Structure-Activity Relationships for Inhibition of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone Lung Tumorigenesis by Arylalkyl Isothiocyanates in A/J Mice

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ABSTRACT

Phenethyl isothiocyanate (PEITC), 3-phenylpropyl isothiocyanate (PPhITC), 4-phenylbutyl isothiocyanate (PBITC), and the newly synthesized 5-phenylpentyl isothiocyanate (PPITC), 6-phenylhexyl isothiocyanate (PPhITC), and 4-(3-pyridyl)butyl isothiocyanate (PyBITC) were tested for their abilities to inhibit tumorigenicity and DNA methylation induced by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the lungs of A/J mice. Mice were administered isothiocyanates by gavage for 4 consecutive days at doses of 5, 1, or 0.2 μmol/day prior to administration of 10 μmol of NNK by i.p. injection. Mice were sacrificed 16 weeks after NNK administration and pulmonary adenomas were quantitated. 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. PPhITC, PBITC, PPhITC, and PyBITC were all considerably more potent inhibitors of NNK lung tumorigenesis than PEITC. While virtually no differences in inhibitory activity could be ascertained for PPhITC, PBITC, and PPhITC, PyBITC appeared to be the most potent tumor inhibitor of all of the compounds. At a dose of 0.2 μmol/day, PhITC pretreatment reduced tumor multiplicity by 85%. PyBITC, an analogue of both NNK and PhITC, was ineffective as an inhibitor. Using the same protocol, the compounds were found to have qualitatively similar inhibitory effects on NNK-induced DNA methylation when administered at 1 μmol/day. These results extend our previous findings that increased alky I chain length enhances the inhibitory activity of an arylalkyl isothiocyanate toward NNK lung tumorigenesis and demonstrate the exceptional chemopreventive potentials of two new isothiocyanates, PPhITC and PhITC.

INTRODUCTION

Isothiocyanates have been found to be effective inhibitors of tumorigenesis induced by polycyclic aromatic hydrocarbons and nitrosamines (1–3). Among the most extensively investigated isothiocyanates is PEITC, a naturally occurring compound which inhibits lung tumorigenesis induced by the tobacco-specific nitrosamine NNK in both F344 rats (3) and A/J mice (4, 5). Previous work established that increasing the alkyl chain length of an arylalkyl isothiocyanate up to 4 carbons enhances the inhibition of NNK-induced pulmonary adenomas in the A/J mouse model. Thus, PhITC and PBITC have greater inhibitory activity toward NNK-induced pulmonary adenomas than PEITC, while phenethyl isothiocyanate and benzyl isothiocyanate possess no significant inhibitory activity (5). As we are interested in elucidating the structural features of isothiocyanates which improve chemopreventive efficacy, the primary goals of this study were: (a) to examine the relative inhibitory potencies of arylalkyl isothiocyanates with alkyl chain lengths ranging from 2 to 6 carbons; (b) to examine the effect of replacement of the phenyl group of an isothiocyanate with a pyridyl moiety by comparison of the inhibitory activities of PBITC and PyBITC; (c) to determine the effects of doses of isothiocyanates lower than previously tested on NNK lung tumorigenicity; and (d) to examine the relationship between the inhibitory effects of these compounds on NNK-induced lung tumorigenesis and effects on pulmonary DNA methylation.

MATERIALS AND METHODS

Animals

Female A/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were fed AIN-76A semipurified diet with 5% corn oil (Dyets: Bethlehem, PA) and maintained under the following standard conditions: 20 ± 2°C, 50 ± 10% relative humidity, and a 12-h light, 12-hr dark cycle. Animals were used in experiments at 7 weeks of age.

Instrumentation

NMR spectra were obtained in CDCl3 on a Bruker Model AM 360 WB spectrometer using trimethylsilylane as an internal standard. CI-MS were obtained on a Hewlett-Packard Model 5988A spectrometer. High resolution EI-MS was obtained on a VG-11-250 spectrometer. The HPLC system used for 7-mGua, O6-mGua, and guanine analysis consisted of a Rheodyne injection valve, two Whatman Partisol-10 strong cation exchange columns linked in series, a Perkin-Elmer Model 250 binary LC pump, a Perkin-Elmer LS-240 fluorescence detector, and a Hitachi D-2000 chromato-integrator.

