J Toxicol Pathol 2017; 30: 209–216

Original Article

Dosimetry for lung tumorigenesis induced by urethane, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and benzo[a]pyrene (B[a]P) in A/JJmsSlc mice

Saeko Onami[1*], Chigusa Okubo[1], Asuka Iwanaga[1], Hiroaki Suzuki[1], Hajime Iida[1], Yurie Motohashi[1], Yuki Tomonari[2], Seiji Otake[3], Hiroyuki Tsuji[1], and Hiroyuki Yoshimura[1]

[1] Scientific Product Assessment Center, R and D Group, Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
[2] Pathology Department, Nonclinical Research Center, LSI Medience Corporation, 14-1 Sunayama, Kamisu, Ibaraki 314-0255, Japan
[3] Safety Assessment Department, Nonclinical Research Center, LSI Medience Corporation, 14-1 Sunayama, Kamisu, Ibaraki 314-0255, Japan

Abstract: Some chemicals are known to be lung carcinogens in rodents. While many studies using two-stage models have administered medium or high doses to mice, few have tested lower doses. The dose dependence of urethane, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and benzo[a]pyrene (B[a]P), three well-known lung carcinogens at high doses, has not been sufficiently reported in lower dose ranges. Our study evaluated the tumorigenicity of urethane, NNK, and B[a]P at 26 weeks after a single intraperitoneal administration of each compound within medium to low dose in male and/or female A/JJmsSlc (A/J) mice. Dose-dependent tumorigenesis was demonstrated histopathologically for the three compounds. These results suggested that the tumorigenicity of these chemicals is dose dependent in A/J mice, even at lower doses than previously reported. (DOI: 10.1293/tox.2017-0005; J Toxicol Pathol 2017; 30: 209–216)

Key words: urethane, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, benzo[a]pyrene, lung tumorigenesis

Introduction

Rodent initiation/promotion models (two-stage models) have been used to evaluate potential tumor promotion activities of chemicals and to elucidate tumorigenesis during acute and semi-chronic phases. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) recommended conducting a long-term carcinogenesis study to evaluate potential carcinogenicity in rodents, along with a second study, in either a short or medium term (initiation/promotion study) rodent test system. This protocol was suggested instead of chronic exposure studies in two species[1]. Two-stage models have frequently been used to evaluate skin, liver, lung, and colon carcinogenesis.

Air pollutants and nanoparticles have been concerned a potential risk with regard to impairment of the respiratory organs. The current study protocol for lung carcinogenesis needs to include more animals and a longer study period. The medium-term carcinogenesis screening procedure facilitates elucidation of the potential risk of these materials to the lung. Regarding the medium-term study protocol, medium-term rat studies have been conducted that have targeted liver carcinogenesis and multiple organs (DMBDD model) in rats[2]. In those models, 20 to 100 mg/kg of initiators were administrated to F344 rats more than once. In the multi-organ model, N-bis(2-hydroxypropyl)-nitrosamine (DHPN) is administrated in drinking water as an initiator; however, DHPN is difficult to obtain.

Urethane, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and benzo[a]pyrene (B[a]P) showed lung carcinogenicity in animal models, and they are frequently used in chemoprevention studies and two-stage models[3–6]. However, such studies have employed higher doses that generally have caused an approximately 100% incidence of adenoma[6]. Ethyl carbamate, also known as urethane, NNK, and B[a]P are classified as "probably carcinogenic to humans (group 2A)," "carcinogenic to humans (group 1)," and "carcinogenic to humans (group 1)," respectively, by the International Agency for Research on Cancer[3–5]. In mice, urethane induced malignant tumors by various routes of ad-

Received: 19 January 2017, Accepted: 6 March 2017
Published online in J-STAGE: 31 March 2017
*Corresponding author: S Onami (e-mail: saeko.onami@jt.com)
©2017 The Japanese Society of Toxicologic Pathology
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: https://creativecommons.org/licenses/by-nc-nd/4.0/).

ministration in the lung, lymphoid tissue, mammary gland, leukocytes, forestomach, heart, liver, Harderian gland, skin, ovary, and adrenal gland[3]. NNK induced malignant tumors in the lung, forestomach, liver, and skin[4]. B[a]P induced malignant tumors in the lung, forestomach, liver, lymphoid tissue, skin, and sarcoma in the skin (injection site)[5]. While many studies used two-stage models and middle- or high-dose administration of test chemicals to mice, few examined lower doses. Our study evaluated the applicable dose range (dose dependence) of three compounds as the initiators in a two-stage model by assessing carcinogenesis caused by administration of each at relatively low doses. A low dose application of B[a]P was reported to not induce lung tumors in male mice[7]. Therefore, in our study, only female mice were used to test B[a]P, while the other compounds were tested in mice of both sexes.

In the two-stage model using rodents, especially liver and colon two-stage carcinogenesis models, hepatic cancer and colon cancer were reported to be induced in a shorter term in rats[2]. However, period shortening was not established in mouse strains except for genetically engineered mice (transgenic and knockout mice)[8]. Generally, a carcinogenesis model using A-strain mice would have shown tumor induction within a short term compared with other strains. The three carcinogens used in the present study have been frequently used in many carcinogenesis studies as representative initiators. Regarding NNK, Yamakawa *et al.*[9] and others have reported many carcinogenesis studies using A-strain mice, however, few authors have reported carcinogenesis studies for urethane and B[a]P[6].

