Effects of a series of organosulfur compounds on mitotic arrest and induction of apoptosis in colon cancer cells

Danhua Xiao,1 John T. Pinto,3 Gregg G. Gundersen,2 and I. Bernard Weinstein1

1Herbert Irving Comprehensive Cancer Center, 2Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University Medical Center, New York, New York; and 3Burke Medical Research Institute, White Plains, New York

Abstract

We previously reported that the garlic-derived compound S-allylmercaptocysteine (SAMC) causes growth inhibition, mitotic arrest, and induction of apoptosis in SW480 human colon cancer cells by inducing microtubule depolymerization and c-Jun NH2 terminus kinase-1 activation. In the present study, we compared the aforementioned effects of SAMC to those of a series of garlic-derived and other organosulfur compounds. Among the 10 compounds tested, only SAMC, diallyl disulfide (DADS), and S-trityl-L-cysteine (trityl-cys) cause significant inhibition of cell growth with IC50 values of 150, 56, and 0.9 μmol/L, respectively. These three compounds also induce G2-M cell cycle arrest and apoptosis. Further studies reveal that, like SAMC, the garlic-derived compound DADS exerts antiproliferative effects by binding directly to tubulin and disrupting the microtubule assembly, thus arresting cells in mitosis and triggering mitochondria-mediated signaling pathways that lead to apoptosis. However, the synthetic compound trityl-cys exerts its effect on M-phase arrest and growth inhibition by mechanisms that involve spindle impairment but do not involve disruption of microtubule structure or dynamics. Furthermore, trityl-cys does not induce marked loss of mitochondrial membrane potential or release of cytochrome c, but it does induce caspase-3 activation and poly(ADP-ribose) polymerase cleavage. Structure-function analysis suggests that both the allyl and the disulfide moieties are important features for the antiproliferative effects of SAMC and DADS. These findings may be useful in the identification, synthesis, and development of organosulfur compounds that have anticancer activity. [Mol Cancer Ther 2005;4(9):1388–98]

Introduction

Garlic has been used for centuries for disease prevention and treatment by several ethnic cultures. Epidemiologic investigations have found that risk of developing stomach, colon, and prostate cancers seems to be inversely related to garlic consumption (1, 2). Experimental animal and laboratory studies provide convincing evidence that garlic and some of its organosulfur components are effective inhibitors of a variety of cancers and cancer cells in culture, including those of breast, colon, skin, uterine, esophagus, and lung (2, 3). However, the specific component(s) of garlic that underlies the specific cellular and molecular events, which govern the anticancer properties, are not known with certainty. Depending on conditions of its cultivation, garlic can contain at least 33 different organosulfur compounds in addition to amino acids, vitamins, and micronutrients. The allyl sulfur compounds formed by enzymatic activity when garlic is minced or crushed, such as alliin, water-soluble S-allylmercaptocysteine (SAMC) and S-allylcysteine (SAC), and oil-soluble diallyl disulfide (DADS) and diallyl sulfide (DAS), probably account for the majority of these anticancer effects (4, 5). Previous studies from our laboratory showed that SAC inhibits growth, arrests cells at M phase of the cell cycle, and induces apoptosis in at least two human colon cancer cell lines (6). Further studies revealed that these effects of SAC are associated with depolymerization of cellular microtubules, indicating that SAC represents a new chemical class of microtubule-disrupting agents (7). However, a similar response was not observed with SAC (7), which differs from SAMC by having a thioether rather than a disulfide moiety in its structure (Fig. 1).

Because of this rather unique effect of SAMC on microtubule depolymerization, the present study was designed to compare the effects of SAMC to that of a series of garlic-derived and other organosulfur compounds containing allyl, thioether or disulfide moieties, or S-cysteinyl derivatives on SW480 human colon cancer cells and NIH3T3 fibroblasts. Our results show that like SAC, DADS binds to tubulin directly, suppresses microtubule dynamics and disrupts microtubule assembly, interferes with mitotic spindle formation, arrests cells in mitosis, and triggers signaling pathways that lead to apoptosis. This is the first evidence that DADS, a constituent that accounts for

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Requests for reprints: I. Bernard Weinstein, Herbert Irving Comprehensive Cancer Center, College of Physicians and Surgeons, Columbia University Medical Center, 701 West 168th Street, Room 1509, New York, NY 10032. Phone: 212-305-6921; Fax: 212-305-6889.

E-mail: ibw1@columbia.edu

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compounds that have anticancer activity.

identification and/or synthesis of novel organosulfur tubulin. These findings may be useful in the future to arrest cells in mitosis, but without binding directly to cysteine (trityl-cys) is a potent and novel agent that also our study indicates that the synthetic compound j-SAMC and DADS. In addition, the growth inhibition, induction of apoptosis, and G2-M cell cycle arrest produced by SAMC and DADS. In addition, our study indicates that the synthetic compound S-trityl-L-cysteine (trityl-cys) is a potent and novel agent that also arrests cells in mitosis, but without binding directly to tubulin. These findings may be useful in the future identification and/or synthesis of novel organosulfur compounds that have anticancer activity.

