

Cancer Chemopreventive Potential of Sulforamate, a Novel Analogue of Sulforaphane That Induces Phase 2 Drug-metabolizing Enzymes¹

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ABSTRACT

Chemoprevention involves the use of natural or synthetic substances to reduce the risk of developing cancer. Two dietary components capable of mediating chemopreventive activity in animal models by modulation of drug-metabolizing enzymes are sulforaphane, an aliphatic isothiocyanate, and brassinin, an indole-based dithiocarbamate, both found in cruciferous vegetables. We currently report the synthesis and activity of a novel cancer chemopreventive agent, (\pm)-4-methylsulfinyl-1-(*S*-methylthiocarbamyl)-butane (trivial name, sulforamate), an aliphatic analogue of brassinin with structural similarities to sulforaphane. This compound was shown to be a monofunctional inducer of NAD(P)H:quinone oxidoreductase [quinone reductase (QR)], a Phase II enzyme, in murine Hepa 1c1c7 cell culture and two mutants thereof. Induction potential was comparable to that observed with sulforaphane (concentration required to double the specific activity of QR, $\sim 0.2 \mu\text{M}$), but cytotoxicity was reduced by about 3-fold ($\text{IC}_{50} \sim 30 \mu\text{M}$). In addition, sulforaphane, as well as the analogue, increased glutathione levels about 2-fold in cultured Hepa 1c1c7 cells. Induction of QR was regulated at the transcriptional level. Using Northern blotting techniques, time- and dose-dependent induction of QR mRNA levels were demonstrated in Hepa 1c1c7 cell culture. To further investigate the mechanism of induction, HepG2 human hepatoma cells were transiently transfected with QR-chloramphenicol acetyltransferase plasmid constructs containing various portions of the 5'-region of the QR gene. Sulforaphane and the analogue significantly induced ($P < 0.0001$) CAT activity at a concentration of $12.5 \mu\text{M}$ by interaction with the antioxidant responsive element (5–14-fold induction) without interacting with the xenobiotic responsive element. Moreover, both compounds significantly induced mouse mammary QR and glutathione *S*-transferase activity (feeding of 3 mg/mouse intragastric for 4 days), whereas the elevation of hepatic enzyme activities was less pronounced. Both sulforaphane and the analogue were identified as potent inhibitors of preneoplastic lesion formation in carcinogen-treated mouse mammary glands in organ culture (84 and 78% inhibition at $1 \mu\text{M}$, respectively). On the basis of these results, the sulforaphane analogue can be regarded as a readily available promising new cancer chemopreventive agent.

INTRODUCTION

Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue (1, 2). Induction of Phase II drug-metabolizing enzymes such as GST⁴ (EC 2.5.1.18) or QR (EC

1.6.99.2) is considered a major mechanism of protection against chemical stress and initiation of carcinogenesis (3). According to their enzyme induction pattern, compounds that induce drug-metabolizing enzymes have been classified by Talalay and associates into bifunctional inducers (planar aromatic compounds) that elevate both Phase I and Phase II enzymes and monofunctional inducers (diphenols, thiocarbamates, isothiocyanates, and 1, 2-dithiol-3-thiones) that selectively elevate Phase II enzymes (4, 5). As an example, sulforaphane, an aliphatic isothiocyanate, was isolated from broccoli as a potent plant-derived monofunctional inducer of Phase II detoxification enzymes (6) and was shown to significantly inhibit DMBA-induced mammary carcinogenesis in rats (7).

As an approach for the detection of novel cancer chemopreventive agents, plant extracts and pure compounds were tested for induction of QR in cultured Hepa 1c1c7 murine hepatoma cells and two mutants thereof (8, 9). As a result, brassinin, an indole-based dithiocarbamate found in cruciferous vegetables such as Chinese cabbage, was shown to mediate significant chemopreventive activities, including the inhibition of carcinogen-induced lesion formation in a MMOC, reduction of the formation of DMBA/phorbol ester-induced papillomas in the two-stage mouse skin carcinogenesis model, and prevention of chemically induced mammary tumor formation in rats (10, 11). Subsequently, brassinin was demonstrated to induce QR and GST activity and mRNA levels in H4IIE rat hepatoma cells and in rat organs.⁵ This induction was found to be regulated at the transcriptional level by interaction with the XRE found in both Phase I and Phase II drug-metabolizing enzymes, and the ARE of QR and GST Ya (12). On the basis of this induction pattern, brassinin can be regarded as a bifunctional enzyme inducer with similarities to compounds such as indole-3-carbinol (13, 14).

Although Phase I induction and functionalization of xenobiotics might be required for complete detoxification of these compounds by the action of Phase II enzymes, induction of Phase I enzymes is considered a potential cancer risk factor due to the activation of carcinogens to ultimate carcinogens (15). On the basis of the promising chemopreventive activities of both brassinin and sulforaphane, we decided to synthesize a hybrid of these compounds containing the methylsulfinylbutane moiety found in sulforaphane linked to the dithiocarbamate side chain of brassinin. We hypothesized that this modification of the brassinin structure would result in a monofunctional enzyme inducer with chemopreventive activity. We further speculated that replacement of the reactive isothiocyanate group of sulforaphane by a dithiocarbamyl group might result in a reduction of cytotoxic effects mediated by sulforaphane. In this report, we summarize the synthesis of this novel compound, (\pm)-4-methylsulfinyl-1-(*S*-methylthiocarbamyl)-butane, given the trivial name sulfora-

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⁴ The abbreviations used are: GST, glutathione *S*-transferase; QR, quinone reductase, NAD(P)H:quinone oxidoreductase; DMBA, 7,12-dimethylbenz(a)anthracene; MMOC, mouse mammary organ culture; XRE, xenobiotic responsive element; ARE, antioxidant

responsive element; HPLC-MS, high-performance liquid chromatography mass spectrometry; GSH, reduced glutathione; m.p., melting point; m, multiplet; s, singlet; d, doublet; brs, broad singlet; tBHQ, *tert*-butylhydroquinone; β -NF, β -naphthoflavone; CD, concentration required to double the specific activity of QR; CI, chemopreventive index, defined as the ratio of the IC_{50} and the CD; CAT, chloramphenicol acetyltransferase.