A sex difference is observed in the mortality of lung cancer in Japan, and a sex difference is also observed in a mouse carcinogenesis model[10]. Both sexes were used in this study to elucidate the pathogenesis/background factors for lung cancer. However, only female mice were used for B[a]P due to the capacity of the animal rooms. The aim of this study was to evaluate dose dependency within the ranges of low to medium doses of the initiators in a two-stage model using A-strain mice.

Materials and Methods

Test chemicals

Urethane (CAS: 51-79-6) and B[a]P (CAS: 50-32-8) were from Sigma-Aldrich Corporation (St. Louis, MO, USA). NNK (CAS: 64091-91-4) was from Toronto Research Chemicals (Toronto, ON, Canada).

Animals

Six-week-old male and female A/JJmsSlc (A/J) mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in polycarbonate cages (five mice per cage) with soft chips for bedding under a 12 hr light/12 hr dark cycle at 23 ± 3°C and 35.0–75.0% relative humidity, with air flow set at 12 air changes/hr. Animals received a certified rodent diet (CR-LPF, Oriental Yeast, Tokyo, Japan) and fresh tap water (City of Kamisu, Ibaragi, Japan) *ad libitum*.

Table 1. Study Design

Group	Sex	Initiator	Target dose (mg/head)	No. of animals
1	Male	Urethane	0 (vehicle)	40
2			0.2	40
3			1.0	40
4			5.0	40
5	Female		0 (vehicle)	40
6			0.2	40
7			1.0	40
8			5.0	40
9	Male	NNK	0 (vehicle)	40
10			0.25	40
11			0.50	40
12			1.0	40
13			2.0	40
14	Female		0 (vehicle)	40
15			0.25	40
16			0.50	40
17			1.0	40
18			2.0	40
19	Female	B[a]P	0 (vehicle)	40
20			0.25	40
21			0.50	40
22			1.0	40

The study was conducted in a contract laboratory accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). General procedures for animal care and housing complied with the "Guide for Care and Use of Laboratory Animals and Public Health Service Policy on Humane Care and use of Laboratory Animals" (National Research Council, 1996). The study protocol was approved by the Institutional Animal Care and Use Committee of the test facility prior to commencing the experiment.

Experimental design

After a 1- or 2-week acclimatization period, 360 adult male A/J mice and 520 adult female A/J mice were used for the experiment. Mice were allocated, using a body weight-based randomization process, to a total of 22 groups, according to the study design summarized in Table 1. Briefly, each animal received a single intraperitoneal administration of one of the initiators, at one of the indicated doses, on day 1. The highest doses of these chemicals were selected based on previous testing[6, 7, 10–12]. The animals were observed daily for clinical signs and mortality, and body weights were measured weekly. Following an overnight fast at 26 weeks after dosing, all mice were anesthetized with sevoflurane and weighed. They were then sacrificed by exsanguination from the abdominal aorta and caudal vena cava and subjected to necropsy.

Histopathology

After weighing, lungs were instilled with 10% neutral buffered formalin under a pressure of 20 cm water. After

fixation, all five lobes of each lung were embedded in paraffin. They were sectioned in steps (each at a 3 mm interval) into 3-μm-thick sections. Sections from all lobes were stained with hematoxylin and eosin and examined under a light microscope. Animals found dead or moribund were also analyzed as much as possible. For each mouse, 11 to 16 slides were evaluated in detail. Hyperplasia and adenoma were scored for all slides. The multiplicity of hyperplasia and adenoma were calculated for each group, based on the total number of lesions observed divided by the number of mice bearing tumors.

Statistical analysis

Body and lung weights were analyzed for distribution normality using the Shapiro-Wilk test, with homogeneity of variances confirmed by Bartlett's test. For multiple comparisons of treated groups against the vehicle, Dunnett's test for parametric data or Dunnett's multiple comparison test for nonparsmetric data was performed, as appropriate.

The incidence of histopathological findings was analyzed by the Fisher's exact probability test. Multiplicities of histopathological findings were analyzed by the Shirley-Williams test. The dose-dependence of the incidence of histopathological findings was analyzed by the Cochran-Armitage trend test, and that of the multiplicities of histopathological findings was analyzed by the Jonckheere-Terpstra trend test.

Statistical analysis was performed using BellCurve for Excel, Version 2.0.2 (Social Survey Research Information Co., Ltd., Tokyo, Japan), or JMP, Version 10.0.2 (SAS Institute, Cary, NC, USA), and differences were considered to be significant at $p<0.05$.

Results

General conditions

There were no deteriorations in the general conditions observed in urethane-treated groups, except for in three mice. Two females receiving 0.2 mg and one receiving 5 mg were found dead at 14, 26, and 24 weeks, respectively. One mouse was diagnosed with malignant lymphoma, considered to be the cause of death, but no cause of death was determined for the others. There were no deteriorations in the general conditions observed in NNK groups, except for in one mouse. One male receiving 2 mg NNK became moribund at 18 weeks and was immediately sacrificed. A potential cause of death was intranasal adenocarcinoma. There were no deteriorations observed in the general conditions in B[a]P-treated groups, except for in one mouse. One female receiving the vehicle became moribund at 18 weeks and was immediately sacrificed, with no cause of death determined.