Materials and Methods

Garlic-Derived and Other Organosulfur Compounds

SAMC and SAC were supplied by Wakunaga of America, Co., Ltd. (Mission Viejo, CA). Stock solutions (10 μmol/L) were prepared fresh in PBS. DAS and DADS were purchased from Sigma-Aldrich Biotechnology (Saint Louis, MO). Stock solutions (100 mmol/L) were prepared in DMSO. The S-cysteinyland analogues cysteine, cystine, reduced and oxidized glutathione, S-propylglutathione, benzyl-S-cysteine, and trityl-cys were purchased from ICN Biomedicals, Inc. (Irvine, CA). Stock solutions (1–100 mmol/L) were prepared in water or DMSO, according to the solubility. The structural formulae of these compounds are depicted in Fig. 1.

Cell Lines and Culture Conditions

The SW480 human colon adenocarcinoma and NIH3T3 mouse fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA). SW480 cells were grown in DMEM with 10% fetal bovine serum and NIH3T3 cells were grown in DMEM with 10% calf serum (Life Technologies, Grand Island, NY).

Cell Proliferation Assay

Cells were seeded in six-well plates at a density of 3 × 10⁴ cells/well, grown for 36 to 48 hours, and then exposed to increasing concentrations (50–250 μmol/L) of each compound for 48 hours. Cell numbers were determined using a Coulter Counter (Beckman Coulter, Miami, FL) and results were expressed as a percentage of the control (untreated) culture. All assays were done in triplicate. Data were analyzed in Excel and the IC₅₀ values were determined graphically from cell survival curves.

Cell Cycle and Apoptosis Analyses

Propidium iodide staining was used to analyze DNA content and cell cycle distribution (7). After cells were exposed to each test compound for 24 (cell cycle analysis) or 48 hours (apoptosis analysis), both adherent and floating cells were harvested, fixed with 70% ethanol, and incubated with propidium iodide (0.05 mg/mL) and 1 μg/mL RNase A (Sigma) at room temperature in the dark for 30 minutes. The cells were then analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Apoptotic cells were considered to constitute the sub-G₁ population and the percentage of nonapoptotic cells in each phase of the cell cycle was determined. Cells were also stained with a mitotic protein monoclonal antibody (4 μg/mL; Upstate Biotechnology, Lake Placid, NY) and a FITC-conjugated secondary antibody (4 μg/mL; Rockland, Gilbertsville, PA) to distinguish M-phase cells from those in G₂ (8). All experiments were repeated and yielded similar results.

Tubulin Turbidity Assay

In vitro tubulin polymerization was monitored by the turbidity assay (7). To examine the effect of each test compound on microtubule disassembly, replicate samples of 50 μL of 5 mg/mL pure tubulin (Cytoskeleton, Denver, CO) was allowed to polymerize to a steady state in GTP-PEM buffer plus 10% glycerol in a 96-well plate by incubation at 37°C for 30 minutes. Twenty microliters of prewarmed test compound solution were then pipetted into the polymer stock wells and the plate was reincubated at 37°C for an additional 30 minutes. To examine the effect of each compound on de novo tubulin polymerization, pure tubulin (5 mg/mL) was preincubated with each test compound in 50 μL GTP-PEM buffer plus 10% glycerol in a 96-well plate at 0°C. Polymerization was initiated by placing the plate into a 37°C incubator. The change in absorbance at 340 nm was recorded using a Spectramax O.D. Reader (Molecular Devices, Sunnyvale, CA). Results are presented as percentage of absorbance, with 100% representing the A₃₄₀ value at 30 minutes when the tubulin polymerization reached a steady state. In all assays, the A₃₄₀ of each test compound itself was subtracted from the total absorbance.

Indirect Immunofluorescence Microscopy

The immunostaining method has been described previously (7). NIH3T3 cells growing on glass coverslips were exposed to the indicated compound for 20 hours and fixed with methanol at −20°C. Cells were then incubated with antibodies to tyrosinated α-tubulin (Tyr-tu, 1:10 dilution) and detyrosinated tubulin (Glu-tu, 1:400; ref. 9) at 37°C for 1 hour, and incubated with corresponding FITC- and rhodamine-conjugated secondary antibodies and 4,6-diamidino-2-phenylindole (Chemicon International Inc., Temecula, CA) at 37°C for 45 minutes, and refixed with 4% formaldehyde. The coverslip slides were observed for interphase microtubules and mitotic spindles, with
a Nikon Optiphot microscope. Images were captured with a MicroMax camera (Princeton Scientific Instruments, Monmouth Junction, NJ) and analyzed in Adobe Photoshop.

**Protein Extraction and Western Blotting**

After treatment with the indicated compounds, cell lysates were prepared (10). Samples containing 50 μg protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with corresponding primary and secondary antibodies. Results were detected using an ECL system (Amersham, Piscataway, NJ). The primary antibodies for cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology (Beverly, MA) and the primary antibody to actin (loading control) was obtained from Sigma.

