Effects of benzyl isothiocyanate and 2-phenethyl isothiocyanate on benzo[a]pyrene and 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone metabolism in F-344 rats

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A mixture of dietary benzyl isothiocyanate (BITC) and 2-phenethyl isothiocyanate (PEITC) inhibits lung tumorigenesis by a mixture of benzo[a]pyrene (B[a]P) and 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK) in A/J mice. Previous studies indicated that inhibition of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) releasing DNA adducts of NNK by PEITC in the lung was responsible for inhibition of tumorigenicity. We have now extended these investigations to F-344 rats treated with 2 p.p.m. B[a]P in the diet and 2 p.p.m. NNK in the drinking water. The effects of BITC (1 µmol/g diet), PEITC (3 µmol/g diet), and a mixture of BITC plus PEITC (1 and 3 µmol/g diet) on DNA and hemoglobin (Hb) adducts of B[a]P and NNK, and on two urinary metabolites of NNK, were examined. DNA adducts were quantified after 8 and 16 weeks of treatment. Hb adducts were quantified in blood samples withdrawn every 2 weeks. 4-(Methylnitrosamo)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide NNAL-Gluc were measured in urine every 4 weeks. PEITC or BITC significantly reduced levels of HPB releasing DNA adducts of NNK in lung at 8 and 16 weeks, but there was no effect of BITC. There were no effects of any of the treatments on levels of HPB releasing DNA adducts of NNK in liver, or on DNA adducts of B[a]P in either lung or liver. PEITC or BITC plus PEITC significantly inhibited the formation of Hb adducts of NNK from 2–12 weeks of treatment while there were no effects on Hb adducts of B[a]P. There was a significant increase in levels of NNAL and NNAL-Gluc in the urine of the rats treated with PEITC or BITC plus PEITC. These results demonstrate that dietary PEITC, or a mixture of BITC plus PEITC, inhibit the formation of HPB releasing adducts of NNK in the rodent lung, leading to inhibition of tumorigenesis.

Introduction

Isothiocyanates are among the most effective chemopreventive agents known. It is not unusual to achieve complete inhibition of carcinogenesis in laboratory animals treated with isothiocyanates (1). For example, 2-phenethyl isothiocyanate (PEITC, Figure 1) completely inhibits lung tumor induction in rats by the tobacco-specific carcinogen 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone (NNK, Figure 1) and in hamsters by N-nitrosobis-(2-oxopropyl)amine (2–4). 3-Phenylpropyl isothiocyanate completely inhibits esophageal tumor induction in rats by the tobacco-specific nitrosamine N-nitrosornornicotine (5). Benzyl isothiocyanate (BITC, Figure 1) and sulforaphane are two other isothiocyanates with well-established chemopreventive activity in laboratory animals (1,6,7). All of these isothiocyanates occur naturally as conjugates in cruciferous vegetables (8).

Lung cancer is the leading cause of cancer death in the world, being responsible for ~1.2 million deaths per year (9). Cigarette smoking causes 90% of lung cancer (10). Prevention of smoking initiation and improved methods of smoking cessation are the best ways to prevent lung cancer. However, these approaches have stalled in the last decade (11) and, even after cessation, former smokers maintain an elevated risk to develop lung cancer (12). Chemoprevention is an alternative approach to reduce lung cancer incidence in current and former smokers. Our goal is to develop a mixture of chemopreventive agents targeted against lung cancer.

Three recent epidemiologic studies demonstrated that isothiocyanates are protective against lung cancer. London et al. (13) showed that individuals with detectable isothiocyanates in their urine were at decreased risk of lung cancer, and the protective effect was seen particularly in individuals with homozygous deletion of GSTM1 or both GSTM1 and GSTT. Zhao et al. and Spitz et al. obtained similar results (14,15). GSTM1 and GSTT1 are involved in the metabolism of isothiocyanates (16). It has been hypothesized that deletion of these enzymes would result in higher levels of free isothiocyanates in tissues and therefore more effective chemoprevention (17).

Benzo[a]pyrene (B[a]P, Figure 1) and NNK are two of the most important lung carcinogens in tobacco smoke (18). BITC inhibits lung tumor induction by B[a]P and other polycyclic aromatic hydrocarbons in mice while PEITC inhibits lung tumor induction by NNK in mice and rats (1). A mixture of BITC and PEITC, administered in the diet, inhibits lung tumor induction by a mixture of B[a]P and NNK in A/J mice (19). Taken together with the epidemiologic data, these results underline the efficacy and potential suitability of isothiocyanates for chemoprevention of lung cancer.