Body weights

In urethane-treated groups, statistically significant decreases in body weight were observed in males at 8–10, 17–18, and 20–22 weeks and in females at 1–11, 15–16, and 18 weeks after receiving 5.0 mg urethane compared with the vehicle group, as shown in Fig. 1 (A and B). The body weights of the NNK-treated groups are shown in Fig. 1 (C and D). Statistically significant decreases compared with the vehicle group were observed in the following groups: at 1–2, 8, and 22 weeks, males receiving 1.0 mg NNK; at 1–11, 15–16, and 18 weeks, males receiving 2.0 mg NNK; and at 1–26 weeks, females receiving 2.0 mg NNK. The body weights of the B[a]P-treated groups are shown in Fig. 1 (E). Statistically significant decrease was observed at 7 weeks in females receiving 1.0 mg B[a]P compared with the vehicle group. For all three chemicals, body weight gains were suppressed, relative to those in the vehicle-treated groups, in the highest dose groups. However, the levels of suppression were within 10% of the total weight gains in the vehicle groups (Table 2).

Lung weights

The final body weights and absolute and relative lung weights of the urethane-, NNK-, and B[a]P-treated groups summarized in Table 2. In the urethane-treated groups, no statistically significant differences in lungs weights were observed among the groups. In the NNK-treated groups, statistically significant increases in absolute and relative lung weights were observed in female mice treated with 2 mg NNK, the highest dose. At sacrifice, the relative lung weights, which were normalized to body weights, were also significantly higher in the females receiving the highest NNK dose. In the B[a]P-treated groups, no statistically significant differences in lung weights were detected among the groups.

Necropsy observations

Lung nodules were observed on the lung surface in all groups, including cage controls and vehicle groups (data not shown). The nodules were macroscopically similar in size and shape among the chemically induced groups, cage controls, and vehicle-treated groups.

Histopathology

Urethane-induced lung tumorigenesis in male and female mice was dose dependent. Lung lesions, which were evaluated microscopically, are summarized in Table 3. Proliferative lesions in the lungs were classified as bronchiolar-alveolar hyperplasia or adenoma, and no malignant neoplasms (adenocarcinoma) were observed (Fig. 2). Hypertrophic alterations of Clara cells or type II epithelial cells increased and extended to alveolar walls, and the alterations were comprised of hypertrophic alveolar type II epithelial cells and alveolar macrophages and leukocytes, which are thought to be the early stage changes of hyperplasia. Hyperplasia and adenoma were observed with slight enlargement of nuclei, and vacuolation was observed in rare cases, although mitosis and polymorphism of nuclei were not observed. Inflammatory cells were slightly infiltrated around the adenoma. Hyperplasia and adenoma in the lungs were detected in all groups. The incidences of hyperplasia and adenoma were significantly increased in both sexes in a urethane dose-dependent manner. In 1.0 and 5.0 mg urethane-

