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Alkenyl group is responsible for the disruption of microtubule network formation in human colon cancer cell line HT-29 cells

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Alk(en)yl trisulfides (R-SSS-R′) are organosulfur compounds produced by crushed garlic and other *Allium* vegetables. We found that these compounds exhibit potent anticancer effects through the reaction with microtubules, causing cell cycle arrest. Nine alk(en)yl trisulfides including dimethyl trisulfide, diethyl trisulfide, dipropyl trisulfide (DPTS), dibutyl trisulfide, dipentyl trisulfide, diallyl trisulfide (DATS), dibutenyl trisulfide, dipentenyl trisulfide and allyl methyl trisulfide were synthesized and added to cultures of HT-29 human colon cancer cells at a concentration of 10 μM. The trisulfides with alkenyl groups such as DATS, but not those with alkyl groups, induced rapid microtubule disassembly at 30–60 min as well as cell cycle arrest during the mitotic phase approximately at 4 h after the treatment. Both DATS-induced microtubule disassembly and the cell cycle arrest were cancelled by the simultaneous treatment of the cancer cells with 2 mM L-cysteine, glutathione (GSH) or *N*-acetyl-L-cysteine. Reciprocally, L-buthionine-(S,R)-sulfoximine (500 μM), an inhibitor of GSH synthesis, enhanced the power of DATS in inducing the cell cycle arrest. These results indicate that alk(en)yl trisulfide react with sulfhydryl groups in cysteine residues of cellular proteins such as microtubule proteins. Thus, the present study provides evidence that trisulfides with alkenyl groups have potent anticancer activities, at least in part, directed toward microtubules. These findings suggest that alkenyl trisulfides and their structurally related compounds may provide novel and effective anticancer agents.

Introduction

Alk(en)yl trisulfides (R-SSS-R′) are organosulfur compounds produced by crushed garlic and other *Allium* vegetables. These compounds have been shown to exhibit a significant protective effect against cancer in experimental carcinogenesis models (1). Recent studies revealed that these compounds can suppress the proliferation of a variety of cancer cell lines and lead to apoptotic cell death (2–14).

Diallyl trisulfide (DATS) is a characteristic flavor produced by garlic, and contains three sulfur atoms with two allyl groups (CH_2=$CHCH_2$-SSS-CH_2CH=CH_2); and as we reported earlier (10), it causes growth inhibition, mitotic arrest and induction of apoptosis in both HCT-15 and DLD-1 human colon cancer cells through its microtubule-depolymerizing effect. It has been also reported that DATS generates reactive oxygen species in prostate cancer cells, but not normal prostate cell lines, and induces apoptosis of these cells without any affection to normal prostate cell lines (8,12,13). There are several studies suggesting that organosulfur compounds function as an antimicrotubule agent similarly to *Vinca* alkaloids. For example, Li *et al.* (14) reported that *Z*-ajoene, an organosulfur compound generated in oil-macerated crushed garlic, induced a complete disassembly of the microtubule network, and increased the number of cells arrested at the early mitotic stages. Xiao *et al.* (4,9) identified the key cellular events that lead to apoptosis as well as to microtubular depolymerization after the treatment of colon cancer cells with *S*-allylmercaptocysteine. However, there is little information as to the mechanisms underlying the induction of microtubule disruption by organosulfur compounds.

Our previous studies showed that DATS induced the microtubule disassembly through oxidative modification of specific cysteine residues present in the β-tubulin molecule (10). On the other hand, neither diallyl monosulfide nor diallyl disulfide showed such effect. Thus, the number of sulfur atoms in allyl sulfides was thought to be an important factor in exhibiting their antiproliferative microtubule-disassembling activity. In this study, we further developed various structures of alk(en)yl trisulfides with different side chains to clarify the structural and functional relationships of these sulfides especially with respect to their reactivity with microtubule molecules and potency to cause cell cycle arrest. Our present findings indicate that the trisulfides with alkenyl groups such as DATS, but not those with alkyl groups such as dipropyl trisulfide (DPTS), induced rapid microtubule disassembly as well as cell cycle arrest during the mitotic phase in HT-29 human colon cancer cells. Both microtubule disassembly and cell cycle arrest induced by DATS were cancelled by the simultaneous treatment of the cancer cells with sulfhydryl reagents such as L-cysteine, glutathione (GSH) or *N*-acetyl-L-cysteine. L-buthionine-(S,R)-sulfoximine (BSO), an inhibitor of GSH synthesis, reciprocally enhanced DATS-induced cell cycle arrest during the G_2/M phase. These results indicate that the alk(en)yl trisulfide-induced microtubule disassembly and subsequent cell cycle arrest were caused by the reaction between alk(en)yl trisulfide and sulfhydryl groups in cysteine residues in cellular proteins such as those constituting microtubules.

Abbreviations: BSO, L-buthionine-(S,R)-sulfoximine; DATS, diallyl trisulfide; DPTS, dipropyl trisulfide; FBS, fetal bovine serum; GSH, glutathione; PBS, phosphate-buffered saline.

