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Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates

(chemoprotection/quinone reductase/enzyme induction/dimethylbenzanthracene/rat mammary tumors)

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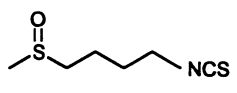
ABSTRACT **Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane] was recently isolated from one variety of broccoli as the major and very potent inducer of phase 2 detoxication enzymes in murine hepatoma cells in culture. Since phase 2 enzyme induction is often associated with reduced susceptibility of animals and their cells to the toxic and neoplastic effects of carcinogens and other electrophiles, it was important to establish whether sulforaphane could block chemical carcinogenesis. In this paper we report that sulforaphane and three synthetic analogues, designed as potent phase 2 enzyme inducers, block the formation of mammary tumors in Sprague–Dawley rats treated with single doses of 9,10-dimethyl-1,2-benzanthracene. The analogues are *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane. When sulforaphane and *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane were administered by gavage (75 or 150 μmol per day for 5 days) around the time of exposure to the carcinogen, the incidence, multiplicity, and weight of mammary tumors were significantly reduced, and their development was delayed. The analogues *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane were less potent protectors. Thus, a class of functionalized isothiocyanates with anticarcinogenic properties has been identified. These results validate the thesis that inducers of phase 2 enzymes in cultured cells are likely to protect against carcinogenesis.**

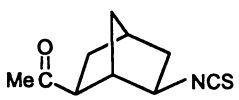
Enzymes that metabolize xenobiotics play a major role in regulating the toxic, mutagenic, and neoplastic effects of chemical carcinogens. Much evidence indicates that the activities of phase 2 detoxication enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases, and epoxide hydrolase) in particular can modulate the response of animals and their cells to carcinogen exposure. Induction of these enzymes by a wide variety of chemicals (including components of the diet) results in protection against toxicity and neoplasia (1). To identify such protective inducers and to measure their potencies, a simple cell culture system has been developed in our laboratory (2, 3). This system depends on determining the specific activities of quinone reductase in murine hepatoma cells grown in 96-well microtiter plates and exposed to a range of concentrations of the inducers. Such measurements not only have reliably predicted the ability of compounds to induce phase 2 enzymes in rodent tissues *in vivo* but also have identified several chemoprotectors against carcinogenesis. By use of this system, sulforaphane [(−)-1-isothiocyanato-4-(methylsulfinyl)butane, **1**] was recently isolated from Saga broccoli as the major phase 2 enzyme inducer present in organic solvent extracts of this vegetable.

Sulforaphane is of interest for three reasons: (*i*) it occurs naturally in a widely consumed vegetable; (*ii*) it is a very potent inducer of phase 2 enzymes; and (*iii*) it is a monofunctional inducer (4)—i.e., it elevates phase 2 detoxication enzymes without significantly changing the synthesis of cytochromes P-450 (5). These findings allowed the design and the systematic synthesis of a large number of structurally related isothiocyanates (6). It was found that the methylsulfinyl (CH_3SO-) function of sulforaphane could be replaced by a methylcarbonyl (i.e., acetyl) group without significantly affecting inducer potency and that, in the most potent inducers, the isothiocyanate function and the acetyl group were separated by three or four carbons of an aliphatic or cyclo-aliphatic chain. Several isomeric norbornyl isothiocyanates substituted with acetyl groups were found to approach or equal the potency of sulforaphane as a phase 2 enzyme inducer (6). The merits of these norbornyl isothiocyanates [*exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, **2**; *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, **3**; and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane, **4**] are that they can be more easily synthesized (from commercial 2-acetyl-5-norbornene) than sulforaphane and that they are probably more stable toward chemical and biological oxidation–reduction reactions.

We report here that sulforaphane and synthetic cyclic isothiocyanate analogues block mammary tumor development in Sprague–Dawley rats treated with 9,10-dimethyl-1,2-benzanthracene (DMBA) (7, 8). These findings identify a class of functionalized isothiocyanates as enzyme inducers that block carcinogenesis, and further strengthen the view that the search for phase 2 enzyme inducer activity from natural sources can successfully identify chemoprotectors against cancer (1–3). These results also encourage further efforts at rational design and laboratory synthesis of even more potent chemoprotectors.



