

Phenethyl Isothiocyanate and Sulforaphane and their *N*-Acetylcysteine Conjugates Inhibit Malignant Progression of Lung Adenomas Induced by Tobacco Carcinogens in A/J Mice

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Abstract

We have shown previously that naturally occurring isothiocyanates derived from cruciferous vegetables and their *N*-acetylcysteine conjugates inhibit lung adenoma formation induced by tobacco carcinogens in A/J mice at the post-initiation stage. The tumor-inhibitory activity by these compounds is linked with activation of activator protein and induction of apoptosis in lung tissues, suggesting that these compounds may also inhibit the development of adenomas to adenocarcinomas in lung. In this study, the chemopreventive activity of phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates during progression of lung adenomas to malignant tumors was investigated in A/J mice. Mice were divided into 14 groups and treated with a mixture of 3 μ mol benzo(*a*)pyrene [B(*a*)P] and 3 μ mol 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) given by gavage once weekly for 8 weeks. Twenty weeks after the beginning of carcinogen administration, a total of 20 mice in the treatment groups were sacrificed with an average yield of 7.3 ± 4.5 lung adenomas per mouse. The remaining mice in each group were fed diets containing phenethyl isothiocyanate (3 and 1.5 mmol/kg diet), sulforaphane (3 and 1.5 mmol/kg diet), phenethyl isothiocyanate-*N*-acetylcysteine (8 and 4 mmol/kg diet), sulforaphane-*N*-acetylcysteine (8 and 4 mmol/kg diet) during weeks 21 to 42. Four mice in each of the high-dose treatment groups were sacrificed during weeks 28 and 36 and the bioassay was terminated during week 42; lung tissues were harvested for histopathologic examination of tumors and for cell proliferation (proliferating cell nuclear antigen) and apoptosis (caspase-3) assays using immunohistochemical staining. At termination, the incidence of adenocarcinoma in the 3 mmol/kg diet phenethyl isothiocyanate group and 8 mmol/kg diet phenethyl isothiocyanate-*N*-acetylcysteine group was reduced to 19% and 13%, respectively, compared with 42% in the carcinogen-treated control group. At the lower doses, phenethyl isothiocyanate and its *N*-acetylcysteine conjugate also inhibited the incidences of lung adenocarcinoma, however, the decreases were not statistically significant. The lung tumor incidences in groups treated with sulforaphane-*N*-acetylcysteine in the diet were also significantly

reduced to 11% or 16%. Furthermore, the malignant lung tumor multiplicity was significantly reduced from 1.0 tumor/mouse in the carcinogen-treated control group to 0.3 in the sulforaphane low-dose group, 0.3 and 0.4 in the two sulforaphane-*N*-acetylcysteine groups, and 0.4 in the phenethyl isothiocyanate high-dose group. The malignant tumor multiplicities in other treatment groups were also reduced (0.5-0.8 tumors/mouse), but not significantly. Unlike lung adenocarcinomas, both incidences and multiplicities of lung adenomas were not much affected by treatment with isothiocyanates or their conjugates. Immunohistochemical examination of the lung tumors from all time points indicated that significant reduction in proliferating cell nuclear antigen and induction of apoptosis (terminal nucleotidyl transferase-mediated nick end labeling and caspase-3) were observed in the isothiocyanate and isothiocyanate-*N*-acetylcysteine-treated groups that showed inhibition of the development of lung adenocarcinomas. The results of the study provide a basis for future evaluation of the potential of phenethyl isothiocyanate and sulforaphane and their conjugates as chemopreventive agents in smokers and ex-smokers with early lung lesions. (Cancer Res 2005; 65(18): 8548-57)

Introduction

Lung cancer is the leading cause of cancer death in both men and women in the U.S., and cigarette smoking is a major etiologic factor (1). Lung cancer has a low survival rate and chemotherapy for advanced non-small cell lung cancer is generally ineffective, with considerable toxicity (2). Although the lung cancer risk in ex-smokers gradually declines over the years, it remains substantial, compared with those who have never been smokers (3-5). Thus, approaches to the prevention of lung cancer need to be developed so that they can be implemented to benefit this high-risk population, especially efforts targeted to the progression of preneoplastic and benign neoplastic lesions to more malignant lung tumors. The use of chemopreventive agents for this purpose has been proposed (6, 7), yet, no effective chemopreventive agent has thus far been identified in human trials (8). Part of the reason for this is the scarcity of information from studies in animals. To our knowledge, only a limited number of agents, i.e., black tea polyphenol constituents (9), budesonide (10, 11), lycopene (12), and myo-inositol (11) have been studied for their chemopreventive activity during the progression phase after benign lung tumors had already developed.

Isothiocyanates occur as glucosinolates in a wide variety of cruciferous vegetables. Hydrolysis of glucosinolates by myrosinase when these plants are crushed and chewed produces isothiocyanates that

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are ultimately absorbed in the intestinal tract and excreted as thiol conjugates via the mercapturic acid pathway (13, 14). The conjugation of isothiocyanate with thiol is a reversible reaction; the thiol conjugates of isothiocyanates can dissociate by releasing parent isothiocyanates under physiologic conditions (15). Therefore, isothiocyanate conjugates can be considered prodrugs of parent isothiocyanate compounds. Isothiocyanates are versatile chemopreventive agents, because a number of isothiocyanates and their *N*-acetylcysteine conjugates have been shown to inhibit lung tumor development when given prior to or during carcinogen exposure (16–21). Mechanisms of isothiocyanates as anticarcinogens of the lung cancer have been attributed to the inhibition of phase I enzymes and/or to the induction of the activity of phase II conjugation enzymes for metabolism of carcinogens (22–25). Isothiocyanates were also effective when given throughout the bioassays, although it is difficult to determine based on results from these studies whether the tumor inhibition was due to its activity at the initiation or post-initiation stages (26). A more recent study showed that the *N*-acetylcysteine conjugates of phenethyl isothiocyanate and benzyl isothiocyanate given in the diet after treatment with benzo(*a*)pyrene [B(*a*)P] inhibited the formation of lung adenomas, indicating that these agents are effective during the post-initiation stage (21). A possible mechanism by which isothiocyanate compounds act as tumor inhibitors during post-initiation stages is through inhibiting the cell cycle and inducing apoptosis by activating mitogen-activated protein kinases and/or activator protein *in vitro* and *in vivo* (21, 27–33).

Although the chemopreventive potential of isothiocyanates is corroborated by data obtained from cell culture and animal studies, it was not until recently that their roles in protecting against cancer had been assessed in humans. Epidemiologic studies have provided evidence for the protective role of dietary isothiocyanate compounds in reducing the risk of lung cancer (34–36). Similar results were obtained for colon and breast cancer (37–39). As isothiocyanate compounds have begun to emerge as promising chemopreventive agents for certain cancers, an important question is whether they are effective in blocking the progression of benign lung tumors to malignant tumors. The observations that certain isothiocyanate compounds, including phenethyl isothiocyanate and sulforaphane, can reduce cell proliferation and induce apoptosis, suggest that they may also be effective during the tumor progression phase, because genetically altered cells are sensitive to dietary and environmental factors, at least in the early stages (40). The A/J mouse is an excellent model to evaluate the chemopreventive agents for lung tumor progression because lung adenomas appear during weeks 16 to 19 after administration of tobacco carcinogens, and adenocarcinomas occur generally during weeks 28 to 36 (41–44). Taking advantage of this model, we have carried out a study in mice treated with 4-methyl-(nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) plus B(*a*)P, two important carcinogens in cigarette smoke, to examine the effects of phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates (structures in Fig. 1) on the development of lung adenocarcinomas after lung adenomas had already formed.

