Diallyl Trisulfide Suppresses the Proliferation and Induces Apoptosis of Human Colon Cancer Cells through Oxidative Modification of β-Tubulin*

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Allyl sulfides are characteristic flavor components obtained from garlic. These sulfides are thought to be responsible for their epidemiologically proven anticancer effect on garlic eaters. This study was aimed at clarifying the molecular basis of this anticancer effect of garlic by using human colon cancer cell lines HCT-15 and DLD-1. The growth of the cells was significantly suppressed by diallyl trisulfide (DATS, HCT-15 IC50 = 11.5 μM, DLD-1 IC50 = 13.3 μM); however, neither diallyl monosulfide nor diallyl disulfide showed such an effect. The proportion of HCT-15 and that of DLD-1 cells residing at the G1/S boundary revealed cell cycle-dependent induction of apoptosis through the transition of the G2/M phase to the G1 phase by DATS. Thus, DATS inhibited tubulin polymerization in an in vitro cell-free system. DATS disrupted microtubule network formation of the cells, and microtubule fragments could be seen at the interphase. Peptide mass mapping by liquid chromatography-tandem mass spectrometry analysis for DATS-treated tubulin demonstrated that there was a specific oxidative modification of cysteine residues Cys-12β and Cys-354β to form S-allylmercaptocysteine with a peptide mass increase of 72.1 Da. The potent antitumor activity of DATS was also demonstrated in nude mice bearing HCT-15 xenografts. This is the first paper describing intracellular target molecules directly modified by garlic components.

Allyl sulfides, e.g. diallyl monosulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS), are characteristic flavor components of the essential oil prepared from garlic (Allium sativum L.). Garlic is widely served around the world, and it has been reported that allyl sulfides inhibit both the initiation and promotion stages of tumorigenesis in experimental carcinogenesis models for various types of cancer (1–5). Recently, several lines of investigation have shown that allyl sulfides suppress cell growth and induce apoptosis in multiple cancer cell lines (6–12). We previously reported that the sulfur-containing volatile oils prepared from garlic and onion inhibit proliferation and induce differentiation of the human promyelocytic leukemia cell line HL-60 (13). However, the molecular mechanisms underlying the antitumorigenesis of allyl sulfides are still not fully understood.

Microtubules are ubiquitous proteins present in eukaryotes as components of the cytoskeleton and play pivotal roles in a variety of cellular processes involving cell division, motility, and intracellular trafficking (14). The microtubules are dynamic polymers composed of αβ-tubulin heterodimers, and they form the mitotic spindles, which are known to introduce the replicated DNA molecules to the respective daughter cell. Thus, antimitotic drugs developed for targeting microtubules have gained great success in cancer chemotherapy (15, 16). Various microtubule-interacting drugs, such as Vinca alkaloid and paclitaxel, cause mitotic arrest prior to the induction of apoptosis in tumor cells. It is quite natural that the suppression of spindle dynamics by these drugs hampers or completely blocks the mitosis of cells, especially at the transition from metaphase to anaphase.

This study was aimed at clarifying the molecular target of allyl sulfides to understand the anticancer mechanism elicited by the consumption of garlic. We show the structure-function relationship of allyl sulfides in the inhibition of human colon adenocarcinoma cell lines at first, and the changes in the cells caused by DATS, which include the disruption of spindle formation, sustainment of the cyclin B1 expression, mitotic arrest, and finally apoptosis. We also report for the first time that the direct modification of specific cysteine residues in β-tubulin molecules by DATS in vitro causes these events. Finally, we show the tumor growth inhibition by DATS in a xenograft mouse model in vivo.

MATERIALS AND METHODS

Cells and Chemicals—Human colon adenocarcinoma cell lines HCT-15 and DLD-1 (obtained from Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were grown and maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) at 37 °C in 95% air and 5% CO2. DAS and DADS were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). DATS was synthesized by using Bunte salt (17). DADS and commercial DAS were purified by high pressure liquid chromatography (Alliance 2695 system; Waters Co., Milford, MA) on an Inertsil ODS-3 column (6 mm × 250 mm; GL Science, Tokyo, Japan).

