ABSTRACT

Phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), and phenyl isothiocyanate (PITC) were tested for their abilities to inhibit lung tumorigenesis and O\(^{6}\)-methylguanine formation in lung DNA induced by the tobacco-specific nitrosamine 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) in A/J mice. Pretreatment with PEITC for 4 consecutive days at daily doses of 5 or 25 \(\mu\)mol inhibited tumor multiplicity induced by a single 10-\(\mu\)mol dose of NNK by approximately 70% or 97%, respectively. The 25-\(\mu\)mol daily dose of PEITC also reduced the percentage of animals that developed tumors by 70%. In contrast, both BITC and PITC failed to significantly reduce tumor multiplicity or the percentages of mice that developed tumors. Using an identical dosing regimen, parallel results were observed in the effects of these isothiocyanates on O\(^{6}\)-methylguanine formation in the lung, in which PEITC at either dose resulted in considerable inhibition at 2 or 6 h after NNK administration, while BITC or PITC had little effect. PEITC was further tested for its ability to inhibit lung microsomal metabolism of NNK. A single administration of PEITC (5 or 25 \(\mu\)mol) resulted in 90% inhibition of NNK metabolism. These results in conjunction with recent results obtained using F344 rats firmly establish PEITC as an effective inhibitor of NNK lung tumorigenesis and suggest that the basis of this inhibition is the reduction of DNA adduct formation caused by the inhibition of enzymes responsible for NNK activation.

INTRODUCTION

PEITC\(^2\) and BITC are naturally occurring constituents of cruciferous vegetables, existing as their glucosinolate precursors, gluconasturtiin and glucotropaeolin, respectively (1–3). The structures of PEITC, BITC, and the synthetic compound PITC are shown in Fig. 1. These three isothiocyanates were found to inhibit mammary tumors induced by DMBA in Sprague-Dawley rats when administered p.o. shortly prior to carcinogen administration; dietary PEITC and BITC were also found to inhibit DMBA-induced tumors of the forestomach and lung of ICR/Ha mice (4). Dietary BITC also proved effective in inhibiting DMBA-induced mammary tumors when administered following carcinogen exposure (5). Glucotropaeolin was found to reduce both pulmonary neoplasia in A/J mice induced by BP and DMBA-induced mammary tumorigenesis in Sprague-Dawley rats (6). More recently, BITC administered shortly before carcinogen treatment was found to inhibit N-nitrosodimethylamine-induced forestomach tumors and BP-induced lung and forestomach tumors in A/J mice (7).

NNK (Fig. 1) is the most potent tobacco-specific carcinogenic nitrosamine known (8). NNK induces lung tumors in all species tested, regardless of the route of administration used (9). These facts suggest a possible role for NNK in the induction of lung cancer in smokers. Recently, we have examined the inhibitory effects of dietary PEITC on NNK-induced tumorigenesis and NNK-induced DNA adduct formation in F344 rats (10). The results indicated that the inhibitory effect of PEITC on NNK lung tumorigenesis could be attributed to its ability to inhibit NNK-induced DNA methylation in rat lung. In the present study, in order to develop a more rapid means of screening inhibitors of NNK tumorigenicity and to verify the importance of DNA adduct formation in NNK tumorigenesis, we have examined the effects of PEITC, BITC, and PITC on NNK-induced pulmonary adenoma formation and NNK-induced O\(^{6}\)-mGua formation in A/J mice. Additionally, we have investigated the effect of PEITC pretreatment on A/J mouse lung microsomal metabolism of NNK.

MATERIALS AND METHODS

Animals. Female A/J mice of 5 to 6 wk of age were obtained from Jackson Laboratories (Bar Harbor, ME) and used in experiments at 6 to 7 wk of age. Mice were grouped 10 per cage in polycarbonate cages with hardwood bedding and were maintained at the following standard conditions: 20 ± 2°C; 50 ± 10% relative humidity; and 12 h/12 h light/dark cycle.

Chemicals. Unlabeled NNK and NNK metabolite standards were synthesized as described previously (11–13). [\(\text{\textsuperscript{5}}\text{H}\text{]}\text{NNK} (1.3 Ci/mmol) and O\(^{6}\)-mGua were purchased from Chemsyn Science Laboratories (Lenexa, KS). PEITC, BITC, and PITC were purchased from Aldrich Chemical Company (Milwaukee, WI). When analyzed by reversed phase HPLC, these isothiocyanates were found to have a purity of at least 99%. Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, EDTA, NAD\(^{+}\), and ammonium sulfate were obtained from Sigma Chemical Company (St. Louis, MO). BCA protein assay reagents were obtained from Pierce Chemical Co. (Rockford, IL).