Materials and Methods

Diets, chemicals, and reagents. Phenethyl isothiocyanate and *N*-acetylcysteine were purchased from Aldrich (Milwaukee, WI). Sulforaphane was synthesized by an improved, safe, and cost-effective route, useful for preparation of multi-gram quantities, based on previously described reports (45–47). The synthesis of sulforaphane is outlined in Scheme 1 and the

details are described below. The *N*-acetylcysteine conjugates of phenethyl isothiocyanate and sulforaphane were prepared using published methods (48, 49). The purity of the test chemicals was verified by their proton nuclear magnetic resonance (NMR) spectra and by high-performance liquid chromatography (>98%). B(*a*)P (purity >97%) and cottonseed oil were obtained from Sigma Chemical Co., (St. Louis, MO). NNK was synthesized by a published method (50). Other reagents used were obtained from commercial sources at the highest purity available.

Synthesis of sulforaphane. Caution! 3-Chloroperbenzoic acid and 1,1'-thiocarbonyldi-2(1*H*)-pyridone are irritating to the eyes, respiratory system, and skin. Therefore, appropriate protective eyewear and clothing should be used. Sodium thiomethoxide has an unpleasant odor, is flammable and corrosive, and causes burns. Hydrazine monohydrate is a toxic, cancer-suspect agent, and dichloromethane is an irritant and a carcinogen and should be handled in a well-ventilated hood.

N-(4-Bromobutyl)phthalimide (**2**).

Acetone (1.4 L) and 1,4-dibromobutane (213.5 mL, 1.8 mol) were added to a 3 L three-necked round-bottomed flask fitted with a mechanical stirrer and reflux condenser. The flask was heated to 40°C and potassium phthalimide (**1**, 185 g, 1 mol) was added slowly over a 15-minute period. The reaction mixture was heated under reflux for 2.5 hours. The reaction mixture was hot-filtered and the acetone removed via rotary evaporation. Unreacted 1,4-dibromobutane was recovered by vacuum distillation (4 mm Hg, 95°C) and reused in subsequent batches. The product *N*-(4-bromobutyl)phthalimide was isolated as a tan solid (130 g, 0.46 mol, 46% yield).

N-(4-Methylsulfonylbutyl)phthalimide (**3**).

Sodium thiomethoxide (54.6 g, 0.78 mol) and methanol (400 mL) were added to a 3 L three-necked round-bottomed flask fitted with a mechanical stirrer, reflux condenser, and nitrogen line. The reaction vessel was cooled to 0°C and **2** (212 g, 0.75 mol), suspended in methanol (600 mL), was added. The reaction mixture was allowed to stir at room temperature overnight, heated to reflux for 2 hours, then hot-filtered and concentrated. The product *N*-(4-methylsulfonylbutyl)phthalimide was dissolved in a mixture of Et₂O and H₂O (75:25, 600 mL). The solution was extracted with Et₂O (3 × 300 mL) and the organics were combined, dried (Na₂SO₄), filtered, and condensed to give 135 g (0.54 mol) of a tan solid (72% yield).

N-(4-Methylsulfonylbutyl)phthalimide (**4**).

CH₂Cl₂ (0.5 L) and **3** (128 g, 0.5 mol) were added to a 3 L three-necked round-bottomed flask fitted with a mechanical stirrer, addition funnel, and

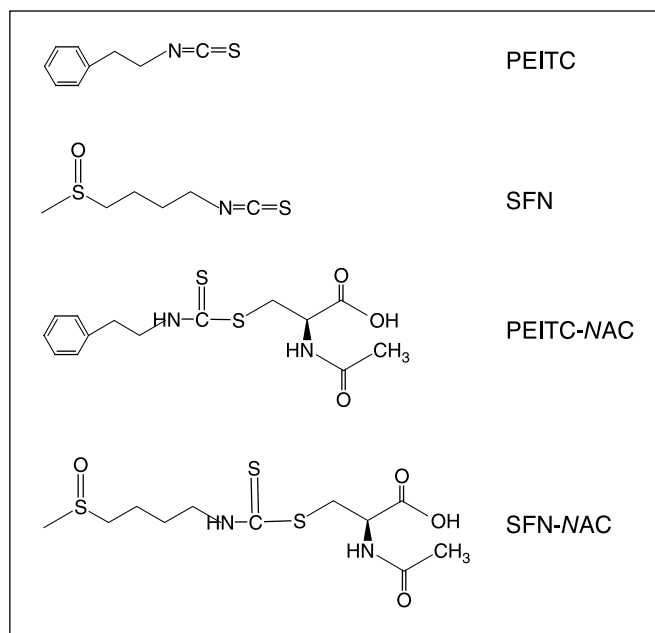


Figure 1. Structures of isothiocyanates and the *N*-acetylcysteine conjugates used in the bioassay.

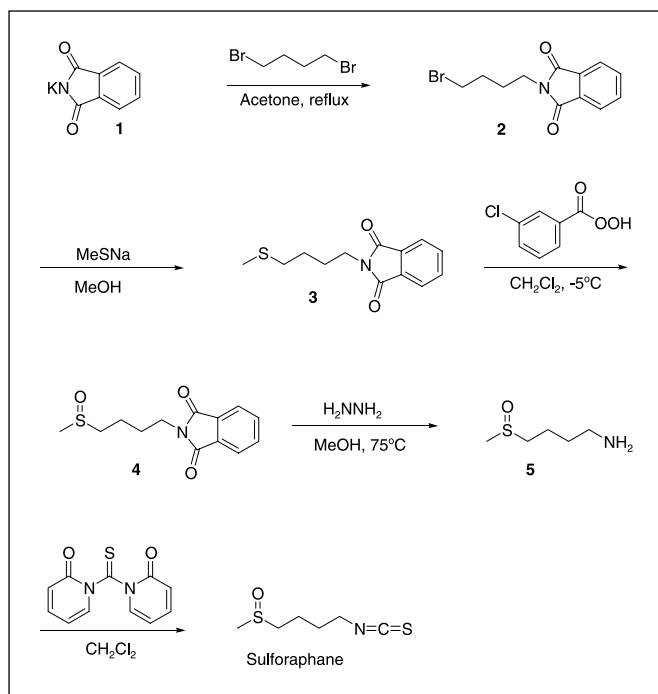
nitrogen line. The reaction vessel was cooled to -5°C and 3-chloroperbenzoic acid (112 g, 0.5 mol), dissolved in CH_2Cl_2 (750 mL), was added via addition funnel over a 15-minute period. The reaction was allowed to stir at -5°C for 2 hours or until complete by TLC. The reaction mixture was poured into 5% aqueous NaHCO_3 (400 mL) and extracted with CH_2Cl_2 (5×300 mL). Organics were combined, dried (Na_2SO_4), filtered, and condensed to give 102 g (0.38 mol) of an off-white solid product, *N*-(4-methylsulfinylbutyl)phthalimide (75% yield). $^1\text{H NMR}$ (CDCl_3) δ 7.79 (m, 2H), 7.67 (m, 2H), 3.69 (m, 2H), 2.86 (m, 1H), 2.75 (m, 1H), 2.62 (s, 3H), 1.85 (m, 4H); MS m/z 266.3 (M + H).

4-Methanesulfinylbutylamine (5).

Methanol (750 mL) and 4 (79.3 g, 0.3 mol) were added to a 2 L round-bottomed flask fitted with a reflux condenser. Hydrazine monohydrate (29 mL, 0.6 mol) was added and the mixture was heated to reflux for 2 hours. After cooling to room temperature, the mixture was diluted with H_2O (400 mL) and the pH was adjusted to 5.0 with 2 N HCl. The precipitate was allowed to settle overnight and then filtered. The filtrate was concentrated and partially purified by ion-exchange column chromatography (Dowex 1X8-200, strongly basic) with H_2O elution. Fractions were combined and basified (pH > 10) with concentrated NaOH. The combined fractions were extracted with CHCl_3 (5×100 mL), dried (Na_2SO_4), filtered, and condensed. The free amine, 4-methanesulfinylbutylamine (24.3 g, 0.18 mol), was isolated as a viscous yellow oil (60% yield). $^1\text{H NMR}$ (D_2O) δ 2.81 (m, 2H), 2.58 (m, 5H), 1.65 (m, 2H), 1.50 (m, 2H); MS m/z 136.0 (M + H).

