Original Article

Anticancer effects of diallyl trisulfide derived from garlic

Taiichiro Seki PhD[1], Takashi Hosono PhD[1], Tomomi Hosono-Fukao PhD[1], Kahoru Inada BS[1], Rie Tanaka BS[1], Jun Ogihara PhD[2] and Toyohiko Ariga PhD[1]

[1] Laboratory of Nutrition and Physiology, Department of Applied Life Sciences, Nihon University Graduate School of Bioresource Sciences, Kanagawa, Japan
[2] Laboratory of Enzymology and Molecular Biology, Department of Bioresource Utilization, Nihon University Graduate School of Bioresource Sciences, Kanagawa, Japan

Alk(en)yl sulfides are characteristic flavor components of garlic. Several lines of epidemiological study indicate that the risk of a certain cancer can be prevented by consumption of garlic. In this manuscript, we examined the anticancer property of garlic-derived alk(en)yl sulfides, and the molecular basis especially for diallyl trisulfide which is a major constituent of the garlic oil. Alk(en)yl sulfides with different numbers of sulfur atom (i.e., mono-, di-, and trisulfide) were synthesized and purified (>99%). The anticancer activity of the alk(en)yl sulfides was primarily examined using human colon cancer cells HCT-15 and DLD-1. The growth of the cells was significantly suppressed by diallyl trisulfide, but neither diallyl monosulfide nor diallyl disulfide showed such an effect. The number of cells arrested at G_2/M phase, the cells with a sub-G_1 DNA content, and the cells with caspase-3 activity were dramatically increased by diallyl trisulfide treatment. Diallyl trisulfide disrupted microtubule network formation of the cells, and microtubule fragments could be seen at the interphase. There was a specific oxidative modification of cysteine residues Cys12β and Cys354β, forming *S*-allylmercaptocysteines in the tubulin molecule. These results suggest that diallyl trisulfide is responsible, at least in part, for the epidemiologically proven anticancer effect for garlic eaters.

Key Words: diallyl trisulfide, garlic, cancer, tubulin, apoptosis

INTRODUCTION

Garlic (*Allium sativum* L.*)* has a long history as being a food having a unique taste and odor along with some medicinal qualities. Phytochemicals present in the garlic have potential pharmacological functionalities against several physiological processes.[1-4] Thus the therapeutic use and application of garlic for prevention of cancer or cardiovascular disease has widely been studied.[2,3]

Alk(en)yl sulfides are characteristic flavour component of garlic. It has been reported that allyl sulfides inhibit both initiation and promotion stages of tumorigenesis in experimental caricinogenesis model for various type of cancer.[2,3] Several lines of studies have shown that allyl sulfides suppress cell growth and induce apoptosis in multiple cancer cell line.[2,3] However, the molecular mechanisms underlying the antitumorigenesis of allyl sulfide are still not fully understood. In addition to these laboratory studies, some epidemiological studies on the relationship between garlic consumption and incidence of cancers suggested that there is a reciprocal relationship between the two with respect to stomach and colorectal cancers.[5,6]

This study was aimed at clarifying the effect of allyl sulfides and the molecular target of allyl sulfides to understand the anticancer mechanism elicited by the consumption of garlic. We initially compared the number of sulfur atom in the allyl sulfides; i.e., we examined the effect of diallyl monosulfide, diallyl disulfide, and diallyl trisulfide for their anticancer activity against human colon cancer cell lines, HCT-15 and DLD-1 cells. We now show the changes in the cells caused by diallyl trisulfide which include the disruption of microtubule network formation and mitotic arrest as well as the induction of apoptosis. It was suggested that the modification of tubulin is a causative of the anti-cancer effect of diallyl trisulfide.[7]

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). Diallyl sulfide and diallyl disulfide were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Diallyl trisulfide was synthesized by using Bunte salt. Diallyl trisulfide and commercial diallyl disulfide were purified by high pressure liquid

Corresponding Author: Dr T. Seki, Nihon University, Graduate School of Bioresource Sciences, Kameino 1866, Fujisawa, Kanagawa 252-8510, Japan.
Tel/Fax: + 81 466 84 3949
Email: tseki@brs.nihon-u.ac.jp
Manuscript received 9 September 2007. Accepted 3 December 2007.

chromatography (Alliance 2695 system; Waters Co., Milford, MA) on an Inertsil ODS-3 column (GL Science, Tokyo, Japan).