⁵ C. Gerhäuser, J. Liu, R. M. Moriarty, C. F. Thomas, R. C. Moon, and J. M. Pezzuto, Brassinin-mediated induction of phase II detoxification enzymes in rat liver and mammary glands, manuscript in preparation.

mate, and its potential to induce QR activity and glutathione levels in cell culture. Furthermore, we have analyzed the mechanism of the observed enzyme induction by Northern blotting techniques and in transient transfection experiments. Finally, we have investigated the induction of Phase II metabolizing enzymes in mouse liver and mammary glands, and the inhibition of DMBA-induced lesion formation in mouse mammary organ culture, as a preliminary assessment of potential cancer chemopreventive activity.

MATERIALS AND METHODS

Chemicals. Sulforaphane was synthesized as described previously (16, 17) or purchased from LKT Labs, Inc. (St. Paul, MN) as a pale yellow oil. The purity was confirmed by HPLC-MS analysis. *tert*-Butylhydroquinone, crystal violet, and digitonin were obtained from Aldrich Chemical Co. (Milwaukee, WI), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All cell culture media and supplements were obtained from Life Technologies (Grand Island, NY).

Synthesis of Sulforamate [(±)-4-Methylsulfinyl-1-(*S*-methylthiocarbonyl)-butane]. The sulforaphane analogue was synthesized as outlined in Fig. 1. Using a modification of the literature procedure (16), (±)-1-amino-4-(methylsulfinyl)butane [4] was synthesized from *N*-(4-bromobutyl)phthalimide [1]. Carbon disulfide (3.0 mmol) was added dropwise to a solution of [4] (0.4 g, 3.0 mmol) in pyridine (15 ml; with stirring at 0°C), followed by triethylamine (0.18 ml, 3.0 mmol). The resulting mixture was stirred at 0°C for 30 min. Iodomethane (0.19 ml, 3.0 mmol) was then added dropwise to the solution, which was stirred at 0°C for 2 h before stirring at room temperature overnight. The bulk of the solvent was removed *in vacuo*, and the residue was taken up in diethyl ether, which was washed with 1N hydrochloric acid. The ether layer was washed with water (50 ml), saturated NaHCO₃ solution (50 ml), and saturated NaCl solution (50 ml), successively. The ether was dried over MgSO₄, filtered, and concentrated on a rotary evaporator. The residue was purified on flash silica gel, and the fractions containing [5] were combined, concentrated *in vacuo*, and recrystallized from ether/petroleum ether to yield 0.4 g (60%) of a white solid, m.p. 95–96°C. For ¹H nuclear magnetic resonance (for the major tautomer in CDCl₃), δ: 1.82 (m, 4H), 2.57 (s, 3H), 2.72–2.75 (m, 2H), 3.74 (d, 2H), 8.46 (brs, 1H). For ¹³C NMR (CDCl₃), δ: 17.97, 20.12, 27.04, 38.44, 46.27, 53.41, 198.9. The formula and analysis are shown below.



Calculated: C 37.30, H 6.71, N 6.21, S 42.68
Found: C 37.19, H 6.68, N 6.37, S 42.51

The purity was confirmed by HPLC-MS analysis.

Determination of QR Activity in Cell Culture. For the analysis of enzyme inducers, QR activity was measured in Hepa 1c1c7 mouse hepatoma cells (18). Briefly, cells were grown in 96-well plates at a density of 2×10^4 cells/ml in 200 μl of α-MEM containing 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B (Life Technologies) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. After a preincubation period of 24 h, the media were changed, test compounds dissolved in 10% DMSO (10 μl, final concentration 0.5%) were added (7 serial 2.5-fold dilutions in a final concentration range of 0.2–50 μM), and the plates were incubated for an additional 48 h. QR activity was determined by measuring the NADPH-dependent menadiol-mediated reduction of 3-(4, 5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide to a blue formazan. Protein was determined by crystal violet staining of an identical set of test plates. Induction of QR activity was calculated from the ratio of specific enzyme activities of compound-treated cells in comparison with a solvent control, and CDs were generated. CDs were compared with IC₅₀s (half-maximal inhibitory concentration of cell viability in micrometers) to obtain a CI. Additional testing in two mutant cell lines derived from wild-type Hepa 1c1c7 cells, TAOC1BPc1, and BPc1, which are either defective in a functional aryl hydrocarbon receptor or unable to translocate the receptor-ligand complex to the nucleus, respectively (19), according to the protocol outlined above, allowed definition of mono- and bifunctional inducers.