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4 The abbreviations used are: DAS, diallyl monosulfide; DADS, diallyl disulfide; DATS, diallyl trisulfide; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidomethyl]propyl]dimethylammonio]-1-propanesulfonic acid.
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Cell Proliferation Assay—HCT-15 and DLD-1 cells were precultured for 48 h and then exposed to DAS, DADS, or DATS for 24 h. The cells were then counted with a hemocytometer. The rate of growth inhibition was calculated based on the control culture, which was treated with vehicle only (0.1% MeSO), taken as 100% growth.

Cell Synchronization—HCT-15 and DLD-1 cells were synchronized at the G1/S boundary by the method known as the double thymidine-hydroxyurea block. The cells were presynchronized at the S phase by incubation with 2.5 mM thymidine for 12 h. Then they were released by changing the medium to the thymidine-free fresh medium and incubating them for 12 h, after which they were resynchronized at the G1/S transition point by incubation with 1 mM hydroxyurea for 12 h. The cells were then washed and incubated in the fresh medium to re-enter the cell cycle.

Cell Cycle Analysis—The cell cycle distribution of HCT-15 and DLD-1 cells was measured by flow cytometry. The harvested cells (~10^6 cells) were fixed with ice-cold 70% ethanol, treated with 500 μg/ml RNase A (Sigma), and subsequently stained with 25 μg/ml propidium iodide (Sigma). Then they were analyzed by using a flow cytometer FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (TreeStar Inc., Ashland, OR).

Western Blot Analysis of Cyclin B1—Total cellular protein (30 μg) was subjected to sodium dodecylsulfate-10% polyacrylamide gel electrophoresis. The proteins separated were electrically transferred to a cellulose nitrate membrane (Advantec Toyo, Tokyo, Japan) for immunoblot analysis. The blot was incubated with mouse anti-cyclin B1 monoclonal antibody (1:2000; Upstate Biotechnology, Inc., Lake Placid, NY) for 1 h and then incubated with horseradish peroxidase-labeled anti-mouse secondary antibody (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The membrane was developed by using a commercial kit (HRP-1000 Immunostain; Konica, Tokyo, Japan).

Fluorometric Assay of Caspase-3 Activity—Cell lysates containing 30 μg of protein were incubated for 60 min at 37 °C in reaction buffer (20 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 10% glycerol, and 1 mM GTP). The tubulin solution was then placed in a thermostatically controlled cuvette at 4 °C for 10 min in the presence or absence of DATS. 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 380 nm and emission at 460 nm.

Tubulin Polymerization Assay—Tubulin was purified from pig brain by use of a phosphocellulose column and dissolved in PIPES buffer (1.5 mg tubulin/ml of 80 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 10% glycerol, and 1 mM GTP). The tubulin solution was then placed in a thermostatically controlled cuvette at 4 °C for 10 min in the presence or absence of DATS. 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.

Indirect Immunofluorescence Microscopy—The cells were cultured on a Thermanox coverslip (Nalge Nunc International, Rochester, NY) and fixed with acetone/methanol (1:1) for 2 min at room temperature. After washing with phosphate-buffered saline, the fixed cells were incubated with mouse anti-β-tubulin monoclonal antibody (1:500; Sigma) for 30 min at room temperature, followed by incubation with Alexa Fluor 488 goat anti-mouse IgG antibody (1:500; Molecular Probes, Inc., Eugene, OR) for 30 min. The specific fluorescence was observed by a confocal microscope (Fluoview, Olympus, Tokyo, Japan).

