% of Mice with tumors
Corn oil + tricaprylin	------	48	0.6 ± 0.9	40
PEITC	1.5	46	0.6 ± 0.9	40
PEITC	15.0	47	0.4 ± 0.7	32
B(*a*)P (8 μmol)	------	41	8.4 ± 6.9	100
PEITC + B(*a*)P	1.5	42	10.5 ± 9.2	100
PEITC + B(*a*)P	5.0	46	12.3 ± 12.0	97
PEITC + B(*a*)P	10.0	42	9.4 ± 6.0	100
PEITC + B(*a*)P	15.0	40	7.7 ± 6.7	91

[a]Groups of 24 male and 24 female A/J mice were administered corn oil or phenethyl isothiocyanate (1.5, 5, 10, and 15 μmol/mouse/day in corn oil) by gavage daily for six consecutive days. At 2 hours after the fourth treatment, mice were given 8 μmol B(*a*)P in tricaprylin ip. Twenty four weeks after B(*a*)P administration, mice were killed and pulmonary tumors counted.

[b]Mean ± S.D.

were administered corn oil vehicle or isothiocyanates (1 μmol in 0.1 ml corn oil) by gavage for 4 consecutive days. At 2 hours after the final pretreatment mice were administered 10 μmol NNK (in 0.1 ml saline) ip. The animals were sacrificed 6 hours after NNK administration and the levels of O^6-MeGua in lung DNA were determined as described in **MATERIALS AND METHODS**. O^6-MeGua formation was not significantly affected by PEITC at a dose of 1 μmol/day (Table III). In contrast, both PPITC and PBITC significantly reduced formation of O^6-MeGua in lung DNA below that of NNK-treated controls and PEITC-pretreated mice. PPeITC and PHITC both significantly reduced O^6-MeGua levels below those of PPITC and PBITC. Therefore, the relative potency for inhibiting NNK-induced lung DNA methylation was found to be: PHITC ≈ PPeITC > PBITC ≈ PPITC > PEITC. This order is in substantial agreement with that observed for inhibitors of NNK-induced lung tumorigenesis.

An explanation for the differences in the abilities of arylalkyl isothiocyanates to inhibit NNK-induced lung tumorigenesis has been discussed [26]. PEITC has been shown to inhibit the cytochrome P-450-dependent microsomal metabolism of nitrosamines, including NNK [25,38]. When added to lung microsomes *in vitro*, the inhibitory activity of arylalkyl isothiocyanates towards NNK α-hydroxylation was found to increase with increasing chain length [38]. With increasing alkyl chain length for a given isothiocyanate, lipophilicity increases and reactivity decreases [26], both of which may affect the delivery of the compound to the lung. In addition, increased chain length may favor binding of an isothiocyanate to the catalytic site(s) of cytochrome P-450 isozyme(s) responsible for NNK α-hydroxylation. At present, at least two cytochrome P-450s, *i.e.*, P-450IA1 and P-450IIB, appear to be involved in NNK metabolism in mouse lung [38].

Table IV illustrates data obtained when PEITC was evaluated for its ability to inhibit B(*a*)P tumorigenesis in A/J mouse lung. Groups of mice were administered PEITC by gavage for 6 consecutive days at doses of 1.5, 5, 10, and 15 μmol/day. A single dose of 8 μmol of B(*a*)P in 0.1 ml tricaprylin was given by ip injection after the fourth dose of PEITC. Mice were sacrificed 24 weeks after B(*a*)P administration and lung tumors were quantitated. When compared to vehicle (corn oil + tricaprylin) controls, PEITC did not influence the occurrence of spontaneous lung tumors in A/J mice. Tumor incidence and multiplicity in the PEITC + B(*a*)P groups were not significantly different from the B(*a*)P control groups. Therefore, when tested in a protocol similar to that used for the NNK experiments, PEITC was found to be ineffective as an inhibitor of B(*a*)P tumorigenesis in A/J mouse lung.

The basis for the lack of inhibitory effect of PEITC towards B(*a*)P-induced lung tumors in A/J mice is unknown. It is possible that differences in the metabolism of NNK and B(*a*)P may account for the observed differences between PEITC activity toward NNK- and B(*a*)P-induced lung tumors. In addition, the treatment regimen and dose levels selected for PEITC and B(*a*)P may have masked the inhibitory potential of PEITC against B(*a*)P-induced lung tumors. Pharmacokinetic studies have shown that PEITC and NNK are rapidly absorbed and eliminated in mice [29,39]. In contrast, B(*a*)P administered ip is eliminated slowly from mice [40]. Therefore, the persistence of B(*a*)P in the animals for long periods after the elimination of the inhibitor may have been responsible for the lack of PEITC activity. Wattenberg [41] demonstrated inhibitory effects of PEITC against DMBA-induced lung and forestomach tumors in mice. In this study [41], the inhibitor and carcinogen were mixed in the diet and administered to mice for a period of four weeks. This treatment regimen may be more appropriate when using carcinogens that are slowly eliminated from animals, and in situations in which it is desirable to maintain a favorable ratio of the concentration of inhibitor to that of the carcinogen.

To date, no chemopreventive agent has been shown to inhibit lung tumorigenesis in strain A mice when administered after the carcinogen, *i.e.*, during the promotion/progression stages of tumor development. Agents found to be effective as inhibitors of lung tumorigenesis have generally been shown to influence the metabolic activation and/or detoxification of carcinogens, *i.e.*, events associated with tumor initiation. In our opinion, efforts should be made to develop a standardized protocol to evaluate chemopreventive agents as inhibitors of both the initia-

tion and progression stages of lung tumor development in strain A mice.

Historically, the strain A mouse lung tumor model has been criticized because of its perceived lack of "relevance" to human lung cancer [1,2,3]. Most tumors arising in the terminal airways of the mouse lung have been classified as benign adenomas, as opposed to the invasive, malignant carcinomas seen in human lung. However, the aforementioned studies of Dixon *et al.* [6] and Foley *et al.* [7] clearly indicate that both spontaneous and chemically induced lung tumors in strain A mice progress from adenoma → carcinoma within adenoma → carcinoma with increasing age of the animals. The histopathological features of mouse lung carcinomas resemble those of adenocarcinomas arising in the terminal airway of human lung. Moreover, mouse lung tumors and at least 40% of human lung adenocarcinomas have at least one molecular marker in common, *i.e.*, activation of the K-*ras* oncogene [14,16]. Recent epidemiological studies have revealed an increase in the frequency of human lung adenocarcinomas [42]; therefore, the strain A mouse lung tumor model may assume more importance as a relevant model for a type of human lung cancer that is becoming more prevalent.

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