Determination of GSH Levels in Cultured Hepa 1c1c7 Mouse Hepatoma Cells. GSH was measured by an enzymatic recycling procedure developed for the sensitive determination of total GSH levels in cells cultured in 96-well plates. GSH was sequentially oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid; Ellman reagent) and reduced by NADPH in the presence of glutathione reductase. The standard protocol described by Griffith (20) was coupled to a NADPH-generating system (glucose 6-phosphate/glucose 6-phosphate dehydrogenase). Because both GSH and NADPH are regenerated, problems occurring with substrate depletion are avoided, and the reaction is linear over a range of 40–800 pmol GSH/well. Briefly, 2×10^4 cells/ml were plated in 96-well plates (200 μl/well). After 24 h, seven serial 2-fold dilutions of test compounds in 0.5% DMSO (final concentration) in fresh medium were added in duplicate. β-NF was used as positive control substance. After incubation for 48 h, plates were washed three times with PBS (pH 7.4) and kept at –85°C until tested. For analysis, cells were lysed by three repetitive freeze-thaw cycles before addition of 40 μl of solution A. [Solution A is 125 μM sodium phosphate buffer (pH 7.5), containing 6.3 mM EDTA.] A freshly prepared reaction mixture (170 μl) consisting of 20 μl of 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in solution A, 10 μl of glutathione reductase solution (50 units in 10 ml of solution A), and 140 μl of the NADPH-generating system (solution B) was then added to each well to start the reaction. Solution B was prepared by diluting 2.5 ml of 0.5 M Tris-HCl (pH 7.4), 330 μl of 150 mM glucose 6-phosphate, 30 μl of 50 mM NADP⁺, and 100 units of glucose 6-phosphate dehydrogenase with distilled water to a total volume of 50 ml. Plates were shaken at room temperature for 5 min on a titer plate shaker. The extent of 2-nitro-5-thiobenzoic acid formation was monitored at 405 nm (maximum at 412 nm) using a BT2000 Microkinetic Reader (Fisher Biotech) 10 min after the initiation of the reaction. The cellular GSH content was calculated in comparison with a GSH standard curve measured on each plate in designated cell-free wells. The influence of protein sulfhydryl groups interfering with the determination of GSH was found to be negligible; therefore, GSH was measured without prior deproteinization of the lysates. The protein content was determined in duplicate plates prepared and treated as described above, using a bicinchoninic acid protein assay kit (Sigma) with BSA (Sigma) as a standard (21). GSH levels were expressed as nmol/mg protein, relative to standards. Cytotoxic activity of test samples was indicated by a reduction of the protein content per well. Generally, compounds were tested at nontoxic concentrations (protein content >50% of control).

Northern Blot Analysis of QR mRNA Expression in Hepa 1c1c7 Mouse Hepatoma Cells. Hepa 1c1c7 mouse hepatoma cells (1×10^5 cells/ml) were cultured in 100-mm tissue culture dishes in 10 ml of α-MEM as described for the induction of QR enzyme activity in Hepa 1c1c7 cell culture. After preincubation for 24 h, enzyme induction was initiated by the addition of test compounds in DMSO (0.1% final concentration). Total RNA extraction using RNazol, size fractionation by 1.2% agarose/0.36 M formaldehyde gel electrophoresis, vacuum blotting to Zeta Probe GT membranes (Bio-Rad, Hercules, CA), and UV cross-linking were described previously (22). Membranes were prehybridized at 65°C in 0.25 M sodium phosphate buffer (pH 7.2) containing 7% SDS for 30–60 min. Hybridization was performed using a 0.9-kb *Pst*I fragment of pDTD55 ³²P-labeled by random-primed labeling using the Prime-a-Gene labeling system (Promega, Madison, WI) as a cDNA probe to detect QR mRNA expression. After hybridization for 24 h, membranes were rinsed 1–2 times with 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS at room temperature, followed by 1–2 washes each at 65°C for 10–15 min using the same buffer containing 5 or 1% SDS, respectively. Hybridized membranes were exposed to X-ray (Fuji RX) film for 3–5 days as indicated. For quantitation of mRNA expression, densitometric scanning of autoradiographs was performed using the Ambis Optical Imaging System of Ambis 100 including Ambis Core Software 4.0 (Scanalytics, Billerica, MA). mRNA levels were expressed as a ratio between treated cells and the control at time 0 in time course experiments, and between treated cells and the solvent control for dose-response analyses.

Transient Transfection of HepG2 Human Hepatoma Cells with QR Promoter-CAT Constructs. HepG2 human hepatoma cells were cultured in RPMI 1640 containing 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate (Life Technologies) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere. Cells were plated in 60-mm tissue culture dishes. After 24 h (50–70% confluence), the medium was changed to Eagle's MEM containing 100 units/ml penicillin G sodium and 100 units/ml

streptomycin sulfate (Life Technologies) supplemented with 10% fetal bovine serum. The cells were transiently transfected with three different plasmid constructs containing various portions of the 5' regulatory region of the rat QR gene linked to the CAT structural gene. Construct pDTD-1097CAT contained 1097 bp of the 5' region of the rat QR gene, including all known regulatory elements of the rat QR gene connected to the CAT structural gene. QR XRE-CAT consisted of the XRE of the rat QR gene containing an aryl hydrocarbon receptor recognition site connected to a heterologous promoter fused to the CAT gene, whereas QR ARE-CAT contained the ARE of the rat QR gene connected to the heterologous promoter fused to the CAT gene (23). A plasmid expressing β -galactosidase (pCH110, Pharmacia, Piscataway, NJ) was cotransfected. The cells were transfected using the calcium phosphate precipitation method (24). Four h after addition of the DNA, cells were treated with 10% glycerol for 3 min and rinsed with PBS. Fresh medium (RPMI 1640) was then added, and the cells were allowed to recover overnight.

Transfected cells were treated with 12.5 μ M sulforaphane, 12.5 μ M sulforamate, 12.5 μ M β -NF, 12.5 μ M or unsupplemented medium as control. The compounds were dissolved in 5 μ l of DMSO (0.1% final concentration). Control dishes received 5 μ l of DMSO. For dose-response analyses, HepG2 cells were transfected with QR ARE-CAT and treated with 0.4, 2, and 10 μ M sulforaphane or sulforamate, respectively, as described above. After 48 h, cells were harvested and lysed. CAT expression in cell lysates was determined using a CAT ELISA kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. CAT activities were normalized for the protein content of each sample determined according to Bradford (25) using BSA (Sigma) as a control, and the ratio between compound-treated and control samples was calculated. All data are expressed as mean \pm SD. Means were compared using Student's *t* test with *n* = 4.