**c-Jun NH2-Terminal Kinase Activity Assay**

The nonradioactive kinase assay has been reported previously (7). After treatment with UV (positive control) or the indicated compounds, cells were scraped off the plate and cell pellets were suspended in M2 lysis buffer. c-Jun NH2-terminal kinase (JNK) was immunoprecipitated by incubation of 200 μg cell protein extract with 2 μg of c-Jun fusion protein beads (Cell Signaling) at 4°C overnight. After four washes with M2 buffer and kinase buffer, cell pellets were suspended in 50 μl kinase buffer with 100 μM ATP and incubated at 30°C for 30 minutes. Samples were then analyzed by Western blotting using a phospho-c-Jun (Ser63) antibody (Cell Signaling) and the NIH image program.4

**Assays of Mitochondrial Membrane Potential**

A JC-1 mitochondrial membrane potential detection kit was utilized according to the instruction manual of Cell Technology, Inc. (Minneapolis, MN). Briefly, after treatment with the indicated compounds, cells were collected, resuspended in JC-1 reagent solution, and incubated at 37°C in a 5% CO2 incubator for 15 minutes. Cells were then washed with and resuspended in assay buffer and analyzed by flow cytometry. The percentage of cells that have lost mitochondrial membrane potential was calculated.

**Determination of Cytochrome c Release**

The method for preparation of cytosolic and mitochondrial fractions from cells was modified from previously described methods (11, 12). Briefly, after treatment with the indicated compounds, cells were collected and washed once with ice-cold PBS. Cell pellets were resuspended in ice-cold buffer A [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 15 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.1 mmol/L phenylmethylsulfonyl fluoride], allowed to swell on ice for 30 minutes, and centrifuged at 20,000 × g at 4°C for 20 minutes. The resulting supernatant fraction served as the soluble cytosolic fraction. The remaining pellets were resuspended in ice-cold buffer B [20 mmol/L HEPES (pH 7.6), 300 mmol/L KCl, 1 mmol/L EDTA, 5 mmol/L DTT, 5% glycerol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.1 mmol/L phenylmethylsulfonyl fluoride], briefly sonicated on ice, and centrifuged at 5,000 × g for 5 minutes. The supernatant fraction served as the mitochondrial fraction. Samples were analyzed by Western blotting using an anti–cytochrome c antibody (BD Biosciences PharMingen, San Diego, CA).

**Statistical Analysis**

Data are expressed as mean ± SD. Comparisons between control and treated cells were made using Student’s t test and P < 0.05 values were considered statistically significant.

**Results**

**Effects of Organosulfur Compounds on Cell Proliferation**

To examine the antiproliferative activity of these compounds, we first investigated their effects on growth of SW480 human colon cancer cells. Exponentially dividing cells were treated with increasing concentrations (50–250 μmol/L) of each compound for 48 hours, cell numbers were counted, and the IC50 values were determined. As summarized in Table 1, SAMC, DADS, and trityl-cys caused marked growth inhibition, with IC50 values of 150, 56, and 0.9 μmol/L, respectively. However, the other organosulfur compounds, including SAC, DAS, reduced and oxidized glutathione, cysteine, cystine, S-propylglutathione, and benzyl-S-cysteine, did not inhibit cell growth. For the subsequent mechanistic studies, we used the IC50 or twice the IC50 concentration of the active compounds, and other compounds were examined at 300 μmol/L.

**Effects of Organosulfur Compounds on Induction of Apoptosis**

To investigate the ability of these compounds to induce apoptosis, SW480 cells were treated with 300 μmol/L SAC, 112 μmol/L DADS, 1.8 μmol/L trityl-cys (2 × IC50 concentrations), or other compounds at 300 μmol/L for 48 hours. Cells were then stained with propidium iodide and the percentage of apoptotic cells was determined as the sub-G1 population in flow cytometric analysis. As summarized in Table 1, SAC, DADS, and trityl-cys induced significant apoptosis (54%, 36%, and 28%, respectively). By contrast, the other organosulfur compounds had no detectable effect on induction of apoptosis. As discussed below, the apoptotic process was also monitored by loss of mitochondrial membrane potential, cytochrome c release, and caspase-3 and PARP activation.

**Effects of Organosulfur Compounds on Cell Cycle Progression**

To determine possible effects of these compounds on cell cycle progression, SW480 cells were treated with 300 μmol/L SAC, 112 μmol/L DADS, 1.8 μmol/L trityl-cys (2 × IC50 concentrations), or other compounds at 300 μmol/L, and then stained with propidium iodide. To evaluate the distribution of actively dividing cells in the cell cycle before the induction of extensive apoptosis, cells were treated for 24 hours rather than 48 hours, and the

4 http://rsb.info.nih.gov/nih-image/
percentage of nonapoptotic cells in each phase of the cell cycle was determined by DNA flow cytometry. Consistent with the results obtained in the cell proliferation and apoptosis induction assays, among these compounds, only SAMC, DADS, and trityl-cys affected cell cycle progression (Table 1). Cells in the G2-M phase increased from 25% in controls to 64%, 45%, and 38%, after treatment with SAMC, trityl-cys, and DADS, respectively. In additional studies using mitotic protein monoclonal antibody staining to specifically evaluate cells in the M phase (8), we found that treatment of cells with SAMC (300 μmol/L) or trityl-cys (1.8 μmol/L) induced a dramatic increase of cells in the M phase (from 2.5% to 35% and 14%, respectively) and only a slight increase of cells in the G2 phase (data not shown), indicating that SAMC and trityl-cys specifically arrest cells at mitosis. However, when tested at 2 × IC50 (112 μmol/L) or 5 × IC50 (280 μmol/L) concentrations, DADS induced accumulation of cells at both the G2 (from 24% to 34% and 59%, respectively) and the M phase (from 2.5% to 4.2% and 10.5%, respectively), indicating that DADS inhibits both the G2 and M phase progression.