Recently, we investigated the mechanism by which a mixture of dietary BITC and PEITC inhibited lung tumorigenesis induced by B[a]P plus NNK in A/J mice (20). The effects of a mixture of BITC and PEITC as well as PEITC alone on DNA adduct formation by B[a]P and NNK were examined (20). A mixture of BITC and PEITC, and PEITC alone, decreased levels of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, Figure 1) releasing DNA adducts of NNK, while there was no effect on levels of O6-methylguanine formed from NNK,
Materials and methods

Chemicals

B[a]P, BITC and PEITC were obtained from Aldrich Chemical (Milwaukee, WI). NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL), r-7,8,9,10-tetra-methoxy-7,8,9,10-tetraydrobenzo[a]pyrene (trans/anti-B[a]P-TME), [13C][H]3, trans/anti-B[a]P-TME, HPB and 4,4'-[2H8]HB were synthesized as described (23–27). r-7,8,9,10-tetrahydroxy-7,8,9,10-tetraydrobenzo[a]pyrene (trans/anti-B[a]P-Trafool, Figure 1) was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). Trans/anti-1H2B[a]P-trafool was a generous gift from Assieh Melikian, American Health Foundation, Valhalla, NY. Pentafluorobenzoyl chloride was obtained from Sigma Chemical (St Louis, MO). All other reagents and solvents were from Fischer Scientific (Hanover Park, IL).

Animal experiments

Male F-344 rats were obtained at age 8 weeks from Charles River, Wilmington, MA. They were housed under standard conditions and maintained on NIH-07 diet (Dyets, Bethlehem, PA) (28). After arrival, they were allowed to acclimate to the animal facility for 2 weeks.

The design of the experiment is illustrated in Figure 2. All rats were given B[a]P in the diet (2 mg/kg diet) and NNK in the drinking water (2 μg/ml) ad libitum. The diets containing B[a]P and isothiocyanates were prepared every 3 weeks and stored at –4°C. The drinking water containing NNK was prepared every 3–4 days. Diets and drinking water were changed in the cages every 3–4 days. There were 12 rats/group. One week prior to carcinogen treatment, the rats were given diet with additions as follows: group 1, none; group 2, 1 μmol BITC/g diet; group 3, 3 μmol PEITC/g diet; group 4, 1 μmol BITC plus 3 mmol PEITC/g diet. Doses of the isothiocyanates were chosen based on previous studies (2,19,29). Food and water consumption were measured every 3–4 days during the experiment. Starting 2 weeks after the first carcinogen treatment, every 2 weeks for 16 weeks, four rats from each group were randomly selected and placed in metabolism cages. Carcinogen treatment was suspended at this time to avoid contamination of urine, but the diets containing the appropriate amounts of isothiocyanates were continued as usual. Twenty-four hour urine samples were collected. Then these rats were returned to the main experiment. Eight weeks after the first carcinogen treatment, every 4 weeks for 16 weeks, three rats from each group were randomly selected and placed in metabolism cages. Carcinogen treatment was suspended at this time to avoid contamination of urine, but the diets containing the appropriate amounts of isothiocyanates were continued as usual. Twenty-four hour urine samples were collected. Then these rats were returned to the main experiment. Eight weeks after the first carcinogen administration, six rats from each group were randomly selected and killed. Sixteen weeks after the first carcinogen administration, the remaining rats were killed. At the final killing, ~5 ml of blood was drawn by cardiac puncture under isofluorane anaesthesia, and lung and liver tissues were harvested and stored at –80°C.