High Performance Liquid Chromatography-Tandem Mass Spectrometry—Phosphocellulose-purified tubulin (1 mg/ml) was incubated at 37 °C for 60 min in the presence or absence of 100 μM DATS. The DATS-treated and native tubulin were digested with modified trypsin (Promega, Madison, WI) and analyzed by liquid chromatography-tandem mass spectrometry by using a MAGIC C18 column (0.15 mm × 50 mm; Michrom Biosources, Auburn, CA). The peptides were eluted over a 20-min period with a linear gradient 5–65% in terms of solvent B (from solvent A (2% (v/v) acetonitrile, 0.1% formic acid) to solvent B (90% (v/v) acetonitrile, 0.1% formic acid) with a flow rate of 0.8 μl/min. The tryptic peptide samples were separated and analyzed with a LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Tandem mass spectrometry data obtained were analyzed by using SEQUEST, a computer program that allows the correlation of experimental data with theoretical spectra generated from known protein sequences (18). In this work, the general criteria for a preliminary positive peptide identification for a doubly charged peptide were a correlation factor greater than 2.5, a delta cross-correlation factor greater than 0.1 (indicating a significant difference between the best match reported and the next best match), a high preliminary scoring, and a minimum of one tryptic peptide terminus. For triply charged peptides, the correlation factor threshold was set at 3.5. All of the matched peptides were confirmed by visual examination of the spectra. All of the spectra were searched against the data in FASTA format generated from pig α-tubulin (NCBI accession number P02550) and pig β-tubulin (NCBI accession number P02554) in the data base of National Center for Biotechnology Information.

Measurement of Cysteine Residues in Tubulin—The number of cysteine residues in the tubulin was determined by titrating the sulfhydryl group in tubulin with 5.5′-dithiobis-2-nitrobenzoic acid. Phosphocellulose-purified tubulin (0.1 mg/ml) was incubated with 10 μM DATS or vehicle at 25 °C for 20 min. After the incubation, tubulin samples were mixed with 5.5′-dithio-bis-2-nitrobenzoic acid (1 mM), and the absorbance at 412 nm was measured. The number of cysteine residues was calculated from the standard curve drawn by using cysteine (Wako Pure Chemical, Osaka, Japan).

Antitumor Effect of DATS on Mice Xenograft Model—All of the animal experiments were performed in accordance with the Guidelines for Animal Experiments of the College of Bioresource Sciences at Nihon University. Tumor xenografts were maintained by serial subcutaneous transplantation of 2 × 2 × 2-mm fragments of HCT-15 tumor into the right subaxillary region of 6-week-old female athymic CAnN.Cg-Foxn1nu/CrlCrlj mice (Charles River Laboratories Japan, Inc., Yokohama, Japan) on day 0. DATS (dissolved in 90% saline, 5% ethanol, and 5% Cremophore EL; Sigma, 6 mg/kg) or vehicle was injected into a tail vein on days 7, 10, 13, 16, 19, 22, and 25 (every 3 days, 7 doses in total). Body weight and tumor sizes were measured every 5 days. Tumor volume was calculated by the equation V = [L × W^2] × 0.52 (where V is volume, L is length, and W is width). The tumors excised were fixed in 4% paraformaldehyde/phosphate-buffered saline for 48 h at 4 °C, embedded in paraffin, sectioned in 1.5-μm-thick sections, and stained with hematoxyline and eosine.

RESULTS

Inhibition of the Growth of Human Colon Cancer Cells by Allyl Sulfoxides in Vitro—We initially examined the effect of allyl sulfides (structures shown in Fig. 1) on the growth of HCT-15 and DLD-1 cells. The cell growth was significantly reduced by DATS in a concentration-dependent manner (IC50 = 11.5 ± 0.8 μM for HCT-15, IC50 = 13.3 ± 0.7 μM for DLD-1). In contrast, neither DAS nor DADS showed any effect on both cell lines. Microscopic observation revealed that most HCT-15 cells in the control cultures were in interphase, with only a small fraction (1–2%) of them in the M phase (Fig. 2B, 0 h). On the contrary, HCT-15...
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The effect of DATS on the proliferation and morphology of colon cancer cells. **A**, comparison of the effect of allyl sulfides on the proliferation of HCT-15 and DLD-1 cells. The cells were incubated with various concentrations of DAS, DADS, or DATS for 24 h. The cell growth was expressed as a percentage of that of the vehicle-treated control. **B**, morphological changes in HCT-15 cells treated with 20 μM DATS for 24 h. The cell morphologies were observed under a light microscope. Scale bar, 50 μm.