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 these cells were analyzed by using a flow cytometer FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and FlowJo software (Tree Star Inc., Ashland, OR).

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, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10 mM dithiothreitol, and 10% sucrose, pH 7.2) containing 50 μM *N*-acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide) (Peptide Institute, Inc., Osaka, Japan). The 7-amino-4-methylcoumarin released was measured by use of a spectrofluorometer with excitation at 380 nm and emission at 460 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 300; Olympus, Tokyo, Japan).

High-performance liquid chromatography-tandem mass spectrometry

Analysis of cysteine residues in the diallyl trisulfide modified-tubulin was performed by using high performance liquid chromatography-tandem mass spectrometry in comparison with native tubulin. Tubulin (1 mg/ml) was incubated at 37°C for 60 min in the presence or absence of 100 μM diallyl trisulfide. Both diallyl trisulfide-treated and untreated tubulin samples were digested with modified-trypsin (Promega, Madison, WI) and analyzed the peptides and their amino acids by LC-MS/MS by using a MAGIC C18 column (0.15 mm × 50 mm, Michrom BioResources, Auburn, CA). Peptides were eluted over a 20 min period with a linear gradient 5-65% of solvent B (90% (v/v) acetonitrile, 0.1% formic acid) in solvent A (2% (v/v) acetonitrile, 0.1% formic acid) to solvent B, with a flow rate of 0.8 μl/min. The tryptic peptides thus separated were analyzed with an LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). MS/MS 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. All spectra were searched against the data in FASTA-format generated from pig α-tubulin (NCBI Accession Number, P02550) and pig β-tubulin (P02554) in the database of National Center for Biotechnology Information (Bethesda, MD).

RESULTS

Inhibition of the growth of human colon cancer cells by allyl sulfides in vitro

The relationship between the number of sulfur in allyl sulfides and their effect on cell proliferation was studied by use of HCT-15 human colon cancer cell line.

Both diallyl monosulfide and diallyl disulfide did not show any effect on the proliferation of HCT-15 cells (see the panels "Vehicle" in Figure 1A). On the other hand, diallyl trisulfide caused reduction in the cell proliferation rate accompanying with striking morphological changes. Shrunk cells with swollen and thick membranes were observed in the culture with diallyl trisulfide for 24 h.

Effect of diallyl trisulfide on cell cycle progression and caspase-3 activity

Flow cytometric analysis was performed to examine the effect of diallyl trisulfide on the cell cycle progression of HCT-15 cells. Under a normal culture condition in the absence of the sulfides, the cell cycle distribution of most HCT-15 cells growing asynchronously was as follows; 51% in G_1 phase, 35% in S phase, and 14% in G_2/M phase, Figure 1B, 0 h. Incubation of HCT-15 cells with 20 μM diallyl trisulfide caused dramatic changes in their cell cycle population especially at G_1 and S phases. The cell population at G_2/M phase markedly increased in a time-dependent manner by the treatment with diallyl trisulfide (Figure 1B). In HCT-15 cells at 12 h after the addition of diallyl trisulfide, approximately 70% of the cells were arrested at G_2/M phase (Figure 1B, 12 h). The cells with sub-G_1 DNA content (DNA <2n), which is an indicator of apoptosis, appeared at 12-16 h after the addition of diallyl trisulfide and increased thereafter (Figure 1B, 12-24 h).

The activity of caspase-3, an effector caspase, also dramatically increased at the time when the cells were arrested at the G_2/M phase (Figure 1C). These data indicate that diallyl trisulfide arrests the cell cycle at G_2/M phase and induces apoptosis.

Disruption of microtubule network formation by diallyl trisulfide

Microtubules are components of cytoskeleton and play pivotal roles in a variety of cellular processes involving cell division. 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 cells. The microtubule-stabilizing agents paclitaxel did not show any effect on the microtubule network formation, whereas microtubule-depolymerizing agents, colcemid and vincristine (not shown), caused the disruption of microtubule network formation. Diallyl trisulfide also caused the disruption of microtubule network formation, and most cells became to have shorter microtubule fragments. These shorter fragments scattered throughout the cytoplasm was quite similar to those in the cells treated