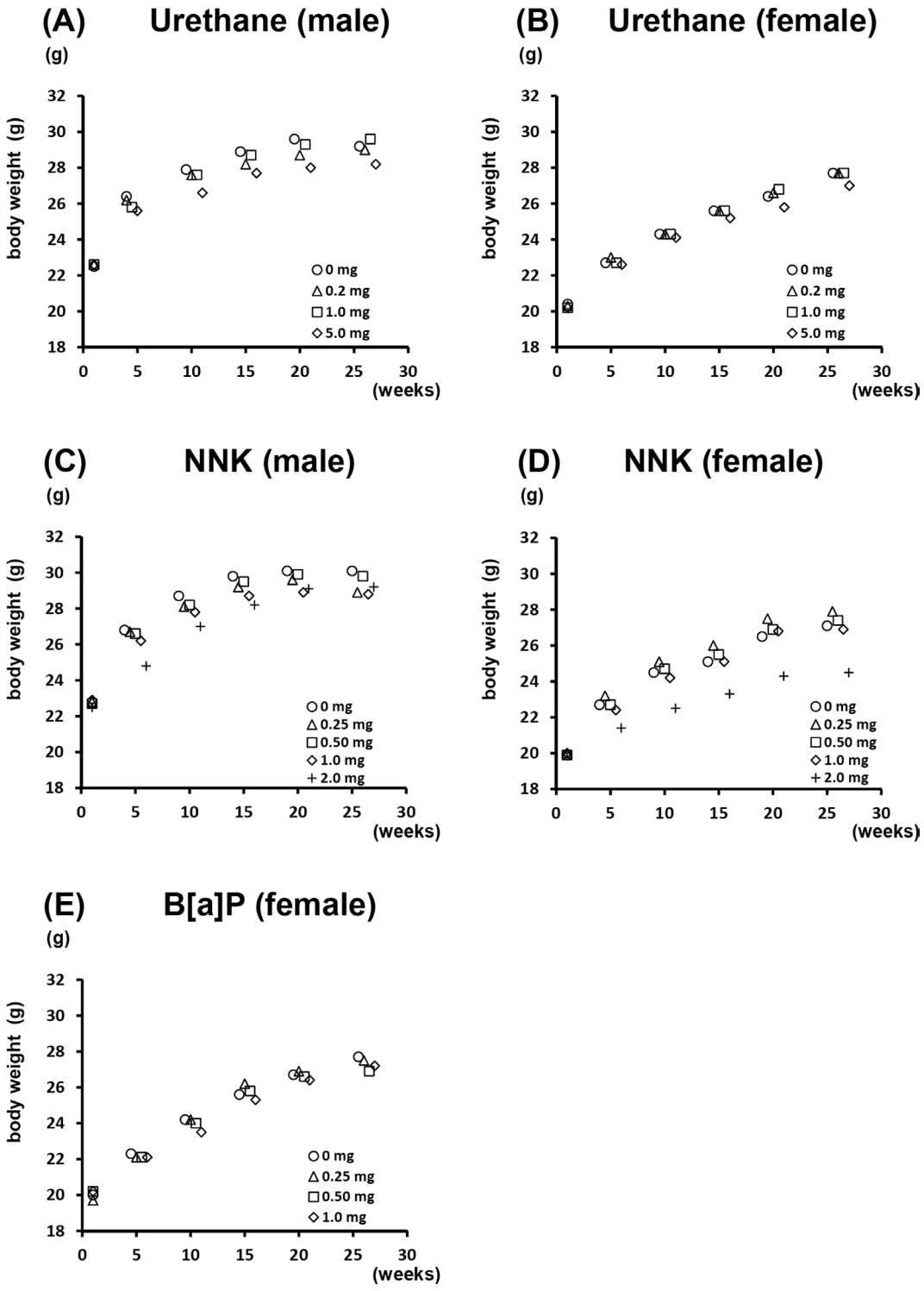

Fig. 1. Body weights of A/J mice at 26 weeks after administration of urethane (A, male, and B, female), NNK (C, male, and D, female), and B[a]P (E, female). (A) Significant differences from the vehicle group (labeled 0 mg) were observed in male mice receiving 5.0 mg urethane: $p<0.05$ at 8–10, 18, and 20–22 weeks and $p<0.01$ at 17 weeks. (B) Significant differences from the vehicle group (labeled 0 mg) were observed in female mice receiving 5.0 mg urethane: $p<0.05$ at 9, 11, 15–16, and 18 weeks and $p<0.01$ at 1–8 and 10 weeks. (C) Significant differences from the vehicle group (labeled 0 mg) were observed in male mice receiving 1.0 mg NNK and male mice receiving 2.0 mg NNK: $p<0.05$ at 2, 8, and 22 weeks and $p<0.01$ at 1 week and $p<0.05$ at 9, 11, 15–16, and 18 weeks and $p<0.01$ at 1–8 and 10 weeks, respectively. (D) Significant differences from the vehicle group (labeled 0 mg) were observed in female mice receiving 2.0 mg NNK: $p<0.05$ at 2, 6, and 25 weeks and $p<0.01$ at 1, 3–5, 7–24, and 26 weeks. (E) Significant differences from the vehicle group (labeled 0 mg) were observed in female mice receiving 1.0 mg B[a]P: $p<0.05$ at 7 weeks.

Table 2. Lung Weights for A/J Mice Treated with Urethane, NNK, or B[a]P

Treatment	Gender	Dose (mg/head)	n	Body weight (g)[a]	Weight change ratio against vehicle group (%)	Absolute	Relative
						Lungs (g)	Lungs (%)
Urethane	Male	Vehicle	40	26.7 ± 2.7	-	0.17 ± 0.02	0.63 ± 0.06
		0.2	40	26.9 ± 1.6	0.4	0.17 ± 0.01	0.62 ± 0.05
		1.0	40	27.2 ± 2.4	−1.6	0.17 ± 0.01	0.62 ± 0.05
		5.0	40	25.6 ± 1.8	3.3	0.17 ± 0.01	0.66 ± 0.05
Urethane	Female	Vehicle	40	25.3 ± 2.5	-	0.16 ± 0.02	0.65 ± 0.06
		0.2	40	25.4 ± 2.5	0.2	0.16 ± 0.01	0.64 ± 0.06
		1.0	40	25.1 ± 2.9	0.2	0.16 ± 0.01	0.65 ± 0.07
		5.0	40	24.6 ± 3.1	2.6	0.17 ± 0.01	0.68 ± 0.08
NNK	Male	Vehicle	40	28.2 ± 3.2	-	0.16 ± 0.01	0.58 ± 0.06
		0.25	40	26.9 ± 2.1	3.8	0.16 ± 0.01	0.62 ± 0.09
		0.50	40	27.9 ± 2.8	0.7	0.16 ± 0.01	0.58 ± 0.05
		1.0	40	26.8 ± 1.9	4.1	0.16 ± 0.01	0.60 ± 0.05
		2.0	40	27.1 ± 2.5	3.0	0.16 ± 0.01	0.60 ± 0.05
NNK	Female	Vehicle	40	24.8 ± 2.3	-	0.15 ± 0.01	0.63 ± 0.06
		0.25	40	25.4 ± 2.7	−3.2	0.16 ± 0.01	0.62 ± 0.06
		0.50	40	25.1 ± 2.6	−1.3	0.16 ± 0.01	0.64 ± 0.06
		1.0	40	24.8 ± 2.9	0.6	0.16 ± 0.01	0.65 ± 0.05
		2.0	40	22.8 ± 2.5 **	9.5	0.17 ± 0.01**	0.74 ± 0.08 **
B[a]P	Female	Vehicle	40	25.1 ± 2.3	-	0.17 ± 0.01	0.67 ± 0.05
		0.25	40	24.9 ± 2.7	0.5	0.16 ± 0.02	0.66 ± 0.06
		0.50	40	24.6 ± 2.7	2.6	0.16 ± 0.01	0.67 ± 0.06
		1.0	40	24.4 ± 2.8	1.6	0.16 ± 0.01	0.68 ± 0.06