**FIGURE 1.** Chemical structure of allyl sulfides used in this study.

**FIGURE 2.** The effect of allyl sulfides on the proliferation of colon cancer cells. **A**, comparison of the effect of allyl sulfides on the proliferation of HCT-15 and DLD-1 cells. The cells were incubated with various concentrations of DAS, DADS, or DATS for 24 h. The cell growth was expressed as a percentage of that of the vehicle-treated control. **B**, morphological changes in HCT-15 cells treated with 20 μM DATS for the times indicated. The cells were stained with Giemsa. Scale bar, 50 μm.

cells cultured in the presence of DATS exhibited mitotic arrest as early as 6 h. More than 60% of the cells were arrested at the M phase by the treatment with DATS for 12 h, and the mitotic morphology was observed for up to 18 h (Fig. 2B, 6, 12, and 18 h). In these cells, characteristic changes such as chromosome aggregation and disappearance of nuclear membrane were observed.

**Inhibition of Cell Cycle Progression and Induction of Apoptosis by DATS**—The effect of DATS on the cell cycle progression of HCT-15 and DLD-1 cells was analyzed further by using flow cytometry. Under normal condition in the absence of DATS, the cell cycle distribution of most HCT-15 and DLD-1 growing asynchronously was as follows: HCT-15 (51% in G1 phase, 35% in S phase, and 14% in G2/M phase) and DLD-1 (69% in G1 phase, 31% in S phase, and 9% in G2/M phase; Fig. 3A, 0 h). When DATS was added to the cultures, the percentages of HCT-15 and DLD-1 cells at G1 and S phase decreased, and the cell population at G2/M phase markedly increased in a time-dependent manner. HCT-15 cells at 12 h after the addition of DATS, ~70% of the cells were at the G2/M phase (Fig. 3A, 12 h). The population of DLD-1 cells in the G2/M phase at 16 h after the addition of DATS reached maximum (Fig. 3A, 16 h). The cells with a sub-G1 DNA content, which is an indicator of apoptosis, appeared at 12–16 h and increased thereafter in HCT-15 and DLD-1 cultures treated with DATS (Fig. 3A, 12, 16, 20, and 24 h). Cyclin B1, a protein known to increase during the transition from the G2 phase to the M phase, was accumulated at 6–12 h after the DATS treatment (Fig. 3B). The time of expressing cyclin B1 in DATS-treated DLD-1 cells was slightly later than that in DATS-treated HCT-15 cells. This delay might be due to the difference in the doubling time of these cells. Caspase-3 activity also dramatically increased after the cells arrested at the G1/S phase (Fig. 3C). Hoechst 33258 staining of HCT-15 cells demonstrated that DATS treatment caused more chromatin condensation and nuclear fragmentation than found in the vehicle-treated control cells (Fig. 3D).