Induction of QR and GST Enzyme Activity in Mouse Liver and Mammary Glands. BALB/c female mice (*n* = 3) were treated with sulforaphane, sulforamate, or vehicle for 4 days (intra-gastric application of 3 mg of the compounds in 0.2 ml of sesame oil/mouse/day). The animals were killed by cervical dislocation; livers and bilateral mammary glands were removed, frozen in liquid nitrogen, and kept at -85°C until analyzed. Bilateral mammary glands of each animal were pooled, homogenized in 1 ml of ice-cold 0.1 M sodium phosphate buffer (pH 6.5), and centrifuged at $30,000 \times g$ for 30 min at 4°C . The clear supernatant was used for determination of enzyme activities. One-half of each liver was homogenized in 5 ml of 0.25 M sucrose and centrifuged at $27,000 \times g$ for 20 min at 4°C . A total of 1.25 ml of the supernatant was treated with 0.25 ml of 0.1 M CaCl_2 in 0.25 M sucrose for 30 min at 0°C and centrifuged as before to remove microsomes. The clear cytosolic supernatant was used for the determination of enzyme activities. Dicumarol-sensitive QR activity was measured in duplicate in 50 μ l of a suitable dilution of the tissue supernatants as described for the cellular system. GST enzyme activity was determined according to Habig *et al.* (26) using 1-chloro-2,4-dinitrobenzene as a substrate. Protein was measured as described by Bradford (25) using BSA (Sigma) as a control. Means of specific enzyme activities were compared using Student's *t* test with *n* = 3.

Inhibition of DMBA-induced Lesion Formation in MMOC. The evaluation of potential inhibitors of DMBA-induced preneoplastic lesion formation in mouse mammary organ culture was described earlier (10, 11).

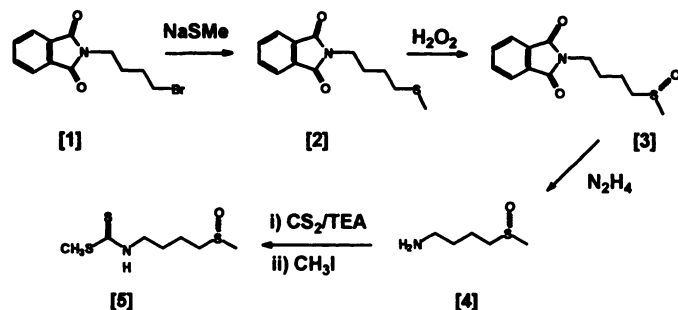


Fig. 1. Synthesis of sulforamate [(\pm)-4-methylsulfinyl-1-(*S*-methylthiocarbonyl)-butane] [5] starting from *N*-(4-bromobutyl)phthalimide [1]. The experimental procedure is outlined in "Materials and Methods."

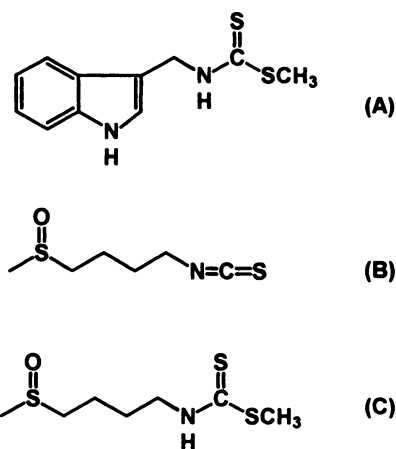


Fig. 2. Structures of brassinin, sulforaphane, and sulforamate. The figure shows brassinin [3-(*S*-methylthiocarbonyl)aminomethyl indole] (A), sulforaphane [($-$)-1-isothiocyanato-4(*R*)-(methylsulfinyl)-butane] (B), and sulforamate [4-methylsulfinyl-1-(*S*-methylthiocarbonyl)-butane] (C).

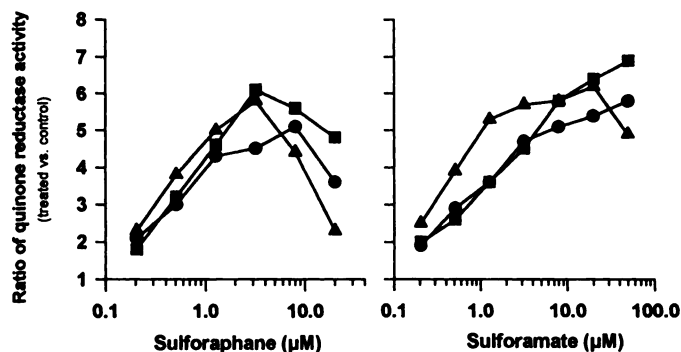


Fig. 3. Induction of QR activity by sulforaphane and sulforamate. Hepa 1c1c7 (●), BP1c1 (■), or TAOc1BP1c1 (▲) mouse hepatoma cells were grown for 24 h and then exposed to sulforaphane and sulforamate in a concentration range of 0.2–20 and 50 μ M, respectively, for 48 h. QR activity in compound-treated cells was measured and compared with the activity of solvent-treated cells (0.5% DMSO final concentration) to calculate the ratio of QR activity. Control values for the cell lines were as follows: Hepa 1c1c7, 75 ± 3 (sulforaphane), 86 ± 3 (sulforamate); BP1c1, 23 ± 2 ; TAOc1BP1c1, 73 ± 4 (nmol/min/mg of protein).

RESULTS

Synthesis of Sulforamate [(\pm)-4-Methylsulfinyl-1-(*S*-methylthiocarbonyl)-butane]. We have developed a 4-step synthesis summarized in Fig. 1 to produce a novel analogue with structural similarities to brassinin, an indole-based phytoalexin from Chinese cabbage (Fig. 2A), and the isothiocyanate sulforaphane (Fig. 2B), found in mustard oils of cruciferous vegetables (6, 27). Sulforamate [(\pm)-4-methylsulfinyl-1-(*S*-methylthiocarbonyl)-butane] (Fig. 2C) was obtained as a white solid after recrystallization from ethyl acetate/petroleum ether.