Sulforaphane, 1



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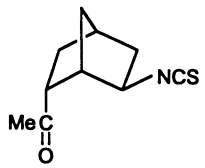

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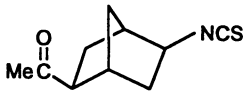

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MATERIALS AND METHODS

Animals and Mammary Tumor Development. Female Sprague–Dawley rats were obtained from Harlan–Sprague–

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Abbreviation: DMBA, 9,10-dimethyl-1,2-benzanthracene.

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Dawley Laboratories at 40 days of age. They were housed in plastic cages (four to five per cage) on Betachip hardwood laboratory bedding (Northeastern Product, Warrensburg, NY) and were fed unrestricted amounts of water and a pelleted AIN-76A diet containing no ethoxyquin (Teklad, Madison, WI). The temperature was 25°C, and 12-hr light/12-hr dark cycles were maintained. All animal experiments were in compliance with National Institutes of Health Guidelines (9) and were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Hygiene and Public Health. The rats were assigned randomly to seven groups: a control group of 25 animals and six treatment groups of 20 animals each. The animals were weighed individually at weekly intervals. At age 47, 48, 49, 50, and 51 days, each animal received by gavage either 0.5 ml of Emulphor EL-620 (Rhone-Poulenc, Cranbury, NJ) alone or the specified doses (75, 100, or 150 μmol daily) of sulforaphane (**1**) or compound **2**, **3**, or **4** in 0.5 ml of Emulphor EL-620. On day 50, 3 hr after administration of the vehicle or protector, all rats also received an intragastric instillation of 8.0 mg of DMBA (Sigma) dissolved in 1.0 ml of sesame oil. This dose of DMBA was selected to produce a substantial tumor incidence, but not one so high as to overwhelm a potential chemoprotective effect. The animals were examined once weekly for the appearance and location of palpable tumors. At age 202 days, i.e., 152 days after carcinogen administration, all animals were euthanized with ether and weighed. The tumors were separated from fat and connective tissue by dissection, weighed, and fixed in buffered 10% formalin. All tumors were identified microscopically by examination of stained sections.

Quantitative Assessment of Tumor Development. Of the 145 rats initially assigned to this experiment, 8 did not survive until we terminated the experiment when the rats were 202 days old. The deaths were distributed among the groups as follows: controls, 2 deaths with tumors; sulforaphane (150-μmol dose), 1 death from gavage accident; sulforaphane (75-μmol dose), no deaths; compound **2** (150-μmol dose), 1 death with tumors; compound **2** (75-μmol dose), 1 death with tumors; compound **3** (100 μmol), 3 deaths, 2 with tumors, 1 without tumors; compound **4** (100 μmol), no deaths.

Since tumor and body weights were not measured on the animals that died during the course of the experiment, we report separately the tumor incidence and multiplicity for all rats (two rats were excluded for reasons given in Table 1) and for the 137 rats surviving to the termination of the experiment (Tables 1 and 2, respectively).

The mean body weights (± SEM) of the animal groups at the beginning of treatment (age 47 days) were between 116 ± 1.7 and 126 ± 1.9 g. The final weights at termination of the experiment are given in Table 2.

The development and characteristics of tumors in each group were assessed in four ways: (*i*) *incidence*, the fraction (percent) of animals that developed tumors; (*ii*) *multiplicity*, the total number of tumors divided by the total number of animals at risk; (*iii*) *total number and weight of tumors* removed from each animal at the termination of the experiment; and (*iv*) *latency of tumor development*. The proportions of tumor-free animals in the control and each treatment group were compared at the time of the weekly examinations of the animals (Fig. 1).

Statistical Analysis of Results. Differences in tumor incidence were evaluated by the Fisher exact test. Tumor multiplicity differences were analyzed by a Poisson distribution model and average rates were compared. The overall progression of tumor development was assessed by Kaplan–Meier analyses followed by logarithmic rank tests.

Chemical Syntheses. The synthetic methods and characterization of the compounds have been described (6). Multigram quantities of compounds **2**–**4** were prepared in one step from commercial 2-acetyl-5-norbornene (a mixture of *exo* and *endo* isomers) obtained from Aldrich.