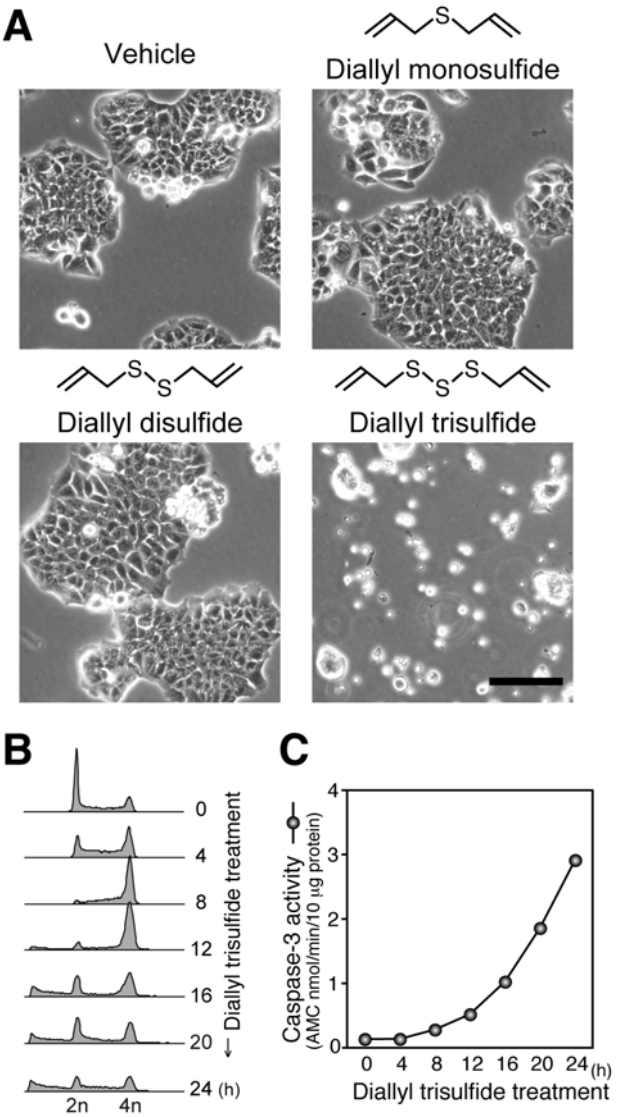

Figure 1. Effect of diallyl trisulfide on the morphology, cell cycle progression and caspase-3 activity of HCT-15 cells. (A) Changes in the morphology of HCT-15 cells treated either with 20 μM of diallyl monosulfide, diallyl disulfide, or diallyl trisulfide for 24 h. Bar, 100 μm. (B) The effect of diallyl trisulfide on the cell cycle distribution. (C) Caspase-3 activity of HCT-15 cells treated with diallyl trisulfide.

with a microtubule-depolymerizing agent, colcemid (Figure 2).

Identification of diallyl trisulfide modification site in the tubulin molecule

Tubulin contains reactive sulfhydryl groups in its molecular structure. To examine wheather diallyl trisulfide directly reacts to the tubulin molecule, both diallyl trisulfide-treated and untreated tubulin samples were digested with trypsin and analyzed the digests by LC-MS/MS. Peptide mass mapping of the diallyl trisulfide-treated tubulin identified 28.8% of it as α-tubulin peptides and 40.0% as β-tubulin peptides. The diallyl trisulfide-modified β-tubulin peptide revealed an increase in mass by 72.1 Da, corresponding to the mass of a fragment molecule derived from diallyl trisulfide, *S*-allylmercaptocysteine; i.e. conversion of a protein sulfhydryl group (protein-SH) to an oxidized form by diallyl trisulfide (protein-SS-allyl). In the β-tubulin, evidence of

