Effects of benzyl isothiocyanate and phenethyl isothiocyanate on DNA adduct formation by a mixture of benzo[a]pyrene and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in A/J mouse lung

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Dietary phenethyl isothiocyanate (PEITC) and a mixture of dietary PEITC and benzyl isothiocyanate (BITC) inhibit lung tumorigenesis in A/J mice induced by a mixture of the tobacco smoke carcinogens benzo[a]pyrene (B[a]P) and 4-(methylitissoamino)-1-(3-pyridyl)-1-butanone (NNK). In this study, we tested the hypothesis that inhibition of tumorigenesis by these isothiocyanates was due to inhibition of DNA adduct formation. We quantified the following pulmonary DNA adducts: N2-[7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene-10-yldeoxyguanosine (BPDE-N2-dG) from B[a]P; and O6-methylguanine (O6-mG) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing adducts from NNK. Initial experiments demonstrated that there were no effects of B[a]P on NNK-DNA adduct formation, or vice versa, and established by way of a time course study the appropriate sacrifice intervals for the main experiment. Dietary PEITC, or dietary BITC plus PEITC, inhibited the formation of HPB-releasing DNA adducts of NNK at several of the time points examined. There were no effects of dietary isothiocyanates on levels of O6-mG or BPDE-N2-dG. These results, which are consistent with previous studies in rats and with tumor inhibition data in mice, support a role for inhibition of HPB-releasing DNA adducts of NNK as a mechanism of inhibition of tumorigenesis by dietary PEITC and BITC plus PEITC. However, the observed inhibition was modest, suggesting that other effects of isothiocyanates are also involved in chemoprevention in this model.

Introduction
Our goal is to develop mixtures of chemopreventive agents as inhibitors of lung cancer induction by carcinogens in tobacco smoke. Such agents could be used potentially to decrease the risk of lung cancer in high risk individuals, namely, addicted smokers and ex-smokers. This is important because lung cancer is the leading cause of cancer death in the USA, with nearly 160 000 deaths expected in 2001 (1). Unfortunately, efforts at prevention of smoking have stalled since 1990, necessitating the development of alternate approaches to prevention of tobacco-related cancer. Chemoprevention is one of these approaches.

We believe that isothiocyanates will be important components of such a chemopreventive mixture. Several isothiocyanates such as benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), and sulforaphane occur naturally in cruciferous vegetables as conjugates and have impressive chemopreventive properties in animal models (3). Considerable amounts of these isothiocyanates are released when the vegetables are chewed (4,5). It is noteworthy in this regard that epidemiologic studies consistently demonstrate that vegetable consumption, including cruciferous vegetables, protects against lung cancer (6,7). Moreover, a recent nested case–control study of lung cancer indicates that isothiocyanates are protective (8). Among the carcinogens in tobacco smoke, benzo[a]pyrene (B[a]P) and 4-(methylitissoamino)-1-(3-pyridyl)-1-butanone (NNK) are believed to play significant roles as causes of lung cancer in smokers (9). Therefore, B[a]P and NNK are our target molecules for development of a chemopreventive mixture. In the A/J mouse and F-344 rat, PEITC inhibits lung tumor induction by NNK (3), while BITC inhibits B[a]P induced lung tumorigenesis in the A/J mouse (3).

We have developed an A/J mouse lung tumor model for evaluation of chemopreventive agents (10). In this model, mice are treated once weekly for 8 weeks by gavage with a mixture of B[a]P (3 µmol) and NNK (3 µmol). Nineteen weeks after the last treatment, the mice are killed and lung tumors are counted. This protocol typically results in about 20 lung tumors per mouse, an amount which can be decreased readily by effective chemopreventive agents. In this model, dietary PEITC (3 µmol/g diet), or BITC (1 µmol/g diet) plus PEITC (3 µmol/g diet), administered from one week prior through one week following B[a]P and NNK treatment, significantly inhibited lung tumor multiplicity by ~40% (11). No inhibition was observed with BITC (1 µmol/g diet) alone, or did dietary PEITC inhibit B[a]P tumorgenesis in this model, leading us to conclude that the observed inhibition by dietary isothiocyanates was due mainly to the effect of PEITC on tumor induction by NNK.