Effects of SAMC, DADS, and Trityl-Cys on In vitro Tubulin Polymerization

Our previous studies provided evidence that the M-phase arrest induced by SAMC is due to a direct effect on microtubule depolymerization (7). To determine whether DADS or trityl-cys have similar effects on tubulin, we in vitro tubulin turbidity assays. Tubulin was first incubated at 37°C for 30 minutes to allow polymerization to reach a steady state, and then each test compound was added to the mixture (Fig. 2A). It is typical in these in vitro turbidity assays to test drugs at considerably higher concentrations than those used in intact cells (13). As expected (13), addition of 10 μmol/L Taxol caused a marked increase of microtubule assembly. On the other hand, addition of 300 μmol/L SAMC (2 × IC50) resulted in ~45% disassembly of polymerized tubulin, and 1,000 μmol/L SAMC resulted in ~75% disassembly. However, even when tested at 20 × IC50 concentration, neither DADS (1120 μmol/L) nor trityl-cys (20 μmol/L) had a significant effect on microtubule polymerization when compared with the DMSO control.

In a second study, we tested these compounds by adding them to the tubulin incubation system at time zero to observe possible effects on the initiation of tubulin polymerization (Fig. 2B). As expected (13, 14), colcemid (3 μmol/L) completely inhibited de novo tubulin polymerization, whereas Taxol (10 μmol/L) did not. SAMC (1,000 μmol/L) also inhibited de novo tubulin polymerization with some fluctuations in the absorbance (Fig. 2B). By contrast, relatively high concentration of trityl-cys (20 μmol/L) did not affect de novo tubulin polymerization. DADS at 560 μmol/L caused ~30% inhibition, whereas at 1,120 μmol/L DADS almost completely inhibited de novo tubulin polymerization (Fig. 2B).

A comparison of the results obtained in Fig. 2A and B indicate that SAMC, DADS, and trityl-cys have different effects on in vitro tubulin polymerization. SAMC induced both microtubule depolymerization and inhibition of de novo tubulin polymerization. DADS inhibited de novo tubulin polymerization but did not induce depolymerization of polymerized tubulin even when tested at a 20 × IC50

Table 1. Growth inhibition, induction of apoptosis, and cell cycle distribution in SW480 human colon cancer cells after treatment with garlic-derived and other organosulfur compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Growth inhibition*</th>
<th>Apoptosis†</th>
<th>Cell cycle distribution‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC50 (μmol/L)</td>
<td>Sub-G1 %</td>
<td>G1 %</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>5</td>
<td>57</td>
</tr>
<tr>
<td>SAMC</td>
<td></td>
<td>150</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td>SAC</td>
<td>+</td>
<td>—</td>
<td>2</td>
<td>56</td>
</tr>
<tr>
<td>DADS</td>
<td>+</td>
<td>+</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>—</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>Cystine</td>
<td>–</td>
<td>+</td>
<td>6</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>5</td>
<td>62</td>
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<tr>
<td>GSH</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>5</td>
<td>61</td>
</tr>
<tr>
<td>Propyl-glu</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>54</td>
</tr>
<tr>
<td>Benzyl-cys</td>
<td>1 Benzyl ring</td>
<td>Sulfide</td>
<td>6</td>
<td>54</td>
</tr>
<tr>
<td>Trityl-cys</td>
<td>3 Benzyl ring</td>
<td>Sulfide</td>
<td>0.9</td>
<td>28</td>
</tr>
</tbody>
</table>

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; benzyl-cys, benzyl-α-cysteine; propyl-glu, S-propylglutathione.

*Cells were treated with 50 to 250 μmol/L of each compound for 48 hours. Cell numbers were then determined by cell counting and the IC50 values were determined from cell survival curves.

†Cells were treated with each compound for 48 hours and then stained with propidium iodide and analyzed by flow cytometry for apoptosis, assessed by the sub-G1 population. SAMC, DADS, and trityl-cys were treated at twice their IC50 concentration; other compounds were treated at 300 μmol/L.

‡Cells were treated with each compound for 24 hours and then stained with propidium iodide and analyzed by flow cytometry for cell cycle distribution. SAMC, DADS, and trityl-cys were treated at twice their IC50 concentration and other compounds were tested at 300 μmol/L.