Values are shown as means ± standard deviation. [a]The body weights were measured at week 26 after an overnight fast before sacrifice. **Significantly different from the vehicle group at $p<0.01$.

Table 3. Histopathological Analysis of Proliferative Lesions in Lungs from A/J Mice Treated with Urethane, NNK, or B[a]P

Treatment	Gender	Dose (mg/head)	n	Hyperplasia		Adenoma	
				Incidence (%)[a]	Multiplicity[a, b]	Incidence (%)[a]	Multiplicity[a, b]
Urethane	Male	Vehicle	40	7/40 (17.5)	1.0 ± 0.0	4/40 (10.0)	1.3 ± 0.5
		0.2	40	7/40 (17.5)	1.4 ± 0.5*	2/40 (5.0)	1.0 ± 0.0
		1.0	40	16/40 (40.0)*	1.0 ± 0.0	15/40 (37.5)**	1.4 ± 0.5
		5.0	40	37/40 (92.5)**	1.8 ± 0.9**	38/40 (95.0)**	2.6 ± 1.5**
Urethane	Female	Vehicle	40	10/40 (25.0)	1.1 ± 0.3	3/40 (7.5)	1.0 ± 0.0
		0.2	40	12/40 (30.0)	1.1 ± 0.3	3/40 (7.5)	1.3 ± 0.6
		1.0	40	21/40 (52.5)*	1.4 ± 0.7	10/40 (25.0) *	1.0 ± 0.0
		5.0	40	37/40 (92.5)**	2.9 ± 1.7**	35/40 (87.5)**	2.8 ± 1.6**
NNK	Male	Vehicle	40	5/40 (12.5)	1.4 ± 0.5	3/40 (7.5)	1.0 ± 0.0
		0.25	40	15/40 (37.5)**	1.1 ± 0.4	9/40 (22.5)	1.1 ± 0.3
		0.50	40	9/40 (22.5)	1.2 ± 0.7	4/40 (10.0)	1.0 ± 0.0
		1.0	40	19/40 (47.5)**	1.6 ± 0.8	15/40 (37.5)**	1.4 ± 0.5
		2.0	40	35/40 (87.5)**	2.8 ± 1.8*	27/40 (67.5)**	2.2 ± 1.3**
NNK	Female	Vehicle	40	5/40 (12.5)	1.0 ± 0.0	5/40 (12.5)	1.0 ± 0.0
		0.25	40	14/40 (35.0)*	1.4 ± 0.5	4/40 (10.0)	1.3 ± 0.5
		0.50	40	26/40 (65.0)**	1.7 ± 1.0*	12/40 (30.0) *	1.2 ± 0.4
		1.0	40	37/40 (92.5)**	3.0 ± 1.6**	29/40 (72.5)**	2.0 ± 1.2**
		2.0	40	40/40 (100)**	8.1 ± 3.1**	39/40 (97.5)**	4.0 ± 1.8**
B[a]P	Female	Vehicle	40	6/40 (15.0)	1.0 ± 0.0	7/40 (17.5)	1.0 ± 0.0
		0.25	40	19/40 (47.5)**	1.2 ± 0.4	4/40 (10.0)	1.0 ± 0.0
		0.50	40	26/40 (65.0)**	1.8 ± 1.0**	11/40 (27.5)	1.0 ± 0.0
		1.0	40	39/40 (97.5)**	3.3 ± 1.8**	18/40 (45.0)**	1.6 ± 1.1*

[a]Microscopically confirmed with step-sectioned specimens (3 mm intervals); all 5 lobes were examined for each mouse. [b]The total number of lesions observed divided by the number of mice bearing tumors (for each group). Each value represents the mean ± SD. *Significantly different from the vehicle group at $p<0.05$. **Significantly different from the vehicle group at $p<0.01$.