Sulfuraphane. Compound 5 (36 g, 0.27 mol), dissolved in CH_2Cl_2 (700 mL), was added to a 2 L round-bottomed flask fitted with a nitrogen line. To this, 1,1'-thiocarbonyldi-2(1*H*)-pyridone (62 g, 0.27 mol) was added slowly in portions over a 10-minute period. The reaction mixture was stirred until the starting amine disappeared (TLC). Once the reaction was complete, it was partitioned with H_2O (200 mL) and extracted with CHCl_3 (2×200 mL). The combined organics were washed with saturated NaCl solution (1×100 mL), dried (Na_2SO_4), filtered, and condensed. Sulfuraphane (23.6 g, 0.13 mol), was purified by flash silica gel column chromatography with elution by CHCl_3 and $\text{CHCl}_3/\text{MeOH}$ (99:1) as a yellow oil which solidified when stored at -20°C (50% yield). $^1\text{H NMR}$ (CDCl_3) δ 3.61 (t, $J = 7.2$ Hz, 2H), 2.72 (m, 2H), 2.60 (s, 3H), 1.92 (m, 4H); MS m/z 178.1 (M + H).

Preparation of diets. For preparation of diets, the *N*-acetylcysteine conjugates were incorporated (12 and 4 $\mu\text{mol/g}$ diet) into AIN-76A diet



Scheme 1. An improved synthesis of sulfuraphane.

(Dyets, Inc., Bethlehem, PA) with 5% corn oil by coating the dextrose used in diet preparation. The *N*-acetylcysteine conjugates were dissolved in 50 mL ethyl acetate, which was added dropwise with a Pasteur pipette to an appropriate amount of dextrose, followed by mixing using a rotary evaporator and subsequent removal of solvent under vacuum. Final traces of ethyl acetate in the dextrose were removed with a vacuum pump (2 hours). Required amounts of coated dextrose were then added to the diet, with dispersion using a Hobart diet mixer. Diets containing phenethyl isothiocyanate and sulfuraphane were prepared by mixing phenethyl isothiocyanate and sulfuraphane in corn oil (3 and 1.5 $\mu\text{mol/g}$ diet) prior to incorporating the oil into the AIN-76A diet. The diets were prepared in 1 to 2 kg batches, and then stored at 4°C in containers purged with nitrogen. Stability studies, using analysis by high-performance liquid chromatography as previously described (21), showed that the isothiocyanate compounds were stable in the diet for at least 1 month under these conditions.

Tumor bioassay. Female strain A mice (The Jackson Laboratory, Bar Harbor, ME), 5 weeks of age, were housed under quarantine in polycarbonate cages (five mice/cage) and provided modified AIN 76A diet (5% corn oil) and acidified drinking water ad libitum. The mice were maintained on a 12-hour light/12-hour dark regimen at $22 \pm 5^{\circ}\text{C}$ and $50 \pm 20\%$ relative humidity. After 1 week in quarantine, the mice were weighed and allocated into 14 groups according to body weight. Groups 1 to 9 were carcinogen-treated groups and contained 20 to 39 mice, whereas control groups 10 to 13 (without carcinogen treatment, but receiving the high-dose test compounds in the diet) were composed of 5 mice per group (see Table 1). Group 14 was an untreated control group (five mice). The treatment protocol and sacrifice schedule are shown in Fig. 2. Mice in groups 1 to 9 received eight weekly doses of 3 μmol NNK and 3 μmol B(a)P combined in 0.1 mL cottonseed oil by gavage. Body weights were recorded at study initiation and 1 week later, then every 4 weeks until termination. Twelve weeks after the carcinogen treatments were completed, four mice in groups 1, 2, 4, 6, and 8 were killed by cervical dislocation to histopathologically examine the formation of lung adenomas. The lung from each mouse was fixed for 12 to 24 hours in 10% buffered formalin, then placed in 70% ethanol. Dietary administration of isothiocyanates and isothiocyanate-*N*-acetylcysteines began early in week 21. Four mice from groups 1, 2, 4, 6, and 8 were also killed during weeks 28 and 36 to monitor tumor development by histopathologic methods and to obtain tissues samples for immunohistochemical assays. Lungs were processed as in the week 20 sacrifice. The remaining mice were killed in week 42; lungs were processed for histopathologic and immunohistochemical examination (see below).

Histopathology. Three transverse step sections of 35 to 40 μm were prepared in an effort to sample all portions of the left lung of each mouse. A series of 10 thin (4 μm) sections were then cut from each step section. Slides 1, 5, and 10 of the thin sections prepared were stained with H&E and examined at $40\times$ magnification. The sections were scored for hyperplasia, adenoma, adenoma with dysplasia, and adenocarcinoma. The total number of lesions in each category in the three transverse sections examined was deemed proportional to the number of lesions occurring in lungs. The following criteria for diagnostic evaluation were used: hyperplasia—alveolar structure of lung is present, proliferation of cells obliterates normal alveolar space. Adenoma—alveolar structure is largely absent, and replaced by proliferated tumor cells, which are homogeneous and slightly enlarged without nuclear-cytoplasm ratio reversal or hyperchromatic nuclei. Adenoma with dysplasia—two populations of cells: one is the same as adenoma (above), whereas the other is characterized by increased size with enlarged nuclei, hyperchromasia, and nuclear-cytoplasmic reversal. Adenocarcinomas—a pattern of homogeneous tumor composed of glandular, papillary, or solid masses of hyperchromatic cells with nuclear-cytoplasmic ratio reversal and increase of mitotic figures.

Immunohistochemistry. Contiguous sections from the H&E sections from the lung of each mouse were used for immunohistochemical analysis. Slide numbers 2, 4, 6, or 9 from the serial sections were used. Immunohistochemistry was only done with three animals from groups 1 to 9 using one slide from each animal on tumor masses that contained both adenoma with dysplasia and adenocarcinoma. The majority of slides were

Table 1. The effect of phenethyl isothiocyanate, sulforaphane, and their *N*-acetylcysteine conjugates on the progression of lung tumorigenesis in A/J mice treated with and without NNK and B(a)P

Group	Number of mice		Adenomas		Adenocarcinomas	
	At beginning	At termination	Tumors/mouse (mean \pm SD)	Incidence (%)	Tumors/mouse (mean \pm SD)	Incidence (%)
NNK + B(a)P-treated						
1. Control	39	9	9.4 \pm 5.5	100	1.0 \pm 1.5	42
2. Phenethyl isothiocyanate (3 μ mol/g)	32	5	8.7 \pm 3.9	100	0.4 \pm 0.9*	19*
3. Phenethyl isothiocyanate (1.5 μ mol/g)	20	8	7.3 \pm 4.5	95	1.2 \pm 1.9	35
4. Sulforaphane (3 μ mol/g)	32	6	8.9 \pm 6.1	93	0.8 \pm 1.5	28
5. Sulforaphane (1.5 μ mol/g)	20	8	9.3 \pm 4.2	100	0.3 \pm 0.7*	20
6. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (8 μ mol/g)	32	9	8.3 \pm 4.0	96	0.5 \pm 1.5	13*
7. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (4 μ mol/g)	20	8	8.2 \pm 3.7	95	0.5 \pm 1.2	21
8. Sulforaphane- <i>N</i> -acetylcysteine (8 μ mol/g)	32	11	9.7 \pm 5.4	100	0.4 \pm 1.0*	16*
9. Sulforaphane- <i>N</i> -acetylcysteine (4 μ mol/g)	20	9	8.5 \pm 4.4	95	0.3 \pm 1.0*	11*
Untreated						
10. Phenethyl isothiocyanate (3 μ mol/g)	5	5	2.0 \pm 3.9	40	0.0 \pm 0.0	0
11. Sulforaphane (3 μ mol/g)	5	5	0.0 \pm 0.0	0	0.0 \pm 0.0	0
12. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (8 μ mol/g)	5	5	0.3 \pm 0.0	25	0.0 \pm 0.0	0
13. Sulforaphane- <i>N</i> -acetylcysteine (8 μ mol/g)	5	4	0.2 \pm 0.5	20	0.0 \pm 0.0	0
14. Untreated	5	5	0.2 \pm 0.5	20	0.0 \pm 0.0	0