Previous studies demonstrate the importance of DNA adduct formation in A/J mouse lung tumor induction by B[a]P or NNK (12–14). However, there are no reports in the literature on DNA adduct formation by a mixture of B[a]P and NNK. Experiments carried out in rats and mice indicate that one of the main mechanisms by which isothiocyanates inhibit tumorgenesis is favorable modification of carcinogen metabolism leading to decreased DNA adduct formation (3,15,16). Therefore, our hypothesis in this study was that chemoprevention by dietary BITC plus PEITC was due to reduction in DNA adduct formation by B[a]P and/or NNK. In this paper, we report the results of our experiments to test this hypothesis.
Materials and methods

Chemicals

B[a]P, PEITC, and BITC were obtained from Aldrich Chemical (Milwaukee, WI). NNK was synthesized (17). All procedures involving B[a]P were performed within glass or Teflon vessels. All metabolite standards were obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). Cottonseed oil and all other biochemical reagents were purchased from Sigma Chemical (St. Louis, MO) or Aldrich Chemical (Milwaukee, WI), unless noted. OmnSolV H2O for DNA hydrolysis and HPLC analysis was obtained from EM Science (Gibbstown, NJ). Optima grade methanol for HPLC was obtained from Fisher Scientific (Springfield, NJ).

Animal studies

Female A/J mice were obtained at age 5–6 weeks from Jackson Laboratories (Bar Harbor, ME). They were housed under standard conditions and maintained on AIN-93G diet (Dyets, Bethlehem, PA). The mice were 7 weeks of age at the time treatments were initiated.

In experiment 1, there were 12 groups of nine mice each. Mice were treated weekly by gavage with either NNK (3 µmol in 0.1 ml cottonseed oil; groups 1, 5 and 9), B[a]P (3 µmol in 0.1 ml cottonseed oil; groups 2, 6 and 10), B[a]P plus NNK (3 µmol each in 0.1 ml cottonseed oil; groups 3 and 7); or 0.1 ml cottonseed oil alone (groups 4, 8 and 12). Mice were killed 24 h after (groups 1–4, 4–8 groups 5–9) or 8 groups (groups 9–12) treatments. Lungs were harvested from each group and divided into three pools of three lungs each. They were frozen in liquid nitrogen and stored at −80°C until the DNA was isolated as described below.

In experiment 2, 90 mice were divided into 10 groups of nine, and were treated weekly for 4 weeks by gavage with B[a]P plus NNK (3 µmol each in 0.1 ml cottonseed oil). The mice in each group were killed 2, 4, 6, 12, 24, 48, 72, 120, 192, or 384 h after the fourth dose of B[a]P plus NNK. At sacrifice, lungs from each group were isolated and stored at −80°C until isolation of DNA. In experiment 3, 126 mice were divided into 21 groups of six mice each. All mice were treated with B[a]P plus NNK (3 µmol each in 0.1 ml cottonseed oil by gavage). Control animals had nothing added to their diets. Groups of six of the control mice were killed 4, 24, or 120 h after a single dose of B[a]P plus NNK, or 24 or 120 h after 4 weekly doses of B[a]P plus NNK, or 24 or 120 h after 8 weekly doses of B[a]P plus NNK. The animals treated with PEITC were given PEITC in AIN-93G diet (3 mg/g diet) from 1 week before carcinogen treatment until killing, and groups of six mice were killed at the same intervals used for the control mice. The animals treated with BITC plus PEITC were given BITC (1 mg/g diet) plus PEITC (3 mg/g diet) from 1 week before carcinogen treatment until killing, and groups of six mice were killed at the same intervals used for the control mice. Food consumption was monitored using metal boxfeeders (18). The diets were prepared monthly and stored at 4°C. At killing, lungs from each group of mice were divided into three pools of two lungs each and stored at −80°C until DNA isolation.

