Inhibition of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA Adduct Formation and Tumorigenicity in the Lung of F344 Rats by Dietary Phenethyl Isothiocyanate

Mark A. Morse, Chung-Xiou Wang, Gary D. Stoner, Swapna Mandal, Philip B. Conran, Shantu G. Amin, Stephen S. Hecht, and Fung-Lung Chung

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595 [M. A. M., C-X. W., S. G. A., S. S. H., F-L. C.] and Department of Pathology, Medical College of Ohio, Toledo, Ohio 43699 [G. D. S., S. M., P. B. C.]

ABSTRACT

F344 rats fed diets containing phenethyl isothiocyanate (PEITC, 3 μmol/g diet), a cruciferous vegetable component, before and during treatment with the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), developed about 50% fewer lung tumors than NNK-treated rats fed control diets. NNK-induced liver and nasal cavity tumors in rats were, however, not affected by this dietary treatment. The effects of PEITC diets on the formation of DNA adducts by NNK were also investigated in these target tissues. DNA methylation and pyridoxobutylation by NNK were both decreased by 50% in lung of rats fed PEITC diets compared to that of rats fed control diets, but the levels of DNA methylation were not affected in liver and nasal mucosa. These results correlated with those from the carcinogenicity bioassay, suggesting that DNA alkylations could be used as indicators for screening inhibitors of NNK tumorigenesis. A slight increase in the number of tumors of the exocrine pancreas was observed in PEITC-fed rats with or without NNK treatments. However, these incidences were not statistically significant when compared to the control groups. The potential toxicity of PEITC at concentrations ranging from 0.75 μmol to 6 μmol/g diet was evaluated in a 13-week study. The only toxicity noted, as a result of this treatment was minimal fatty metamorphosis in the liver. Considering the widespread human exposure to NNK through tobacco use, it is of practical importance to demonstrate inhibition of lung tumors induced by this carcinogen. These results provide a basis for studies designed to discover agents of better efficacy for the prevention of NNK-induced tumorigenesis.

INTRODUCTION

NNK (Fig. 1) is the most potent carcinogenic nitrosamine so far found in tobacco and tobacco smoke (1). It induces lung, nasal cavity, liver, and pancreatic tumors in F344 rats; nasal cavity and lung tumors in hamsters; and lung tumors in mice (2). The organ-specific effect of NNK in the induction of lung tumors in all animal species tested regardless of route of administration strongly supports its possible role in the development of lung cancer among smokers. In the U. S. alone, there are still about 53 million cigarette smokers exposed to NNK daily (3). Therefore, it is of great importance to discover compounds either synthetic or dietary related which can effectively alleviate the carcinogenic action of NNK.

Our previous studies demonstrated that pretreatment of rats with a diet containing PEITC (Fig. 1) reduced the number of tumors of the exocrine pancreas was observed in PEITC-fed rats with or without NNK treatments. However, these incidences were not statistically significant when compared to the control groups. The potential toxicity of PEITC at concentrations ranging from 0.75 μmol to 6 μmol/g diet was evaluated in a 13-week study. The only toxicity noted, as a result of this treatment was minimal fatty metamorphosis in the liver. Considering the widespread human exposure to NNK through tobacco use, it is of practical importance to demonstrate inhibition of lung tumors induced by this carcinogen. These results provide a basis for studies designed to discover agents of better efficacy for the prevention of NNK-induced tumorigenesis.

MATERIALS AND METHODS

Chemicals. Both [1H-CH3]NNK (1.36 Ci/mmol; purity 98%) and [5-3H]NNK (0.93 Ci/mmol; purity 98%) were purchased from Chemsys Science Laboratories, Lenexa, KS. The specific activities of [1H-CH3]-NNK and [5-3H]NNK were modified with unlabeled NNK to 0.13 Ci/mmol and 0.55 Ci/mmol, respectively, before use. Unlabeled NNK was synthesized by a method described previously (8). PEITC was obtained from Eastman Kodak Co., Rochester, NY. Its purity was checked by proton nuclear magnetic resonance and HPLC and was found to be greater than 99%.

Animals. Male F344 rats weighing 150 g (bioassay) or 200–300 g (DNA adduct assays) were obtained from Charles River Breeding Laboratories, Kingston, NY. These rats were housed two or three per cage and maintained under standard conditions (20 ± 2°C; 50 ± 10% relative humidity; 12 h light/dark cycle).