**Induction by DATS of Cell Cycle-dependent Apoptosis through the Transition of G1/S to G2/M Phase**—To determine whether the DATS-induced apoptosis originated from a specific stage of the cell cycle, we synchronized HCT-15 and DLD-1 cells at the G1/S boundary by the thymidine-hydroxyurea double-block method. After arrest of the cells at the G1/S boundary, the culture medium was replaced with fresh medium containing vehicle (thymidine/hydroxyurea → vehicle) or DATS (thymidine/hydroxyurea → DATS). In HCT-15 cells, after releasing from the block at G1/S boundary, the vehicle-treated cells went into the late S phase at 4 h and the G2/M phase at 6 h (Fig. 4A). At 8 h post-release, the vehicle-treated cells already at the G2/M phase entered into the next G1 phase. Most of the cells were located in the G1 phase of the next cycle by 10 h after replacement of the culture medium. On the contrary, the cells released from the G1/S block in the presence of DATS exhibited a delayed cell cycle progression. At 8 h, 80% of the DATS-treated cells were in the G2/M phase. At 10–12 h, 60% of the cells still remained in the G2/M phase. After 10 h, the cells at sub-G1 markedly increased in number in a time-dependent manner. DLD-1 cells showed more delayed cell cycle progression than HCT-15 cells (Fig. 4B). DATS did not induce apoptosis of HCT-15 cells arrested at the G1/S phase, and the cells with a sub-G1 DNA content was 6.3% in the vehicle-treated cells and 7.8% in the DATS-treated cells, respectively. There was no apparent morphological difference between these two groups. These results suggest that induction of apoptosis by DATS requires the cell cycle progression from the G2/M to the G1 phase.

**Inhibition of Tubulin Polymerization by DATS in a Cell-free System**—Based on the finding that DATS inhibited the mitosis of HCT-15 cells (Fig. 2B), we next examined the effect of DATS on the polymerization-depolymerization cycle of tubulin. Polymerization of phosphocellulose-purified tubulin was measured in the presence of glycerol and GTP as an increase in turbidity (absorbance at 340 nm). As shown in Fig. 5A, colcemid, a microtubule-depolymerizing agent, inhibited tubulin polymerization, whereas paclitaxel, a microtubule-stabilizing agent, enhanced it. DATS (10 μM) also completely inhibited microtubule formation. Neither DAS (100 μM) nor DADS (100 μM) showed any effect on the microtubule formation (Fig. 5B). These data indicate that like a microtubule-depolymerizing agent, DATS inhibits tubulin polymerization in a cell-free system.

**Disruption of Cytoplasmic Microtubule Organization by DATS**—The effect of DATS on the microtubule organization was examined by immunostaining of β-tubulin in DLD-1 human colon cancer cells. The normal microtubule distribution and its network formation were observed in the cytoplasm of vehicle-treated DLD-1 cells at interphase (Fig. 5C, Vehicle). Colcemid caused the disruption of microtubule net-
work formation, whereas paclitaxel did not show any apparent influence on microtubule network formation at interphase (Fig. 5C, Colcemid (3 h) and Paclitaxel (3 h)). DATS caused the disruption of microtubule network formation by depolymerization of the microtubules, and most cells had shorter microtubule fragments than those observed in the cells at interphase. The shorter microtubules were scattered throughout the cytoplasm of the cells at 3 h after the DATS treatment (Fig. 5C, DATS (3 h)). Because the mitotic spindle is a highly dynamic structure, it is susceptible to antimitotic agents. DATS inhibited spindle formation, and the nuclear membrane disappeared from the cells treated with DATS. Treatment of DLD-1 cells with DATS caused the accumulation of the cells at prometaphase; the nuclear membranes of the cells disappeared, and there was no spindle formation (Fig. 5C, DATS (12 h)). When the test compounds were added after having reached the maximum turbidity, both DATS and colcemid caused the decrease in the turbidity. On the contrary, paclitaxel further increased the turbidity in the assay system. Taken together, DATS altered the microtubule structure by acting as a microtubule-depolymerizing agent.