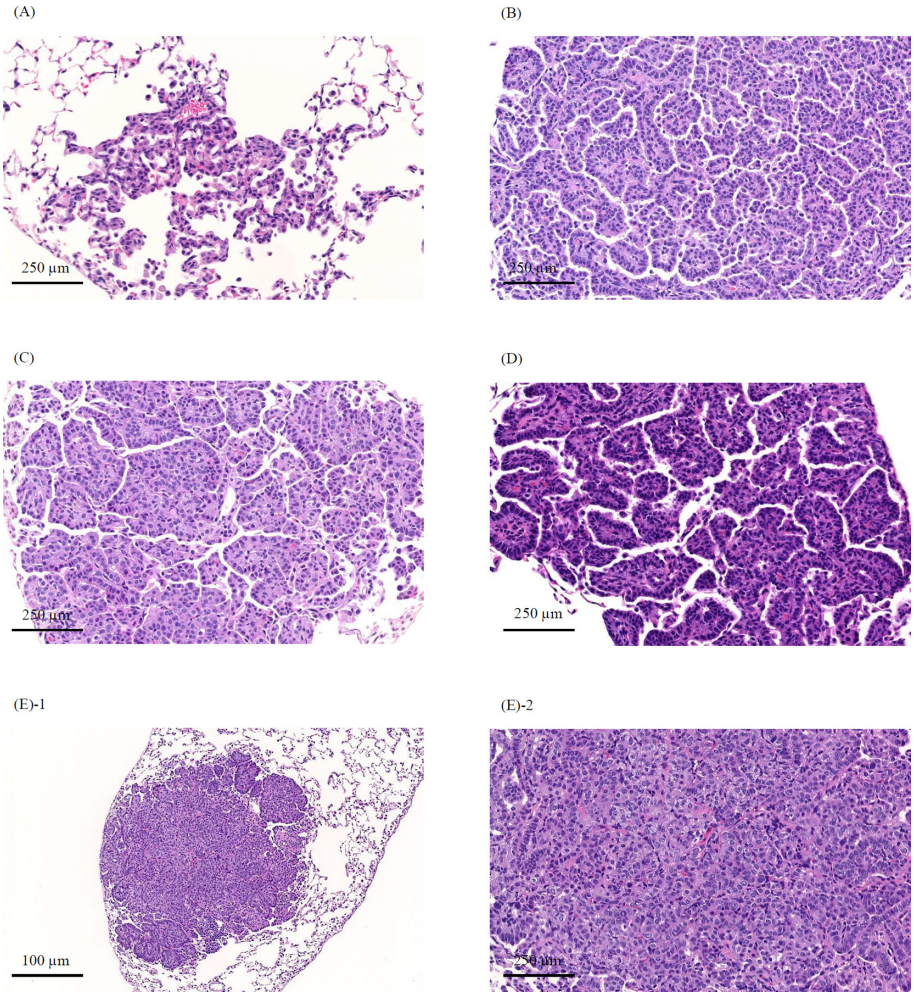

Fig. 2. Lung histopathology showing proliferative lesions (hematoxylin and eosin staining). Representative histopathology of lung lesions in the 26 week study. Bar = 100 or 250 µm. (A) Bronchiolar-alveolar hyperplasia in a female mouse receiving 2.0 mg NNK; (B) adenoma in a vehicle-treated male (the control for urethane-treated groups); (C) adenoma in a female receiving 2.0 mg NNK; (D) adenoma in a female receiving 1.0 mg urethane; (E-1 and E-2) adenoma in a female receiving 1.0 mg B[a]P.

treated groups of both sexes, the incidences of hyperplasia and adenoma were significantly higher than in the corresponding vehicle groups. The multiplicity of hyperplasia values in males treated with 0.2 mg and males and females with 5.0 mg urethane were significantly higher than in the vehicle groups. The multiplicity of adenoma values of both sexes in the groups given 5.0 mg urethane were significantly higher than in the vehicle groups.

With NNK treatment, hyperplasia and adenoma developed in the lungs of mice in all groups. Dose-dependent lung tumorigenesis was observed in both male and female mice. The incidence and multiplicity of lung lesions, which were diagnosed microscopically, are summarized in Table 3. Proliferative lesions in the lungs were classified as bronchiolar-alveolar hyperplasia or adenoma in all mice, including vehicle groups, and no malignant neoplasms (adenocarcinoma) were observed (Fig. 2). Compared with the vehicle groups, the incidence of hyperplasia was significantly higher in the male and female mice treated with 0.25, 1.0, or 2.0 mg NNK and in females receiving 0.50 mg NNK. The incidence of adenoma values were significantly higher in male and female mice treated with 1.0 or 2.0 mg NNK and in females receiving 0.50 mg NNK than in the vehicle groups. The multiplicity of hyperplasia values in males receiving 2.0 mg NNK and females receiving 0.50, 1.0 or 2.0 mg NNK were significantly higher than in the vehicle groups. The multiplicity of adenoma values in males receiving 1.0 mg and females receiving 1.0 or 2.0 mg NNK were significantly higher than with vehicle treatment.