Preparation of hemoglobin from RBC

Hb was isolated from RBC as described previously (30). In brief, the RBC samples were lysed by adding 1 vol H2O and kept on ice for 10 min, followed by addition of 1 vol 0.67 M NaH2PO4, pH 6.5, to a final concentration of 0.2 M. Samples were transferred to 50 ml centrifuge tubes (Nalgene, Rochester, NY) and centrifuged at 25 000 g for 30 min at 4°C. The Hb solution was transferred into a dialysis tube (SpectraPor, Gardena, CA, MW cut-off 15 000) and the cell debris (pellet) was discarded. Hb solutions were dialyzed at 4°C against 20 vol H2O for 3 h. Water was changed every hour. Dialyzed Hb solution was transferred to 50 ml disposable centrifuge tubes and stored at –20°C.
Precipitation of globin

To precipitate globin, 2 ml of Hb solution was added dropwise to 40 ml icecold 1% HCl–acetic. The supernatant was discarded and the globin was washed twice with 100% acetone. Globin was dissolved in 2 ml H2O and the precipitation was repeated twice. The washed globin was dried at 50°C overnight and stored at –80°C.

DNA isolation

DNA was isolated using the Puregene® DNA isolation kit (Genta, Minneapolis, MN) according to the manufacturer’s description. In brief, tissues were homogenized in 3 ml of ‘Cell Lysis Solution’ using 10–50 strokes in a glass homogenizer. To digest the protein, 15 µl ‘Proteinase K Solution’ (20 mg/ml) was added, and samples were incubated at 55°C for 5 h. Then 15 µl ‘RNase A Solution’ was added and the samples were incubated at 37°C for 1 h. Proteins were precipitated by adding 3 ml ‘Protein Precipitation Solution’ followed by centrifugation at 2000 g for 10 min. The pellet was discarded and the supernatant was precipitated by slowly adding 3 ml isopropanol, then transferred to 4 ml siliconized vials. The DNA was rinsed once with 70% EtOH and twice with 100% EtOH (1 ml each), dried under a gentle stream of N2 and stored at –20°C until analysis.

Gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) analysis of HPB releasing DNA and Hb adducts

The samples were analyzed in sets of 24, including three H2O blanks (negative controls) and one H2O blank spiked with 300 fmol HPB (positive control).

Acid hydrolysis of DNA. The procedure was performed essentially as described previously (20,31).

Base treatment of Hb. For the analysis of Hb adducts, 2.0 ml dialyzed Hb solution was used. The Hb concentration was determined by the Drabkin method (Sigma). For the base treatment, 4 N NaOH was added to a solution was used. The Hb concentration was determined by the Drabkin method (Sigma). For the base treatment, 4 N NaOH was added to a

Extractions. The acidic aqueous solutions from DNA or Hb, containing HPB and 4,4'[2H2]HPB, were extracted twice with equal volumes of CH2Cl2. The Hb samples were additionally extracted twice with 1 vol hexanes. The aqueous layer was saved and the pH was adjusted to 7.0 with 1 N NaOH. The samples were extracted three times with equal volumes of CH2Cl2. The organic layers were pooled and the solvent was removed at reduced pressure using a Speedvac concentrator (Thermo Savant, Holbrook, NY).

Derivatization and GC-NICI-MS analysis. These were carried out as described previously (20).

Analyses of NNAL and its glucuronide (NNAL-Gluc) in urine

These analyses were performed essentially as described previously (32). Briefly, 2 ml urine was extracted three times with 1 vol ethyl acetate to obtain NNAL. The aqueous layer containing NNAL-Gluc was incubated overnight with 12 000 U β-glucuronidase type IX-A from Escherichia coli (Sigma) at 37°C. It was then extracted three times with CH2Cl2. To the ethyl acetate and the CH2Cl2 extracts, 2.7 µl iso-NNAL was added as internal standard and the solvents were removed at reduced pressure using a Speedvac concentrator (Thermo Savant, Holbrook, NY).

The samples were purified by HPLC using a 3.9 × 150 mm Bondclone 10 C18 column (Phenomenex, Torrance, CA) with a gradient from 10 to 45% MeOH in 20 min at a flow rate of 1 ml/min. Samples were dissolved in 500 µl 20 mM KH2PO4 buffer containing 50 µg 3-acylpyridine and 50 µg 3-pyridylcarbinol acetate as retention time markers. Fractions between the apices of the UV-markers were collected. Samples were removed with a SpeedVac and stored at –20°C until analysis.

GC-NICI-MS analysis of trans/anti-[B(a)P]-tetraol releasing DNA and Hb adducts

The procedure was similar to the one described by Melikian et al. (33,34) but with a simplified permethylation method (35).