Materials and methods

Chemicals

Nine compounds of alk(en)yl trisulfides (dimethyl trisulfide, diethyl trisulfide, DPTS, dibutyl trisulfide, dipentyl trisulfide, DATS, dibutenyl trisulfide, dipentenyl trisulfide and allyl methyl trisulfide) were synthesized by the method of Milligan *et al.* (15). Alk(en)yl trisulfides were purified by high-performance liquid chromatography (Alliance 2695 system, Waters Co., Milford, MA) on an Inertsil ODS-3 column (6 × 250 mm, GL Science, Tokyo, Japan). The molecular mass of the alk(en)yl trisulfides was determined by using an Agilent 6890/5973N GC/MSD System (Agilent Technologies, Santa Clara, CA). L-cysteine, GSH, *N*-acetyl-L-cysteine and BSO were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Cell culture and treatment with alk(en)yl trisulfides

Human colon adenocarcinoma cell line HT-29, obtained from the American Type Culture Collection, Manassas, VA, was grown and maintained in McCoy's 5A medium (Sigma–Aldrich Co., St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) at 37°C in 95% air and 5% CO_2. The cells were precultured in 10% FBS containing McCoy's 5A medium for 48 h. Alk(en)yl trisulfides were prepared in dimethyl sulfoxide solution at a concentration of 5 mM and then added to fresh medium to give

a concentration of 10 μM. Dimethyl sulfoxide was also added to control culture medium to give a final concentration, 0.2%, which was equivalent to that of alk(en)yl trisulfides in the medium.

Indirect immunofluorescence microscopy of β-tubulin

Cells were inoculated on glass slides coated with type I collagen (Cellmatrix type I-C, Nitta Gelatin Inc., Osaka, Japan), precultured for 48 h and then incubated with a given alk(en)yl trisulfide (10 μM) for 30–120 min or 12 h. The glass slides were washed twice with phosphate-buffered saline (PBS), and the cells were fixed with acetone:methanol (1:1) for 2 min at room temperature. After having been washed with PBS, the fixed cells were incubated with mouse anti-β-tubulin monoclonal antibody (TUB2.1, Sigma–Aldrich Co.) for 30 min at room temperature, after which they were incubated with Alexa Fluor 488-labeled goat anti-mouse IgG antibody (Invitrogen Co., Carlsbad, CA) for 30 min. The nuclei were stained with propidium iodide (Sigma–Aldrich Co.). The specific fluorescence was observed with a confocal microscope (FLUOVIEW FV300; Olympus Co., Tokyo, Japan).

Cell cycle analysis

The cell cycle distribution of HT-29 cells was measured by flow cytometry. The cells were incubated with 10 μM alk(en)yl trisulfides as described above. After two washes with PBS, the cells were harvested by trypsinization (~5×10^5 cells) and fixed overnight with ice-cold 70% ethanol at −20°C. They were then washed twice with PBS prior to being incubated with 500 μg/ml RNase A (Sigma–Aldrich Co.) and 25 μg/ml propidium iodide (Sigma–Aldrich Co.) at room temperature for 20 min. Then, the cell cycle was analyzed by using a flow cytometer, FACSCanto (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR).

Tubulin polymerization assay

Tubulin was purified from pig brain by use of a phosphocellulose column and dissolved in 1,4-piperazinediethane-sulfonic acid buffer (1.5 mg tubulin/ml of 80 mM 1,4-piperazinediethane-sulfonic acid, pH 6.8, 1 mM $MgCl_2$, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 10% glycerol and 1 mM guanosine-5′-triphosphate). The tubulin solution was then placed in a thermostatically controlled cuvette at 4°C for 10 min in the presence or absence of 1 μM DATS or DPTS. In order to initiate tubulin polymerization, the reaction mixture was warmed at 37°C. The tubulin polymerization was monitored by measuring the increase in the absorbance at 340 nm.

Cell growth inhibition assay

HT-29 cells were precultured for 48 h and then exposed to either DATS or DPTS for 16 h. The cell numbers were thereafter counted with a hemocytometer, and the growth inhibition rate was plotted to determine the IC_{50} value, which stands for the drug concentration necessary for reducing cell proliferation by 50%.

Flow cytometric analysis of phosphohistone H3 (pSer10)

Phosphorylation of histone H3 at its serine 10 is causally linked to chromosome condensation and segregation and is required for proper chromosome dynamics and chromosome condensation (16,17). Thus, the phosphorylation of histone H3 at serine 10 (pSer10) is used as a sensitive marker for mitotic cells (18). For the analysis of pSer10, the cells were washed twice with PBS and then fixed with ice-cold 70% ethanol at −20°C overnight. The fixed cells (2×10^5 cells) were washed in PBS and treated with a permeabilization buffer (PBS with 1% FBS and 0.25% Triton X-100) for 5 min on ice. The permeabilized cells were washed with the wash buffer (PBS with 1% FBS), resuspended in the same buffer containing monoclonal antiphosphorylated histone H3 (phosphoserine 10, H3-P, Sigma–Aldrich Co.) and then incubated for 60 min at room temperature. The cells were washed twice with the wash buffer, resuspended in the same buffer containing Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen Co.) and then incubated for 30 min at room temperature in the dark. After the cells had been washed with the wash buffer, the cells were stained at room temperature for 20 min with 10 μg/ml propidium iodide solution containing 500 μg/ml RNase A. The cells were finally subjected to flow cytometric analysis as described above.

Statistical analysis

In Figures 3A and 5B, one-way analysis of variance was employed and used to evaluate the difference among multiple groups. If significance was obtained between the groups, Dunnett's multiple comparison test (Figure 3A) or Tukey–Kramer multiple comparison test (Figure 5B) was used to compare the means between specific groups. The differences were considered statistically significant at $P < 0.01$. Statistical analysis was performed using statistical package GraphPad Prism software (GraphPad Software, San Diego, CA).