As shown in Table 3, lung tumorigenesis induced by B[a]P was dose dependent in female A/J mice. The incidence of hyperplasia values in females treated with 0.25, 0.50, and 1.0 mg B[a]P were significantly higher than in the vehicle-treated group. The incidence of adenoma in females receiving 1.0 mg B[a]P was significantly higher than in the vehicle group. The multiplicity of hyperplasia in females re-

ceiving 0.50 or 1.0 mg B[a]P was significantly higher than in the vehicle group. The multiplicity of adenoma in the group treated with 1.0 mg was also significantly higher than in the vehicle group. The incidences of hyperplasia and adenoma in female A/J mice were significantly increased by B[a]P in a dose-dependent manner. Proliferative lesions in the lungs were classified as bronchiolar-alveolar hyperplasia or adenoma, and no malignant neoplasms (adenocarcinoma) were observed (Fig. 2).

Discussion

Suppression of body weight gain was previously reported in female mice treated with a high dose of NNK. Because this effect became no longer statistically significant by 32 weeks, it was regarded as not being lethal[13]. In our study, the maximal suppression of body weight gain in the highest dose groups was approximately 10%, meeting the criteria for the maximally tolerated dose in a carcinogenicity study. The absolute and relative lung weights were significantly increased in females treated with the highest dose of NNK. In this group, the multiplicities of hyperplasia and adenoma were also higher than in the other groups. These latter parameters might have affected the absolute and relative lung weights. All three compounds tested had dose-dependent effects on the incidences of hyperplasia and adenoma.

The three compounds we tested are known to induce K-*ras* mutations in the process of causing carcinogenesis[14–16]. Chen *et al*.[17] reported that various doses of NNK induced mutations at different sites of the K-*ras* gene. This sensitivity was attributed to a 37-base-pair deletion in the second intron of K-*ras*[18]. The deleted K-*ras* gene is expressed earlier in development than is the wild type gene, presumably providing a longer time frame for selection of K-*ras* mutations during periods when cell proliferation rates are high. Imaida *et al*.[19] described the A/J mouse as a very useful strain that is reliable for modeling *in vivo* lung carcinogenesis and also for identifying chemopreventive agents. For the reason, we selected A/J mice for our study.

Regarding B[a]P, Meng *et al*.[20] reported that the compound increased the mutation fraction of K-*ras* in a dose-dependent manner. Therefore, for both NNK and B[a]P, the compounds altered the mutation repair system, resulting in dose-dependent tumorigenesis. Our results suggested that the three compounds all induced carcinogenesis in a dose-dependent manner at lower concentrations compared with previous reports[6, 10–12].

In a two-stage hepatoma study, the initiator dosage is generally administered at a dose that can induce precancerous lesions (such as GST-P-positive cell lesions in the liver) but is insufficient to induce cancerous lesions. The A/J strain is well known for having spontaneous lung tumors. In a lung carcinogenesis study with these mice, potentially useful compound dosimetry information would be the dose causing a statistically significant increase in hyperplasia without significant adenoma. As an example, this was 0.25 mg for NNK and 0.25 or 0.50 mg for B[a]P in our study.

No cases of adenocarcinoma were observed during our study. With regard to NNK, Belinsky *et al*.[21] suggested that the dose used by them required over 34 weeks for induction of malignant alterations. Yamakawa *et al*.[9] reported that a single administration of 2 mg/head NNK resulted in the development of adenocarcinomas, adenomas, and hyperplasia in the lung after 52 weeks. Ninomiya *et al*.[10] reported that a longer post-administration period was correlated with a higher number of adenocarcinomas. Estensen *et al*.[11] reported that adenocarcinoma development required adequate time and that, in the case of B[a]P, higher doses accelerated pathogenesis. With regard to urethane, Gurley *et al*.[12] showed that adenomas developed by 25 weeks and adenocarcinomas developed by 40 weeks. Furthermore, the number of adenocarcinomas increased in accordance with the period of time after administration[22]. The doses used in our experiment were relatively low, and the study period was semi-chronic. Based on this information, progression of adenomas to adenocarcinomas may be a useful indicator for evaluation of these initiators as promoters.

Generally, high-dose administration has been reasonably well accepted in past carcinogenicity studies, especially those intended to identify hazards. However, it is preferable to select an initiator dose that is within the range giving a linear-dose response in prospective carcinogenicity studies[23], similar to in our experiment. The dosimetry used in the study, which showed precancerous histopathological alterations, could contribute to risk assessment of air pollutants and nanoparticles inhaled into the lung.

In our study, the three test compounds, which were used at lower concentrations compared with previous reports[6, 10–12], conclusively showed dose-dependent effects on the incidences of lung hyperplasia and adenoma. Our data predicted the applicable dose ranges for these agents as initiators in a two-stage carcinogenesis model. The activity of all three chemicals as initiators should be evaluated in further studies.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

References

1. ICH (International Conference on Harmonisation) Guidance for Industry, S1B Testing for Carcinogenicity of Pharmaceuticals. 1–10. 1997.
2. Tsuda H, Futakuchi M, Fukamachi K, Shirai T, Imaida K, Fukushima S, Tatematsu M, Furukawa F, Tamano S, and Ito N. A medium-term, rapid rat bioassay model for the detection of carcinogenic potential of chemicals. Toxicol Pathol. **38**: 182–187. 2010. [Medline] [CrossRef]
3. IARC (International Agency for Research on Cancer) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 96, Lyon. 1281–1378. 2010.
4. IARC (International Agency for Research on Cancer) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 100E, Lyon. 319–331. 2012.