No effects on growth inhibition when tested at up to 250 μmol/L.
concentration. Trityl-cys did not affect either of these processes, indicating that this compound has no direct effect on tubulin. The mechanisms by which DADS and SAMC produce different effects in these assays are not known. One possible explanation might be different binding properties of the two compounds to tubulin. SAMC might bind to both soluble and microtubule tubulin. DADS might bind poorly to microtubule tubulin, but instead bind to soluble tubulin and, therefore, exert a specific inhibitory effect on de novo tubulin polymerization. Another possible explanation relates to different effects of microtubule nucleation and elongation. The tubulin depolymerization assay tests the effects of compounds on microtubule elongation, which mainly depends on the dynamics of the plus ends of microtubules; whereas the de novo tubulin polymerization process requires microtubule nucleation, which involves the formation of new polymer ends (tubulin nuclei); this process is kinetically limiting (15). Therefore, SAMC might inhibit both microtubule nucleation and elongation, whereas DADS might have a more specific inhibitory effect on the formation of tubulin nuclei and the dynamics of the minus ends. Further studies are required to characterize the precise mechanism of binding of these compounds to tubulin and to elucidate the mechanisms by which they differentially affect microtubule nucleation.

**Effects of DADS and Trityl-Cys on Microtubule Assembly and Spindle Formation in Intact Cells**

To extend the above in vitro findings related to microtubule polymerization, we did indirect immunofluorescent microscopy to observe the effects of these compounds on interphase microtubules and mitotic spindles in cultured cells. For these studies, we utilized SW480 human colon cancer cells as well as NIH3T3 mouse fibroblasts because the latter cell line provides a well-characterized model system for examining microtubules (16). The IC₅₀ values for growth inhibition of these compounds on NIH3T3 cells are similar to those obtained with SW480 cells (data not shown). Because Tyr-tu represents the major form of microtubule tubulin in cells and stable microtubules are enriched in Glu-tu (9, 17), we used a Tyr-tu

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**Figure 2.** In vitro effects of SAMC, DADS, and trityl-cys on tubulin polymerization. A, pure tubulin (5 mg/mL) was incubated at 37°C for 30 min, and then the indicated compounds were added. A₃₄₀ values were recorded once per minute for an additional 30 min. B, pure tubulin (5 mg/mL), together with the indicated compounds added at time zero, was incubated at 37°C for 40 min. The process of tubulin polymerization was monitored by recording changes in the A₃₄₀ value. The results are presented as percentage absorbance, with 100% representing the A₃₄₀ value at 30 min when tubulin polymerization had reached a steady state. Values on the right indicate percentage absorbance for each sample at 60 or 40 min. For additional details, see Materials and Methods. These experiments were repeated and gave similar results.
antibody to observe the total microtubule-polymer mass and the fine microtubule structure and a Glu-tu antibody to assess microtubule stability. We previously reported that SAMC (150 μmol/L) caused depolymerization of microtubules in interphase NIH3T3 cells and induced aberrant spindle formation in mitotic cells (7). In the present study, we found that when NIH3T3 cells were treated with 56 μmol/L DADS (the IC50 concentration), there was no significant change of Tyr-tu staining in interphase cells, indicating no significant change in the total microtubule mass in these cells, but the microtubule cytoskeleton network was disorganized (Fig. 3B) when compared with the control cells (Fig. 3A). In addition, the staining of Glu-tu was increased in these cells (Fig. 3B'), indicating that DADS kinetically stabilized the microtubules, or, in another words, suppressed microtubule dynamics. DADS-treated (56 μmol/L) mitotic cells displayed normal spindles. However, “lagging” chromosomes were observed in some of the mitotic cells (Fig. 3B', solid arrow). These chromosomes are delayed or unable to achieve a bipolar attachment to the spindle and, therefore, remain at the spindle pole when most chromosomes are already arranged in the midplate at metaphase. Their presence prevents the transition to anaphase. Therefore, these cells remain blocked in prometaphase and metaphase (18). Indeed, we found an increase of cells in the prometaphase and metaphase in cultures treated with DADS (data not shown). Some of these cells can eventually exit mitosis, often aberrantly, and micronuclei are then found in the daughter cells (18). Examples of these micronuclei are shown in Fig. 3B (dashed arrows). These effects probably explain the dramatic increase of multinucleated cells after treatment with DADS (56 μmol/L; from 2% to 26%, data not shown). Treatment with 112 μmol/L DADS (2 × IC50) caused marked microtubule depolymerization in interphase cells (Fig. 3C). The staining of Glu-tu was weak (Fig. 3C'), apparently due to the depolymerization of both dynamic and stable microtubules. In addition, all of the mitotic cells were arrested at the prophase and prometaphase due to failure of mitotic spindle formation (Fig. 3C').

By contrast to effects of DADS, trityl-cys did not depolymerize or stabilize microtubules in interphase cells, even when tested at 5 × IC50 concentration (4.5 μmol/L; Fig. 3E and E'). However, treatment with 0.9 μmol/L trityl-cys induced multipolar spindles in some of the mitotic cells (Fig. 3D') and treatment with 4.5 μmol/L trityl-cys induced monopolar spindles in most of the mitotic cells, with one spindle pole enclosed in a spherical arrangement of chromosomes (Fig. 3E'; ref. 19). It also induced micronuclei formation at 4.5 μmol/L (Fig. 3E, dashed arrow), but to a much lesser extent than that caused by 56 μmol/L DADS.