Chemicals

Unlabeled NNK was synthesized as described previously (6). PEITC, guanine, and 3-(3-pyridyl)-1-propanol were obtained from Aldrich Chemical Co. (Milwaukee, WI). PPhITC, 5-phenylpentyl chloride, and 6-phenylhexyl chloride were obtained from Fairfield Chemical Co. (Blythewood, SC). PBITC was synthesized as described previously (5). O6-mGua and 7-mGua were obtained from ChemSyn Science Laboratories (Lexena, KS). Ribonuclease A and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade or purer.

Synthesis of PhITC. 4-(3-Pyridyl)butylamine was synthesized as described previously (5). PhITC was synthesized from the crude amine hydrochloride by reaction with thiophosgene as described previously for the synthesis of 4-oxo-4-(3-pyridyl)butyl isothiocyanate (5). The final yield was 10%. 1H NMR: δ 8.43–8.49 (2H, m, pyridyl H2 and H5), 7.50 (1H, ddd, pyridyl H3), 7.24 (1H, dd, pyridyl H4), 3.55 (2H, t, −CH2CH2NCS), 2.67 (2H, t, −CH2CH2NCS), 1.67–1.85 p.p.m. (−CH2CH2CH3, −). Cl-MS, m/e (relative intensity): 193 (M+H+, 100).

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: PEITC, phenethyl isothiocyanate; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PPhITC, 3-phenylpropyl isothiocyanate; PBITC, 4-phenylbutyl isothiocyanate; PPhITC, 5-phenylpentyl isothiocyanate; PhITC, 6-phenylhexyl isothiocyanate; PyBITC, 4-(3-pyridyl)butyl isothiocyanate; 7-mGua, 7-methylguanine; O6-mGua, O6-methylguanine; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; CI-MS, chemical ionization mass spectra; EI-MS, electron impact mass spectra; 7-mGua, 7-methylguanine; O6-mGua, O6-methylguanine; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; CI-MS, chemical ionization mass spectra; EI-MS, electron impact mass spectra; m, multiplet; t, triplet; d, doublet of doublets; dd, doublet of doublets; of doublets.
Synthesis of PEITC and PHITC. 5-Phenylpentyl chloride (5 g, 27.4 mmol) was dissolved in 150 ml of dimethylformamide. Sodium azide (2.67 g, 41.1 mmol) was added and the reaction mixture was stirred for 2 h at 80°C. The reaction mixture was dissolved in 200 ml of CHCl3, washed with H2O (3 × 100 ml), dried over MgSO4, and concentrated in vacuo. The residue was dissolved in 300 ml of CHCl3/toluene 10:1 to yield 4.3 g (83%). CI-MS, m/e (relative intensity): 204 (M+H+, 10) 176 (M-N2+H+, 100) 91 (PhCH2+, 59).

Detection of 5-phenylpentyl azide (4.0 g, 21.1 mmol) was dissolved in 300 ml of isopropyl alcohol. Following addition of sodium borohydride (3.0 g, 79 mmol), the reaction mixture was stirred for 2 h at 80°C. The reaction mixture was dissolved in 200 ml of CHCl3, washed with H2O (6 × 150 ml). The CHCl3 layer was dried over Na2SO4 and concentrated in vacuo to yield 3.8 g of crude 5-phenylpentylamine. 'H NMR (CDCl3), 0 7.15-7.40 (5H, m, phenyl H); 3.52 (2H, t, —CH2N3); 2.75 (2H, t, —CH2Ph); 1.4-1.8 ppm (8H, m, —CH2CH2CH2CH2CH2—). UC NMR: 0 141.5, 128.3, and 125.6 (phenyl C); 51.4 (—CH2N3); 45.0 (—CH2NCS); 35.9 (—CH2Ph); 31.4, 28.8, and 26.7 ppm (—CH2CH2CH2CH2CH2—). CI-MS, m/e (relative intensity): 204 (M+H+, 10) 176 (M-N2+H+, 100) 91 (PhCH2+, 59).