Methods. A/J Mouse Pulmonary Adenoma Protocol. Groups of 20 to 30 female A/J mice were administered PEITC (5 or 25 \(\mu\)mol/day), BITC (5 \(\mu\)mol/day), or PITC (5 \(\mu\)mol/day), or corn oil vehicle by gavage for four consecutive days. On the fourth day, NNK (10 \(\mu\)mol/mouse) was administered 2 h after the final gavage. Groups of 5 animals were sacrificed and pulmonary adenomas were counted. Statistical comparison of tumor multiplicities among the various groups was performed by analysis of variance followed by the Newman-Keuls ranges test. Comparisons of the proportions of animals in groups that developed tumors were performed by the \(\chi^{2}\) test.

Determination of O\(^{6}\)-mGua Levels in A/J Mouse Lung. Groups of 10 mice were administered isothiocyanates (5 or 25 \(\mu\)mol/day) or corn oil by gavage for 4 consecutive days. On the fourth day, NNK (10 \(\mu\)mol/mouse) was administered 2 h after the final gavage. Groups of 5 animals were sacrificed by cervical dislocation at 2 and 6 h following NNK dosing, and the lungs of each animal were excised and stored at -20°C. DNA was isolated from the individual lungs of animals by a modification of the method of Marmur (14) and purified by the method of Sebti et al. (15). This method of DNA purification has been shown to yield...
DNA that is virtually free of RNA contamination. Purified DNA samples were hydrolyzed in 0.1 N HCl for 60 min at 80°C. Pre-HPLC sample purification was accomplished by the use of Gelman Acrodiscs (Ann Arbor, MI). Guanine and O-mGuo were separated and quantitated by strong cation exchange HPLC and fluorescence detection as described previously (16), except that the buffer used in isocratic elution of these compounds was 0.1 M ammonium phosphate (pH 2.0) in 5% methanol. The identities of guanine and O-mGuo were confirmed by coelution with authentic standards.

NNK Metabolism in A/J Lung Microsomes. Groups of 4 female A/J mice were administered a single dose of PEITC (5 or 25 μmol) or corn oil 2 h prior to sacrifice. Excised lungs were homogenized in 1.15% KCl-0.05 M sodium phosphate (pH 7.4) and centrifuged at 9,000 × g for 30 min at 5°C. The supernatants were removed and further centrifuged at 105,000 × g for 60 min at 5°C. The supernatants were discarded, and the pellets were resuspended in buffer and recentrifuged at 105,000 × g for another 60 min. The microsomal pellets were then suspended in 0.25 M sucrose and stored frozen until used. Protein was quantitated by use of the Pierce BCA protein assay.

Samples constituting 200 μg of microsomal protein were incubated in 0.8 ml of solution at 37°C for 60 min. The concentrations of the various solution components were as follows: 100 mM sodium phosphate (pH 7.4); 3.0 mM MgCl2; 1.0 mM EDTA; 1.0 mM NADP+; 5.0 mM glucose 6-phosphate; 3.8 units/ml of glucose-6-phosphate dehydrogenase; and 10.0 μM NNK (1.0 μCi of [5-3H]NNK). Following incubation, 0.2 ml of saturated ammonium sulfate were added to each vial to precipitate protein. Samples were freed of protein prior to HPLC analysis by the use of Amicon Centrifripe tubes (Amicon Corp., Danvers, MA). Separation and quantitation of NNK metabolites were performed by reverse-phase HPLC. The HPLC system used consisted of a Model 5125 Rheodyne injection valve, a Waters automated gradient controller, two Waters 510 pumps, a Knauer UV detector, and a Radiometric Beta Flo-One radioflow detector. An Alltech Versapack C18 column (4.1-mm inner diameter x 300-mm length) was eluted with a linear gradient of 100% of Buffer A (0.02 M sodium phosphate, pH 7.4) to 65% Buffer A:35% methanol over a 70-min period. The identities of the metabolites were established by coelution with authentic UV standards.

RESULTS

Pulmonary Adenoma Assays. As shown in Table 1, a single i.p. administration of NNK at a dose of 10 μmol/mouse resulted in a 100% incidence of pulmonary adenomas with an accompanying multiplicity of 10.7 tumors/mouse. The 5-μmol daily dose (20-μmol total) of PEITC did not significantly reduce the proportion of mice that developed pulmonary adenomas, but resulted in an approximate 70% reduction in tumor multiplicity. The 25-μmol daily dose (100-μmol total) of PEITC resulted in a 70% reduction of the percentage of mice that developed tumors and a nearly complete inhibition of tumor multiplicity. However, pretreatment with BITC for 4 days at 5 μmol/day resulted in no change in the percentage of mice that developed tumors and caused only a 29% reduction in tumor multiplicity which, by the multiple-comparison statistics utilized, was not significantly different from the control tumor multiplicity. Similarly, PITC administration at 5 μmol/day had no significant effects on the percentage of mice with tumors or on tumor multiplicity.