* $P < 0.05$ compared with control after adjustment for survival time.

obtained from the sacrifice at 42 weeks. Only a few were from earlier sacrifices (28-36 weeks). Selected analysis of adenomatous masses was done on at least two tumors of 2 to 3 mm² and 1 to 2 mm in diameter per slide. This dimension was determined by measuring using a millimeter ruler on H&E sections displaying the tumor mass and the formula πr^2 . Histopathology (H&E) confirmed that the tumor mass contained adenoma with dysplasia and adenocarcinoma cells.

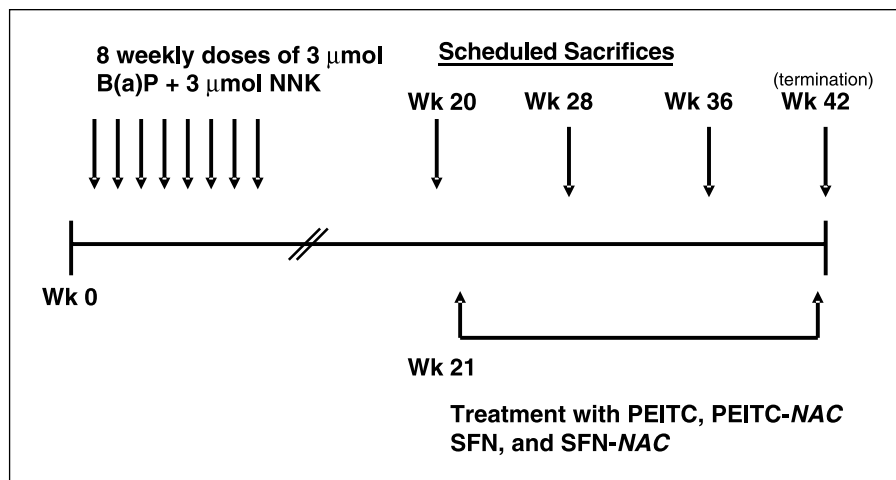
Immunohistochemical expression was determined through immunofluorescent (FITC) detection using a monoclonal specific antibody to proliferating cell nuclear antigen (PCNA; clone; PC10, 1:100 in PBS, Lab Vision, Fremont, CA) and polyclonal antibody (caspase-3, 1:50, Cell Signaling, Beverly, MA). Antibody staining was accomplished using a Ventana Discovery System (Ventana Medical Systems, Tucson, AZ). Slides were deparaffinized, hydrated, and retrieved on the Ventana Discovery System, 100 μ L per slide was applied for 1 hour at 37°C. The secondary fluorescent-conjugated antibody was a goat anti-mouse phycoerythrin (Immunotech, France); it was diluted 1:150 in PBS with 0.1 mol/L glycine.

The incubation was done on the instrument for 1 hour at 37°C. Phycoerythrin fluoresces apple red at 580 nm, allowing for a dual staining of the same slides for the terminal nucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) assay.

Tissue profiling and analysis for PCNA. Distribution of cells expressing PCNA was accomplished using a laser scanning cytometer (Compucyte, Cambridge, MA). Each tissue section was phantom-contoured to optimize detection by reducing background and enhancing threshold and pixel density recognition. A report was produced showing a schematic of the tissue section containing tumor masses using pseudo-coloration. Green was used to distinguish cells expressing PCNA. Each scanned slide contained at least 10,000 cells for analysis. The level of expression was provided by the software analysis found in the instrument (51).

Mebstain apoptosis (TUNEL) assay. Mebstain was used for *in situ* detection of programmed cell death. After the sections had been stained for PCNA via the immunofluorescent technique, the second part of the staining was completed. A Mebstain Apoptosis kit II (Immunotech) was

Figure 2. The treatment schedule in the lung tumor bioassay. After initial weekly dosing of carcinogens for the first 8 weeks, 20 mice were sacrificed on week 20 for the examination of lung adenomas. The remaining mice were then treated with isothiocyanate compounds in the diet from weeks 21 to 42.



conducted as follows. Sections were permeabilized and washed in three changes of PBS, followed by three washes in deionized water. TdT enzyme was applied (45 μ L TdT buffer, 2.5 μ L biotin-dUTP and 2.5 μ L TdT enzyme). Slides were incubated for 1 hour at 37°C, then washed thrice in deionized water; 50 μ L of blocking solution (included in the kit) was then applied, and slides were incubated for 10 minutes at room temperature. Afterwards, the blocking solution was topped off, and another 50 μ L avidin-FITC was applied to slides and followed with incubation for 30 minutes at room temperature (52). After the incubation, the slides were rinsed thrice with PBS, mounted with Vectashield Hardset media (Vector Labs, Burlingame, CA), and coverslipped.

For mebstain analysis, the fluorescence of mebstain-positive cells was detected after contouring, thresholding, and pixel intensity application before scanning. Levels of expression were represented by a percentage mean and SD and SE provided for each assessment as previously described (53).

Caspase-3 antibody staining. Slides were baked at 60°C for 20 minutes, deparaffinized in xylene and hydrated through graded alcohols to distilled water. Antigen retrieval was done in a microwave with citrate buffer (Signal Labs, Dedham MA). The slides were cooled to room temperature prior to staining. To block endogenous peroxidase, a 1% solution of hydrogen peroxide in distilled water was applied for 10 minutes. Slides were then washed in several changes of deionized water, followed by several changes of PBST (PBS with Tween 20). A protein block of 5% normal horse serum was applied to each section for 1 hour at room temperature. Both the primary and secondary antibodies were made using this blocking serum. The primary antibody for caspase-3 was applied at a 1:50 dilution and incubated overnight at 4°C. The next morning, slides were then brought to room temperature and washed in several changes of PBST prior to applying the secondary antibody. A 1:200 dilution of goat anti-rabbit (Vector Labs) was applied and slides were incubated for 30 minutes at room temperature (54). The detection method used was an avidin-biotin complex kit from Vector Labs, with the recommended incubation time of 30 minutes. A 3',3'-diaminobenzidine chromogen (Vector Labs) was used for visualization (54). Slides were counterstained briefly with hematoxylin, washed in running tap water, and then dehydrated through graded alcohols. Several changes of xylene were used to clear the sections, which were then mounted with a permanent resin and coverslipped.

For caspase-3 expression, horseradish peroxidase-positive cells expressing caspase-3 were assessed using Image Pro Plus. Caspase-3 expression was ascertained by random gating onto three areas from each tumor mass. The contrast was enhanced to optimize the assessment for positive expressing cells, and the equalization best fit was also done for each gated image. Processing of the image further included color channeling and segmentation. Measuring expression involved a check for intensity and a record of total number of objects in the field and the number of dark cell (objects). A mean percentage and SD was attained for each group.