6-Phenylhexyl azide and 6-phenylhexylamine were prepared in the same manner as described for their phenylpentyl homologues.

PEITC and PHITC were generated as oils from their corresponding alyalkylamine hydrochlorides by reaction with thiophosgene as described previously for the synthesis of phenylbutyl isothiocyanate (5).

RESULTS

Effects of Isothiocyanates on NNK-induced Pulmonary Adenomas. The structures of the isothiocyanates investigated in this study and NNK are shown in Fig. 1. The results of the A/J mouse pulmonary adenoma bioassay are shown in Table 1. With the exception of PEITC at the 0.2-μmol dose and PPITC at all doses, there were no groups with significantly lower weights than animals treated with corn oil/NNK. Mice administered only 5 μmol of PEITC or PHITC without subsequent NNK administration exhibited normal weight gain and no tumor formation (data not shown). Similar findings on body weight gain and tumor formation were previously reported with PEITC, PPITC, and PBTC (5). Animals pretreated with corn oil only prior to NNK administration developed a tumor multiplicity of 7.9 tumors/mouse with an incidence of 100%. In general, most pretreatments exhibited a dose-dependent inhibition. At a daily dose of 5 μmol/mouse, all isothiocyanates...
tested inhibited NNK-induced lung tumorigenesis. Due to a shortage of compound, PyBITC was not evaluated at this dose. PEITC significantly reduced tumor multiplicity without lowering tumor incidence, as had been found previously at this dose. At a dose of 5 nmol/mouse, PEITC exhibited no inhibitory effects. In fact, PEITC appeared to be the most potent inhibitor at the 0.2 nmol/mouse dose, it is not possible to compare it with isothiocyanates such as PEITC. However, PyBITC is clearly much less potent than the other isothiocyanates tested, including PEITC, PBITC, and PPelTC at the 0.2 nmol daily dose produced inhibitory effects similar to those of PEITC at the 5-nmol dose, with a reduction of tumor multiplicity by at least 47%. Again, PHITC appeared to be the most potent inhibitor, producing a tumor multiplicity that was significantly lower than those of the corn oil/NNK controls and all of the other isothiocyanate-pretreated groups. As before, PyBITC had no significant effects on NNK tumorigenicity. The data above show the relative order of inhibitory potency to be: PHITC > PPelTC > PBITC ≈ PPITC > PEITC. Since PyBITC was not evaluated at the 5-nmol daily dose, it is not possible to compare it with isothiocyanates such as PEITC. However, PyBITC is clearly much less potent than the other isothiocyanates tested, including PPelTC, its corresponding phenyl analogue.

Effects of Isothiocyanates on NNK-induced DNA Methylation. The effects of isothiocyanates on NNK-induced pulmonary and hepatic DNA methylation at a daily dose of 1 µmol in A/J mice are shown in Table 2. Pulmonary DNA methylation was not significantly affected by PEITC at this dose. Both PPelTC and PHITC significantly reduced DNA methylation in lung below that of NNK-treated controls and PEITC-pretreated mice. PPelTC and PHITC both significantly reduced DNA methylation to levels below those of PPITC and PBITC. Thus, the relative order of potency toward inhibition of NNK-induced pulmonary DNA methylation was found to be: PHITC ≈ PPelTC > PBITC ≈ PPITC > PEITC. This relative order is in substantial agreement with that found for inhibition of tumorigenesis.

In contrast to the results in the lung, hepatic 7-mGua levels were not significantly affected by any of the isothiocyanates.