Isolation of DNA from tissues

DNA was isolated by modifications of the Kirby method (19). In brief, the tissues were homogenized in 50 mM Tris, 1 mM EDTA, pH 7.4 and incubated with 1% sodium dodecyl sulfate and proteinase K (300 µg/ml). The homogenates were extracted twice with phenol/chloroform/isomyl alcohol (25:24:1) and then once with chloroform/isomyl alcohol (24:1). DNA was precipitated with ethanol and dissolved in 15 mM sodium citrate, 15 mM sodium chloride, 1 mM EDTA, pH 7.4. The DNA was incubated with RNase A (100 µg/ml) and RNase T1 (2000 U), extracted once with chloroform/isomyl alcohol, and precipitated with ethanol. DNA was dried under a stream of nitrogen and stored at −20°C.

Fluorometric analysis of O(6)-methylguanine (O(6)-mG) in DNA

The method is similar to that described previously (12). Briefly, DNA was dissolved in 10 mM sodium cacodylate buffer, pH 7.0, and the DNA concentration was determined by UV. The solution was heated at 100°C for 30 min to release 7-methylguanine. The partially apurinic DNA was precipitated with 0.1 N HCl. The precipitated DNA was hydrolyzed in 0.1 N HCl at 80°C for 1.5 h, transferred to 2 ml high recovery silanized autosampler vials and analyzed by HPLC with fluorescence detection for O(6)-mG.

O(6)-mG was eluted from two Whatman Partisil (Clifton, NJ)10 SCX columns in a linear gradient with an isocratic solution of 100 mM ammonium phosphate, pH 2.0, and 5% methanol at 1.0 ml/min. Buffer was filtered through a 0.2 µm nylon membrane and sonicated under vacuum prior to use. The concentration of the buffer and/or the percentage of methanol added to the buffer were altered as the columns aged so that the retention time of O(6)-mG was ~30 min.

O(6)-mG was detected by fluorescence (excitation 286 nm, emission 356 nm). The level of O(6)-mG in each sample was determined by comparison with a standard curve generated from the fluorescence areas of authentic O(6)-mG standards analyzed prior to and immediately following the analysis of a set of samples. At 12 h intervals throughout the analysis of a set of samples, the fluorescence of O(6)-mG standard was validated to use the standard curve. The detection limit was 2 fmol B[a]P-tetrol. The DNA from each lung was analyzed for released B[a]P-tetrols in single HPLC runs. The same DNA samples were analyzed for guanine. The levels of B[a]P-tetrols released from DNA were expressed as pmol/mg guanine.

Fluorometric analysis of O(2)-methylguanine (O(2)-mG) in DNA

The procedure was performed essentially as previously described (21). Briefly, lung DNA was dissolved in H2O and the DNA concentration was determined by UV. An average of 94 ± 53 mg DNA was analyzed. Fifty pg (299 fmol) [4,4-D2]HPB as internal standard was added to each sample and the DNA was hydrolyzed by incubation at 80°C for 3 h in a final concentration of 0.1 M NaCl. A 50 µl aliquot from each sample was reserved for analysis of guanine. The aqueous solution of DNA was extracted twice with CH2Cl2. The aqueous layer was saved and the pH was adjusted to 7.0 with 1 N NaOH. The sample was then extracted twice with equal volumes of CH2Cl2, the organic layers were pooled and the solvent removed with a SpeedVac (Holtbrook, NY) concentrator. The residue, containing HPB, was dissolved in 0.5 ml of CH3Cl2. Derivatization was carried out in a hexane solution of trimethylamine freshly prepared as follows: 240 mg trimethylamine hydrochloride dissolved in 20 ml 0.8 N NaOH, and extracted with 20 ml hexane. The hexane layer was dried (Na2SO4). One ml of this solution was added to each sample. The reaction was started by adding 0.5 ml pentfluoroanisole chloride solution (20 µl pentfluoroanisole chloride in 10 ml CH3Cl2) and was carried on for 1 h at room temperature. The solvent was removed using a SpeedVac concentrator.