Release of B(a)P-tetraols from DNA and globin. B(a)P-tetraols were released from DNA by mild acid hydrolysis. In brief, ~100 µg DNA was dissolved in 600 µl H2O. Two picomole trans/anti-[1]H3B[a]P-tetraol was added as internal standard. Samples were hydrolyzed in 0.1 N HCl, 4 h at 80°C. To release B(a)P-tetraols from globin, 50 µg globin was dissolved in 3 ml H2O. The globin solutions were transferred to an 8 ml vacuum hydrolysis vial, 2 pmol trans/anti-[1]H3B[a]P-tetraol was added as internal standard, and B(a)P-tetraols were released by hydrolysis with 0.1 N HCl, 3 h, 80°C under vacuum. After hydrolysis, the globin was precipitated by neutralization with 0.4 N NaOH. The released B(a)P-tetraols were extracted five times with 1 vol ethyl acetate and the combined organic layers were concentrated to dryness on a SpeedVac and stored at –20°C.

HPLC clean up #1. The released B(a)P-tetraols were further purified by reverse phase HPLC using a 4.6 × 250 mm 5 µ UltraSphera ODS C18 column (Beckman, Fullerton, CA). The procedure was as follows: 20% MeOH in H2O for 10 min, then increase linearly to 55% MeOH in 5 min, and hold for 20 min. The flow rate was 1 ml/min. The samples from globin extracts were dissolved in 70 µl MeOH, while for the DNA samples, the total hydrolysis solution was injected. The retention time of trans/anti-[B(a)P]-tetraol (27–29 min) was determined before each set of samples was injected. Fractions containing trans/anti-[B(a)P]-tetraol and trans/anti-[1]H3B[a]P-tetraol were collected from 1 min before until 4 min after their elution positions. Solvents were removed with a SpeedVac concentrator and samples were stored at –20°C until derivatization.

Derivatization of trans/anti B(a)P-tetraol to trans/anti-B(a)P-TME. The derivatization was carried out at room temperature. Samples were dissolved in 100 µl DMSO. A magnetic miniature stirrer and ~2 mg of dry NaH were added and the mixture was stirred for 2 min. Then 50 µl CH3I was added and the reaction was allowed to proceed for 15 min with stirring at room temperature. The reaction was quenched by adding 500 µl H2O. Trans/anti-B(a)P-TME was extracted with benzene (3 × 1 ml). The benzene solution and the samples were stored at –20°C.

HPLC clean up #2. Trans/anti-B(a)P-TME was purified by reverse phase HPLC using the ODS C18 column operated with a gradient from 70 to 100% MeOH in H2O in 30 min at a flow rate of 1 ml/min. Samples were dissolved in 50 µl MeOH containing 350 µg of hexanophenone and octanophenone as retention time markers. The entire sample was injected. Fractions between apices of the UV markers were collected. Solvents were removed with a SpeedVac and the residues were transferred to 300 µl insert vials with 3 × 250 µl MeOH and stored at –20°C until analysis.

GC-NICI-MS. Samples were dissolved in 10 µl benzene containing 150 fmol trans/anti-[1]H4B[a]P trans/anti-B(a)P-TME as injection standard. Five microliters were injected in splitless mode.

Analysis was performed on a Finnigan TSQ 7000 instrument (Finnigan-MAT/Thermoquest, San Jose, CA) interfaced with a CTC A200SE autosampler (Leap Technologies, Carrboro, NC) and a HP5890 series II gas chromatograph (Agilent, Wilmington, DE). A DB-17 MS (30 m, lD 0.25 mm, 0.15 µm) capillary GC column (J&W Scientific, Folsom, CA), connected to a 2 m × 0.530 mm fused silica uncoated deactivated retention gap, was interfaced to the CI source operated in negative ion mode. The oven temperature was held at 60°C for 1 min, then increased to 300°C at 20°C/ min. The temperature was held at 300°C for 15 min. The MS parameters were as follows: ion-source temperature, 150°C; emission current, 700 mA; electron energy, 150 eV. Ultra high-purity methane was the reagent gas. The molecular ions m/z 376 (trans/anti-[B(a)P]-TME, analyte), m/z 380 (trans/anti-[1]H3B[a]P-TME, injection standard) and m/z 384 (trans/anti-[1]H3B[a]P-TME, internal standard) were monitored.

Statistical analyses

Adduct and urinary metabolite levels were compared using analysis of variance. When the overall F-test was significant, subsequent pairwise comparisons were tested using Student’s t-test. A P value of 0.05 or less was considered statistically significant.