Table 1 Effects of arylalkyl isothiocyanates on NNK-induced lung tumorigenicity in A/J mice

| Treatment       | Pretreatment | Lung O'-mGua | Liver 7-mGua
<table>
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<tr>
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<tr>
<td>Corn oil/NNK</td>
<td>17.6 ± 0.9</td>
<td>997 ± 67</td>
<td>177 ± 12</td>
</tr>
<tr>
<td>PEITC/NNK</td>
<td>17.2 ± 0.2</td>
<td>890 ± 39</td>
<td>145 ± 7</td>
</tr>
<tr>
<td>PPITC/NNK</td>
<td>13.8 ± 0.4</td>
<td>852 ± 43</td>
<td>150 ± 9</td>
</tr>
<tr>
<td>PBITC/NNK</td>
<td>13.4 ± 0.5</td>
<td>960 ± 66</td>
<td>158 ± 9</td>
</tr>
<tr>
<td>PPelTC/NNK</td>
<td>11.1 ± 0.2</td>
<td>786 ± 40</td>
<td>138 ± 8</td>
</tr>
<tr>
<td>PHITC/NNK</td>
<td>9.9 ± 0.1</td>
<td>907 ± 53</td>
<td>155 ± 6</td>
</tr>
</tbody>
</table>
| Corn oil/saline | 15.4 ± 0.2   | 221 ± 0.4    | 1 ± 0.1

* Groups of 15 A/J mice were administered corn oil vehicle or isothiocyanates (1 µmol in 0.1 ml corn oil) by gavage for 4 consecutive days. At 2 h after the final pretreatment, mice were administered 10 µmol NNK (in 0.1 ml saline) i.p. Mice were killed 6 h after NNK administration and the liver and lungs of each animal were excised. Following DNA isolation and purification, O'-mGua and 7-mGua were 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).

** Mean ± SE.
(Table 2). Only PPeITC produced a significant reduction in O6-mGua levels of hepatic DNA when compared to controls.

Stability and Lipophilicity of Isothiocyanates. Table 3 shows the half-life ($t_{1/2}$) and partition coefficient for each isothiocyanate. The $t_{1/2}$ for each isothiocyanate is a function of its stability as well as its reactivity with bovine serum albumin. In this system, PyBITC had a half-life of 1.5 h, PEITC had a half-life of 1.7 h, and the rest of the isothiocyanates had half-lives ranging from 2.2 to 2.5 h. Predictably, the octanol:H2O partition coefficients of the arylalkyl isothiocyanates increased with increasing alkyl chain length, ranging from 3.4 for PEITC to 4.6 for PHITC. PyBITC was the most polar of all of the isothiocyanates, with a partition coefficient of only 2.7.

DISCUSSION

One of the principal goals of this study was to determine the differences in inhibitory activity of PEITC and its longer chain homologues. PEITC has been previously shown to inhibit NNK-induced pulmonary adenomas in A/J mice when administered at daily doses of 5 or 25 µmol/mouse (4, 5). In the current study, PEITC had no significant effect on pulmonary adenoma formation at 1 µmol/mouse, and appeared to enhance tumor formation when administered at a dose of 0.2 µmol/mouse. This latter finding is inconsistent with the known effects of PEITC at higher doses; we are currently retesting PEITC at this dose. In contrast, its longer chain homologues (with the exception of PyBITC) were extremely effective at doses of 1 or 0.2 µmol/day. While the inhibitory effects of PPITC, PBITC, and PPeITC were virtually indistinguishable from each other, PHITC did appear to be more potent than the other isothiocyanates. PHITC greatly inhibited NNK tumorigenicity at the 0.2-µmol dose, a total dose (0.8 µmol) more than 10-fold lower than the dose of carcinogen (10 µmol) used. Another interesting comparison is found with PBITC and PyBITC. Replacement of the phenyl ring in PBITC with a pyridyl ring yields a compound devoid of inhibitory activity at 1 or 0.2 µmol/day, while PBITC is quite effective at either dose.