Unreacted pentfluoroanisole chloride was removed from the samples by reverse phase HPLC. The HPLC conditions were as follows: A 4.6 mm × 12.5 cm Whatman Partisil 5 ODS 3 column was eluted with a 900 µl injection. The total volume in each autosampler vial was brought to 1.0 ml with H2O.

HPLC analysis was performed with a Hewlett Packard Series 1100 auto- sampler coupled to a Waters Millipore automated gradient controller and nitrogen gas was used as the solvent delivery linear gradient. The gradient solvents were switched by a Valco Instruments switching valve to flow over a Beckman Ultrasphere ODS 4.6 mm × 25 cm analytical column to separate the four B[a]P-tetrol isomers. The B[a]P-tetrols were detected by an Shimadzu (Columbia, MD) RF-10AXL fluorescence detector linked to a PeakSimple (Alltech Deerfield, IL) Chromatography Data System. The fluorescence excitation wavelength was set at 344 nm and the emission wavelength at 398 nm. The fluorescence corresponding to the eluted peaks of B[a]P-tetrol isomer was integrated. The level of each B[a]P-tetrol in the samples was determined by comparison with a standard curve generated from the fluorescence areas of authentic B[a]P-tetrol standards analyzed prior to and immediately following the analysis of a set of samples. At 12 h intervals throughout the analysis of a set of samples, the fluorescence of B[a]P-tetrol standard was validated to use the standard curve. The detection limit was 2 fmol B[a]P-tetrol. The DNA from each lung was analyzed for released B[a]P-tetrols in single HPLC runs. The same DNA samples were analyzed for guanine. The levels of B[a]P-tetrols released from DNA were expressed as pmol/mg guanine.

Gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) analysis of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) released from DNA

The procedure was performed essentially as previously described (21). Briefly, lung DNA was dissolved in H2O and the DNA concentration was determined by UV. An average of 94 ± 53 mg DNA was analyzed. Fifty pg (299 fmol) [4,4-D2]HPB as internal standard was added to each sample and the DNA was hydrolyzed by incubation at 80°C for 3 h in a final concentration of 0.1 M NaCl. A 50 µl aliquot from each sample was reserved for analysis of guanine. The aqueous solution of DNA was extracted twice with CH2Cl2. The aqueous layer was saved and the pH was adjusted to 7.0 with 1 N NaOH. The sample was then extracted twice with equal volumes of CH2Cl2, the organic layers were pooled and the solvent removed with a SpeedVac (Holtbrook, NY) concentrator. The residue, containing HPB, was dissolved in 0.5 ml of CH3Cl2. Derivatization was carried out in a hexane solution of trimethylamine freshly prepared as follows: 240 mg trimethylamine hydrochloride dissolved in 20 ml 0.8 N NaOH, and extracted with 20 ml hexane. The hexane layer was dried (Na2SO4). One ml of this solution was added to each sample. The reaction was started by adding 0.5 ml pentfluoroanisole chloride solution (20 µl pentfluoroanisole chloride in 10 ml CH3Cl2) and was carried on for 1 h at room temperature. The solvent was removed using a SpeedVac concentrator.

Unreacted pentfluoroanisole chloride was removed from the samples by reverse phase HPLC. The HPLC conditions were as follows: a 4.6 mm × 12.5 cm Whatman Partisil 5 ODS 3 column was eluted with a
gradient program of 35% methanol in H$_2$O for 10 min followed by a linear gradient from 35% to 75% methanol in 15 min at 1 ml/min. For the HPLC clean up, the sample residue was dissolved in 70 µl methanol/tetrahydrofuran (1:1) containing 25 µg/ml pentanophenone and hexanophenone as retention time markers. The entire sample was injected with a Hewlett Packard Series 1100 autosampler. The fraction between the apices of the retention time marker compounds was collected and dried in a SpeedVac concentrator. The residue was transferred to a silanized conical vial using three aliquots of 100 µl tetrahydrofuran.