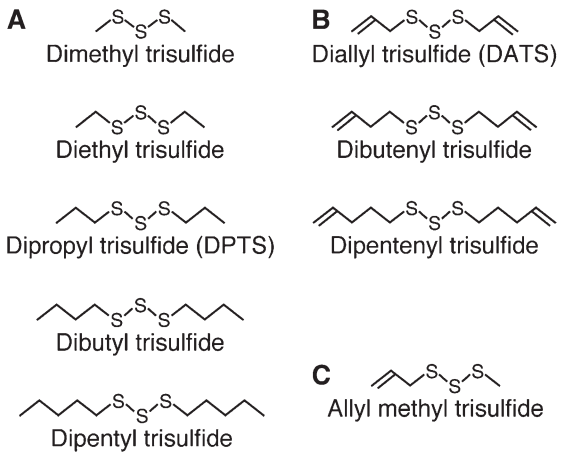


Fig. 1. Chemical structures of alk(en)yl trisulfides used in this study. These compounds can be divided into three groups: (**A**) alkyl trisulfides, (**B**) alkenyl trisulfides and (**C**) mixed alk(en)yl trisulfides.

Results

Alk(en)yl trisulfides disturb microtubule network formation in HT-29 human colon cancer cells

We have shown previously that DATS treatment caused to disarrange the microtubule structures by acting as a microtubule-depolymerizing agent and to induce cell cycle arrest during the G_2/M phase (10). In this study, to determine the effects of alkenyl or alkyl trisulfides on microtubule formation, we synthesized and purified nine kinds of alk(en)yl trisulfides (Figure 1). Figure 2 shows the effect of these trisulfides on microtubules in HT-29 human colon cancer cells. The normal microtubule distribution and its network formation were observed in the cytoplasm of untreated control HT-29 cells, as shown in Figure 2A. On the contrary, treatment of HT-29 cells with the series of alkenyl trisulfides caused clear disarrangement of the microtubular network, represented as a smeared image due to disassemble of the fibrous network of microtubules (Figure 2C, arrowheads). Such a change could be seen at 30 min (DATS and dibutenyl trisulfide) or at 60 min (dipentenyl trisulfide). There was no obvious change in the microtubule network of the cells treated with the series of alkyl trisulfides (Figure 2B). Among these alkenyl compounds, only DPTS gave an abnormal smeared image of the microtubule network at 120 min after the addition of the agent (Figure 2B, DPTS 120 min, arrowhead). Allyl methyl trisulfide, an asymmetric alk(en)yl trisulfide, also gave similar results to those from DATS (Figure 2D, arrowhead). These disruptions of tubulin structure observed in the DPTS- or allyl methyl trisulfide-treated HT-29 cells were transient and the normal tubulin structures were observed in the cells at 12 h after the treatment (data not shown). We next examined the effect of DATS and DPTS on tubulin polymerization in a cell-free system *in vitro* (Figure 2E). Polymerization of phosphocellulose-purified tubulin was measured as an increase in turbidity (absorbance at 340 nm) in the presence of glycerol and guanosine-5′-triphosphate. The preincubation of tubulin with DATS (1 μM) inhibited microtubule polymerization (Figure 2E, closed squares). DPTS (1 μM) showed weaker effect on the microtubule polymerization than DATS (Figure 2E, triangles). These results indicate that the microtubule network disarranging effect of trisulfide compounds was dependent on their side alk(en)yl chains, i.e. alkenyl structures, but not alkyl ones, preferentially disrupted the microtubule network formation.

Alk(en)yl trisulfides arrest the cell cycle at G_2/M phase

Since the alkenyl trisulfides acted on the HT-29 cells to cause their microtubular disarrangement, we next analyzed their effect on the cell cycle progression of these cells. The cells were cultured for 12 h in the presence or absence of a given 10 μM alkyl or alkenyl trisulfides and then analyzed with a flow cytometer. Vehicle-treated control cells