Results

An overview of DNA and Hb adduct formation from NNK and B(a)P is presented in Figure 3. NNK is metabolically activated to α-hydroxymethylNNK, which is converted spontaneously to a pyridyllobutyl diamonox hydridox. This intermediate alkylates DNA and Hb. Treatment of the DNA with acid releases HPB from pyridyllobutyl DNA adducts. Treatment of the Hb with base releases HPB from pyridyllobutyl Hb adducts. NNK is also metabolically activated by α-hydroxylation at the methylene carbon, leading ultimately to methyl DNA adducts. Competing with the metabolic activation of NNK is conversion to NNN7, which in tum is metabolized to NNAL-Gluc. NNAL can bealso be reconverted to NNK, or can be metabolically activated by α-hydroxylation. B(a)P undergoes a three-step metabolic activation process leading
to (7R,8S)-dihydroxy-(95,10R)-epoxy-7,8,9,10-tetrahydrobenzo [a]pyrene (BPDE), which forms adducts with both DNA and Hb. These are released by acid hydrolysis as B[a]P-tetraols, with the major isomer being trans-anti-B[a]P-tetraol. In this study, we quantified HPB releasing DNA and Hb adducts of NNK, NNAL and NNAL-Gluc in urine, and trans/anti-B[a]P-tetraol releasing DNA and Hb adducts of B[a]P. Further details of NNK and B[a]P metabolism and adduct formation have been described (36,37).

Table I summarizes food and water consumption, body weights and daily doses of B[a]P, NNK and isothiocyanates.

<table>
<thead>
<tr>
<th>Addition to diet</th>
<th>Weeks on diet</th>
<th>Mean ± SD</th>
<th>Daily doses (mg/kg body wt/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Food consumption (g/day)</td>
<td>Water consumption (ml/day)</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>19.2 ± 1.8</td>
<td>22.5 ± 3.4</td>
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<tr>
<td></td>
<td>8</td>
<td>19.0 ± 2.2</td>
<td>24.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18.8 ± 1.8</td>
<td>24.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>19.4 ± 1.4</td>
<td>25.5 ± 1.3</td>
</tr>
<tr>
<td>BITC</td>
<td>4</td>
<td>18.3 ± 1.2</td>
<td>21.4 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>17.2 ± 1.4</td>
<td>21.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17.1 ± 1.5</td>
<td>25.1 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>19.5 ± 0.9</td>
<td>25.8 ± 1.8</td>
</tr>
<tr>
<td>PEITC</td>
<td>4</td>
<td>18.6 ± 1.3</td>
<td>22.0 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>18.3 ± 2.5</td>
<td>22.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>17.9 ± 0.9</td>
<td>24.8 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>20.6 ± 0.8</td>
<td>26.0 ± 2.4</td>
</tr>
<tr>
<td>BITC + PEITC</td>
<td>4</td>
<td>19.0 ± 1.8</td>
<td>21.3 ± 2.7</td>
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<tr>
<td></td>
<td>8</td>
<td>17.8 ± 2.1</td>
<td>23.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>18.6 ± 1.0</td>
<td>25.0 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>18.8 ± 0.8</td>
<td>25.3 ± 1.5</td>
</tr>
</tbody>
</table>

Table II summarizes DNA and Hb adducts in F-344 rats treated with B[a]P plus NNK and isothiocyanates.

<table>
<thead>
<tr>
<th>FPB releasing adducts, mean ± SD</th>
<th>B[a]P-tetraol releasing adducts, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groupa</td>
<td>Duration (weeks)</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>BITC</td>
<td>8</td>
</tr>
<tr>
<td>PEITC</td>
<td>24.4 ± 2.0</td>
</tr>
<tr>
<td>BITC + PEITC</td>
<td>31.7 ± 5.8</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
</tr>
<tr>
<td>BITC</td>
<td>30.2 ± 7.2</td>
</tr>
<tr>
<td>PEITC</td>
<td>29.1 ± 9.3</td>
</tr>
<tr>
<td>BITC + PEITC</td>
<td>30.7 ± 11.3</td>
</tr>
</tbody>
</table>

Table II and Figure 4A summarize adduct levels for each group after 8 and 16 weeks of treatment. HPB releasing DNA adducts were significantly higher in lung than liver. PEITC and BITC plus PEITC significantly reduced HPB releasing DNA adduct levels in lung after 8 and 16 weeks of treatment by 50%. PEITC and BITC plus PEITC reduced levels of HPB releasing DNA adducts in lung to levels similar to those seen in the liver DNA (Figure 4A). There were no effects of the isothiocyanates on levels of HPB releasing DNA adducts in liver.