Statistical analysis. Tumor incidence was compared among the groups using the χ^2 test, adjusted for multiple comparisons with the Bonferroni correction (55). Tumor multiplicity was compared between the high and low dose of each treatment compound and the control using one-way ANOVA followed by Dunnett's multiple comparisons procedure (56). Logistic regression and analysis of covariance (ANCOVA) models were alternatively used to adjust for survival time when analyzing incidence and multiplicity, respectively. Overall survival was evaluated through life table analysis in order to account for animals that died or were sacrificed before the end of the study. The survival function for each group was estimated separately using the Kaplan-Meier product limit method for censored data (57). The estimated survival distributions were then compared using the log-rank test (58). Repeated measures ANOVA models (59) were used to assess group differences in body weight measured over time. Student's *t* test for unpaired data was used for statistical analysis of data from the immunohistochemical studies.

Results

Body weights and overall survival. Although NNK/B(a)P-treated mice had a slight loss in body weights as compared with mice

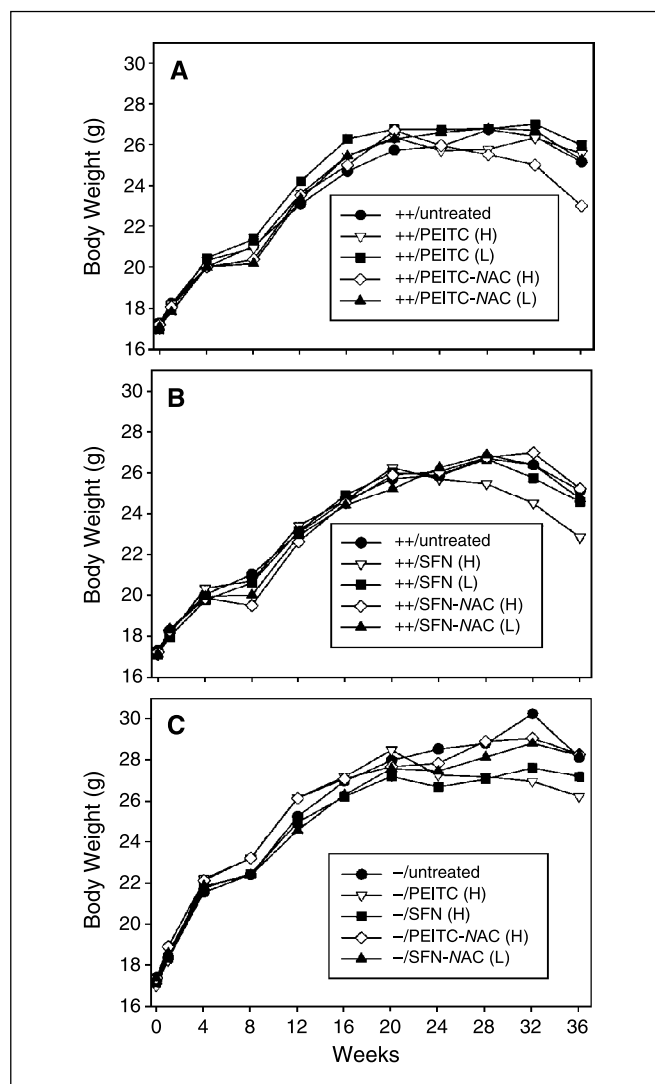


Figure 3. Mean body weight curves for the experimental groups (++, carcinogen treatment). A, carcinogen-treated A/J mice receiving dietary phenethyl isothiocyanate or phenethyl isothiocyanate-*N*-acetylcysteine. B, carcinogen-treated A/J mice receiving dietary sulforaphane or sulforaphane-*N*-acetylcysteine. C, A/J mice not treated with carcinogens but receiving dietary isothiocyanates (3 μ mol/g), isothiocyanate-*N*-acetylcysteines (8 μ mol/g) or no treatment.

without carcinogen treatment, the mean body weights in all groups; carcinogen-treated (Fig. 3A and B) versus untreated (Fig. 3C), were not significantly different throughout the course of the study (Fig. 3). Figure 3A and B show that weekly oral administration with NNK/B(a)P for the first 8 weeks caused a slower growth during that period, then growth recovered after the carcinogen treatment ended. A noticeable drop in body weight occurred during weeks 28 to 36 in mice of group 6 (phenethyl isothiocyanate-*N*-acetylcysteine, 8 mmol/kg diet) and group 4 (sulforaphane, 3 mmol/kg diet), although the mean difference between the weights of the carcinogen groups treated with the high dose of isothiocyanate compounds (groups 2, 4, 6, and 8) and their corresponding isothiocyanate controls (groups 10-13) was only 1.9 g at week 28. None of the weight decreases in these groups exceeded 10% of the weight in group 1 during this period. No deaths occurred in any carcinogen-treated group prior to week 20. The survival rates after adjusting for scheduled sacrifices of

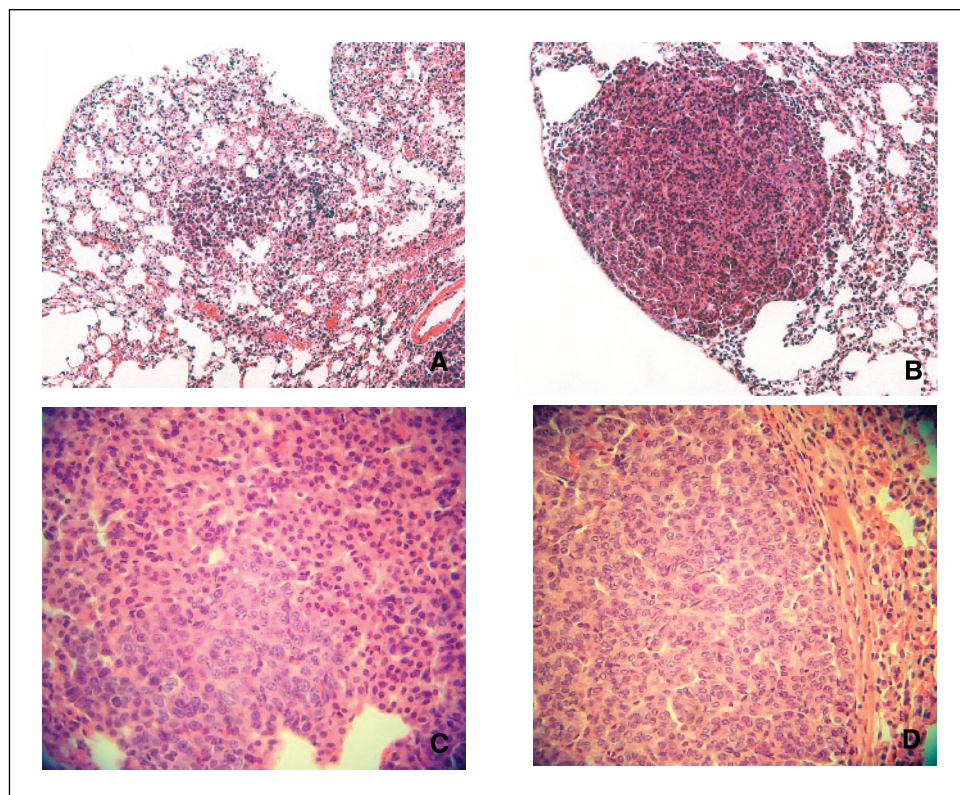
animals treated with carcinogens and test compounds in groups 2 to 9 at termination, ranging from 25% to 55%, were not significantly different from the carcinogen-treated control (group 1). On termination at 42 weeks, the survival rate of the mice in the high-dose dietary control groups (groups 10-13) without carcinogens was 80% to 100%, comparable to that in the untreated control group (group 14).