Bioassay. Rats at 8 weeks of age were randomized into four groups, each of which consisted of 20 to 40 rats. These four groups are summarized in Table 1. The PEITC diet (3 μmol/g diet) was prepared weekly as described below and stored at 4°C before use. The stability of PEITC in the diet was examined by ethyl acetate extraction of a known quantity of diet containing PEITC. Following concentration of the extracts, the samples were analyzed by HPLC on an Alltech Versapack C18 column eluted with 30% MeOH in H2O isocratically. Under the storage conditions described above, PEITC was stable in the diet for at least 10 days. Groups 2 and 3 were fed PEITC diets ad libitum for 21 weeks while Groups 1 and 4 were given only the NIH-07 diet. After the first week of feeding, NNK (1.76 mg/kg b.w.) was administered by s.c. injection 3 times weekly for 20 weeks. The total dose of NNK administered was 106 mg (0.5 mmol)/kg b.w. Food consumption was measured twice weekly for 21 weeks. Body weights were initially recorded once or twice weekly, then once every other week after 21 weeks. The experiment was terminated after 104 weeks.
INHIBITION OF NNK LUNG TUMORIGENESIS BY PEITC

Fig. 1. Structures of PEITC and NNK.

when 70% mortality was observed in the NNK-treated group. Gross lesions and representative samples of all major organs of all animals used in the bioassay were fixed in 10% buffered formalin and processed for microscopic examination. Statistical significance was determined by the χ² test.

DNA Adduct Assays. Groups of six rats were fed either NIH-07 diet or NIH-07 diet containing 3 μmol/g diet PEITC ad libitum for 2 weeks. In the dose-response study, groups of four rats were fed NIH-07 diet containing 1.5, 6.0, and 10 μmol/g diet PEITC for 2 weeks. The experimental diet was prepared by thoroughly mixing NIH-07 diet with the appropriate amount of PEITC using a mechanical mixer. It was stored at 4°C before use. Beginning on the 11th day of feeding, rats were treated with 0.3 mg/kg b.w. of NNK for 4 consecutive days by s.c. administration. The total dose of NNK administered to each rat was 2.4 mg/kg which contained 0.5 mCi [3H-CH₃]NNK and 2.5 mCi [5-3H]-NNK. Five times greater radioactivity was used for [5-3H]NNK in order to obtain detectable amounts of 4-hydroxy-1-(3-pyridyl)-1-butanone which was released by acid hydrolysis of DNA. In the dose-response study, rats were administered [3H-CH₃]NNK; 4 h after the last NNK dosing, rats were sacrificed and DNA was isolated from liver, lung, and nasal mucosa using the Marmur procedure (9). DNA was purified by the method of Sebti et al. (10). Purified DNA samples were subjected to acid hydrolysis (4). The hydrolysate of each sample was used for the analysis of 7-mGua and 4-hydroxy-1-(3-pyridyl)-1-butanone by HPLC. Simultaneous analysis of 7-mGua and 4-hydroxy-1-(3-pyridyl)-1-butanone was achieved by injecting 50% of the acid hydrolysate from each liver DNA sample and 90% from each lung or nasal mucosa DNA sample. The HPLC system employed for the analysis consisted of a Model 7125 Rheodyne injector, a Waters automated gradient controller, two Waters 510 pumps, a Knauer UV detector, and a Beta Flo-One radioflow detector. All determinations were made using two Whatman Partisil-10 SCX columns connected in sequence. The sample was eluted with 0.2 M ammonium phosphate (pH 2.0), isocratically at a flow rate of 1 ml/min. The retention times of 7-mGua and 4-hydroxy-1-(3-pyridyl)-1-butanone were 12 and 19 min, respectively. Unlabeled 7-mGua was added as a UV marker and guanine was analyzed from a small aliquot of acid hydrolysate using the same HPLC conditions. Results were expressed as nmoi of 7-mGua or keto alcohol/mol of guanine.