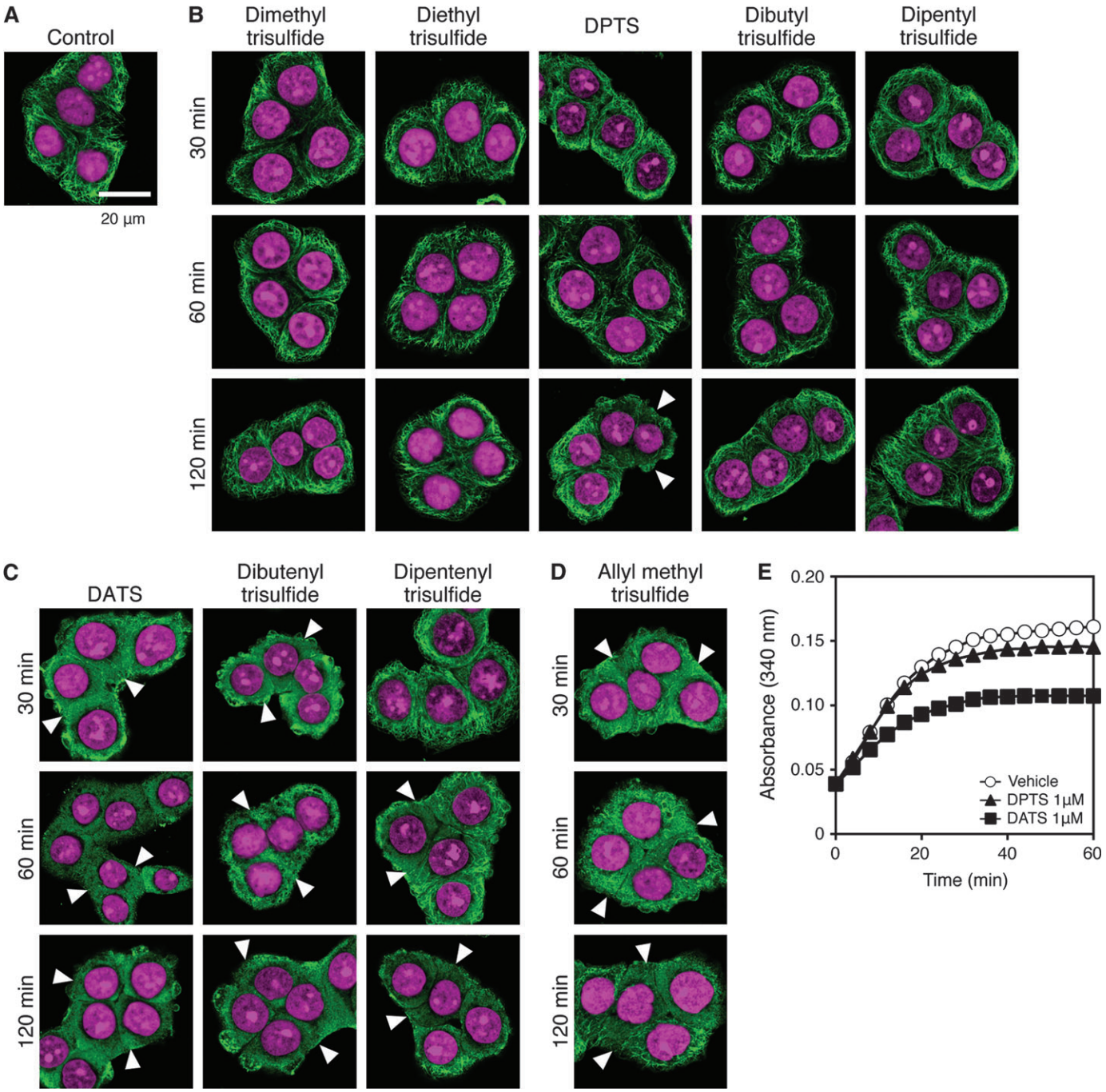
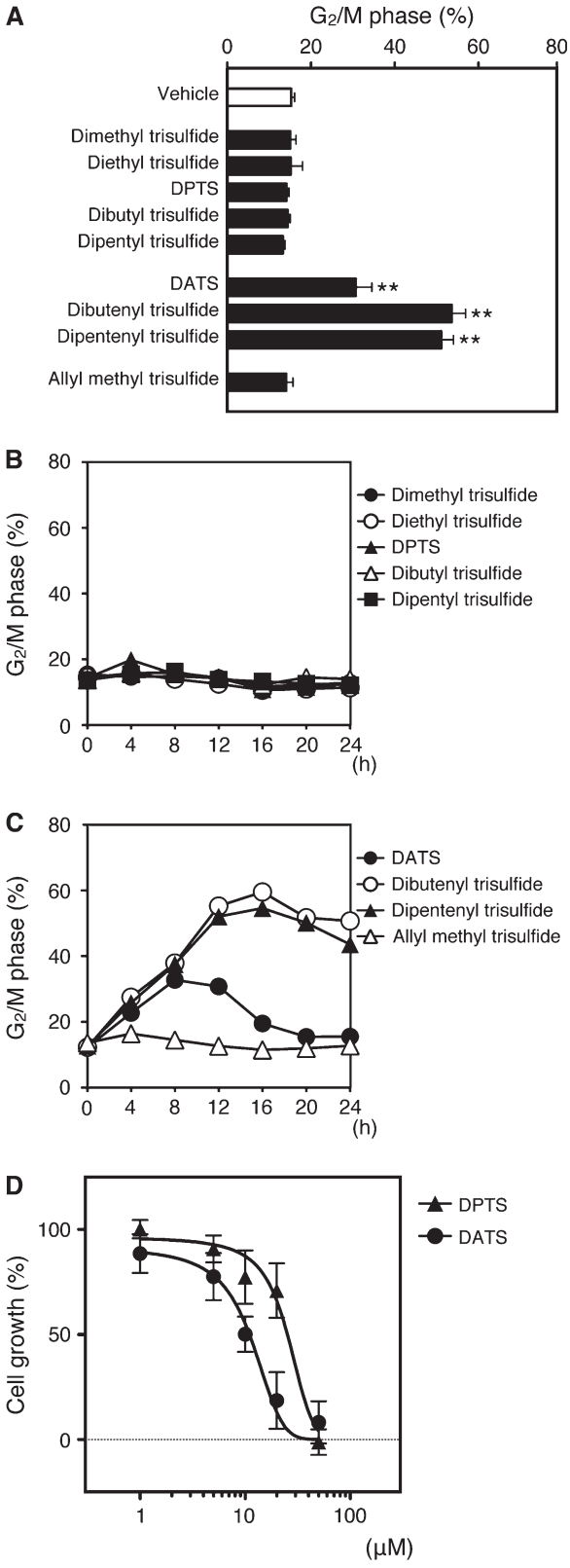