Identification of DATS Modification Site in the Tubulin Molecule—Tubulin contains reactive sulfhydryl groups in its molecular structure (19). To examine whether DATS directly reacted with the tubulin molecule, we digested both DATS-treated and native tubulin samples with trypsin and analyzed the digests by liquid chromatography-tandem mass spectrometry. Peptide mass mapping of the DATS-treated tubulin identified 28.8% of it as \( \beta \)-tubulin peptides and 40.0% as \( \alpha \)-tubulin peptides. The DATS-modified \( \beta \)-tubulin peptide revealed an increase in mass by 72.1 Da, corresponding to the mass of a fragment molecule derived from DATS, S-allylmercaptocysteine, i.e. conversion of a pro-
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Antiproliferative Mechanisms of DATS: Role in Cell Cycle Progression and Apoptosis

In the study, the authors investigated the antiproliferative effects of DATS on human colon cancer cells. They observed that DATS inhibited cell proliferation and induced apoptosis. The mechanism behind this effect was found to be the modification of cysteine residues in tubulin, specifically at Cys-12β and Cys-354β.

To elucidate the antiproliferative mechanisms of DATS, the authors examined the effects of DATS on cell cycle progression and analyzed the induction of apoptosis. They found that DATS caused cell cycle arrest at the M phase, leading to apoptotic cell death. The authors also observed that DATS inhibited spindle formation, which is crucial for cell division.

Further experiments were conducted to determine the role of mitochondria in the induction of apoptosis by DATS. The authors found that DATS caused mitochondrial membrane permeabilization, leading to the release of pro-apoptotic proteins and the induction of apoptosis.

The authors concluded that the oxidative modification of cysteine residues in tubulin by DATS plays a key role in the antiproliferative and apoptotic effects of DATS. This finding provides a potential mechanism for the therapeutic application of DATS in cancer treatment.

DISCUSSION

The results of this study support the hypothesis that DATS induces apoptosis by the oxidative modification of cysteine residues in tubulin. The modification of cysteine residues at Cys-12β and Cys-354β is crucial for the disruption of microtubule network formation and the induction of apoptosis.

The oxidative modification of cysteine residues in tubulin by DATS was confirmed by mass spectrometry. The authors found that the incubation of tubulin with 10 µM DATS for 20 min resulted in the oxidation of sulfhydryl groups at Cys-12β and Cys-354β. This modification was also observed in the in vivo study, where the growth of HCT-15 xenografts was significantly reduced in mice administered DATS compared to control mice.

The ability of DATS to induce apoptosis was further supported by the observation that DATS caused an increase in mitochondrial membrane permeability and a decrease in the number of sulfur atoms in allyl sulfides. This suggests that the number of sulfur atoms in allyl sulfides is an important factor for exhibiting antiproliferative activity.

The mechanism by which DATS modifies tubulin remains to be fully elucidated. However, the authors propose that the oxidative modification of cysteine residues in tubulin by DATS plays a critical role in the antiproliferative and apoptotic effects of DATS. Further studies are needed to understand the detailed mechanisms involved in the oxidative modification of tubulin by DATS and its therapeutic potential in cancer treatment.
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FIGURE 6. Analysis of the modification of tubulin by liquid chromatography-tandem mass spectrometry. A, collision-induced dissociation spectrum of the DATS-modified peptide ([M + 2H]⁺ 225.1 m/z 919.65) with the sequence 3ENVHQAGQCGNQGAK19. The b ions (‘b10, and ‘b13–16) and y ions (‘y14–16, and ‘y16) were observed to increase 72.1 Da, suggesting that the DATS-oxidative modification site is in the sequence on cysteine 12 of β-tubulin. B, [M + 2H]⁺ 225.1 m/z 522.47 peptide with the sequence 351TAVC*CDIPPR359. The b ions (‘b4–7) and y ions (‘y6–7) were observed to increase 72.1 Da, suggesting that the DATS-oxidative modification site is in the sequence on cysteine 354 of β-tubulin.