5. IARC (International Agency for Research on Cancer) IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 100F, Lyon. 111–144. 2012.
6. Vikis HG, Rymaszewski AL, and Tichelaar JW. Mouse models of chemically-induced lung carcinogenesis. Front Biosci (Elite Ed). **5**: 939–946. 2013. [Medline] [CrossRef]
7. Singh SV, Benson PJ, Hu X, Pal A, Xia H, Srivastava SK, Awasthi S, Zaren HA, Orchard JL, and Awasthi YC. Gender-related differences in susceptibility of A/J mouse to benzo[a]pyrene-induced pulmonary and forestomach tumorigenesis. Cancer Lett. **128**: 197–204. 1998. [Medline] [CrossRef]
8. Fukushima S. Medium-term tests for carcinogens and carcinogenic hazard evaluation for humans. Journal of Occupational Safety and Health. **1**: 141–149. 2008. [CrossRef]
9. Yamakawa K, Yokohira M, Nakano Y, Kishi S, Kanie S, and Imaida K. Activation of MEK1/2-ERK1/2 signaling during NNK-induced lung carcinogenesis in female A/J mice. Cancer Med. **5**: 903–913. 2016. [Medline] [CrossRef]
10. Ninomiya F, Yokohira M, Kishi S, Nakano Y, Yamakawa K, Inoue T, Kuno T, and Imaida K. Gender-dependent effects of gonadectomy on lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female and male A/J mice. Oncol Rep. **30**: 2632–2638. 2013. [Medline]
11. Estensen RD, Jordan MM, Wiedmann TS, Galbraith AR, Steele VE, and Wattenberg LW. Effect of chemopreventive agents on separate stages of progression of benzo[alpha]pyrene induced lung tumors in A/J mice. Carcinogenesis. **25**: 197–201. 2004. [Medline] [CrossRef]
12. Gurley KE, Moser RD, and Kemp CJ. Induction of lung tumors in mice with urethane. Cold Spring Harb Protoc. **2015**: pdb.prot077446. 2015. [Medline] [CrossRef]
13. Kuno T, Yokohira M, Matsuda Y, Suzuki S, Hashimoto N, Yamakawa K, Saoo K, and Imaida K. Lack of modifying potential of 8-methoxypsoralen in the promotion or progression stages of lung carcinogenesis in A/J female mice. Oncol Rep. **20**: 767–772. 2008. [Medline]
14. Gray DL, Warshawsky D, Xue W, Nines R, Wang Y, Yao R, and Stoner GD. The effects of a binary mixture of benzo(a) pyrene and 7H-dibenzo(c,g)carbazole on lung tumors and K-ras oncogene mutations in strain A/J mice. Exp Lung Res. **27**: 245–253. 2001. [Medline] [CrossRef]
15. Hisamoto A, Kondo E, Kiura K, Okada T, Hosokawa S, Mimoto J, Takigawa N, Tabata M, and Tanimoto M. Point mutation of K-ras gene in cisplatin-induced lung tumours in A/J mice. Lung Cancer. **58**: 15–20. 2007. [Medline] [CrossRef]
16. You M, Candrian U, Maronpot RR, Stoner GD, and Anderson MW. Activation of the Ki-ras protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. Proc Natl Acad Sci USA. **86**: 3070–3074. 1989. [Medline] [CrossRef]
17. Chen B, Liu L, Castonguay A, Maronpot RR, Anderson MW, and You M. Dose-dependent ras mutation spectra in N-nitrosodiethylamine induced mouse liver tumors and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone induced mouse lung tumors. Carcinogenesis. **14**: 1603–1608. 1993. [Medline] [CrossRef]
18. Chen B, Johanson L, Wiest JS, Anderson MW, and You M. The second intron of the K-ras gene contains regulatory elements associated with mouse lung tumor susceptibility. Proc Natl Acad Sci USA. **91**: 1589–1593. 1994. [Medline] [CrossRef]
19. Imaida K, Yokohira M, Hashimoto N, and Kuno T. Risk analysis of environmental chemicals on lung carcinogenesis. Asian Pac J Cancer Prev. **11**: 9–12. 2010. [Medline]
20. Meng F, Knapp GW, Green T, Ross JA, and Parsons BL. K-Ras mutant fraction in A/J mouse lung increases as a function of benzo[a]pyrene dose. Environ Mol Mutagen. **51**: 146–155. 2010. [Medline]
21. Belinsky SA, Devereux TR, Foley JF, Maronpot RR, and Anderson MW. Role of the alveolar type II cell in the development and progression of pulmonary tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the A/J mouse. Cancer Res. **52**: 3164–3173. 1992. [Medline]
22. Nuzum EO, Malkinson AM, and Beer DG. Specific Ki-ras codon 61 mutations may determine the development of urethan-induced mouse lung adenomas or adenocarcinomas. Mol Carcinog. **3**: 287–295. 1990. [Medline] [CrossRef]
23. Hayashi Y. Scientific basis for risk analysis of food-related substances with particular reference to health effects on children. J Toxicol Sci. **34**(Suppl 2): SP201–SP207. 2009. [Medline] [CrossRef]