Fig. 2. Effects of alk(en)yl trisulfides on microtubule formation. HT-29 cells were treated with alkyl trisulfides (panel **B**), alkenyl trisulfides (panel **C**) or mixed alk(en)yl trisulfides (panel **D**) for the times as indicated. Panel **A** shows untreated control cells. Morphology of the microtubules formed in the cells was observed by the indirect immunofluorescence method using anti-β-tubulin antibody, as described in the text. Scale bar 20 µm. Panel **E** shows assembly of phosphocellulose-purified tubulin measured by turbidity assay, in which tubulin (1.5 mg/ml) mixed with 1 µM DATS or DPTS at 4°C was warmed at 37°C to initiate the polymerization. The increase in absorbance was monitored at 340 nm.

growing naturally or asynchronously showed a typical cell cycle distribution, i.e. 41.7 ± 0.4% in G_1 phase, 44.2 ± 0.7% in S phase and 13.2 ± 0.9% in G_2/M phase. The presence of alkyl trisulfides did not significantly affect the cell cycle, which maintained the distribution of its G_2/M phase with rates between 13 and 16% of all the cells. On the other hand, alkenyl trisulfides markedly increased the G_2/M phase arrest as compared with vehicle-treated cells ($P < 0.01$). The trisulfide having both alkyl and alkenyl groups in the same molecule, i.e. allyl methyl trisulfide, did not change the G_2/M phase cells percent. We next examined the time course of the changes in G_2/M phase distribution after treatment with 10 µM alk(en)yl trisulfides (Figure 3B and C). The cell cycle arresting effects of alkenyl trisulfides such as DATS, dibutenyl trisulfide and dipentenyl trisulfide were markedly different during the time course for up to 24 h, i.e. the population at the G_2/M phase increased as the culture time with the alkenyl trisulfides progressed (Figure 3C). DATS treatment reached maximum at 8–12 h and then gradually returned to the initial pattern (Figure 3C, closed circles). The population of the cells at G_2/M phase in dibutenyl or dipentenyl trisulfide-treated cultures reached maximum at 16 h and then slightly decreased (Figure 3C, open circles or

closed triangles). The restoration of the cell cycle suggests that alkenyl trisulfide-induced cell cycle arrest was transient, implying the existence of sulfide clearance mechanisms in the cells. On the contrary, alkyl trisulfides such as dimethyl trisulfide, diethyl trisulfide, DPTS, dibutyl trisulfide and dipentyl trisulfide did not change the cell cycle pattern over the 24 h examined (Figure 3B).



The IC_{50} values for growth inhibition were calculated to be 11.0 ± 1.4 μM for DATS and 25.2 ± 3.5 μM for DPTS, showing the growth inhibitory activity of DPTS was weaker than that of DATS. Other alkenyl trisulfides also showed growth inhibition at low concentration (<10 μM) in comparison with alkyl trisulfides (data not shown). These growth-inhibiting potencies correlate with the potencies for microtubule disruption and the inhibition of tubulin polymerization. The trisulfides with alkenyl groups showed both properties (i) anti-microtubule and (ii) cell cycle arrest at G_2/M phase. Thus, the

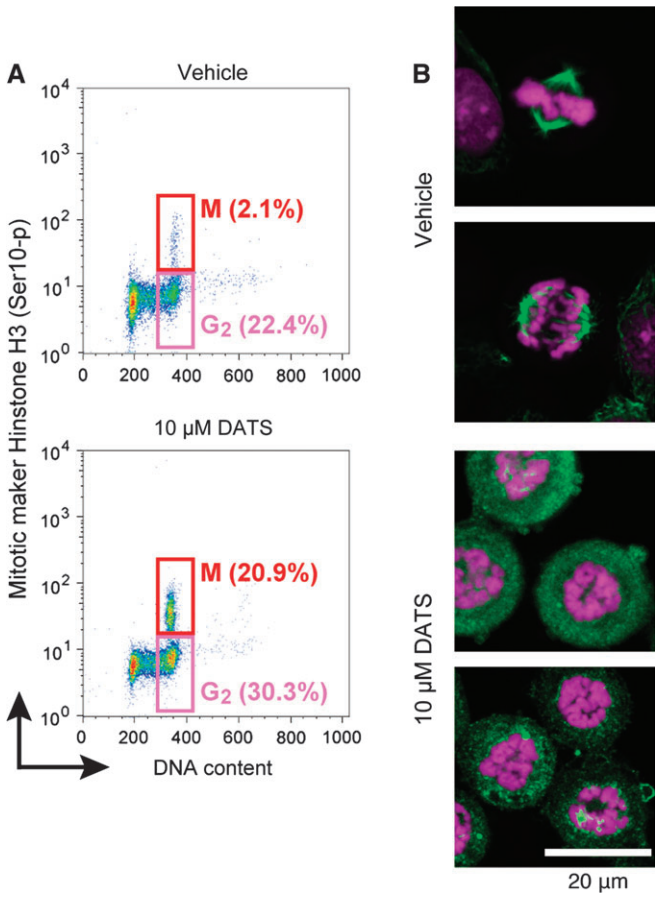


Fig. 4. Induction of mitotic arrest by DATS through the inhibition of mitotic spindle formation. Detection of Ser10-phosphorylated histone H3, a sensitive marker for cells at the M phase, was detected in HT-29 cells cultured with DATS (10 μM) for 12 h by using a flow cytometer (panel **A**). Spindle formation of HT-29 cells cultured with vehicle or DATS (10 μM) was assessed by the immunofluorescence method using anti-β-tubulin antibody, as described in Materials and Methods (green; panel **B**). The nucleus was counterstained with propidium iodide (magenta). Scale bar 20 μm.