Toxicity Study. In a 13-week study, groups of 16 male F344 rats weighing 100-120 g were fed AIN-76 diets containing PEITC at concentrations of 0, 0.75, 1.5, 3.0, and 6.0 μmol/g diet. Body weights were recorded weekly, and food consumption was measured twice weekly for the duration of the study. At the end of 13 weeks, a clinical evaluation was conducted for each group based on the hematological profile, serological examination, and urinalysis of the animals. At necropsy, all body organs were grossly examined and their weights recorded. The tissues were then fixed in 10% buffered formalin and processed for histopathological examination.

RESULTS

The intake of PEITC in the bioassay was approximately 8.8 mg/rat/day, based on an average consumption of 18 g of diet/rat/day. At this dose, there were no significant differences in survival and body weights between PEITC-treated groups and the control groups throughout the 2-year bioassay.

Table 1 shows the tumor incidences in lung, liver, and nasal cavity. The tumor incidences induced by NNK alone were within the expected range based on previous bioassay results (11). Rats fed PEITC before and during NNK treatments developed only 43% lung tumor incidence compared to 80% in the group fed control diets. However, the PEITC diet did not alter the incidences of tumors in liver or nasal cavity induced by NNK. The majority of the NNK-induced tumors observed in lung were adenocarcinomas. It appeared that both benign and malignant tumors in lung were inhibited by PEITC.

Tumor incidences at other sites are listed in Table 2. The group given the PEITC diet alone did not develop tumor incidences significantly different from those of the control group. The number of rats with pancreatic tumors in PEITC groups with or without NNK treatment were slightly higher than those of the NNK group or the control group. However, these differences were not statistically significant. Mammary fibroadenomas and forestomach papillomas were observed in the NNK-treated group; the incidences of these tumors appeared to be reduced in the PEITC-treated group. However, neither the occurrence of these tumors nor PEITC-caused inhibition was statistically significant.

The potential adverse effects of PEITC diets to animals were investigated in a 13-week toxicity study. Dietary administration of PEITC at the concentrations tested did not produce any deleterious effects on the survival, body weight or food intake of the animals in the various groups. Clinical evaluation did not demonstrate any adverse changes in the hematological profile, serum chemistry or urinalysis suggestive of toxicity. In addition, gross examination at necropsy and organ:weight ratios of PEITC-treated rats did not show any significant differences from control animals. However, histopathological analyses revealed centrilobular and midzonal fatty metamorphosis in the liver of rats treated with PEITC at all dose levels. This lesion was confirmed by oil Red O staining and by electron microscopy.

To determine the effects of PEITC diets on the formation of DNA adducts by NNK, we chose to use experimental conditions analogous to those used in the bioassay. The total dose of NNK administered to each rat in the DNA adduct assay (2.4 mg/kg b.w.) was comparable to each single dose (1.76 mg/kg b.w.) in

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Incidence of lung, liver, and nasal cavity tumors after treatment with NNK, NNK + PEITC, and PEITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>No. of rats</td>
</tr>
<tr>
<td>NNK</td>
<td>40</td>
</tr>
<tr>
<td>NNK + PEITC</td>
<td>40</td>
</tr>
<tr>
<td>PEITC</td>
<td>20</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
</tr>
</tbody>
</table>

¹ Squamous cell papillomas, transitional-cell papillomas, polyps.
² Squamous cell carcinomas.
³ One animal had squamous cell carcinoma and 11 had adenocarcinoma.
⁴ P < 0.05 compared to NNK group.
the bioassay. These treatments resulted in DNA methylation and pyridyloxobutylate via metabolic α-hydroxylations of NNK in all target tissues. The structures of DNA adducts resulting from pyridyloxobutylation have not yet been characterized. However, a recent investigation showing the release of 4-hydroxy-1-(3-pyridyl)-1-butanone following acid hydrolysis of rat target tissue DNA suggested that DNA pyridyloxobutylation occurs in vivo (12). While O'-mGua was not detectable at this low NNK dose, 7-mGua was readily measured and was used as an indicator of total DNA methylation. Table 3 shows the effects of 2 weeks' feeding of PEITC diets on DNA methylation and pyridyloxobutylate in liver, lung, and nasal mucosa. The levels of 7-mGua were not affected in DNA of the liver, lung, and nasal mucosa of rats fed this diet. In lung, however, 7-mGua was reduced from 10.4 to 5.9 µmol/mol guanine, a reduction of nearly 50%. The level of DNA pyridyloxobutylate was also decreased to a similar extent in lung, and to a lesser degree in the liver. These results indicated that both DNA methylation and pyridyloxobutylate were inhibited in lung of PEITC-fed rats.