It should be noted that both BITC and PITC proved too toxic to be tested at a dose of 25 μmol for 4 consecutive daily administrations. At this dose, we found that PITC resulted in 100% mortality after two consecutive administrations, while BITC yielded nearly 25% mortality after two administrations. No apparent toxicity occurred upon administration of BITC or PITC at a daily dose of 5 μmol. Also, neither the 5-μmol dose nor the 25-μmol dose of PEITC resulted in any overt toxicity. Thus, besides being a better inhibitor of NNK tumorigenesis in A/J mice than either BITC or PITC, PEITC appears to be considerably less toxic.

O-mGuo Assays. In an effort to relate the effects of PEITC, BITC, and PITC on NNK lung tumorigenicity in vivo, NNK:DNA adduct formation, the effects of these isothiocyanates on NNK-induced O-mGuo in A/J mouse lung DNA were investigated. The same dosing regimen used in the pulmonary adenoma assays was used in the O-mGuo assays. As shown in Table 2, at 2 h after NNK administration, both doses of PEITC resulted in an approximate two-thirds reduction of O-mGuo when compared to that of controls. However, neither BITC nor PITC had any significant effect on O-mGuo levels at 2 h. At 6 h after NNK administration, the 5-μmol daily dose of PEITC resulted in an 87% reduction of O-mGuo levels, while the 25-μmol daily dose of PEITC yielded O-mGuo levels that were undetectable. (Given lung DNA yields of 350 to 500 μg/mouse, the limit of detection in our system is approximately 1.0 μmol of O-mGuo/mmol of guanine.) At 6 h, BITC and PITC pretreatment again resulted in no significant reduction in O-mGuo

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Daily dose (μmol)</th>
<th>WT at sacrifice (g)</th>
<th>% of mice with tumors/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>30</td>
<td>23.8</td>
<td>100</td>
</tr>
<tr>
<td>PEITC 5</td>
<td>18</td>
<td>23.0</td>
<td>89</td>
</tr>
<tr>
<td>PEITC 25</td>
<td>20</td>
<td>24.2</td>
<td>100</td>
</tr>
<tr>
<td>BITC 5</td>
<td>20</td>
<td>24.4</td>
<td>7.6 ± 0.5 (1)</td>
</tr>
<tr>
<td>PITC 5</td>
<td>20</td>
<td>23.5</td>
<td>9.5 ± 1.2 (1)</td>
</tr>
</tbody>
</table>

* Mean ± SE.

† Means with different numbers in parentheses are statistically different (P < 0.05) from one another as determined by analysis of variance followed by the Newman-Keuls ranges test.

Significantly (P < 0.01) less than that of Group 1 as determined by the χ² test.
levels. On the whole, the effects of the isothiocyanates on NNK-induced O2-imethylguanine formation were in good agreement with their effects on NNK lung tumorigenicity.

**NNK Microsomal Metabolism Assays.** Based on its ability to effectively inhibit both NNK-induced tumorigenicity and O-mGua formation, PEITC was further tested for its ability to inhibit the microsomal metabolism of NNK. Table 3 shows the effects on lung microsomal NNK metabolism of pretreatment with single doses of 5 or 25 μmol of PEITC, 2 h prior to sacrifice. Microsomes from untreated mice yielded four possible metabolites of NNK; two were identified as NNAL and keto alcohol [4-hydroxy-1-(3-pyridyl)-1-butanone], respectively, while two early eluting peaks could not be precisely identified on the basis of coelution with UV standards. Keto alcohol is [4-hydroxy-1-(3-pyridyl)-1-butanone], respectively, while two early eluting peaks could not be precisely identified on the basis of coelution with UV standards. Keto alcohol is.

**DISCUSSION**

In the pulmonary adenoma assay protocol utilized in this study, a single i.p. dose of 10 μmol of NNK induced a readily detectable lung tumor response (100% incidence in controls, with 10.7 tumors/mouse) in just 16 wk. In previous work, NNK (total dose, 110 μmol) was administered to A/J mice over a 7.3-wk period followed by a 30-wk period prior to sacrifice. This treatment resulted in tumor formation in 100% of the mice, with a multiplicity of 19.7 tumors/mouse (17). Repetitive administration of NNK (3 times weekly for 20 wk) to F344 rats at total doses of 0.3 and 0.5 mmol/kg resulted in lung tumor formation in 75% and 80% of rats 2 yr after initiation of the dosing regimen (10, 18). Thus, the current protocol provides a simple, rapid means of testing potential inhibitors of NNK tumorigenicity.