Fig. 3. Cell cycle progression of HT-29 cells cultured in the presence of alk(en)yl trisulfides having various structures. HT-29 cells were cultured in the presence or absence of alkyl trisulfides (dimethyl trisulfide, diethyl trisulfide, DPTS, dibutyl trisulfide and dipentyl trisulfide), alkenyl trisulfides (DATS, dibutenyl trisulfide and dipentenyl trisulfide) or mixed ank(en)yl trisulfide (allyl methyl trisulfide) at a concentration of 10 μM for 12 h. Then, the cell cycle distribution in G_2/M phase was analyzed by using a flow cytometer, as described in Materials and Methods (panel **A**). Values are the mean ± SE of three independent experiments. ** ($P < 0.01$) represents the significant differences from value of control group (vehicle) determined by Dunnett's multiple comparison test. Time-course estimations of the effects of alkyl trisulfides or alkenyl trisulfides (10 μM) on the cell cycle progression are shown in panels **B** and **C**, respectively. Panel **D** shows growth inhibition curves for HT-29 cells cultured with DATS or DPTS. Data represent the average ± SEM of three experiments.

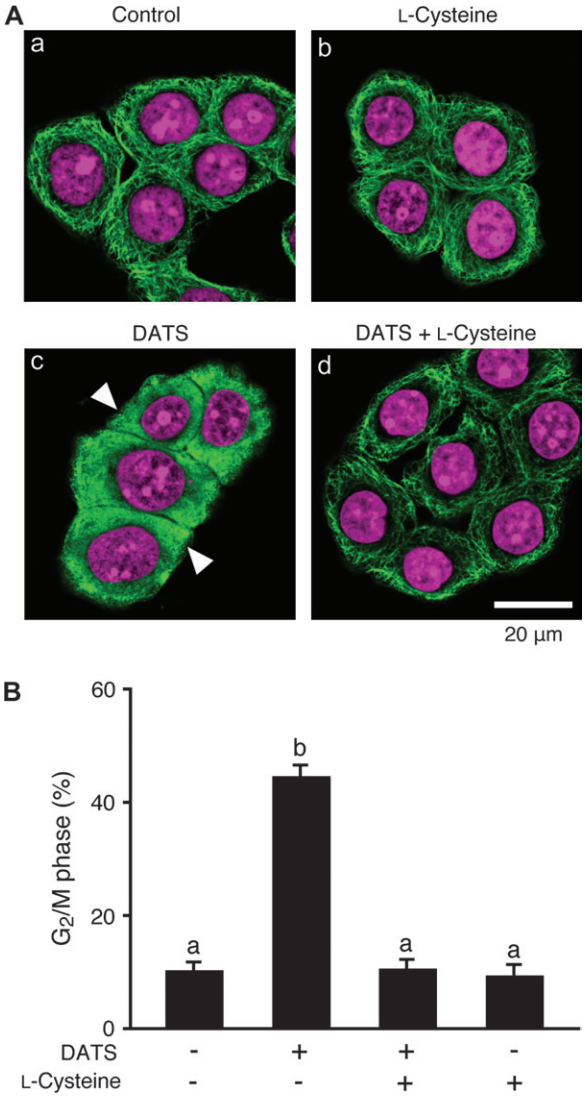


Fig. 5. DATS-induced microtubule disarrangement and cell cycle arrest are cancelled by the pretreatment of the cell with L-cysteine. Panel **A**: HT-29 cells were cultured with vehicle (**a**), 2 mM L-cysteine (**b**), 10 μM DATS (**c**) or both 10 μM DATS and 2 mM L-cysteine (**d**) for 12 h. The microtubule distribution of the cells was assessed by the immunofluorescence method using anti-β-tubulin antibody (green). The nucleus was counterstained with propidium iodide (magenta). Scale bar 20 μm. Panel **B**: HT-29 cells were preincubated with 2 mM L-cysteine for 2 h prior to the DATS treatment. Then, the cells were further incubated with or without 10 μM DATS for 12 h. The cell cycle distribution of the cells was analyzed by using a flow cytometer. Values are the mean ± SE of three independent experiments. The letters on the bars show the statistical relations and if different from each other, they differ with $P < 0.01$ in Tukey–Kramer's multiple comparison test.

microtubule protein is suspected to be one of the molecular targets of the alkenyl trisulfides.