To further study the dose-dependent effects of PEITC on DNA methylation by NNK, rats were fed diets containing levels of PEITC ranging from 1.5 µmol/g diet to 10 µmol/g diet for 2 weeks. There was no apparent toxicity in rats fed levels of PEITC higher than 3 µmol/g diet. Table 4 shows a dose-related effect of PEITC diets on the inhibition of DNA methylation in lung. Diets containing 6.0 and 10 µmol/g diet of PEITC inhibited DNA methylation in lung by 30% and 43%, respectively. However, at 1.5 µmol/g diet, DNA methylation in rat lung was not inhibited. PEITC diet at all concentrations exerted little, if any, effect on DNA methylation in liver and nasal mucosa, with the exception of a 20% reduction that was observed in hepatic DNA at 10 µmol/g diet.

**Table 3 DNA methylation and pyridyloxobutylate in NNK-treated rats fed control or PEITC diets**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Lung 7-mGua</th>
<th>Liver 7-mGua</th>
<th>Nasal Mucosa 7-mGua</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>10.4 ± 1.3</td>
<td>20.6 ± 0.9</td>
<td>22.8 ± 0.7</td>
</tr>
<tr>
<td>PEITC</td>
<td>20</td>
<td>5.9 ± 0.6</td>
<td>12.7 ± 0.1</td>
<td>31.5 ± 1.1</td>
</tr>
</tbody>
</table>

Abbreviation for 4-hydroxy-1-(3-pyridyl)-1-butanone. Mean ± SE of six rats.

**Table 4 DNA methylation in NNK-treated rats fed diets containing PEITC at various concentrations**

<table>
<thead>
<tr>
<th>PEITC concentrations (µmol/g diet)</th>
<th>7-mGua (µmol/mo guanine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>0</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>1.5</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td>6.0</td>
<td>6.3 ± 0.9</td>
</tr>
<tr>
<td>10.0</td>
<td>5.4 ± 0.5</td>
</tr>
</tbody>
</table>

See Table 3.

**DISCUSSION**

In this study we have shown that feeding PEITC in the diet results in an approximate 50% reduction in the incidence of NNK-induced lung tumors in F344 rats. This is the first demonstration of inhibition of NNK tumorigenesis by any compound. While PEITC effectively reduced the incidence of lung tumors in rats, it had no significant effects on NNK-induced liver or nasal cavity tumors. Several factors could account for the selective inhibition of lung tumors by PEITC in rats. These include effects on the tissue disposition of NNK, the detoxification of NNK, and/or the specific effects of PEITC on cytochrome P-450 isozymes responsible for NNK activation. Judging from its structure, PEITC and its metabolites are unlikely to act as scavengers of reactive intermediates generated in the metabolic activation of NNK. The binding of isothiocyanates to proteins through SH or NH2 groups has been well documented (13). It is conceivable that this type of conjugation could occur between PEITC and the specific cytochrome P-450 isozyme(s) in lung which is responsible for NNK activation. Further studies are needed in order to understand the biochemical mechanisms for the inhibition of NNK-induced lung tumors and DNA alkylation in the PEITC-treated rats.

Previously we have shown that hepatic demethylation and DNA methylation of NNK were inhibited in rats fed PEITC diets (4), whereas, in the present study, the same dietary treatment failed to decrease hepatic DNA methylation. The only difference between these experiments was that the NNK dose used in this study (2.4 mg/kg b.w.) was considerably lower than that used in previous experiments (85 mg/kg b.w.). A plausible explanation for the differential effects of PEITC on the hepatic DNA methylation by NNK is the existence of high and low affinity forms of cytochrome P-450 isozymes for NNK activation as suggested by Belinsky et al. (14). The inhibition by PEITC of hepatic DNA methylation at a high dose of NNK but not at the low dose of NNK might be due to the fact that the low affinity form (high Ks) is concentrated in rat liver.