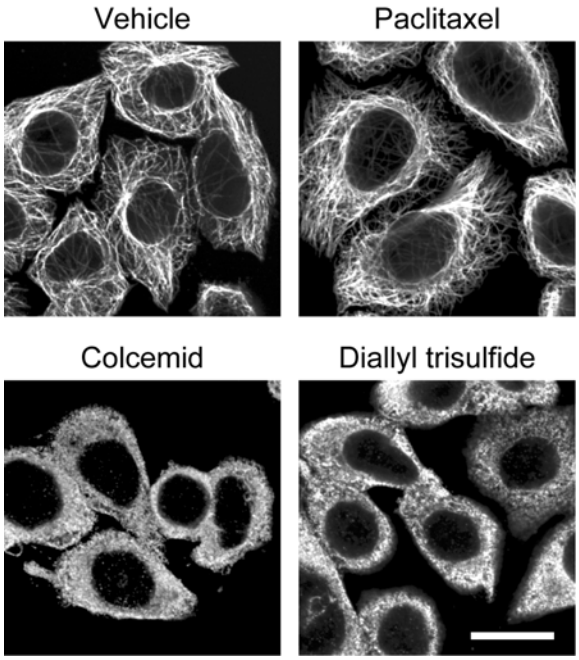

Figure 2. Effect of diallyl trisulfide on the microtubule formation. DLD-1 cells were treated either with diallyl trisulfide (20 μM), paclitaxel (100 nM), or colcemid (100 ng/ml) for 24 h. Morphological changes of the microtubule formed in the cells are observed by immunofluorescence method using anti-β-tubulin antibody. Bar, 20 μm.

cysteine residue modification was successfully proven by the detection of peptide ^{3}EIVHIQAGQCGNQIGAK^{19} ($[M + 2H]^{2+}$ of *m/z* 919.65) and peptide ^{351}TAVCDIPPR^{359} ($[M + 2H]^{2+}$ of *m/z* 522.47). Cys12β and Cys354β were identified as the residues that increased the peptide mass of 72.1 Da, suggesting that diallyl trisulfide-modified residues are included in the cysteine-containing peptide fragments. On the other hand, diallyl trisulfide-related modification of cysteine residues other than Cys12β or Cys354β could not be detected in either α- or β-tubulin (data not shown). These data suggest that among the tubulin structure, only 2 amino acid residues, Cys12β and Cys354β, are the specific amino acid residues that are oxidized by diallyl trisulfide.

DISCUSSION

To elucidate the relationship between the structure and function of allyl sulfides, we examined the effect of diallyl monosulfide, diallyl disulfide and diallyl trisulfide on the replication of human colon cancer cells. The reason why the diallyl trisulfide is more potent than disulfide or mono sulfide remains unclear. Because diallyl trisulfide is known to react with sulfhydryl group, modification of cysteine would be crucial for inhibiting cell growth. We next performed cell cycle analysis as well as assaying caspase activity to clarify the antiproliferative effect of diallyl trisulfide. Diallyl trisulfide caused cell cycle arrest at G_2/M phase. Immunofluorescence microscopy using anti-β-tubulin antibody clearly showed that diallyl trisulfide disrupted the microtubule network formation (Figure 2). Inhibition of the spindle formation by diallyl trisulfide, and the following cell cycle arrest at mitotic phase were also observed (data not shown). The mitotic arrest caused

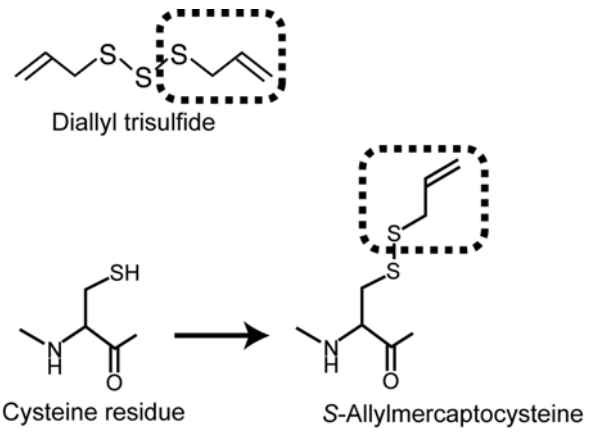

Figure 3. Oxidative modification of the cysteine residues to *S*-allylmercaptocysteine by diallyl trisulfide.