Alkenyl trisulfides induced mitotic arrest by inhibiting mitotic spindle formation

As mentioned above, the alkenyl trisulfides affected the cell cycle and caused cell cycle arrest during the G_2/M phase. We next tried to clarify which phase was the real target of the sulfides, G_2 phase or M phase. Because ordinary flow cytometry based on the quantification of DNA content of cells does not distinguish between M phase cells and G_2-phase ones, we performed flow cytometry by measuring the level of a sensitive mitotic cell marker, phosphorylated Ser10 in histone H3 (18). The cells at G_2 phase have an unphosphorylated Ser10 residue and contain 4*n* DNA. On the other hand, the cells at M phase possess a phosphorylated Ser10 and contain 4*n* DNA. As shown in Figure 4A, the number of cells with phosphorylated Ser10 in their histone H3 was increased ~10-fold more in the culture with DATS for 12 h than in the culture without DATS (vehicle-treated cells). The mitotic phase consists of the stages of nuclear division (mitosis) and cytoplasmic division [cytokinesis (19)]. The segregation of the replicated chromosomes is brought about by a complex cytoskeletal machine, the so-called mitotic spindle. The upper two panels in Figure 4B show the mitotic spindle formation in the cells from metaphase to anaphase. DATS inhibited the spindle formation, and most cells had short microtubule fragments, which were scattered throughout the cytoplasm [Figure 4B, lower two panels (photos taken at the culture times comparable with those for the upper two panels)]. Since these DATS-treated HT-29 cells had no nuclear membrane, they were probably in the mitotic phase. Thus, the target was this phase, not G_2.

Disarrangement of microtubules and cell cycle arrest induced by trisulfide is completely blocked by L-cysteine

We reported previously that DATS-induced microtubule disarrangement was due to the microtubule depolymerization attributed to oxidative modification of specific cysteine residues in the β-tubulin molecule (10). So, we examined if the alkenyl trisulfide-induced microtubule disarrangement could be attenuated by coincubation of the cells with L-cysteine. The cells were precultured in medium containing 2 mM L-cysteine for 1 h and then 10 μM DATS was added; then they were cultured for 2 h. DATS-induced microtubule network depolymerization [Figure 5A(c), arrowhead] was completely cancelled by the pretreatment of the cells with L-cysteine[Figure 5A(d)]. Incubation of the cells with 10 μM DATS for 12 h caused ~4.5-fold increase in the percentage of cells at the G_2/M phase (44.6 ± 1.8% in G_2/M phase, Figure 5B). L-Cysteine also completely blocked the DATS-induced cell cycle arrest at the G_2/M phase (9.4 ± 1.7% by the coincubation with L-cysteine). These results strongly suggest that exogenous alkenyl trisulfides modify growth-relating proteins at their sulfhydryl groups and hamper their normal functions.

DATS-induced cell cycle arrest was sustained by the depletion of GSH

The effect of cellular GSH depletion on the DATS-induced cell cycle arrest was studied. BSO depletes cellular GSH by inhibiting the activity of γ-glutamylcysteine synthetase, and at the same time, it enhances the cytotoxic effects of DNA topoisomerase inhibitor or alkylating agents (20–24). The cells were incubated with or without 500 μM BSO for 24 h and then treated with 10 μM DATS or DPTS. The treatment of the cells with BSO alone did not show any effect on the cell cycle distribution (data not shown). Pretreatment of the cells with BSO enhanced the effect of DATS and sustained the cell cycle arrest during the G_2/M phase for a longer time than without BSO. The population of the cells during the G_2/M phase at 12 h was 59% in the cultures with both DATS and BSO and 42% in those with DATS alone (Figure 6A and C). On the other hand, BSO showed little effect on the size of the population of DPTS-treated cells during the G_2/M phase (Figure 6B and D). These results indicate that the depletion of cellular GSH enhanced the effect of alkenyl trisulfide on the cell cycle.

Discussion

Recent studies revealed that DATS, a garlic-derived allyl sulfide compound, has a potent antiproliferative effect on several human cancer cell lines (2,5,8,10–13). However, neither diallyl monosulfide nor diallyl disulfide showed such effect on the cells (10). As part of our basic study to determine the nature of more potent anticancer principles in garlic and its related organosulfur compounds, we compared the effect of nine kinds of alk(en)yl trisulfides on microtubule network formation as well as on cell cycle progression by using HT-29 human colon cancer cells. Among the alk(en)yl trisulfides, DATS, dibutenyl