Induction of QR Activity and Glutathione Levels in Mouse Hepatoma Cell Culture. Sulforaphane was identified earlier as a potent inducer of QR activity in mouse hepatoma cell culture and in murine liver, forestomach, glandular stomach, small intestine, and lung (6). Therefore, we tested the potential of the analogue to induce QR activity in Hepa 1c1c7 mouse hepatoma cells and two mutant cell lines in comparison with the activities of sulforaphane. Induction profiles of both compounds are shown in Fig. 3. Sulforamate induced QR activity in a dose-dependent manner in the concentration range of 0.2–50 μ M with a maximum of 7-fold induction at the highest concentration tested. Maximum enzyme induction mediated by sulforaphane was observed in a concentration range of 4–8 μ M. As indicated

by a reduction of QR activity, sulforaphane was cytotoxic at concentrations above $\sim 8 \mu\text{M}$ in all three cell lines, whereas sulforamate displayed weak toxicity only with TAOc1BP^{c1} cells at the highest concentration tested ($50 \mu\text{M}$). Both compounds strongly induced QR enzyme activity in the wild-type as well as in mutant cell lines, indicative of a monofunctional induction pattern. As summarized in Table 1, CDs obtained with sulforamate were found in a concentration range similar to that obtained with sulforaphane in all three cell lines. IC₅₀s, as an indication of cytotoxicity, were about 3–4-fold lower than those obtained with sulforaphane. Therefore, the CI (defined as the ratio between the IC₅₀ and the CD) was about 3-fold higher when sulforamate was used as an inducer. In addition to the induction of QR, both sulforaphane and sulforamate significantly induced glutathione levels in cultured Hepa 1c1c7 cells in a dose-dependent manner, with a maximal 2-fold increase at $10 \mu\text{M}$ (Fig. 4).

Induction of QR mRNA. Studies to determine the mechanism of induction of QR enzyme activity mediated by sulforaphane and sulforamate included the analysis of effects on QR mRNA levels in Hepa 1c1c7 cell culture. Cells were treated with $10 \mu\text{M}$ sulforaphane, $10 \mu\text{M}$ sulforamate, or DMSO as solvent control for 12, 24, 48, and 72 h. QR mRNA levels measured by Northern blotting techniques increased within the first 24 h of incubation, and maxima of 8.8- and 9.9-fold induction of QR mRNA levels were observed, respectively, after an incubation time of 24 h, which declined by 48 and 72 h. Both compounds showed a similar induction profile (Fig. 5). Dose-dependent effects were analyzed at the time of maximum induction (24 h) in a concentration range of 0.4 – $25 \mu\text{M}$ with sulforaphane and 0.4 – $50 \mu\text{M}$ with sulforamate. As illustrated in Fig. 6, QR mRNA was significantly induced in comparison with the solvent control by treatment with either compound in the concentration range of 0.4 – $2 \mu\text{M}$, and induction reached a plateau at higher concentrations. When H4IIE rat hepatoma cells were used for analysis, maximum induction of QR mRNA levels was observed after 12 h of incubation with sulforaphane or sulforamate at $10 \mu\text{M}$. Dose-dependent induction was analyzed in a concentration range of 0.4 – $25 \mu\text{M}$ sulforaphane and 0.4 – $50 \mu\text{M}$ sulforamate, respectively, after a 12-h incubation period. Maximum induction was observed at $10 \mu\text{M}$, which declined at higher concentrations of each compound due to changes in the kinetics of enzyme induction at these concentrations (data not shown).

Determination of Regulatory Elements in Transient Transfection Assays. To determine if the increase in QR mRNA levels was due to transcriptional regulation of the QR gene by sulforaphane and the analogue, the induction of QR was analyzed in transient transfection experiments. HepG2 human hepatoma cells were transfected with three plasmid constructs containing various portions of the 5' regulatory region of the rat QR gene linked to the CAT structural gene. As shown in Fig. 7, sulforaphane, sulforamate, β -NF, and tBHQ significantly ($P < 0.0001$) induced CAT levels in HepG2 cells transfected with the pDTD-1097CAT construct (containing all of the known regulatory elements of the rat QR gene connected to the CAT structural gene). Sulforaphane induced CAT expression with about twice the potency of the analogue. A more detailed analysis revealed that

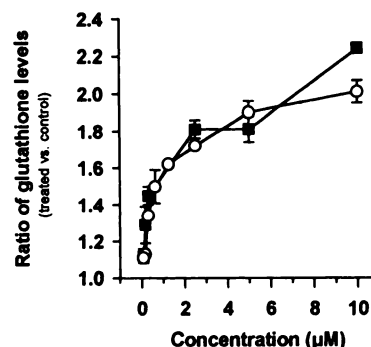


Fig. 4. Induction of glutathione levels by sulforaphane and sulforamate. Cultured Hepa 1c1c7 mouse hepatoma cells were treated with sulforaphane (■) or sulforamate (○) in a concentration range of 0.08 – $10 \mu\text{M}$ for 48 h. Glutathione levels of compound-treated cells were analyzed and compared with a solvent control (0.5% DMSO final concentration) to calculate the ratio of GSH induction. GSH level of solvent control: $53 \pm 0.3 \text{ nmol/mg}$ protein.

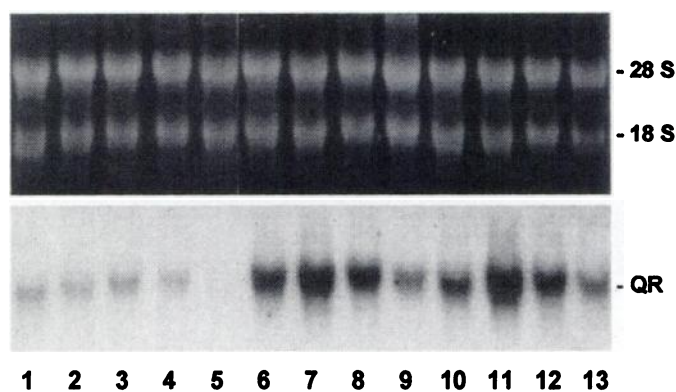


Fig. 5. Northern blot analysis of QR mRNA levels in Hepa 1c1c7 cells after treatment with sulforaphane or sulforamate for various time periods. A total of $20 \mu\text{g}$ of RNA were loaded per lane. Top, ethidium bromide-stained gel. Bottom, hybridization with a QR cDNA probe. Lane 1, time 0; Lanes 2–5, treatment with DMSO (0.1% final concentration) for 12, 24, 48, and 72 h, respectively; Lanes 6–9, treatment with $10 \mu\text{M}$ sulforamate for 12, 24, 48, and 72 h, respectively; Lanes 10–13, treatment with $10 \mu\text{M}$ sulforaphane for 12, 24, 48, and 72 h, respectively. RNA isolation and fractionation, blotting, and hybridization conditions are described in "Materials and Methods." After washing, hybridized membranes were exposed to X-ray film for 5 days. The autoradiographs were scanned to quantify QR mRNA induction relative to control at time 0. For Lanes 2–5 (DMSO), the ratio (treated:control) was calculated as 0.9, 1.1, 0.7, and 0.1, respectively; Lanes 6–9 (sulforamate): 6.2, 8.8, 6.1, and 2.4, respectively; Lanes 10–13 (sulforaphane): 4.2, 9.9, 5.7, and 2.6, respectively.