In general, the differences found in pulmonary DNA methylation among the various treatment groups qualitatively reflected the differences found among these groups in susceptibility to NNK tumorigenesis. A reduction in O6-mGua levels 6 h after NNK administration has been shown to be a reasonably predictive indicator of the inhibitory potentials of modulators of NNK lung tumorigenesis (4, 10). However, the magnitude of the decreases in pulmonary DNA methylation in the current study was considerably less than the corresponding effect of each isothiocyanate on NNK lung tumor multiplicity at a pretreatment dose of 1 µmol/day. PPITC, PBITC, PPeITC, and PHITC all decreased tumor multiplicity by at least 85%, yet the decreases in pulmonary DNA methylation at 6 h ranged from 22 to 44% for these compounds. Without a detailed time course of DNA methylation, it is difficult to assess the significance of these differences. It is possible that examination at later time points may yield inhibition of DNA methylation more similar to the inhibition of tumorigenicity observed in groups pretreated with isothiocyanates. Alternatively, there may be a threshold value of O6-mGua formation in lung DNA such that levels of O6-mGua below this value result in no tumor formation. Finally, it may be that inhibition of pulmonary DNA methylation is unimportant in the inhibition of NNK-induced lung tumorigenesis. This last possibility seems unlikely, given the known correlation of DNA methylation to NNK tumorigenesis in A/J mice (4, 10, 11).

Interestingly, virtually no inhibitory effect on NNK-induced DNA methylation was observed in the livers of mice pretreated with isothiocyanates. Previous work with PEITC in F344 rats yielded similar results, as dietary PEITC (3-µmol/g diet) inhibited NNK-induced DNA methylation and tumorigenicity in lung, but had no effects on hepatic DNA methylation or liver tumor incidence (3). The inability of isothiocyanates to inhibit NNK activation in liver may be a consequence of distribution and dose level, since there is considerably more enzymatic activity to be inhibited in the liver compared to the lung. Alternatively, there may be differences in the distribution or level of expression of the cytochrome P-450 isozyme(s) responsible for NNK activation in liver and lung. At present, there is some evidence that isoforms of P450I1A1 and P450I1B may be involved in NNK metabolism by murine lung (12), and that isoforms of P450IIB4 and P450I1A1 may be involved in NNK activation in rat lung (13). A recently discovered difference between liver and lung involves P4501B content, which appears to be expressed in much higher levels in lung than in liver (14). Although this P-450 isozyme is involved in the metabolism of N-nitrosodibutylamine, it is not known what role, if any, this isozyme plays in the metabolism of NNK. Obviously it is necessary to firmly establish which isozyme(s) of P-450 are responsible for NNK activation.

The fact that these isothiocyanates differ in their abilities to inhibit NNK-induced lung tumorigenesis is of considerable interest. The absence of any induction in hepatic DNA methylation argues against decreased distribution of nitrosamine to the lung as a result of induction of hepatic $\alpha$-hydroxylation pathways, as appears to be the case for the inhibitory effects of indole-3-carbinol on NNK tumorigenesis (9). PEITC inhibits the microsomal metabolism of nitrosamines, including NNK (4, 12, 15, 16). When added to pulmonary microsomes in vitro, the inhibitory potency of an arylalkyl isothiocyanate toward NNK $\alpha$-hydroxylation increases with increasing alkyl chain length as is seen for inhibition of NNK lung tumorigenicity (12). With increasing alkyl chain length for a given isothiocyanate, lipophilicity increases and reactivity decreases, both of which may affect the delivery of compound to the target tissue (the lung). Also, increased alkyl chain length may favor binding of an isothiocyanate to the catalytic site(s) of the cytochrome P-450 isozyme(s) responsible for NNK $\alpha$-hydroxylation. The failure of PyBITC to inhibit NNK lung tumorigenesis may be due in part to its relatively low stability and lipophilicity.

Administration of isothiocyanates p.o. for 4 consecutive days may be unnecessary, since it appears that inhibition of enzymes responsible for NNK activation is involved. Thus, the final dose...
of isothiocyanate may provide the majority of the inhibitory activity. Experiments are in progress to examine the inhibitory potentials of a single-dose pretreatment versus the 4-dose pretreatment protocol used in the present study.

The present work confirms that increased alkyl chain length in an arylalkyl isothiocyanate is an important structural feature in the inhibition of NNK-induced lung tumorigenesis in A/J mice. The results demonstrate that PHITC, the 6-carbon homologue, is the most potent inhibitor of NNK lung tumorigenicity in A/J mice found thus far, possibly due to its increased lipophilicity and stability. Future studies will focus extensively on the precise mechanism of isothiocyanate-mediated inhibition of NNK tumorigenicity, as well as an examination of PHITC and some of its homologues with different animal models and other classes of carcinogens.

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