In weeks 2–12, HPB releasing Hb adduct levels were 40–50% lower in the groups treated with PEITC or BITC plus...
PEITC compared with controls (Table II, Figure 5A). The differences were significant at each time point ($P < 0.05$). The 50% inhibition of HPB releasing Hb adducts was similar to the inhibition observed for HPB releasing DNA adducts in lung. After week 12, HPB releasing Hb adduct levels declined in the B[a]P plus NNK and B[a]P plus NNK/BITC treated groups. At weeks 14 and 16, there were no significant differences in HPB releasing Hb adduct levels among the groups.

**NNAL and NNAL-Gluc in urine**

PEITC caused a significant increase in the excretion of NNAL, NNAL-Gluc and NNAL plus NNAL-Gluc at all time points analyzed (Figure 6). BITC reduced levels of NNAL, NNAL-Gluc and NNAL plus NNAL-Gluc at the 8 week time point. However, at 4, 12 and 16 weeks, the levels were not different from those of the control group. Treatment with BITC plus PEITC resulted in a delayed increase of NNAL, NNAL-Gluc and NNAL plus NNAL-Gluc. Levels were significantly increased at 12 and 16 weeks, similar to the groups treated with PEITC.

**Trans/anti-B[a]P-tetraol releasing DNA and Hb adducts of B[a]P**

DNA (97.1 ± 32.5 mg) was analyzed for trans/anti-B[a]P-tetraol releasing adducts. The recovery of trans/anti-[2H8]B[a]P-tetraol was 28.5 ± 15.2%. None of the H2O blanks contained detectable trans/anti-B[a]P-TME and the precision of the spiked positive controls was CV <10% ($n = 8$).

There were no effects of the isothiocyanates on levels of trans/anti-B[a]P-tetraol releasing DNA adducts in lung or liver (Table II, Figure 4B) or on levels of trans/anti-B[a]P-tetraol releasing Hb adducts (Table II, Figure 5B).

**Discussion**

Our results are consistent with those of our previous study in A/J mice (20). In that study, we observed reductions of HPB releasing DNA adducts of NNK in the lungs of mice gavaged with B[a]P plus NNK, once weekly for 8 weeks, and given PEITC or a mixture of BITC and PEITC in the diet. Reductions in adduct levels were observed 24 and 120 h after the forth and eighth weekly treatments with B[a]P plus NNK, but the extents of the reductions were variable and not always significant. In the present study, we observed consistent significant reductions of HPB releasing DNA adducts in lung, in rats treated for 8 or 16 weeks with B[a]P in the diet and NNK in the drinking water, and either PEITC or a mixture of BITC and PEITC in the diet. As the reduction in adduct levels was about the same in the rats treated with PEITC or BITC plus PEITC, but there was no effect of BITC, the results demonstrate that adduct inhibition was due to PEITC. This is consistent with previous studies in which we have shown that PEITC inhibits HPB releasing DNA adduct formation in the lungs of rats treated with NNK (38,39). In contrast, we observed no effects of PEITC or a mixture of BITC and PEITC on BPDE-N2-dG adducts of B[a]P in either mice or rats.

In A/J mice treated weekly by gavage with a mixture of B[a]P plus NNK, addition of PEITC or BITC plus PEITC to the diet, at the doses used here, reduced lung tumor multiplicity by ~40% (19). No inhibition was observed with dietary BITC. In experiments with single carcinogens administered by the same protocol, PEITC inhibited lung tumorigenesis induced by NNK but not by B[a]P (19). Therefore, we concluded that the inhibitory effect of a mixture of dietary BITC and PEITC on lung tumorigenesis by B[a]P plus NNK in A/J mice was due mainly to the effect of PEITC on NNK. The results of our previous DNA adduct experiments in mice, taken together with the present results, strongly support this conclusion.