GC-NICI-MS analysis was performed with a model 5890 GC (Hewlett-Packard, Palo Alto, CA) interfaced with a TSQ 7000 mass spectrometer (Thermoquest, San Jose, CA). A 0.25 mm i.d. × 30 m 50% methylphenylsilicone (DB-17) bonded phase column (0.15 µm film thickness) (J&W Scientific, Folsom, CA) connected to a 2 m × 0.32 mm i.d. noncoated deactivitated retention gap was used. The oven program was as follows: 35°C for 1 min, then 20°C/min to 150°C, then 4°C/min to 205°C, then hold 20 min at a constant flow of 2.5 ml/min helium. For GC–NICI–MS analysis, samples were resuspended in 10 ml of toluene containing 6 fmol/µl HPB-tetrafluorobenzoate as injection standard. The MS was operated in the NICI mode with a methane pressure of 3000 mtorr. The source temperature was 150°C. The molecular ions of HPB-tetrafluorobenzoate (injection standard, m/z 359) and [4,4]D$_2$-HPB-tetrafluorobenzoate (internal standard, m/z 361) were monitored. Two to four H$_2$O blanks were analyzed with each set of 24 samples. The mean value of the H$_2$O blanks was 73 ± 21 fmol HPB (n = 28). The mean H$_2$O blank value was subtracted from each sample value. The average recovery of [4,4D$_2$]HPB was 34 ± 14%.

The levels of HPB-releasing adducts in mouse lung DNA were expressed as pmol/µmol guanine.

**Fluorometric analysis of guanine**

This method is similar to that described previously (20). Guanine from about 2 µg DNA per injection was eluted from two Whatman Partisil 10 SCX columns in tandem with an isotropic solution of 100 mM ammonium phosphate, pH 2.0, at 1.0 ml/min. Buffer was filtered through a 0.2 µm nylon membrane and sonicated under vacuum prior to use. Detection was by fluorescence (excitation 286 nm, emission 366 nm). The level of guanine in each sample was determined by comparison with a standard curve generated from the fluorescence areas of guanine standards analyzed at 12 h intervals throughout the analysis of a set of samples. Determinations were carried out in duplicate, with variations typically <2%.

**Statistical analysis**

Comparisons between DNA adduct levels in different treatment groups were made using Student’s t-test.

**Results**

The prevalent established pathway of DNA adduct formation by B[a]P yields BPDE-N$^2$-dG (Figure 1) (22). This adduct was measured in the lung DNA of B[a]P-treated mice by acid hydrolysis of the DNA followed by HPLC–fluorometric quantitation of the released B[a]P-tetraols. NNK forms DNA adducts by two main pathways (Figure 1) (23).

α-Hydroxylation at the methylene carbon leads to methyl DNA adducts, among which O$^2$-mG is thought to be important in tumor induction in mice. This adduct was quantified by HPLC–fluorometry. α-Hydroxylation at the methyl carbon of NNK produces pyridloxybutylated DNA adducts, which can be hydrolyzed to HPB. The released HPB was quantified by GC-NICI–MS–MS.

In experiment 1, our goal was to determine whether formation of these adducts in pulmonary DNA was different when B[a]P and NNK were administered to A/J mice individually.
versus in combination. Mice were treated with B[a]P, NNK, or B[a]P plus NNK once weekly for 8 weeks and killed 24 h after the 1st, 4th, or 8th weekly treatment. The results of this analysis are illustrated in Figure 2A-C. Figure 2A demonstrates that B[a]P-tetraols released from BPDE-N2-dG were detected only in mice treated with B[a]P or B[a]P plus NNK. Adduct levels increased significantly between 1 and 4 weeks (P < 0.01) and were similar after 4 and 8 weeks of treatment. There were no significant effects of NNK on BPDE-N2-dG levels. Figure 2B shows that O6-mG was detected only in mice treated with NNK or B[a]P plus NNK. Adduct levels increased significantly between 1 and 4 weeks (P < 0.05) and were similar after 4 and 8 weeks of treatment. There were no significant effects of B[a]P on O6-mG levels. Figure 2C shows similar results for HPB-releasing adducts, although there was no significant increase in adduct levels with time, probably due to the relatively large variation. A small amount of HPB was detected in the lung DNA of the group treated once with B[a]P, presumably due to assay background. Collectively, the results of this experiment demonstrate that adduct formation by B[a]P and NNK is similar when the compounds are administered individually compared to when they are given together.