Figure 3. In vivo effects of DADS and trityl-cys on interphase microtubules and mitotic spindles in NIH3T3 cells. Cells were treated with DMSO (control, A–A'), 56 μmol/L DADS (B–B'), 112 μmol/L DADS (C–C'), 0.9 μmol/L, trityl-cys (D–D'), or 4.5 μmol/L trityl-cys (E–E') for 20 h, fixed with methanol, and immunostained for Tyr-tu (green), Glu-tu (red), and DNA (blue), and visualized by immunofluorescent microscopy. Cells in the top panel (A–E) are interphase cells, showing the staining of Tyr-tu and DNA. Cells in the middle panel (A'–E') are the same cells as in the top panel, but show staining of Glu-tu. Cells in the bottom panel (A''–E'') are mitotic cells and indicate staining of Tyr-tu and DNA. Dashed arrow, micronuclei in interphase cells; solid arrow, lagging chromosomes in mitotic cells.
Taken together, these findings suggest that at a low concentration (IC₅₀), DADS exerts its antiproliferative effects, at least in part, by suppression of microtubule-spindle dynamics, thus interfering with the normal function of mitotic spindles, whereas at a higher concentration (2 × IC₅₀) it can impair microtubule polymerization and inhibit mitotic spindle formation. On the other hand, trityl-cys exerts its effect on M-phase arrest and growth inhibition by mechanisms that involve spindle impairment but do not involve disruption of microtubule structure or dynamics. Studies with SW480 cells indicate that these compounds produced effects on interphase microtubules and mitotic spindles similar to those described above with NIH3T3 cells (data not shown).

Unlike SAMC, DADS and Trityl-Cys Do Not Induce JNK1 Activation

To study the signaling pathways involved in apoptosis induced by these organosulfur compounds, we first investigated JNK1 because this kinase plays an important role in mediating the apoptosis induced by some antimitotic drugs (20, 21). We previously reported that treatment of SW480 cells with SAMC caused a rapid and sustained activation of JNK1 and that this activation seems to play an important role in the early phase of apoptosis induced by SAMC (7). However, in the present study, we found that treatment with the 2 × IC₅₀ concentration of DADS (112 µmol/L) or trityl-cys (1.8 µmol/L) for the indicated times, and cell lysates were fractionated to obtain the cytosolic and mitochondrial fractions. Cell fractions were analyzed by Western blotting using an anti–cytochrome c antibody. An antibody to actin was used as a loading control. 

Figure 4. Effects of SAMC, DADS, and trityl-cys on signal transduction pathways in SW480 cells. A, JNK1 activity. Cells were treated with UV radiation (positive control), DADS (112 µmol/L), or trityl-cys (1.8 µmol/L) for the indicated times, and JNK kinase assays were done as described in Materials and Methods. Activation fold is expressed relative to the control value obtained with untreated cell. B, loss of mitochondrial membrane potential. Cells were treated with SAMC (300 µmol/L), DADS (112 µmol/L), or trityl-cys (1.8 µmol/L) for 24 or 48 h. Cells were then harvested, stained with the JC-1 reagent, and analyzed by flow cytometry. The percentage of cells that have lost mitochondrial membrane potential was calculated. Data are expressed as means of three independent assays, *P < 0.05, comparing the indicated group to untreated control. C, release of cytochrome c. Cells were treated with SAMC (300 µmol/L), DADS (112 µmol/L), or trityl-cys (1.8 µmol/L) for the indicated times, and cell lysates were fractionated to obtain the cytosolic and mitochondrial fractions. Cell fractions were analyzed by Western blotting using an anti–cytochrome c antibody. An antibody to actin was used as a loading control. D, caspase-3 activation and PARP cleavage. Cells were treated with SAMC (300 µmol/L), DADS (112 µmol/L), or trityl-cys (1.8 µmol/L) for the indicated times. Cells were then harvested and cell extracts were analyzed by Western blotting using antibodies specific for cleaved caspase-3 (activated form) and cleaved PARP. An antibody to actin was used as a loading control. All experiments were repeated and yielded similar results.
space into the cytoplasmic compartment. This triggers activation of downstream events that lead to apoptosis (22). Loss of mitochondrial membrane potential provides an early indication of the initiation of cellular apoptosis, and is a marker for commitment to programmed cell death (23). Using a JC-1 mitochondrial membrane potential detection kit, we measured cellular mitochondrial membrane potential in SW480 cells after treatment with SAMC (300 μmol/L), DADS (112 μmol/L), or trityl-cys (1.8 μmol/L). We found that SAMC strongly induced loss of mitochondrial membrane potential. The percentage of cells with loss of mitochondrial membrane potential increased from 7% in control cells to 17% and 34% after treatment with SAMC for 24 and 48 hours, respectively (Fig. 4B). DADS induced the loss of mitochondrial membrane potential to a similar extent as SAMC. However, trityl-cys did not induce loss of mitochondrial membrane potential at 24 hours, and at 48 hours caused only a modest increase in cells with induced loss of mitochondrial membrane potential (from 9% to 16%).