RESULTS

Administration of sulforaphane or of the 2-acetylnorbornyl isothiocyanates **2**, **3**, and **4** reduced the incidence, multiplicity, and weights and delayed the development of the mammary tumors evoked by a single dose of DMBA in female Sprague–Dawley rats (Tables 1 and 2; Fig. 1). There were clear-cut differences in the potencies of the chemoprotective compounds.

In the control group, not receiving any protector, the incidence of mammary tumors for all animals was 68.0% (Table 1). If the two animals that did not survive to the termination of the experiment (rat age, 202 days) were censored, the tumor incidence in the control group was very similar, 65.2% (Table 2). The corresponding tumor multiplicities (total number of tumors per number of animals at risk) were 1.56 (all animals) and 1.43 (survivors to termination),

Table 1. Protective effects of sulforaphane and norbornyl isothiocyanates **2**, **3**, and **4** on incidence and multiplicity of mammary tumors in DMBA-treated female Sprague–Dawley rats

Treatment group	No. of rats: In group	No. of rats: With tumors	Tumor incidence, % (% of control)	No. of tumors: Total	No. of tumors: Multiplicity (% of control)
Control	25	17	68.0 (100)	39	1.56 (100)
Sulforaphane					
75 μmol	20	7	35.0* (51.4)	9	0.45† (28.8)
150 μmol	19‡	5	26.3* (38.7)	5	0.26† (16.7)
Compound **2**					
75 μmol	20	5	25.0* (36.8)	6	0.30† (19.2)
150 μmol	20	5	25.0* (36.8)	7	0.35† (22.4)
Compound **3**					
(100 μmol)	19§	9	47.3 (69.6)	14	0.74† (47.4)
Compound **4**					
(100 μmol)	20	8	40.0 (58.8)	8	0.40† (25.6)

A total of 145 rats were entered into the experiment. Each received 8 mg of DMBA at age 50 days. There were initially 25 controls and 20 animals in each of the six treated groups. The above analysis is based on 143 animals (see below).
*$P < 0.05$ for differences from controls (Fisher exact test).
†$P \leq 0.01$ for differences from controls (Poisson distribution model).
‡One rat died immediately after gavage and is not included.
§One rat died without palpable tumors at age 167 days and is not included.

Table 2. Protective effects of sulforaphane and norbornyl isothiocyanates 2, 3, and 4 on incidence, multiplicity, and weights of mammary tumors in DMBA-treated female Sprague–Dawley rats: Analysis of survivors to termination of the experiment at rat age 202 days

Treatment group	No. of rats: In group	No. of rats: With tumors	Mean (± SEM) final body weight, g	Tumor incidence, % (% of control)*	Total no. of tumors in group*	Tumor multiplicity (% of control)*	Mean tumor weight, g (% of control)
Control	23†	15	287 ± 4.6	65.2 (100)	33	1.43 (100)	2.79 (100)
Sulforaphane							
75 μmol	20	7	272 ± 5.1	35.0 (53.8)	9	0.45 (31.4)	1.24 (44.4)
150 μmol	19‡	5	265 ± 4.9	26.3 (40.3)	5	0.26 (18.2)	0.68 (24.4)
Compound 2							
75 μmol	19§	4	268 ± 5.1	21.0 (32.2)	5	0.26 (18.2)	1.10 (39.4)
150 μmol	19§	4	274 ± 7.0	21.0 (32.2)	5	0.26 (18.2)	1.12 (40.1)
Compound 3							
(100 μmol)	17¶	7	276 ± 4.4	41.2 (63.3)	12	0.71 (49.7)	2.49 (89.2)
Compound 4							
(100 μmol)	20	8	286 ± 4.8	40.0 (61.3)	8	0.40 (27.9)	1.98 (71.0)

*The results shown in these columns differ from those in Table 1 because only the 137 rats that survived to termination of experiment are analyzed.
†Two animals died with tumors before termination of the experiment and are not included.
‡One rat died immediately following gavage and is not included.
§One rat in each group died with tumors before termination of experiment and is not included.
¶Three rats died (two with tumors) before termination of the experiment and are not included.

respectively. The mean weight of the control tumors at termination of experiment was 2.79 g (Table 2).