Previous studies have demonstrated that certain inhibitors of tumorigenesis yield a similar inhibition in DNA adduct formation. Anderson et al. (19) showed that butylated hydroxyanisole, a potent inhibitor of BP-induced lung neoplasia in A/J mice, decreased BP:DNA adduct formation in A/J mouse lung. Bull et al. (20) demonstrated that both disulfiram and pyrazole, inhibitors of 1,2-dimethylhydrazine-induced colon tumorigenesis (21), decreased O-mGua formation in the colon of 1,2-dimethylhydrazine-treated rats. Our results are consistent with these findings, since PEITC inhibited both tumorigenicity and O-mGua formation induced by NNK in mouse lung. Metabolic α-hydroxylation of NNK results in both methylation and pyridyloxobutylation of DNA (22). In this study, however, we did not examine the effects of isothiocyanates on pyridyloxobutylation of DNA.

Recent work by Belinsky and coworkers has indicated the importance of the promutagenic adduct O-mGua in NNK tumorigenesis of F344 rat lung. O-mGua was found to be much more persistent than either 7-methylguanine or O-methylthymidine (23). Additionally, O-mGua was found to be most highly accumulated in Clara cells (24), the presumed progenitor cells of NNK-induced rat lung neoplasia (25, 26). The parallel effects of PEITC, BITC, and PITC on NNK mouse lung tumorigenesis and O-mGua formation lend further credence to the importance of this adduct in NNK tumorigenesis.

The present studies serve as a basis for the elucidation of structure-activity relationships of isothiocyanates towards inhibition of NNK tumorigenesis. The two-carbon alkyl chain homologue PEITC was clearly more potent in reducing NNK tumorigenesis and O-mGua formation than either BITC or PITC. Interestingly, the toxicities of these isothiocyanates appear to be inversely related to alkyl chain length. PEITC, the least toxic of these three isothiocyanates, had no apparent toxic effects at the 25-μmol daily dose level, while both BITC and PITC proved too toxic to test at this dose. A number of studies have shown that the relative potencies of these three isothiocyanates in inhibition of tumorigenesis or of tumorigenic parameters could vary. In previous experiments conducted by Wattenberg (4), both BITC and PITC inhibited DMBA-induced mammary tumors in Sprague-Dawley rats as effectively if not more effectively than PEITC. In the same study, dietary BITC and PEITC appeared to inhibit DMBA-induced pulmonary adenomas equally well in ICR/Ha mice. In previous work conducted in our laboratory (16), PEITC and PITC inhibited NNK demethylation in F344 rat liver microsomes when administered acutely via gavage or chronically in the diet. Additionally, both compounds inhibited NNK-induced in vivo methylation of hepatic DNA when administered acutely or chronically to rats. In the same study, BITC had no effect on NNK hepatic microsomal metabolism when administered acutely, and actually induced NNK demethylation after chronic administration. Ultimately, the doses of isothiocyanates used,
the time of their application in relationship to carcinogen administration, their availability to a given target tissue, and the precise nature of their effects on enzymes of carcinogen activation may play substantial roles in determining their inhibitory activities in a given system.

The inhibitory activity of PEITC can readily be explained by its inhibition of NNK metabolism. While no dose-response relationship was evident in the inhibition of NNK metabolism by PEITC, it is possible that such an effect could be manifested by a more long-lived effect at the higher dose of PEITC; such a phenomenon could only be detected by the examination of microsomes prepared from mice sacrificed at several different time points following PEITC administration.

In summary, we have demonstrated that PEITC effectively inhibits NNK tumorigenicity and NNK-induced O\(^4\)-mGua formation in the lungs of A/J mice, while BITC and PITC have virtually no effect on either parameter. The inhibitory activity of PEITC appears to be related to its inhibitory effects on NNK metabolism in A/J mouse lung microsomes. These results, when combined with our previous results in F344 rats (10), firmly establish PEITC as an effective inhibitor of NNK lung tumorigenesis in rodents. Future studies in our laboratory will focus on developing more potent and less toxic isothiocyanate inhibitors via structural modifications of PEITC.

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Effects of Aromatic Isothiocyanates on Tumorigenicity, $O_6$-Methylguanine Formation, and Metabolism of the Tobacco-specific Nitrosamine 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J Mouse Lung

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