Consistent with the DNA adduct data, we observed significant decreases in levels of HPB releasing Hb adducts of NNK in rats treated with PEITC or BITC plus PEITC, but not with BITC. There were no effects of the isothiocyanates on levels of Hb adducts of B[a]P, which is also consistent with the DNA adduct data. Hb and DNA are traps for electrophiles formed in the metabolic activation of B[a]P and NNK. These results indicate that PEITC inhibits the metabolic activation of NNK to pyridyloxobutylating agents, resulting in a decrease in both DNA and Hb adducts. The observed decrease in DNA adduct levels is therefore unlikely to be due to effects on DNA repair. Neither PEITC nor BITC plus PEITC appears to have any significant effect on the metabolic activation of B[a]P to BPDE.

Levels of HPB releasing Hb adducts of NNK decreased between 12 and 16 weeks in the rats treated with B[a]P plus NNK or with B[a]P plus NNK and BITC. In a previous study, we also observed a decrease in Hb adduct levels in rats during chronic treatment with NNK, 2 p.p.m, in the drinking water (2). The decrease occurred during months 5–9 of treatment. The origin of this effect is not known at present. Previous studies demonstrated that chronic NNK treatment of rats resulted in a decrease in lung microsomal α-methylene hydroxylation of NNK as well as levels of pulmonary Oβ-methyguanine, a product of α-methylene hydroxylation (39,40). Further studies demonstrated that this was due to NNK treatment and not to the age of the rats (40). However, no such effects were observed on α-methyl hydroxylation, the pathway which leads to the formation of the HPB releasing Hb and DNA adducts studied here (39,40). Therefore, effects of NNK on its own metabolism are unlikely to explain this observation, and further studies are required.

Dietary PEITC, or BITC plus PEITC, significantly inhibited levels of HPB releasing DNA adducts of NNK in lung but not in liver. These results are consistent with a previous study in which we showed that chronic treatment of rats with PEITC inhibited pulmonary but not hepatic metabolic activation of NNK, by both α-hydroxylation pathways (40). In tandem with the inhibitory effects of PEITC on pulmonary HPB releasing DNA adducts of NNK observed in the present study, we saw a substantial increase in levels of urinary NNAL and NNAL-Gluc. These results indicate a link between inhibition of
pulmonary NNK α-hydroxylation and increased urinary NNAL and NNAL-Gluc. We have recently shown that NNAL accumulates in rat lung, possibly due to binding of (S)-NNAL to a receptor site (41). PEITC is known to inhibit pulmonary metabolism of NNAL to NNK, as well as α-hydroxylation of NNAL and NNK (40). Inhibition of these metabolic steps in the lung could lead to further accumulation of NNAL and ultimately to increased urinary excretion of NNAL. There was no effect of BITC on HPB releasing DNA adducts of NNK in lung or liver, nor was there any consistent effect of BITC on urinary NNAL or NNAL-Gluc levels. Collectively, these results indicate that pulmonary NNK and NNAL metabolism is a factor in urinary NNAL levels. These conclusions are consistent with a previous study, which compared NNK metabolism in vivo in the rat with its metabolism by primary lung and liver cells (42). The authors concluded that, at low doses of NNK, the profile of urinary metabolites is determined mainly by pulmonary metabolism. These results suggest that levels of NNAL and NNAL-Gluc in human urine could be a biomarker of NNK activation in the lung, and therefore could be used to determine the effects of chemopreventive agents such as PEITC.

HPB releasing DNA adducts of NNK were significantly higher in lung than liver after 8 and 16 weeks of treatment. These results are consistent with the known organoselectivity of NNK for induction of lung tumors in rats and with the importance of the lung in NNK metabolism, as discussed above. Previous studies indicated that HPB releasing DNA adducts and O6-methylguanine are important in lung tumor induction by NNK in rats (36,39,43). However, there have been few comparisons of HPB releasing adduct levels in rat liver and lung. A study by Murphy et al. (44) compared levels of HPB releasing DNA adducts of NNK in rat lung and liver.
after 4 daily i.p. injections of radiolabeled NNK. At low doses (150 µg/kg/day or less) the amount of HPB released from lung DNA was greater than from liver DNA, whereas at higher doses HPB releasing DNA adduct levels in liver exceeded those in lung. The daily dose in the present experiment was ~150 µg/kg/day. Therefore, our results are consistent with those of Murphy et al., although the routes of administration were different. Morse et al. (38) found higher levels of HPB releasing DNA adducts of NNK in liver than in lung after treatment of rats with four consecutive daily s.c. doses of 600 µg/kg body wt NNK, consistent with Murphy’s study. Similar results were obtained by Peterson et al. (45). The present study is the only one to compare levels of pulmonary and hepatic HPB releasing DNA adducts of NNK using a protocol similar to that which induces lung tumors in rats, e.g. chronic treatment with 2 p.p.m. NNK in the drinking water.