In experiment 2, we evaluated the time course of adduct persistence after 4 weeks of treatment with B[a]P plus NNK. We chose the 4 week time point because the results of experiment 1 indicated that adduct formation had reached a plateau at that point. However, we needed further information on adduct persistence to determine the appropriate sacrifice intervals after B[a]P plus NNK treatment. The results of this experiment, in which B[a]P-tetraols released from BPDE-N2-dG, and O6-mG, were quantified, are summarized in Figure 3A,B. Levels of B[a]P-tetraols and O6-mG did not change markedly over the entire 2–384 h time course.

These results set the stage for experiment 3, the design of which is illustrated in Figure 4. PEITC or BITC plus PEITC were administered in the diet. The 24 h and 120 h sacrifice intervals after 4 and 8 weeks of treatment were chosen based on the results of experiment 2. We also included a 4 h sacrifice interval after the first treatment because previous studies had shown that O6-mG formation peaked at this time point after one treatment with NNK. B[a]P-tetraols, O6-mG, and HPB-releasing adducts were quantified.

The results are summarized in Figure 5A–I. Figure 5A shows little effect of dietary PEITC or BITC plus PEITC on B[a]P-tetraols after one treatment with B[a]P plus NNK; a small but significant increase was seen at the 120 h interval. Similar results were observed after 4 and 8 weeks (Figure 5B,C). Figure 5D–F show no significant effect of dietary isothiocyanates on O6-mG levels. There were apparent increases in some groups, but these were not significant. Figure 5G–I demonstrate decreases, some significant (P < 0.05), in levels of HPB-releasing adducts in most groups of mice treated with dietary PEITC, or BITC plus PEITC, for 4 or 8 weeks.

**Discussion**

The results of this study partially support our hypothesis that isothiocyanates diminish levels of DNA adducts, thereby decreasing B[a]P plus NNK-induced lung tumorigenesis. Levels of HPB-releasing DNA adducts of NNK decreased in several of the groups treated with dietary PEITC, or dietary BITC plus PEITC, and it was these treatments that provided the greatest protection against lung tumor development in our earlier experiments (11). Inhibition of adduct formation was particularly consistent at the 24 h time points (Figure 5G–I). These results support the proposal, based on the results of the tumor inhibition studies, that inhibition of B[a]P plus NNK-induced lung tumorigenesis by dietary PEITC is due mainly to the effect of PEITC on NNK. Inhibition of tumor formation in this model was the same when either dietary PEITC, or a mixture of dietary BITC and PEITC, was used, and dietary BITC had no effect. In addition, dietary PEITC had no effect on lung tumor induction by B[a]P (11). The results presented here demonstrate that dietary PEITC, or BITC plus PEITC, had similar inhibitory effects on HPB-releasing adducts of NNK. Moreover, dietary PEITC had no inhibitory effect on BPDE-N2-dG levels. Collectively, these results demonstrate a role for HPB-releasing DNA adducts of NNK in tumorigenesis in this model and indicate that inhibition of this pathway is important in chemoprevention by dietary PEITC.

Previously, we demonstrated that inhibition of HPB-releasing adducts of NNK is the major mechanism by which PEITC inhibits lung tumorigenesis in F344 rats treated with PEITC and NNK (24). In that study, PEITC was administered in the
diet at 3 µmol/g diet while NNK was given three times weekly for 20 weeks by subcutaneous injection. HPB-releasing DNA adducts of NNK were measured in DNA of pulmonary cell types and whole lung at various intervals during NNK and PEITC treatment. Consistent decreases in levels of HPB-releasing adducts were seen in the presumed target cells—alveolar type II cells—as well as in Clara cells and whole lung. The inhibitory effects were stronger than in the present study, dietary PEITC-mediated inhibition of HPB-releasing DNA adducts formed in rats treated with 2 µ.p.m. NNK in the drinking water (25). Our results, which demonstrate a role for PEITC treatment. Consistent decreases in levels of HPB-releasing adducts were seen in the presumed target cells—alveolar type II cells—as well as in Clara cells and whole lung. The null effect of the isothiocyanate treatments on O6-mG levels was quite surprising. Previous studies demonstrated convincingly that O6-mG is important in lung tumorigenesis in A/J mice treated with a single dose of NNK, and that O6-mG levels can be decreased by PEITC (12,26,27). However, consistent with our previous data in the rat.