by microtubule-interfering agents has been found to precede apoptotic cell death. Actually, the hypothesis that cell cycle arrest at mitosis is the primary signal to induce apoptosis has been widely accepted.[8,9] In our experiment, diallyl trisulfide also caused mitotic arrest prior to the induction of apoptosis in HCT-15 cells. Microtubules are ubiquitous protein present in eukaryotes as components of cytoskeleton and play pivotal roles in a variety of cellular the processes involving cell division, motility, and intracellular trafficking. 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 cells. Thus, antimitotic drugs developed for targeting microtubules have gained great success in cancer chemotherapy. We demonstrated that the direct oxidative modification of tubulin at Cys12β and Cys354β by diallyl trisulfide (Figure 3). This modification is assumed to be a cause of disruption of microtubule network formation. 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.[10] Our mass spectrometric study demonstrated that diallyl trisulfide is able to oxidize the sulfhydryl groups of tubulin at Cys12β and Cys354β to form disulfide; i.e., protein-SS-allyl. α-Tubulin has 12 cysteine residues and β-tubulin has 8 cysteine residues in the molecule. Cys354β is located near the colchicine-binding site.*Vinca* alkaloids also bind with a domain so called "*Vinca* domain" containing Cys12β, which is thought to be located close to the exchangeable GTP-binding site.[11] It has been reported by a mutagenesis study using *Saccharomyces cerevisiae* that these two residues, Cys12β and Cys354β, play important roles in maintaining the structure and function of tubulin. Taken together, oxidative modification of Cys12β and Cys354β by diallyl trisulfide causes the dysfunction of tubulin. Based on the findings from the studies *in vitro*, we examined the effect of diallyl trisulfide on the tumour growth in mice as a xenograft model *in vivo*.[7] As a result, diallyl trisulfide potently suppressed the growth of the xenograft without any obvious side effect.

In summary, we demonstrated for the first time that a phytochemical derived from garlic, diallyl trisulfide, bound to specific cysteine residues in β-tubulin to form *S*-allylmercaptocystein and that this could be the sole cause of cell cycle arrest and successive apoptosis with activation of caspase-3. These results suggest that diallyl trisulfide is responsible, at least in part, for the epidemiologically proven anticancer effect for garlic eaters.

ACKNOWLEDGMENTS

This work was supported by grants from Nihon University College of Bioresource Sciences (General Research Grant to T.S.), and the programs Grants-in Aid for Scientific Research (B) (to T.A.) and (C) (to T.S.), Grant-in-Aid for Young Scientists (B) (to T.H.) from the Japan Society for the Promotion of Science (JSPS). T.H. and T.H-F. were supported by a JSPS Fellowship and a Fellowship from the COE Program in the 21st Century in Japan.

AUTHOR DISCLOSURES

Taiichiro Seki, Takashi Hosono, Tomomi Hosono-Fukao, Kahoru Inada, Rie Tanaka, Jun Ogihara and Toyohiko Ariga, no conflicts of interest.

REFERENCES

1. Ariga T, Oshiba S, Tamada T. Platelet aggregation inhibitor in garlic. Lancet. 1981;1:150-1.
2. Ariga T, Seki T. Antithrombotic and anticancer effects of garlic-derived sulfur compounds: a review. Biofactors. 2006; 26:93-103.
3. Ariga T, Seki T. Functional Foods from Garlic and Onion. In: Shi J, Ho CT, Shahidi F, editors. Asian Functional Foods. New York: CRC Press, 2005. p. 433-490.
4. Fukao T, Hosono T, Misawa S, Seki T, Ariga T. The effects of allyl sulfides on the induction of phase II detoxification enzymes and liver injury by carbon tetrachloride. Food Chem Toxicol. 2004;42:743-9.
5. Milner JA. A historical perspective on garlic and cancer. J Nutr. 2001; 131(3s): 1027S-31S.
6. Fleischauer AT, Arab L. Garlic and cancer: a critical review of the epidemiologic literature. J Nutr. 2001;131:1032S-40S.
7. Hosono T, Fukao T, Ogihara J, Ito Y, Shiba H, Seki T, Ariga T. Diallyl trisulfide suppresses the proliferation and induces apoptosis of human colon cancer cells through oxidative modification of β-tubulin. J Biol Chem. 2005;280:41487-93.
8. Ling YH, Jiang JD, Holland JF, Perez-Soler R. Arsenic trioxide produces polymerization of microtubules and mitotic arrest before apoptosis in human tumor cell lines. Mol Pharmacol. 2002;62:529-38.
9. Kuo CC, Hsieh HP, Pan WY, Chen CP, Liou JP, Lee SJ, Chang YL, Chen LT, Chen CT, Chang JY. BPR0L075, a novel synthetic indole compound with antimitotic activity in human cancer cells, exerts effective antitumoral activity in vivo. Cancer Res. 2004;64:4621-8.
10. Islam MN, Iskander MN. Microtubulin binding sites as target for developing anticancer agents. Mini Rev Med Chem. 2004;4:1077-104.
11. Gupta S, Bhattacharyya B. Antimicrotubular drugs binding to vinca domain of tubulin. Mol Cell Biochem. 2003;253:41-7.