Incidence and multiplicity of lung adenomas. After weekly doses of NNK and B(a)P from weeks 1 to 8, mice were maintained on the regular AIN-76A diet until week 20. At week 20, four mice were sacrificed from groups 1, 2, 4, 6, and 8 to examine lung adenomas. In these mice, we found 100% incidence of lung adenomas with an average of 7.3 ± 4.5 tumors per mouse based on the histopathologic method described in the Materials and Methods, and with 16.7 ± 5.9 tumors per mouse on the surface of the lung. The incidence and multiplicity observed in these mice are in agreement with that reported in other studies using the same treatment protocol (44). The remaining mice were given test compounds in the diet beginning from week 21 until termination during week 42 (Fig. 2). The lung adenoma incidence and multiplicity at termination, presented in Table 1, were determined based on examination of three tissue slides from each lung (Fig. 4). The number of lung adenomas in the carcinogen-treated control mice (group 1) was 9.4 tumors per mouse; in the groups treated with carcinogens and test compounds (groups 2-9), the mean lung adenoma multiplicity ranged from 7.3 to 9.7 per mouse. The incidences were 100% in the carcinogen-treated control group versus 94% to 100% in the carcinogen-treated groups with test compounds. Neither the multiplicity nor the incidence of lung adenomas in the carcinogen-treated groups with and without test compound differed significantly. As expected in the A/J mouse model, a few spontaneous lung adenomas with incidence ranging from 0% to 25% were observed in

groups not treated with carcinogens (groups 10-14). The only exception was group 10, which had a 40% incidence. The multiplicities of adenomas in groups 10 to 13 did not differ significantly from the nontreated animals in group 14. Table 2 summarizes the multiplicity and incidence of hyperplasia and adenoma with dysplasia in all groups. Again, there were no significant differences of these lesions among the carcinogen-treated control group and groups 2 to 9. Among the control groups not treated with carcinogens (groups 10-14), no significant differences in the multiplicity and incidence of adenomas were observed.

Incidence and multiplicity of adenocarcinomas. Contrary to the lung adenomas, the progression to adenocarcinomas was strikingly inhibited in about half of the carcinogen-treated groups given the isothiocyanate compounds in the diet. The results are summarized in Table 1. Significant reductions in multiplicity or incidence or both as compared with group 1 were observed in group 2 (phenethyl isothiocyanate, 3 $\mu\text{mol/g}$ diet), group 5 (sulforaphane, 1.5 $\mu\text{mol/g}$ diet), group 6 (phenethyl isothiocyanate-*N*-acetylcysteine, 8 $\mu\text{mol/kg}$ diet), and groups 8 and 9 (sulforaphane-*N*-acetylcysteine, 8 and 4 $\mu\text{mol/g}$ diet, respectively). In group 2, the multiplicity was reduced by 60% and the incidence by 55%. In group 5, the multiplicity was reduced by 70%, but the reduction in incidence was not significant. In group 6, the incidence was reduced by 68%, but the reduction in the multiplicity was not significant. In group 8, the multiplicity was reduced by 60% and the incidence by 62%; and, in group 9, the multiplicity was reduced by 70% and the incidence by 74%. The multiplicity and incidence of lung adenocarcinomas in most of the other carcinogen groups treated with isothiocyanates or isothiocyanate-*N*-acetylcysteines were also reduced, however, the reductions were not statistically significant. No adenocarcinomas appeared in any of the control groups not treated with the carcinogens (groups 10-14).

Figure 4. Photomicrographs of typical lung lesions in carcinogen-treated mice. *A*, hyperplasia—the normal alveolar structure is replaced by alveoli with hyperplastic foci with extensive proliferation of the alveolar epithelium (H&E $\times 10$). *B*, adenoma—alveolar spaces are occupied by well-differentiated hyperplastic cells, with compression of adjacent alveoli (H&E $\times 10$). *C*, adenoma with dysplasia—focal dysplasia, the hyperchromatic areas, are present within the adenoma (H&E $\times 20$). *D*, adenocarcinoma—there is a complete loss of alveolar structure; with replacement by glandular, papillary, or solid masses of hyperchromatic, pleomorphic cells (H&E $\times 20$).



Cell proliferation analysis (PCNA). To address the cellular mechanism(s) by which the isothiocyanate compounds inhibit the development of lung adenocarcinomas, we obtained tissue profile expressions of PCNA, a measure of cell proliferation. PCNA expression was reduced in groups which showed significant tumor reduction (Table 3), including the treatment with phenethyl isothiocyanate (3 $\mu\text{mol/g}$), sulforaphane (1.5 $\mu\text{mol/g}$), phenethyl isothiocyanate-*N*-acetylcysteine (8 $\mu\text{mol/g}$), sulforaphane-*N*-acetylcysteine (8 $\mu\text{mol/g}$), and sulforaphane-*N*-acetylcysteine (4 $\mu\text{mol/g}$), from $44.4 \pm 1.9\%$ in NNK/B(a)P control group to $23.9 \pm 10.4\%$ (group 2, $P = 0.07$), $17.3 \pm 11.6\%$ (group 5, $P \leq 0.05$), $25.8 \pm 10.1\%$ (group 6, $P = 0.08$), $18.6 \pm 5.4\%$ (group 8, $P < 0.008$), and 17.0 ± 4.7 (group 9, $P = 0.004$), respectively. This reduction in PCNA expression was shown to be remarkable after examination of the tissue profiling (Fig. 5). The carcinogen-treated control tissues showed a high density of PCNA-expressing cells localized in tumor masses with a lower distribution of PCNA-positive cells dispersed throughout the tissue. In contrast, treatments with some of these compounds produced a totally different pattern of PCNA-positive cells; localization to tumor masses was markedly depressed and more dispersed but fewer cells were noted to express PCNA.

Meibstain (TUNEL) and caspase-3 analysis. To determine the relative percentage of apoptotic cells in the lung, we also obtained tissue profiling expressions of TUNEL-positive cells. Control values for NNK and B(a)P treatment indicated that $12.7 \pm 3.0\%$ of the tissues contained cells were TUNEL-positive. On the other hand, phenethyl isothiocyanate (3 $\mu\text{mol/g}$), $37.1 \pm 13.8\%$ ($P = 0.008$); sulforaphane (1.5 $\mu\text{mol/g}$), $29.4 \pm 5.8\%$ ($P < 0.02$); phenethyl isothiocyanate-*N*-acetylcysteine (8 $\mu\text{mol/g}$), $34.7 \pm 5.9\%$ ($P \leq 0.01$); sulforaphane-*N*-acetylcysteine (8 $\mu\text{mol/g}$), $53.7 \pm 15.5\%$ ($P \leq 0.04$); and sulforaphane-*N*-acetylcysteine (4 $\mu\text{mol/g}$), $49.7 \pm 11.8\%$ ($P \leq 0.02$), showed a significant increase compared with the control levels. To further substantiate the induction of apoptosis, another marker, caspase-3, was examined. The results indicated $4.7 \pm 1.9\%$ of the cells in the NNK and B(a)P treatment control tissues expressed caspase-3. Treatments with phenethyl isothiocyanate

(3 $\mu\text{mol/g}$), sulforaphane (3 $\mu\text{mol/g}$), sulforaphane (1.5 $\mu\text{mol/g}$), phenethyl isothiocyanate-*N*-acetylcysteine (4 $\mu\text{mol/g}$), sulforaphane-*N*-acetylcysteine (8 $\mu\text{mol/g}$), and sulforaphane-*N*-acetylcysteine (4 $\mu\text{mol/g}$) significantly enhanced the percentage of expression to 19.5 ± 5.5 ($P = 0.03$), 18.6 ± 5.1 ($P = 0.03$), 29.3 ± 4.7 ($P = 0.005$), 27.6 ± 5.9 ($P \leq 0.01$), 21.9 ± 5.1 ($P < 0.01$) and 29.5 ± 4.4 ($P = 0.004$), respectively (Table 3). These results show that the simultaneous enhancement of apoptosis and reduction in PCNA expression by these isothiocyanate compounds seemed to correlate with the tumor-inhibitory activity, supporting the notion that these isothiocyanate compounds suppress lung tumor progression through inhibition of PCNA and the induction of apoptosis, perhaps by the caspase-3-dependent pathway.