**SAMC and DADS, but not Trityl-Cys, Induce Release of Cytochrome c**

To further examine the role of mitochondria in induction of apoptosis by these compounds, we then measured the extent of mitochondrial release of cytochrome c using a subcellular fractionation method. As shown in Fig. 4C, treatment of SW480 cells with SAMC (300 μmol/L) induced release of cytochrome c at 24 hours, and to a greater extent at 48 hours. DADS (112 μmol/L) also strongly induced the release of cytochrome c from mitochondria to the cytosol, as evidenced by the significant increase of cytochrome c in the cytosolic fraction at 24 and 48 hours and a corresponding decrease of cytochrome c in the mitochondrial fraction at 24 hours. However, trityl-cys (1.8 μmol/L) did not induce an increase of cytochrome c in the cytosolic fraction (Fig. 4C). Taken together with the data on loss of mitochondrial membrane potential, these results suggest that the mitochondrial pathway plays an important role in the apoptosis induced by SAMC and DADS, but seems to be less important in the action of trityl-cys.

**All Three Compounds Induce Caspase-3 Activation and PARP Cleavage**

Caspases are central components in the induction of apoptosis by various agents. They are activated from proforms to functional forms by partial cleavage events. Caspase-3, a prevalent caspase, is ultimately responsible for the majority of apoptotic processes. It causes the cleavage or degradation of several important substrates, including PARP (22, 24). Therefore, we examined the effects of SAMC, DADS, and trityl-cys on caspase-3 activation and PARP cleavage by Western blotting, using specific antibodies that recognize cleaved and activated forms of caspase-3 and cleaved PARP. We found that treatment of SW480 cells with 300 μmol/L SAMC, 112 μmol/L DADS, or 1.8 μmol/L trityl-cys induced activation of caspase-3 as well as its endogenous substrate PARP within 24 to 48 hours (Fig. 4D). These results suggest that despite apparent differences in the role of mitochondria, activation of caspase-3 and PARP may play a role in the process of apoptosis induced by all three compounds. However, the effect of trityl-cys on caspase-3 activation was weak compared with that obtained with SAMC or DADS (Fig. 4D), indicating the presence of a caspase-independent pathway that also leads to PARP cleavage and apoptosis after cells are treated with trityl-cys.

**Discussion**

Previous studies from our laboratory revealed that the garlic-derived water-soluble allyl compound SAMC exerted antiproliferative effects on several human colon cancer cell lines. However, similar effects were not observed with the structurally related compound SAC (6, 7). To further explore structure-function relationships in this chemical class of agents, in the present study we investigated the effects of a series of garlic-derived and other organosulfur compounds on growth inhibition, cell cycle progression, and induction of apoptosis in SW480 human colon cancer cells. As summarized in Table 1, the allyl disulfide compounds SAMC and DADS inhibited cell growth, induced apoptosis, and arrested the cell cycle at G2-M, whereas their allyl monosulfide analogues SAC and DAS were devoid of these effects. These results suggest that the disulfide moiety plays a critical role in the biological effects of SAMC and DADS. Nevertheless, the presence of a disulfide moiety per se is insufficient for exerting the aforementioned effects because cystine and oxidized glutathione did not exert these effects (Table 1). Therefore, both the allyl and the disulfide moieties are important for the activities of SAMC and DADS.

Data from other laboratories also indicate that garlic-derived organosulfur compounds differ in their capacity to inhibit cell growth and that efficacy depends on the presence of both the allyl and disulfide moieties. Sundaram et al. (25, 26) reported that DADS was effective in inhibiting the growth of human colon, skin, and lung cancer cells, whereas its saturated analogue dipropyl disulfide was not active. Their studies also found that SAC was not effective, suggesting that the disulfide component is essential, which is consistent with our findings. The differential anticancer effects of allyl- and propyl-containing organosulfur compounds have also been examined in animal studies. Diallyl trisulfide, DADS, and DAS inhibited benzo(a)pyrene-induced forestomach and lung cancers and stimulated glutathione S-transferase activity in the forestomach, colon, and liver of mice, whereas their saturated analogues dipropyl disulfide and dipropyl sulfide did not (27–30). We should emphasize that the present studies are confined to cell culture systems. However, further studies are required to determine whether differences in the in vivo metabolism of these organosulfur compounds may explain the different biological effects (31–33).
There is limited pharmacokinetic data on garlic-derived organosulfur compounds in rodents and humans, especially with respect to tissue distribution and tumor uptake. Oral administration of garlic-derived allyl sulfides can inhibit carcinogenesis in various animal models (3, 4), thus indicating the bioavailability of these compounds. After a single p.o. dose of 200 mg/kg DADS to rats, the compound was rapidly absorbed and extensively metabolized. Oxidative metabolites of DADS were found in the stomach, liver, plasma, and urine. The maximum plasma concentrations of some of the metabolites can be as high as 300 to 1,400 μmol/L (34). The maximum plasma concentration of SAC has also been reported to be >200 μmol/L after p.o. administration of a 50 mg/kg dose to rats (35). Therefore, the organosulfur compound concentrations we used in our cell culture studies are comparable with those seen in the serum of animals and to those used in previous cell culture assays (36). It is also possible that after p.o. consumption, organosulfur compounds might be concentrated in the intestinal mucosa (37), thus enhancing their effects on colon cancer prevention.