sulforaphane and sulforamate significantly ($P < 0.0001$) induced CAT-expression by interaction with the ARE, without interacting with the XRE. Again, sulforaphane was about 2.5-fold more potent in inducing CAT expression. β -NF, a well-known bifunctional enzyme inducer that was used as a positive control substance, induced CAT expression by interaction with all three plasmid constructs, whereas the antioxidant tBHQ, a monofunctional inducer similar to sulforaphane and sulforamate, did not enhance CAT expression when QR XRE-CAT was used for transient transfection. Effects of sulforaphane

Table 1 Effect of sulforaphane and sulforamate on QR activity in Hepa 1c1c7 hepatoma cells and Hepa 1c1c7 mutants

Cell line	Sulforaphane			Sulforamate		
	CD \pm SD (μM) ^a	IC ₅₀ \pm SD (μM) ^b	CI ^c	CD \pm SD (μM) ^a	IC ₅₀ \pm SD (μM) ^b	CI ^c
Hepa 1c1c7	0.23 ± 0.03	9.9 ± 1.9	42	0.26 ± 0.06	34.9 ± 2.7	137
BP ^{c1}	0.36 ± 0.1	11.0 ± 1.2	31	0.34 ± 0.12	36.0 ± 3.4	107
TAOc1BP ^{c1}	0.17 ± 0.05	8.7 ± 1.8	51	0.25 ± 0.06	34.3 ± 2.4	140

^a Mean value of the concentration required to double the specific activity of QR ($n = 2$ –4).

^b Mean value of the half-maximal inhibitory concentration of cell viability ($n = 4$).

^c CI, defined as the ratio between IC₅₀s and CDs.

and sulforamate on CAT expression after transient transfection with the QR-ARE construct were analyzed in a dose-dependent manner. Both compounds mediated significant ($P < 0.0001$) induction of CAT expression in a concentration range of 0.4–10 μM (Fig. 8). Again, the sulforaphane-mediated response was about 2 times greater than that of sulforamate at all concentrations tested.

In Vivo Induction of Drug-metabolizing Enzymes. In a preliminary experiment, we further tested the potential of sulforaphane and sulforamate to induce steady-state levels of QR and GST in mouse liver and mammary glands. Compounds were dissolved in sesame oil and administered intragastrically for 4 days at a dose of 3 mg per mouse. Due to limited compound availability, three mice per group were used. In mammary glands, QR activity was significantly ($P < 0.005$) elevated 2.5- or 2.6-fold, respectively, after treatment with sulforaphane or sulforamate. Hepatic QR activity was induced 1.3- or 1.2-fold, respectively; however, this induction was not significant in comparison with the control group. The results of the effects on mammary and hepatic GST activity are summarized in Table 2. Similar to the effects on QR, the steady-state levels of mammary GST

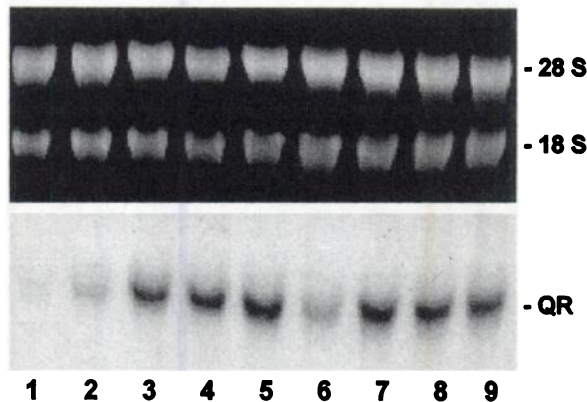


Fig. 6. Northern blot analysis of QR mRNA levels in Hepa 1c1c7 cells after treatment with sulforaphane or sulforamate at various concentrations for 24 h. A total of 20 μg of RNA were loaded per lane. *Top*, ethidium bromide-stained gel. *Bottom*, hybridization with a QR cDNA probe. Lane 1, DMSO (0.1% final concentration); Lanes 2–5, treatment with 0.4, 2, 10, and 50 μM sulforamate, respectively; Lanes 6–9, treatment with 0.4, 2, 10, and 25 μM sulforaphane, respectively. RNA isolation and fractionation, blotting, and hybridization conditions are described in "Materials and Methods." After washing, hybridized membranes were exposed to X-ray film for 3 days. The autoradiographs were scanned to quantify QR mRNA induction relative to control treated with DMSO. For Lanes 2–5 (sulforamate), the ratio (treated:control) was calculated as 2.1, 6.9, 6.6, and 7.7, respectively; Lanes 6–9 (sulforaphane): 3.2, 6.8, 6.1, and 6.0, respectively.