Builes are highly dynamic polymers that are responsible for the accurate chromosome segregation during mitosis through the formation of the bipolar mitotic spindle. Thus, drugs that disrupt microtubule network formation have been applied for the treatment of malignant tumors (15, 16). Each microtubule-interacting agent has its own putative binding site in microtubules, e.g. colchicine-binding site, Vinca alkaloid-binding site, paclitaxel-binding site, and other unknown sites (30). By using mass spectrometry, we demonstrated that DATS can oxidize the sulphydryl group of tubulin (Cys-12β and Cys-354β) to disulfide (formation of protein-SS-allyl). Tubulin has 20 cysteine residues: α- and β-tubulin containing 12 and 8 cysteine residues, respectively (31, 32). Cys-354β is near the colchicine-binding site (33). Vinca alkaloids also bind with a domain so called “Vinca domain” containing Cys-12β, which is thought to be located close to the exchangeable GTP-binding site (34). Gupta et al. (35) recently demonstrated by a mutagenesis study using Saccharomyces cerevisiae that Cys-12β and Cys-354β residues play important roles in maintaining the structure and function of tubulin. Taken together, oxidative modification of Cys-12β and Cys-354β by DATS causes the dysfunction of tubulin.

The oxidative modification of cysteine residues by DATS (formation of protein-SS-allyl) is thought to be reversible, because the inhibited spindle formation by DATS was attenuated at least within 16 h of culture after the challenging of DATS (data not shown). In fact the cells arrested by DATS at the M phase went into the next G1 phase and underwent apoptosis (Fig. 3A); thus DATS would act to delay the cell cycle progression at the M phase (Fig. 4). Protein sulphydryl groups are known to be easily modified by S-glutathionylation (formation of protein-SSG), which is a reversible oxidation, to form a protein disulfide bond (protein-SS-protein) (36, 37). Post-translational modification of protein by oxidative stress can regulate protein function in the same manner as phosphorylation (38). Chaperones, cytoskeletal proteins, cell cycle regulators, signal transduction proteins, and metabolic or redox enzymes are also regulated by oxidative modification (36, 39, 40). A cytoskeletal protein, tubulin, can also be S-glutathionylated (41). The intracellular thiol homeostasis is maintained by the thioredoxin and glutaredoxin systems, which utilize NADH as reducing equivalents to reduce proteins (42). Thus, oxidative modification by DATS forming the protein-SS-allyl may be restored by these redoxins and glutaredoxin systems as well as S-glutathionylation (protein-SSG), and the disulfide bond formation between the proteins may be possible (protein-SS-protein).

Based on the findings from the studies in vitro, we also examined the effect of DATS on the tumor growth in mice as a xenograft model in vivo. DATS potently reduced the tumor size in comparison with vehicle-administered control mice; a 70% reduction in the tumor size was observed. These results strongly suggest that DATS suppresses the tumor cell growth even in vivo by the mechanisms observed in vitro.

In summary, we demonstrated for the first time that DATS, one of the phytochemicals derived from garlic, bound to specific cysteine residues in β-tubulin molecule to form S-allylmercaptocysteine and that this could be the sole cause of cell cycle arrest and successive apoptosis with the activation of caspase-3. In other words, this is the first finding that a garlic-derived anticarcinogenic sulfide binds chemically with one of the most important proteins for cell growth. We also demonstrated that DATS inhibited significantly the growth of human colon carcinoma.
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A

B

FIGURE 7. Antitumor activity of DATS in nude mice bearing HCT-15 xenografts. A, animals randomly divided into two groups were administered DATS (6 mg/kg body weight intravenously, once every 3 days for 18 days) or vehicle. The size of tumors was measured by the method described under "Materials and Methods." The data are expressed as the means ± S.E. of eight mice. B, the sections of tumors collected at 17 days post implantation are the sections prepared from vehicle-administered nude mice; the right two panels (Vehicle) are the sections collected at 17 days post implantation are the sections prepared from DATS-administered nude mice. The arrows indicate the necrotic region in the tumor. Scale bar, 500 μm.

cells in nude mice in vivo. Garlic is widely served as a unique spicy vegetable around the world, and several lines of evidence, obtained by both laboratory and epidemiological research, have proven the anticancer effect of garlic (43, 44). DATS is responsible, at least in part, for the effect, and it might thus be a lead compound for designing novel anti-cancer drugs.

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