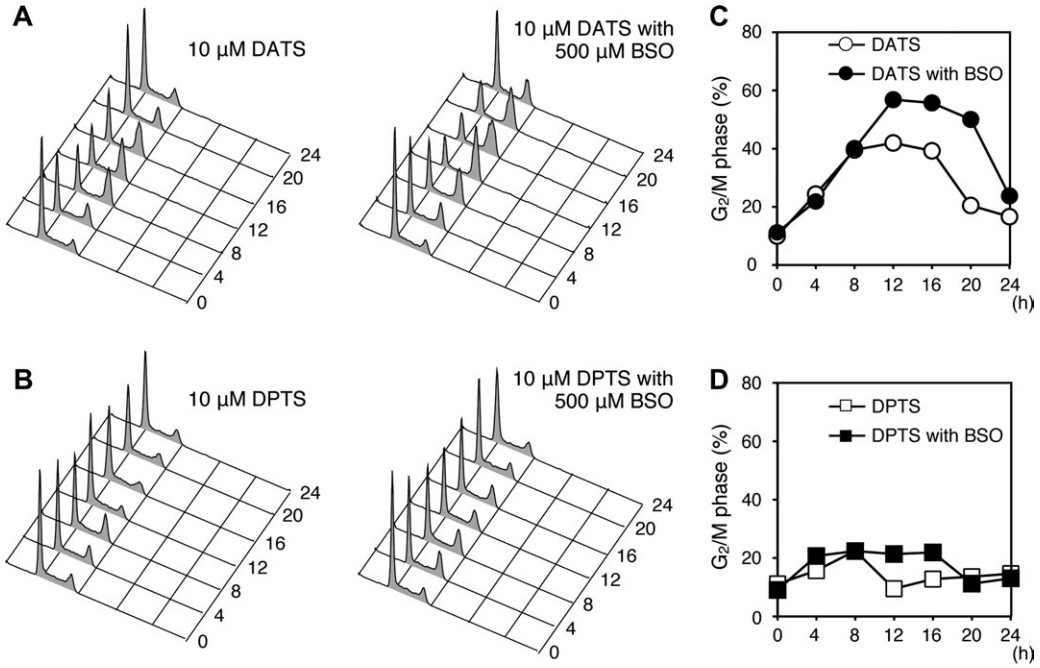


Fig. 6. BSO enhanced and sustained the DATS-induced cell cycle arrest but had little influence on DPTS-treated cells. HT-29 cells were pretreated with 500 μM BSO for 24 h and then the cells were treated with 10 μM DATS (**A** and **C**) or DPTS (**B** and **D**) for the times as indicated. The cell cycle distribution of the cells was analyzed by using a flow cytometer.

trisulfide and dipentenyl trisulfide, all unsaturated sulfide compounds, caused marked disruption of microtubules and prominent cell cycle arrest during the mitotic phase; whereas the saturated sulfide compounds showed very little effect (Figures 2 and 3). DPTS and allyl methyl trisulfide showed disruption of microtubule formation at 30–120 min after the treatment; however, they did not influence the cell cycle progression in the time-course study (Figure 3B and C). Both DPTS and allyl methyl trisulfide caused only a transient change in microtubule formation at 120 min and it recovered to the normal structure at 12 h (data not shown). Such transient effect of DPTS might be due to its lower reactivity with sulfhydryl group than that of DATS.

Other organosulfur compounds, *Z*-ajoene and *S*-allylmercaptocysteine, which are known to induce microtubule disarrangement, also have alkenyl structures (4,9,14). These results indicate that with respect to the side chain structures of trisulfide compounds, the two alkenyl groups, but not the alkyl group, play a critical role in both microtubule disassembly and cell cycle arrest. However, the mechanisms by which trisulfides cause the microtubule network disarrangement has not yet been clarified. In our previous study, DATS caused the sulfhydryl groups of tubulin molecules to undergo a thiol–disulfide exchange reaction, resulting in the dysfunction of these molecules (10). Thiol–disulfide exchange is a chemical reaction, in which a nucleophilic group attacks a sulfur atom of a disulfide bond (25). Cysteine contains a thiol group that exhibits nucleophilicity. Incubation of the combined solution of GSH (1 mM) and DATS (100 μM) at pH 7.4 for 10 min caused a thiol-disulfide exchange between the sulfhydryl group of GSH and DATS, and generated allyl mercaptan and *S*-allylmercaptoglutathione; however, DPTS (100 μM) did not undergo such a reaction. Taken together, our present data strongly suggest that the reactivity of alkenyl trisulfides toward the sulfhydryl group of endogenous structures depends on the side chain groups attached to the trisulfide moiety.

Since cysteine residues of proteins serve either structural or functional roles, modification of certain such residues greatly changes the function of proteins bearing these residues (26–28). This modification should be taken place with following three possible occasions: (i) the direct participation of the sulfhydryl moiety of cysteine in oxidation–reduction (redox) reactions, (ii) affinity for transition metal ions or (iii) its nucleophilic property and facile reaction with electrophiles. These properties may also be critically important for designing novel pharmacological agents (26). Oxidative stress, due to nitric oxide, oxidized GSH or hydrogen peroxide, may also modify specific proteins by the process of *S*-nitration and *S*-glutathiolation (29–31). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 has also been reported to covalently modify some cellular proteins such as nuclear factor-kappa B, AP-1, thioredoxin and tubulin (32–36). These reactions finally lead to protein–protein disulfide bond formation (37). Redox-sensitive proteins function in molecular chaperoning, translation, glycolysis, cytoskeletal structuring, cell growth and signal transduction (37). DATS-induced microtubule disarrangement and the resulting cell cycle arrest were completely cancelled by sulfhydryl agents (Figure 5). This protective effect was obtained not only with cysteine but also with other sulfhydryl group-containing agents such as GSH or *N*-acetyl-L-cysteine (DATS + GSH: 10.1 ± 1.7; DATS + *N*-acetyl-L-cysteine: 9.5 ± 1.3 versus vehicle in Figure 5). These data strongly suggest that some sulfhydryl modification of proteins including tubulin by alkenyl trisulfide is required for their effectiveness.

Reciprocally, the depletion of cellular GSH by BSO potentiated the effects of DATS on the cell cycle arrest (Figure 6). Similar results have been reported for arsenic trioxide (As_2O_3), i.e. BSO treatment enhances the arsenic trioxide-induced anticancer activities (38–40). As observed for DATS, cytotoxicity of arsenic trioxide is cancelled by the sulfhydryl group provided by GSH, cysteine or dithiothreitol (41). The cellular sulfhydryl-containing molecules might be a determinant of susceptibility of cancer cells to the alkenyl trisulfides.

In summary, the present study provides novel evidence that the trisulfides with unsaturated structures have potent anticancer activities, at least in part, by targeting microtubules. These findings suggest that alkenyl trisulfides and their structurally related compounds may be a novel and effective chemical class of anticancer agents.

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