Sulforaphane administered in five doses of either 75 or 150 μmol blocked tumor development in a dose-dependent manner. At the higher dose of sulforaphane, the tumor incidence and multiplicity for all animals in the group were reduced to 38.7% and 16.7% of control values, respectively (Table 1). The magnitude of this protective effect was almost identical (40.3% and 18.2%, respectively) when only those animals surviving to the termination of the experiment were analyzed (Table 2). Sulforaphane also reduced the tumor weights to 44.4% and 24.4% of controls at the lower and higher doses, respectively (Table 2). Treatment with sulforaphane significantly delayed the development of tumors during the course of the experiment in comparison to the control group (P = 0.016 and 0.0022 at low and high doses of sulforaphane, respectively) (Fig. 1).

Norbornyl isothiocyanate 2 was an equally potent chemoprotector at the 75- and 150-μmol doses, irrespective of whether incidence, multiplicity, tumor weight, or latency of tumor development was considered (Tables 1 and 2; Fig. 1). With these doses the protective effect appears to have attained a plateau. Since 75-μmol doses of compound 2 reduced tumor incidence and multiplicity even more markedly than the same dose of sulforaphane, it is possible that 2 may be a more potent chemoprotector than sulforaphane.

Compounds 3 and 4, both tested at five doses of 100 μmol, also blocked tumor formation, but these effects did not reach statistical significance for some indicators of protection (Tables 1 and 2). Thus, compound 3 was clearly the least potent

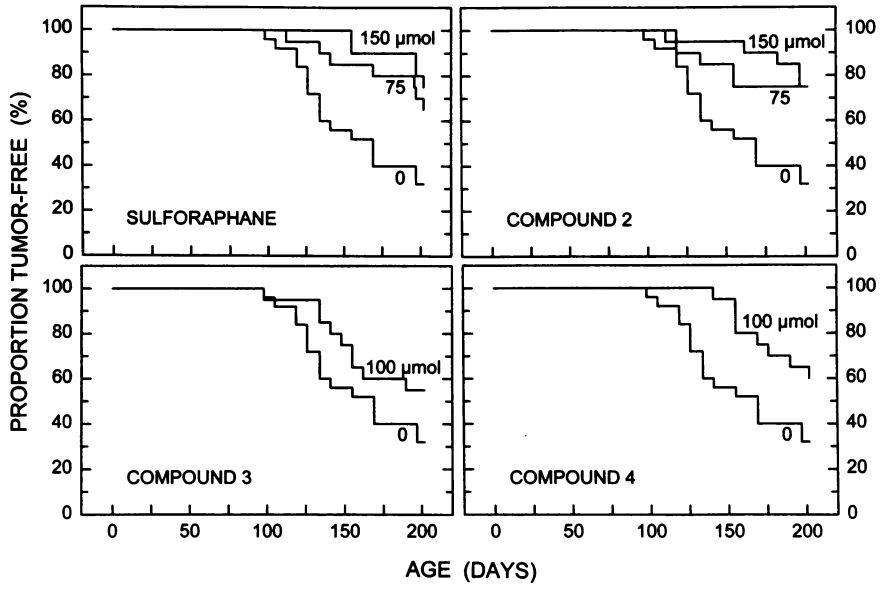

FIG. 1. Effect of treatment with sulforaphane (1) and norbornyl isothiocyanates (2–4) on mammary tumor development in female Sprague–Dawley rats that received 8 mg of DMBA at age 50 days. The proportion of tumor-free animals is shown at weekly intervals. The progression of tumor development in the control animals that received no protector (designated 0) is repeated in each panel. Sulforaphane: 75 μmol (P = 0.016); 150 μmol (P = 0.0022). Compound 2: 75 μmol (P = 0.023); 150 μmol (P = 0.0074). Compound 3: 100 μmol (P = 0.13). Compound 4: 100 μmol (P = 0.023). Kaplan–Meier incidence curves were analyzed by the logarithmic rank test. The P values refer to comparisons of differences in rate of tumor development in treated and control groups. All differences between treated and control groups are significant except for compound 3. The numbers of animals analyzed in each group are shown in Table 1.

protector. Although **3** produced some reduction in tumor incidence and multiplicity in comparison to controls, there was barely any reduction in tumor weights or significant prolongation in latency of tumor development. Compound **4** was apparently more potent than **3** but less potent than sulforaphane or compound **2** (Tables 1 and 2).