The slight increase in the incidence of the rare exocrine
pancreatic tumors observed in groups treated with PEITC diets is worthy of attention. The highest incidence of pancreatic tumors was observed in rats treated with both NNK and PEITC. However, it is difficult to assess the significance of these findings because of the low incidences and small number of animals used in our bioassay. A recent study showed that low doses of NNK administered in drinking water induced significant incidences of pancreatic tumors in F344 rats (15). Furthermore, the same type of tumors were observed in rats treated with both NNK and sinigrin, the parent glucosinolate of allyl isothiocyanate (16).

The only significant toxic effects of PEITC found in this study were fatty changes in the liver at 13 weeks. However, histopathological examination of the livers of animals harvested from the 2-year bioassay revealed no differences in both PEITC-treated and control animals. Fatty metamorphosis has been reported with allyl isothiocyanate following oral administration in mice (17), and after i.p. injection into rats (18). In general, it is considered to be a reversible lesion; however, as a severe form of injury, this lesion may be a harbinger of cell death (19). Therefore, this result may be a cause for concern, especially at high doses of PEITC.

Two types of DNA modifications, methylation and pyridyloxobutylation, occur in vivo as a result of NNK treatment (4, 12). These DNA lesions are caused by α-hydroxylation of NNK at the methylene carbon or the methyl carbon, respectively. The relative importance of these DNA adducts in NNK carcinogenesis is unclear. The preferential formation and persistence of O6-mGua in specific rat lung cells following NNK treatment suggests that DNA methylation is important in NNK-induced lung tumorigenesis (14, 20). In the present study the levels of O6-mGua were too low to be detected, presumably due to highly efficient repair at this low NNK dose. However, 7-mGua was detected readily in all target tissues examined. Both DNA methylation and pyridyloxobutylation were reduced in the lungs of rats fed PEITC. These results strongly suggest that the protective effect of PEITC diet against NNK-induced lung tumorigenesis is due to its ability to inhibit adduct formation. However, the decrease of pyridyloxobutylation in hepatic DNA was not reflected in the reduction of liver tumor incidence in PEITC-treated rats. Two possible explanations are that inhibition of tumors requires reduction in the formation of both adducts, or alternatively, that DNA methylation is more important than pyridyloxobutylation in NNK tumorigenesis. Our results showed that the effects of PEITC on NNK tumorigenesis were in good agreement with its effects on DNA methylation, indicating that DNA methylation could be used as a means of screening potential inhibitors of NNK carcinogenicity.

PEITC is a product of hydrolysis of glucosinortiin which is commonly found in turnips and rutabagas (21). The enzyme myrosinase which catalyzes the hydrolysis has been found in cruciferous vegetables as well as in intestinal bacteria (21). In view of widespread human exposure to these compounds, it is important to demonstrate the inhibition of NNK lung tumorigenesis by a naturally occurring dietary constituent as this could be applied to an intervention study in a specific human population. However, caution must be taken in relating the effects of a single compound of a specific diet to that of an entire diet. We have recently shown that sinigrin and indole-3-carbinol, two major components in cruciferous vegetables, exerted opposing effects on hepatic demethylation and DNA methylation by NNK. Sinigrin diets inhibited demethylation and DNA methylation, whereas, indole-3-carbinol induced them (16). Another factor to be considered in relating these results to the human situation is that the doses of both inhibitor and carcinogen used in the bioassay, on a body weight basis, were considerably higher than the doses to which humans are exposed. However, it is interesting to note that the weight ratio of inhibitor to carcinogen received by each animal in the bioassay was about 50 to 1 and the average yearly intake of NNK through cigarette smoking is estimated at 1 mg/person in the U. S. (22).

The present study demonstrates that rats fed PEITC diets develop considerably fewer NNK-induced lung tumors than the rats fed control diets. The inhibition of lung tumor incidence by dietary PEITC was consistent with the reduction in DNA adduct formation by NNK, suggesting that the DNA adduct assay could be an effective method for screening inhibitors of NNK tumorigenesis. The demonstrated inhibition of NNK-induced lung tumorigenesis in this study is important in view of widespread human exposure through tobacco use and its relevance to human lung cancer development. These results also provide a basis for studies of the structure-activity relationships of PEITC and other related compounds and in-depth biochemical mechanism studies involving the possible roles of cytochrome P-450 enzyme systems in this inhibition. The goals of these studies are to produce more potent and less toxic inhibitors as likely candidates for human intervention in selected high risk populations such as heavy smokers.

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REFERENCES


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