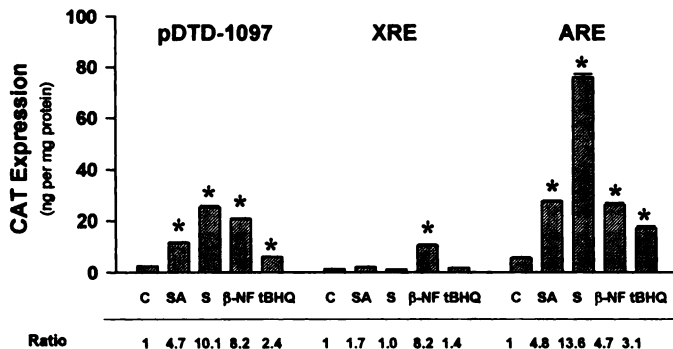


Fig. 7. Quantitation of CAT expression in HepG2 cells transfected with QR-CAT constructs. Transfected cells were treated with 10 μM sulforamate (SA), 10 μM sulforaphane (S), 10 μM β -NF, 20 μM tBHQ, or DMSO (0.5% final concentration) as control (C). Cells were transfected with pDTD-1097 CAT, QR XRE-CAT (XRE), or QR ARE-CAT (ARE). CAT expression (ng CAT/mg protein) was determined using a CAT ELISA, and the ratio of CAT expression between treated cells and control was calculated (Ratio). Experimental details are described in "Materials and Methods." *, mean significantly different from control ($P < 0.0001$) using Student's *t* test with $n = 4$.

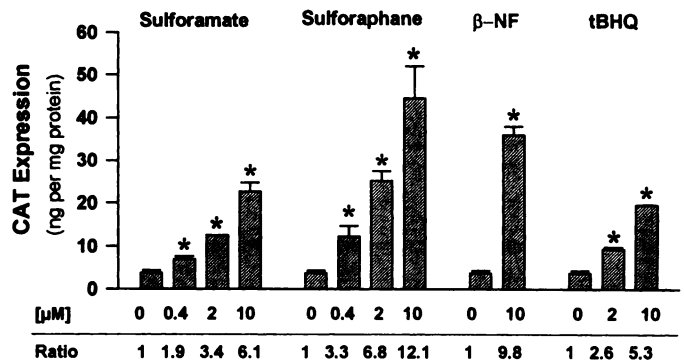


Fig. 8. Dose-dependent expression of CAT in HepG2 cells transfected with QR-ARE-CAT. Transfected cells were treated with 0.4, 2, and 10 μM sulforamate or sulforaphane, respectively, 10 μM β -NF, 2 and 10 μM tBHQ, or DMSO (0.5% final concentration) as control (designated as concentration 0 μM). CAT expression (ng CAT/mg protein) was determined using a CAT ELISA, and the ratio of CAT expression between treated cells and control was calculated (Ratio). Experimental details are described in "Materials and Methods." [μM], final concentration of test compounds in μM . *, mean significantly different from control ($P < 0.0001$) using Student's *t* test with $n = 4$.

activity were significantly ($P < 0.0005$) elevated 2.3- or 2.8-fold, respectively, when mice were treated with sulforaphane or the analogue for 4 days. However, only a slight, nonsignificant increase of hepatic GST activity was observed (1.1-fold induction mediated by either sulforaphane or the analogue; Table 2).

Inhibition of DMBA-induced Lesion Formation in Mouse Mammary Organ Culture. The biological activity of sulforaphane and the analogue was further investigated in a MMOC. This *in vitro* model is known to demonstrate a good correlation with animal models in detecting potential cancer chemopreventive agents. The results are summarized in Table 3. Sulforaphane and sulforamate were identified as two of the most potent inhibitors of DMBA-induced lesion formation in mammary organ culture analyzed thus far. The effect was dose-dependent in a concentration range of 0.01–10 μM . However, signs of toxicity were observed in glands treated with sulforaphane at concentrations of 1 and 10 μM . When glands were treated with sulforamate, toxic effects were observed at the highest concentration tested (10 μM). It is interesting that both compounds were more active than brassinin in preventing DMBA-induced lesion formation in MMOC.

DISCUSSION

In this report, we have described the synthesis and biological activities of a new potential cancer chemopreventive agent, sulforamate. The concept of chemoprevention was based in part on epidemiological observations that suggested that high intake of fruit and vegetables could be associated with a reduced risk of cancer (28), and, notably, cruciferous vegetables, including broccoli, cabbage, cauliflower, and Brussels sprouts, seemed particularly beneficial in preventing carcinogenesis (29). Investigation of constituents responsible for these activities revealed the presence of high amounts of sulfur-containing compounds, such as isothiocyanates (R-NCS) and their glucosinolate precursors (27, 30), and indole-based constituents, such as indole-3-carbinol, 3,3'-di-indolylmethane, and indole-3-acetonitrile (14, 31). These compounds were found to induce drug-metabolizing enzymes in cell culture and rodent tissue, and chemopreventive activity was attributed to the increased detoxification of xenobiotics and carcinogens, at least in part (32, 33). Recently, sulforaphane, an aliphatic isothiocyanate, has been isolated from broccoli as the major inducer of Phase II drug-metabolizing enzymes with potent *in vivo* chemopreventive properties (6, 7, 34). In addition, sulforaphane was

Table 2 Effect of sulforaphane and sulforamate on mammary and hepatic QR and GST activity in mice

Test group	QR ^a		GST ^a	
	Mammary gland	Liver	Mammary gland	Liver
Control	49.1 ± 7.2	223.0 ± 56.1	129.9 ± 7.7	2093 ± 443
Sulforamate	129.3 ± 20.6 ^b	262.6 ± 12.2	362.5 ± 45.1 ^c	2327 ± 131
Sulforaphane	122.5 ± 5.1 ^b	292.0 ± 70.4	299.1 ± 13.7 ^c	2384 ± 575

^a Mean ± SD (nmol/min/mg protein).

^b Mean significantly ($P < 0.005$) different from control (Student's *t* test with $n = 3$).

^c Mean significantly ($P < 0.0005$) different from control (Student's *t* test with $n = 3$).

found to inhibit cytochrome P450 isoenzyme 2E1, which is responsible for the activation of a variety of genotoxic chemicals (35).