Fig. 5. Levels of DNA adducts in A/J mouse lung. Mice were treated by gavage once, or weekly for 4 or 8 weeks with B[a]P plus NNK (3 µmol each in 0.1 ml cottonseed oil). Groups of mice were killed 4, 24, or 120 h after the first treatment, or 24 or 120 h after the 4th and 8th treatments. The mice were treated with PEITC (3 µmol/g diet, ■), or PEITC (3 µmol/g diet) plus BITC (1 µmol/g diet) (▲) from 1 week prior to the first B[a]P plus NNK treatment until sacrifice. Control animals had no additions to the diet (□). (A–C) levels of B[a]P-tetraols released from BPDE-I/-dG; (D–F) levels of O6-mG; (G–I) levels of HPB released from pyridyloxobutylated DNA. Data represent mean ± SD (n = 3 unless no error bar is shown, then n = 2). *P < 0.05.
of B[a]P (0.4 mmol/kg) 4 h later, and killed after 24–120 h (28). They also observed no effect of PEITC on tumorigenesis by B[a]P. Collectively, it is clear that PEITC has little or no effect on B[a]P-DNA adduct formation or tumorigenicity.

In this study, inhibition of DNA adduct formation, when observed, was relatively modest. These results suggest that there are effects of isothiocyanates other than modification of DNA adduct formation that are important in chemoprevention. Prominent among these is induction of apoptosis. There is now a considerable body of evidence indicating that isothiocyanates induce apoptosis in various systems. BITC and PEITC both induce sustained activation of c-Jun N-terminal kinase, and this is associated with induction of apoptosis in various cell types (29). Treatment with isothiocyanates under conditions of apoptosis induction causes rapid and transient induction of caspase-3/CPP32-like activity (30). As discussed above, PEITC induces apoptosis in JB6 cells through a p53-dependent pathway (31). PEITC induces apoptosis in human leukemia cells and inhibits cell growth in this system (32, 33). A recent study demonstrates that PEITC further increases apoptosis induced in the respiratory tract of rats by cigarette smoke (34). BITC and sulforaphane, a related naturally occurring isothiocyanate, induce apoptosis in human colon cancer cells (35, 36). Future studies should examine the role of apoptosis induction under conditions of inhibition of tumorigenesis by isothiocyanates.

There were some limitations to this study. First, we examined only three specific DNA adducts. While there is substantial evidence that these adducts are important in carcinogenesis by B[a]P and NNK, it is possible that other adducts, not measured here, may also contribute. For example, depurinating adducts of B[a]P and O2-methylthymidine from NNK, may be involved in tumorigenesis (37, 38). There are other DNA adducts of B[a]P that are unidentified (39, 40). In addition, we measured HPB-releasing adducts as a class, but individual pyridyloxobutylating adducts, such as O6-[4-oxo-4-(3-pyridyl)butyl] deoxyguanosine, may be important (41). All of our adduct measurements were carried out in whole lung, but it is possible that adducts in individual cell types may be modified differently, thus obscuring the overall effect (24).

In summary, the results of this study partially support our hypothesis that modification of DNA adduct levels is involved in inhibition of B[a]P plus NNK-induced pulmonary tumorigenesis by dietary isothiocyanates in the A/J mouse. However, the effects we observed were generally modest. The results indicate that protective mechanisms other than modification of DNA adduct formation contribute to inhibition of pulmonary carcinogenesis in this model.

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