Discussion

Although the development of benign lung tumors to malignancy is arguably the most critical stage during the development of lung cancer, there is a scarcity of chemoprevention studies on progression of lung carcinogenesis. In this study, we evaluated the tumor-inhibitory activities of phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates on the development of malignancy from benign tumors in the lung of A/J mice after administration of NNK and B(a)P, two potent carcinogens in cigarette smoke that are believed to be involved in lung cancer in smokers (60). The results show that phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates given in the diet after lung adenomas have already developed could inhibit the progression to adenocarcinomas. The inhibitory effects of these compounds are likely to be associated with a combination of reduced cell proliferation and induced apoptosis. These findings are important for future research of lung cancer chemoprevention and therapy in smokers and ex-smokers with early lesions.

To our knowledge, only two other agents have been reported to show chemopreventive activity on the progression of lung tumors in A/J mice (9–11). When black tea extract was given beginning

Table 2. The effect of phenethyl isothiocyanate, sulforaphane and their *N*-acetylcysteine conjugates on the formation of hyperplasia and adenoma with dysplasia in lungs of A/J mice treated with and without NNK and B(a)P

Group	Hyperplasia		Adenoma with dysplasia	
	Multiplicity (mean \pm SD)	Incidence (%)	Multiplicity (mean \pm SD)	Incidence (%)
NNK + B(a)P-treated				
1. Control	1.5 \pm 1.5	26 of 36 (72)	2.9 \pm 2.7	27 of 36 (75)
2. Phenethyl isothiocyanate (3 $\mu\text{mol/g}$)	2.2 \pm 1.9	24 of 32 (75)	2.7 \pm 2.6	26 of 32 (81)
3. Phenethyl isothiocyanate (1.5 $\mu\text{mol/g}$)	2.0 \pm 1.6	16 of 20 (80)	2.8 \pm 2.7	17 of 20 (85)
4. Sulforaphane (3 $\mu\text{mol/g}$)	2.2 \pm 2.2	23 of 32 (72)	2.4 \pm 2.2	25 of 32 (78)
5. Sulforaphane (1.5 $\mu\text{mol/g}$)	2.3 \pm 2.1	16 of 20 (80)	3.4 \pm 2.7	18 of 20 (90)
6. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (8 $\mu\text{mol/g}$)	2.2 \pm 1.8	28 of 31 (90)	1.9 \pm 2.1	22 of 31 (71)
7. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (4 $\mu\text{mol/g}$)	1.4 \pm 2.0	11 of 19 (58)	1.8 \pm 1.5	15 of 19 (79)
8. Sulforaphane- <i>N</i> -acetylcysteine (8 $\mu\text{mol/g}$)	1.9 \pm 1.5	24 of 31 (77)	1.9 \pm 1.5	24 of 31 (77)
9. Sulforaphane- <i>N</i> -acetylcysteine (4 $\mu\text{mol/g}$)	2.1 \pm 1.5	15 of 19 (79)	2.6 \pm 2.6	13 of 19 (68)
Untreated				
10. Phenethyl isothiocyanate (3 $\mu\text{mol/g}$)	0.2 \pm 0.5	1 of 5 (20)	0.4 \pm 0.9	1 of 5 (20)
11. Sulforaphane (3 $\mu\text{mol/g}$)	0.0 \pm 0.0	0 of 5 (0)	0.0 \pm 0.0	0 of 5 (0)
12. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (8 $\mu\text{mol/g}$)	0.3 \pm 0.5	1 of 4 (25)	0.0 \pm 0.0	0 of 4 (0)
13. Sulforaphane- <i>N</i> -acetylcysteine (8 $\mu\text{mol/g}$)	0.2 \pm 0.5	1 of 5 (20)	0.2 \pm 0.5	1 of 5 (20)
14. Untreated	0.0 \pm 0.0	0 of 5 (0)	0.2 \pm 0.5	1 of 5 (20)

Table 3. The effects of treatment of phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates on PCNA, TUNEL, and caspase-3, in comparison with their inhibitory activities against lung tumor progression in NNK/B(a)P-treated A/J mice

Group	Percentage of cells expressed			Relative tumor inhibitory potency*	
	PCNA	TUNEL	Caspase-3	Multiplicity	Incidence
1. Control	44.4 ± 1.9	12.7 ± 3.0	4.7 ± 1.9		
2. Phenethyl isothiocyanate (3 μmol/g)	23.9 ± 10.4	37.1 ± 13.8 [†]	19.5 ± 5.5 [‡]	+	+
3. Phenethyl isothiocyanate (1.5 μmol/g)	43.8 ± 0.8	14.6 ± 6.2	11.4 ± 5.3	–	–
4. Sulforaphane (3 μmol/g)	39.0 ± 10.4	16.3 ± 9.2	18.6 ± 5.1 [‡]	–	–
5. Sulforaphane (1.5 μmol/g)	17.3 ± 11.7 [‡]	29.4 ± 5.8 [‡]	29.3 ± 4.7 [†]	+	–
6. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (8 μmol/g)	25.8 ± 10.1	37.4 ± 5.9 [†]	5.1 ± 2.2	–	+
7. Phenethyl isothiocyanate- <i>N</i> -acetylcysteine (4 μmol/g)	44.4 ± 15.0	13.6 ± 8.3	27.6 ± 5.9 [†]	–	–
8. Sulforaphane- <i>N</i> -acetylcysteine (8 μmol/g)	18.6 ± 5.4 [†]	53.7 ± 15.5 [‡]	21.9 ± 5.1 [†]	+	+
9. Sulforaphane- <i>N</i> -acetylcysteine (4 μmol/g)	17.0 ± 4.7 [†]	49.7 ± 11.8 [‡]	29.5 ± 4.4 [†]	+	+

*+, a significant inhibition of lung adenocarcinoma formation as compared with group 1 ($P < 0.05$); –, no significant inhibition.

[†] $P < 0.01$, compared with group 1.

[‡] $P < 0.05$, compared with group 1.

16 weeks after a single dose of NNK, significant decreases in the incidence and multiplicity of adenocarcinomas were observed in groups provided black tea extract, compared with the NNK-treated control mice. As in the present study, the incidence of adenomas in the NNK plus black tea group was not affected. Moreover, cell proliferation was reduced in adenomas and adenocarcinomas by tea treatment (9). The other agent is budesonide, a glucocorticoid drug. When it was given to A/J mice that were initiated with vinyl carbamate or B(a)P, the growth of carcinomas was decreased. The PCNA labeling index was also reduced in both tumor and nontumor tissue, suggesting that budesonide inhibited the transition from adenoma to adenocarcinoma by decreasing cell proliferation (10, 11).

The A/J mouse model has been extensively used in the study of chemopreventive agents for lung tumor initiation (17, 19, 25). We have previously shown in A/J mice that phenethyl isothiocyanate-*N*-acetylcysteine given in the diet after B(a)P treatment also significantly inhibits the development of lung adenomas (18, 21). However, its potential against tumor progression has not been fully explored. The present study showed that the A/J mouse is a useful model to investigate chemopreventive agents for lung cancer progression, because of the rapid development of lung adenomas and adenocarcinomas that are readily diagnosable and quantifiable. We did, however, encounter a few problems in the bioassay; (a) carcinogen treatment caused a small, although not significant, loss in body weight, suggesting that doses of NNK and B(a)P may be lowered to minimize the toxicity. Nevertheless, the body weights were comparable among all carcinogen-treated groups throughout the bioassay until malignancies began to appear; (b) a high incidence of forestomach tumors occurred in the carcinogen-treated groups as a result of multiple doses of gastric administration of B(a)P, suggesting that alternative routes of administration of B(a)P may be used, although i.p. injection also caused tumors at the injection site (44); and (c) a relatively low survival rate at termination was observed among the carcinogen-treated groups, which may very well be attributed to the total tumor burden in these animals. The comparable survival rates and body weights among the carcinogen-treated groups with and without test compounds (group 1 versus

groups 2-9) suggest that there was little toxicity associated with the isothiocyanate compounds at the doses used. These findings are in agreement with the maximum tolerated doses compiled from our previous bioassays in A/J mice which are estimated to be 12 μmol/g diet for phenethyl isothiocyanate-*N*-acetylcysteine, 15 μmol/g for sulforaphane-*N*-acetylcysteine, and 4 and 8 μmol/g for phenethyl isothiocyanate and sulforaphane, respectively.⁶