The molecular mechanisms by which SAMC and DADS produce their antiproliferative effects seem to be similar, but not identical. We previously reported that the mitotic arrest and induction of apoptosis caused by SAMC are associated with marked effects on microtubules. Therefore, SAMC seems to function as an antitubulin agent (7). In the present study, we obtained evidence that DADS also exerts its antiproliferative effects by targeting microtubules. It suppresses microtubule dynamics and interferes with spindle-microtubule functions at a low concentration (IC_{50}), and causes depolymerization of microtubules and inhibition of spindle formation at a higher concentration (2 × IC_{50}; Fig. 3). Based on these findings, we propose the following model for the action of these two compounds (Fig. 5). After

**Figure 5.** Hypothetical scheme depicting pathways by which the garlic-derived compounds SAMC and DADS and the synthetic organosulfur compound trityl-cys cause mitotic arrest and induce apoptosis in cells.
entering cells, both SAMC and DADS interact directly with tubulin to inhibit microtubule dynamics, thus interfering with the normal function of mitotic spindles and arresting cells at mitosis. This disruption of microtubule structures could also trigger signaling pathways that are regulated by microtubules, and thus lead to apoptosis in the treated cells. In the case of SAMC, these events include activation of JNK1 (7). Presumably, DADS triggers other signaling pathways, which remain to be determined. Apparently, both SAMC and DADS induce apoptosis through the mitochrondrial pathway, as evidenced by the loss of mitochondrial membrane potential and the release of mitochondrial cytochrome c (Fig. 4B and C). Mitochondria play important roles in the apoptotic process induced by other microtubule-interfering agents (38, 39). Caspase-3 and PARP activation are also involved in the apoptosis induced by SAMC and DADS (Fig. 4D). We should, however, stress that our studies do not exclude the possibility that SAMC and DADS target cellular molecules in addition to microtubules and thereby trigger other signaling pathways that induce apoptosis (2). For example, SAMC might also induce a caspase-independent pathway, which we previously reported (7). DADS might also target mitotic checkpoint proteins, which could lead the cell into an aberrant mitotic exit and a G1-like multinucleated state (40). The latter process could contribute to the increased G2 phase and the increase in multinucleated cells we observed after treating cells with DADS (Fig. 3B).

A number of years ago, trityl-cys (NSC 83625) was found to be a potent inhibitor of cell growth when tested in the National Cancer Institute drug screening program. Its profile of activity against a panel of 60 malignant cell lines suggested that it has antimitic activity (41). However, mechanistic studies were not conducted. The present study indicates that trityl-cys is a very potent inhibitor of the growth of human colon cancer cells, with an IC50 value of 0.9 μmol/L, which is consistent with the original National Cancer Institute data. Furthermore, we found that this compound arrests cells at G2-M, predominantly at mitosis, which is consistent with the prediction in the National Cancer Institute study. Our studies indicate that, in contrast to SAMC and DADS, the M-phase arrest and spindle abnormalities caused by trityl-cys are not due to a direct effect on microtubules. During the preparation of this paper, other investigators reported that trityl-cys inhibited the subcellular activity of the human Eg5 protein, a member of the microtubule motor protein kinesin superfamily that is required for the formation of bipolar spindles during mitosis (42). The authors also found that even when tested at 100 μmol/L in HeLa cells, trityl-cys did not affect the normal microtubule network in interphase cells, yet it induced monopolar spindles and caused mitotic arrest (42). These results are consistent with those we obtained in SW480 and NIH3T3 cells, and indicate that trityl-cys specifically targets a spindle-related protein rather than tubulin to exert its effects on mitotic arrest and growth inhibition. Because trityl-cys has no detectable effects on interphase cells but specifically acts on mitotic cells, it may have high selectivity for more rapidly dividing cancer cells than normal cells and may, therefore, produce fewer side effects than known antitubulin drugs (43). Because trityl-cys only induced a moderate loss of mitochondrial membrane potential and did not cause release of cytochrome c (Fig. 4), we hypothesize that this compound induces apoptosis through a pathway other than the mitochondria-mediated caspase-dependent pathway. Presumably, this pathway leads to the PARP cleavage and apoptosis we observed in cells treated with trityl-cys. The caspase-independent pathway is of interest because of its reported role in mediating apoptosis following mitotic arrest induced by other agents (44–46). Additional studies are also required to determine whether trityl-cys has other critical cellular targets in addition to Eg5.

In summary, the present study provides the first evidence that DADS, a major oil-soluble constituent of garlic, exerts its antiproliferative effects, at least in part, by targeting microtubules. These results, coupled with our previous findings that another garlic-derived compound SAMC also acts as an antitubulin agent (7), are of interest because other microtubule-interfering agents have been found to be highly effective in cancer therapy (18). In addition, the present study reveals that an active allyl moiety linked to a disulfide moiety seems to play a critical role in the growth inhibition, G2-M cell cycle arrest, and induction of apoptosis produced by these two compounds. Therefore, allyl sulfides present in garlic or related synthetic organosulfur compounds may prove to be effective agents for cancer prevention and/or therapy. This structure-function correlation may also be useful in the identification and/or synthesis of novel anticancer compounds. Furthermore, the synthetic organosulfur compound trityl-cys is a highly potent inhibitor of the growth of colon cancer cells and causes mitotic arrest and induction of apoptosis via a novel mechanism. These findings suggest that this compound and structurally related compounds may provide a new and effective chemical class of anticancer agents.

References


Molecular Cancer Therapeutics

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