DISCUSSION

Sulforaphane, a naturally occurring isothiocyanate, and several structurally related synthetic 2-acetylnorbornyl isothiocyanates were tested for anticarcinogenic activity in this study, because they had previously been shown to be potent monofunctional inducers of phase 2 enzymes in cultured cells and in mouse tissues *in vivo* (5, 6). Since monofunctional induction has been proposed as a predictor of chemoprotective activity (7) and several structurally unrelated organic isothiocyanates have been shown to block chemical carcinogenesis and to induce Phase 2 enzymes (10, 11), we examined synthetic sulforaphane and the 2-acetylnorbornyl isothiocyanates **2–4** for chemoprotective activity. The present experiments demonstrate that all of these compounds, when administered around the time of carcinogen exposure, reduced—to varied degrees—the incidence, the multiplicity, and the weight of mammary tumors that developed in female Sprague–Dawley rats treated with DMBA. These agents also delayed tumor development. These observations further bolster the validity of the prediction that chemical compounds that induce phase 2 enzymes are promising candidates for achieving chemoprotection.

Sulforaphane was isolated as the principal and very potent phase 2 enzyme inducer from one variety of broccoli (5, 11). Our findings raise the issue to what extent sulforaphane and the many related inducers that are abundant in plants consumed by humans contribute to the chemoprotective activity of vegetables in man (12). There is insufficient information at present to draw conclusions on this matter.

Although our experiments do not provide a rigorous basis for comparing the relative potencies of sulforaphane (**1**) and compounds **2–4** as chemoprotectors, we can obtain some estimate of these potencies and relate them to the phase 2 enzyme inducer potencies (as measured by the concentrations required to double quinone reductase activities in murine hepatoma cells—i.e., the so-called CD values) (5, 6). These CD values are as follows: 0.2 μM (sulforaphane); 0.3 μM (compound **2**); 0.8 μM (compound **3**); and 0.4 μM (compound **4**). A similar order of potency is reflected in the protective potencies of these compounds in blocking DMBA-induced mammary tumors. Compound **3** is the least potent protector. Sulforaphane and compound **2** are approximately equipotent, and **2** may be even more potent than sulforaphane. Sulforaphane and **2** are more potent than **3** or **4**. It is therefore gratifying that measurements of inducer potencies in our cell culture assay not only correctly predicted anticarcinogenic activity but also provided a reasonable index of potency.

The reasons for the apparent differences in potencies of the compounds tested are not clear. The possibly higher potency of **2** in comparison to sulforaphane might be attributed to the fact that the isothiocyanate group of **2** is secondary whereas that of sulforaphane is primary. Consequently, the former is likely to be less reactive and might therefore resist metabolic disposal or other promiscuous intracellular reactions with nucleophiles to which all isothiocyanates are susceptible. The differences in potencies of **2**, **3**, and **4** are more difficult to explain. In compounds **2** and **4** the functional groups are *exo*, whereas in compound **3** the 2-acetyl group is *endo* and, therefore, more protected. One aspect of this structure–activity relation is noteworthy: the nearly equivalent effects of methylsulfinyl (CH_3SO-) and methylcarbonyl (CH_3CO-) functions on both inducer and chemoprotective potencies of these agents.

The mechanisms of the chemoprotective actions of these compounds are not fully understood. Although isothiocyanates induce protective phase 2 enzymes, and the functionalized isothiocyanates used in these experiments are especially potent in this regard, it is becoming increasingly clear that some isothiocyanates also block activation of carcinogens by inhibiting phase 1 enzymes (10, 13). Whether sulforaphane and the 2-acetylnorbonyl isothiocyanates inhibit carcinogen activation is not known. Clearly, agents that are monofunctional inducers of phase 2 enzymes and block carcinogen activation by inhibiting phase 1 enzymes are likely to be ideal chemoprotectors.

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