Generally, isothiocyanates mediate toxic responses due to the high reactivity of the isothiocyanate moiety (27). Detoxification reactions include the reversible conjugation to glutathione, catalyzed by the GST family of isoenzymes (36–38). The resulting dithiocarbamate formation was described as the first step in the metabolic pathway of isothiocyanates. Conjugation of benzyl isothiocyanate to glutathione resulted in greatly reduced toxicity, especially at high glutathione concentrations (39). Similar results were obtained for phenylalkyl isothiocyanate-cysteine conjugates (40). Thus, isothiocyanate conjugation led to reduced toxicity; on the other hand, it was speculated that isothiocyanate thiol conjugates could be regarded as a transport form of isothiocyanates (3).

As a result of our program to identify novel cancer chemopreventive agents from plants, we have reported the anticarcinogenic activities of brassinin, a nontoxic indole-based dithiocarbamate naturally occurring in Chinese cabbage (*Brassica campestris* spp. *pekinensis*; Ref. 10). On the basis of structural similarities of brassinin to compounds such as indole-3-carbinol and indole-3-acetonitrile, brassinin was found to induce drug-metabolizing enzymes in cell culture and rodent tissues. Similar to effects of indole-3-carbinol (13, 41), brassinin also induced the activity of cytochrome P450 isoenzyme 1A1 in mouse hepatoma cell culture (42). Although indole-3-carbinol and related compounds demonstrate significant chemopreventive activity, induction of Phase I metabolizing enzymes is considered a risk factor in the activation of carcinogens (15). As an example, indole-3-carbinol was found to induce liver carcinogenesis in rainbow trout when applied in the postinitiation stage (43, 44). On the basis of this

potential cancer risk of indole-based compounds and the relative toxicity of isothiocyanates, we conceptualized a hybrid molecule of brassinin and sulforaphane by replacing the isothiocyanate group of sulforaphane with the dithiocarbamate moiety of brassinin, thus exchanging the indole ring with a methylsulfinyl-butane side chain. This novel sulforaphane analogue was anticipated to be less toxic than sulforaphane and to lack Phase I enzyme-inducing properties. The compound was synthesized and tested for its ability to induce Phase II drug-metabolizing enzymes in cell culture and *in vivo* models, and the activity was compared with effects mediated by sulforaphane. Relative to sulforaphane, replacement of the isothiocyanate group by a stable dithiocarbamate reduced the toxic effect in cell culture by a factor of three, but potential to induce QR in Hepa 1c1c7 mouse hepatoma cell culture was not reduced. Identical results in two mutant cell lines less responsive to bifunctional inducers like indole-3-carbinol, which induce Phase I as well as Phase II drug-metabolizing enzymes, indicated that the analogue was devoid of cytochrome P450-activating properties. This observation was further investigated in transient transfection experiments. Human hepatoma cells were transfected with DNA constructs containing various portions of the 5' upstream promoter region of the QR gene fused to the structural gene of CAT. The analogue was found to induce CAT expression through activation of the ARE without significantly activating the XRE, responsive to inducers of cytochrome P450. Transcriptional regulation of the induction of QR, as an example of Phase II detoxification enzymes, was further demonstrated using Northern blotting techniques.

In addition, when administered to BALB/c mice, significant induction of Phase II enzymes was demonstrated in mammary glands, but both sulforaphane, as well as the analogue, had little effect on hepatic QR and GST. The latter was unexpected because hepatic induction has been demonstrated previously on administration of similar doses of sulforaphane doses (6), but differences in experimental conditions, such as the use of BALB/c mice and the dose regimen used, probably account for the reduced response intensity. However, promising chemopreventive activity was detected in a MMOC model, wherein both compounds were identified as potent inhibitors of DMBA-induced preneoplastic lesion formation. Also, as demonstrated in cell culture, the analogue demonstrated lower toxicity than sulforaphane.

In conclusion, we have described the synthesis of a novel analogue of sulforaphane, designated as sulforamate, characterized its potential as an inducer of Phase II drug-metabolizing enzymes and glutathione levels in murine hepatoma cell culture, and demonstrated significant chemopreventive activity (inhibition of DMBA-induced lesion formation in mouse mammary organ culture). On the basis of these results and reduced cytotoxicity in comparison with sulforaphane, sulforamate can be regarded as a readily available promising new cancer chemopreventive agent that warrants further investigation.

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We thank Dr. C. Pickett for providing pDTD55 as a source of the QR cDNA probe used in Northern blotting experiments and for providing the three QR

Table 3 Effect of sulforaphane, sulforamate, and brassinin on the development of DMBA-induced mammary lesion formation (MMOC)

Thoracic pairs of mammary glands were incubated with growth-promoting hormones in the presence of increasing concentrations of test compounds for 10 days. The glands were incubated for an additional 14 days with insulin-containing medium. DMBA was included in the medium for 24 h on the third day of culture to induce preneoplastic mammary lesions. Glands were fixed in 10% formalin, stained with alum carmine, and evaluated for the presence of mammary lesions. The incidence of lesion formation (the percentage of glands with mammary lesions relative to the total number of glands per group) in the compound-treated groups was compared with that of untreated (DMBA control) glands, and the percentage of inhibition was calculated.

Treatment group	Concentration (μM)	% Incidence	% Inhibition ^a
DMBA control	None	80 (12/15)	ND
Sulforaphane	10	Toxic (0/15)	ND
	1	13 (2/15)	83.7
	0.1	35 (5/14)	56.3
	0.01	53 (8/15)	33.7
DMBA control	None	80 (12/15)	ND
Sulforamate	10	Toxic (0/15)	ND
	1	18 (2/11)	77.5
	0.1	40 (6/15)	50.5
	0.01	69 (9/13)	13.7
DMBA control	None	60 (9/15)	ND
Brassinin	10	20 (3/15)	66.6
	1	33 (5/15)	44.5
	0.1	47 (7/15)	22.2
	0.01	60 (9/15)	0

^a ND, not determined.

plasmid constructs pDTD-1097CAT, QR XRE-CAT, and QR ARE-CAT used in transient transfection experiments. We also thank Dr. J. P. Whitlock, Jr., for supplying Hepa 1c1c7 cells and two mutant cell lines, Dr. D. H. Barch for support in performing transient transfection experiments, and J. Graham for HPLC-MS analyses of sulforaphane and sulforamate.

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