In the A/J mouse lung, few adenomas or adenocarcinomas arise de novo; instead, the adenomas arise from foci of hyperplasia, whereas adenocarcinomas develop from adenomas, with continuing cell proliferation accompanied by mutations and karyotypic instability (40-42). For example, studies have shown that in vinyl carbamate or NNK-treated A/J mice, adenomas arose from type II epithelial cells lining the alveoli. The initial lesions were diagnosed as hyperplasia; the areas of hyperplasia continued to undergo cellular proliferation, with eventual obliteration of contiguous alveolar spaces, forming spherical masses of cells classified as adenomas. Adenocarcinomas arise from adenomas by continued cellular proliferation of anaplastic cells with nuclear and cytologic atypia. The number of lung adenomas decreased with time, whereas the size and multiplicity of adenocarcinomas increased, indicating that most of the adenocarcinomas arose by continued genetic alterations and atypical cell division in adenomas.

This study showed that the incidences of hyperplasia, adenomas, and adenocarcinomas at 42 weeks were 72%, 100%, and 42%, respectively, with a total dose of 24 μmol NNK and B(a)P, each in eight weekly administrations. These tumor incidences are twice as high as that from a single dose of 10 μmol NNK reported previously (41, 42). This study showed that one or less adenocarcinoma per mouse was found at termination out of an average of 7.3 adenomas per mouse at week 20 in the carcinogen-treated groups, suggesting that, at most, only a small number of adenomas, about 14%, induced by a mixture of NNK and B(a)P had actually progressed to malignancy. Only 7.3 to 9.6 adenomas per mouse were found in all the groups at termination regardless of the treatment, suggesting

⁶ C.C. Conaway and F-L. Chung, unpublished data.

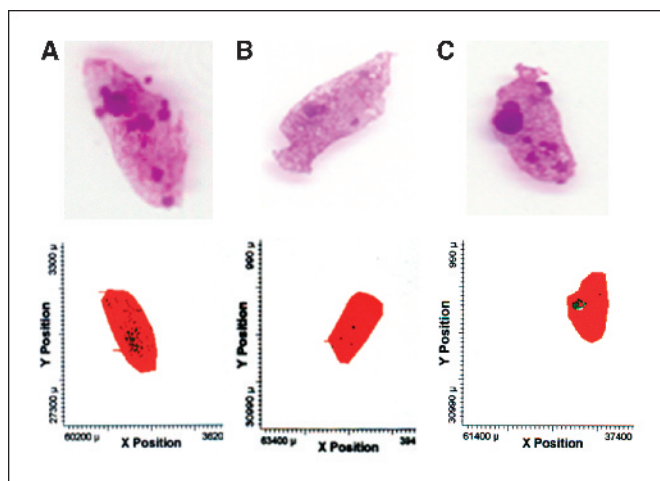


Figure 5. Tissue profiling of tumor mass (*top*) and PCNA-positive cells (*bottom*) of lung sections from: A, the carcinogen-treated group (group 1); B, sulforaphane-*N*-acetylcysteine (4.0 μ mol/g, group 9), treatment-inhibited lung tumor development; C, phenethyl isothiocyanate (1.5 μ mol/g, group 3), treatment had no tumor inhibition. Tissues were stained with hematoxylin and examined with 4 \times digital photomicroscopy to show tumor masses, or with a monoclonal antibody for PCNA and examined with 10 \times digital photomicroscopy by laser scanning cytometry to show the distribution of cells in S phase (dark spot).

that the isothiocyanate compounds did not have any significant effect on the regression of lung adenomas. The results also indicate that only a selected population of highly proliferating adenoma cells was more sensitive to the treatment with the isothiocyanates and their conjugates.

Increased cell proliferation is a hallmark of tumor development, and agents that reduce cell proliferation will slow down the tumorigenic process. Induction of apoptosis is one of the critical functions elicited by cancer chemotherapeutic or chemopreventive agents by eliminating initiated cells. A number of isothiocyanates and their conjugates, including phenethyl isothiocyanate and sulforaphane, have been shown to reduce PCNA and induce apoptosis in cultured cells (28–33). Recent studies in cell culture have shown that sulforaphane can trigger these activities by its functions as an inhibitor of histone deacetylase (61) and a disruptor of mitotic microtubules (62), and the apoptosis induced by phenethyl isothiocyanate could be attributed to either *c-Jun*-NH₂-kinase activation (63) or the Fas-mediated pathway (64). Our studies have previously shown that phenethyl isothiocyanate-*N*-acetylcysteine induces apoptosis in the lung of A/J mice treated B(a)P, and this activity seems responsible for its inhibition of lung adenoma formation (21). Data from the present study also support this mechanism in the inhibition of lung adenocarcinomas by these agents in that tissue. In a recent study, we observed that human lung cancer cells either transfected with *c-Jun* or treated with a phorbol ester tumor promoter are sensitized to apoptosis induction and G₂-M arrest by phenethyl isothiocyanate-*N*-acetylcysteine, suggesting that these effects are targeted to cells with stimulated growth (65).

These findings are important because they not only indicate that the isothiocyanate compounds may inhibit the development of lung adenocarcinomas by exerting their activity on a specific cell population that has gained growth advantage to transform into malignant tumors, but also suggest the chemopreventive potential of these compounds in human lung cancer.

Thiol conjugates of isothiocyanates, formed via the mercapturic acid pathway in humans and in rodents, have several desirable properties when compared with the parent isothiocyanates. Most notably, the maximum tolerated doses for *N*-acetylcysteine conjugates of phenethyl isothiocyanate and sulforaphane are at least two times greater, on a molar basis, than those for the isothiocyanates, therefore, greater amounts of conjugates can be given without toxicity or significant reductions in body weight gain. The parent isothiocyanate compounds are typically lipid-soluble, highly pungent, and can be irritating to the eyes and mucous membranes, whereas the solubility of thiol conjugates in aqueous media is greatly increased, and they are less pungent and irritating. The *N*-acetylcysteine conjugates gradually dissociate >pH 6 to yield the parent isothiocyanates and free thiols (15); thus, they may be considered prodrug forms of isothiocyanates. Although detailed efficacy and pharmacokinetic data to compare the conjugates with the parent isothiocyanates have not yet been obtained, the activity of thiol conjugates of isothiocyanates as inhibitors at the tumor initiation and post-initiation level has been clearly shown in animal studies (19, 21, 27). In this study, we have further shown that the *N*-acetylcysteine conjugates of phenethyl isothiocyanate and sulforaphane are promising candidate prodrugs of the parent isothiocyanate compounds.

In conclusion, this study shows that phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates inhibit the growth of lung carcinomas from benign tumors by reducing cell proliferation and inducing apoptosis in the tobacco carcinogen-treated A/J mice. These findings, together with the earlier observations that these compounds inhibit the development of lung adenomas during the initiation and post-initiation stages, suggest that they are versatile agents in different stages of early lung cancer development. As mentioned above, we have also shown, in a separate study (65), the activity of phenethyl isothiocyanate-*N*-acetylcysteine to reduce cell growth and induce apoptosis in fast-dividing human lung A549 cells transfected with *c-Jun*, further supporting the chemopreventive and perhaps therapeutic potential of these agents in the treatment of human lung cancer.

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