PEITC and BITC plus PEITC, reduced levels of HPB releasing DNA adducts in lung by ~50%, from 1.2–2.0 to 0.4–0.9 fmol/µg DNA. These residual adduct levels in lung, which were unaffected by the isothiocyanate treatments, were approximately the same as the adduct levels observed in liver (0.4–0.5 fmol/µg DNA), independent of isothiocyanate treatment (Figure 4A). This suggests that the HPB releasing DNA adducts were either PEITC-sensitive or PEITC-insensitive. The PEITC-sensitive adducts are found only in lung and are reduced by PEITC treatment. It is unlikely that there are structural differences between HPB releasing DNA adducts in lung and liver. It is more likely that this observation is related to effects of PEITC on cytochrome P450 (P450 (45)) enzymes in rat lung and liver. PEITC is known to specifically inhibit α-methyl hydroxylation of NNK in rat lung (40,46). The effects of PEITC on P450s are complex and dependent on route of administration, dose, and timing (47). PEITC is known to inhibit P450 2B-mediated activity in rat lung, but it can induce P450 2B1 in rat liver. PEITC can also inhibit P450 1A2 and 3A related activities in rat liver (47). These P450s, as well as P450 2A3, which is present in rat lung but not liver, are involved in NNK α-methyl hydroxylation (36,48,49). There may be differential effects of PEITC on these P450s, which produce the PEITC-sensitive and PEITC-insensitive DNA adducts.

The results of this study are fully consistent with our previous investigation in which we determined levels of HPB releasing Hb adducts and urinary NNAL and NNAL-Gluc in rats treated with NNK (2 p.p.m. in drinking water) with or without dietary PEITC (3 µmol/g diet) (2). This was a carcinogenicity study in which PEITC completely inhibited lung tumor induction by NNK. Adduct measurements and urinary metabolite determinations were carried out at intervals during the experiment. Levels of HPB releasing Hb adducts were 1.8 times lower in the PEITC-treated animals than in the controls, throughout the 2 year experiment. Although, as mentioned above, levels of HPB releasing Hb adducts decreased in the control animals during the course of the experiment, a corresponding decrease was also seen in the PEITC-treated rats, which is somewhat different from the present results. Levels of NNAL and NNAL-Gluc in urine also increased markedly (four to six times) as in the present study. Our results indicate that PEITC or BITC plus PEITC would inhibit lung tumor formation in rats treated with NNK in the drinking water and B[a]P in the diet.

There were no effects of BITC on biomarkers of B[a]P metabolic activation in this study. Evidently, the dose of BITC, which is limited to 1 µmol/g diet ( ~9 mg/kg body wt/day) by considerations of palatability, is insufficient. In A/J mice, 1 µmol/g diet BITC (15 mg/kg body wt/day) had no effect on lung tumor induction by B[a]P plus NNK (19). On the other hand, gavaged BITC is a very effective inhibitor of B[a]P-induced lung tumorigenesis in A/J mice (50,51). In a recent study, we observed 64 and 91% inhibition of lung tumor multiplicity by gavage of 6.7 or 13.4 µmol of BITC (50 or 100 mg/kg body wt), respectively, 15 min before each of 8 weekly treatments with 3 µmol B[a]P (52). We observed modest inhibition of B[a]P–DNA adduct formation in mice treated with BITC by gavage prior to B[a]P administration (53). The single high dose of BITC may have other effects,
such as induction of apoptosis, which are also important in lung tumor inhibition.

In summary, our results clearly show that PEITC or a mixture of BITC plus PEITC inhibits the formation of HPB releasing DNA adducts in the lungs of rats treated with B[a]P plus NNK. BITC had no effect on adducts derived from B[a]P or NNK. These results are consistent with previous studies, which assessed the inhibitory effects of these isothiocyanates on lung tumor formation, demonstrating the central role of inhibition of NNK metabolic activation by PEITC as a mechanism of chemoprevention.

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