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Cytotoxic and Antitumor Activities of Thiosulfinates from *Allium tuberosum* L.

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In this study we isolated crude thiosulfinates from *Allium tuberosum* L. using CH_2Cl_2 and then with silica gel column chromatography purified *S*-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate from the crude thiosulfinates. Subsequently, in vitro cytotoxicities against human cancer cells and in vivo antitumor activities of the thiosulfinates were investigated. Their cytotoxicities were strong in human cancer cells, in the order of *S*-methyl 2-propene-1-thiosulfinate, crude thiosulfinates, and *S*-methyl methanthiosulfinate. When thiosulfinates were administered consecutively for 7 days at 10, 30, and 50 mg/kg ip, in mice, we found significant increases in the life spans of mice that had been inoculated with Sacorma-180 tumor cells. The crude thiosulfinates also induced apoptosis in MCF-7 cancer cells. These results suggest that thiosulfinates from *Allium tuberosum* L. inhibit the proliferation of cancer cells via apoptosis and have antitumor activities.

KEYWORDS: ***Allium tuberosum*** **L.; thiosulfinates; cytotoxicity; antitumor; apoptosis**

INTRODUCTION

The *Allium* species are fascinating plants, as large numbers of *Allium* vegetables are consumed by many countries and are used in many cuisines such as American, European, Chinese, Japanese, and Korean (*1*). In addition, alliums have been used since ancient times as pharmaceuticals. *Allium tuberosum* (AT), which contains thiosulfinates, is used in folk medicines as well as to flavor foods. The thiosulfinates of *Allium* species are unstable intermediates in the enzymatically initiated degradation of *S*-alk(en)yl-L-cystene sulfoxide (*2*). AT is a major ingredient of leek *Kimchi* in Korea, which has long been used as a medicinal food and folk remedy for the treatment of abdominal pain, diarrhea, hematemesis, snakebite, and asthma (*3*).

Since the first detailed chemical report on thiosulfinates, many chemists and pharmacists have tried to separate and identify individual compounds by GC–MS, LC–MS, and NMR spectroscopy because of the striking biological activities (*4, 5*) and interesting organosulfur chemistry of these compounds (*6*). Although GC and GC–MS analyses have provided excellent resolution and identification, many of the *Allium* compounds seen by GC may have been artifacts of the analysis (*7*) because of the high reactivity associated with the weak S–S bond (a bond energy of 46 kcal mol^{-1} or less) (*8*). Several weaknesses also exist with the HPLC method, such as retention time variations and the present limitation of LC–MS in characterizing volatile compounds (*9*). In a previous study, we first reported on the separation of two thiosulfinates from AT using simple chromatography (*10*).

Several studies have indicated that a high consumption of leeks was associated with a reduced risk for colorectal cancer (*11–13*). In a case-controlled study in Japan and Hawaii, high consumption of AT was associated with a decreased risk of colorectal cancer, as well as a subset of cancers that occur in the lower part of the rectum (*11, 12*).

In this paper, we report on in vitro antiproliferation activities, via apoptosis, of thiosulfinates from AT against human cancer cells, as well as on in vivo antitumor activities of the compounds.

MATERIALS AND METHOD

Isolation of Thiosulfinates. The thiosulfinates from AT were isolated using a previous method (*10*). The aerial parts of the plant were chopped and repeatedly extracted with CH_2Cl_2 (3 × 4 L) at room temperature. The extract was partitioned between CH_2Cl_2 and 5% aqueous lead acetate and then filtered. The filtrate was separated, and the aqueous layer was reextracted with CH_2Cl_2. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and then concentrated to give a thick mass. This was then chromatographed in a silica gel column (3.2 cm × 38 cm, 230–400 mesh), eluting with a gradient of CH_2Cl_2–hexane [2:1 (500 mL), 4:1 (500 mL), and then 1:0 (300 mL); 20 mL each]. Fractions 41–46 and 48–61 were evaporated to give *S*-methyl 2-propene-1-thiosulfinate (compound **2**, **Figure 1**) [R_f = 0.24 (hexane/EtOAc

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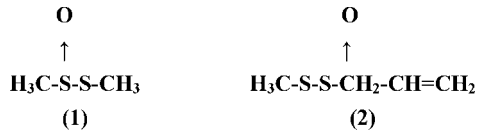

Figure 1. Structures of *S*-methyl methanethiosulfinate and *S*-methyl 2-propene-1-thiosulfinate purified from *Allium tuberosum* L (AT).

= 2:1)] and *S*-methyl methane thiosulfinate (compound **1**, **Figure 1**) [R_f = 0.11 (hexane/EtOAc = 2:1)] as bright-yellow oils, respectively.

Cell Culture and Cell Proliferation. The MCF-7 (human breast cancer cell), SW480, HepG2 (human liver cancer cell), LNCaP.FGC (human prostate cancer cell), and sarcoma-180 tumor cells were purchased from the Korea Cell Line Bank, Seoul National University. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μg/mL) (GIBCO BRL, Life Technologies, Grand Island, NY) in a humidified atmosphere with a 5.5% CO_2 incubator, at 37 °C (*14*).

Cell proliferation was determined by a sulforhodamine B (Sigma, St. Louis, MO) assay. The cancer cells were seeded at a concentration of 5×10^4 cells/well in 48-well tissue culture plates and incubated with various concentrations of thiosulfinates for different time periods. After treatment, the medium was aspirated and 10% trichloroacetic acid was added. After 1 h of incubation at 4 °C, the plates were washed 5 times with distilled water and air-dried. The cells were stained with 0.4% (w/v) SRB at room temperature for 1 h and then washed 5 times using 1% acetic acid. Bound SRB was solubilized with 10 mM Tris, and absorbance was measured at 540 nm (*15*).

Cell number was also counted as another measure for cell growth. Briefly, after incubation with or without thiosulfinates, the cells were detached by 0.025% trypsin EDTA at 37 °C for 2 min and then resuspended in PBS. The number of suspended cells was counted with a hemacytometer (*16*).

Detection of Morphological Apoptosis. Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using 4,6-diamidino-2-phenylindole (Sigma, St. Louis, MO) staining. Briefly, the cells were seeded in six-well plates at seeding densities of 5×10^5 cells and then treated with thiosulfinates (10, 20, and 30 μg/mL) for 24 h. After being harvested, the cells were washed once with PBS, resuspended in PBS containing 0.1% Triton X100 (to induce holes in the cell membranes and increase permeability), and incubated for 10 min on ice. After centrifuging at 1500*g* for 5 min, the precipitate was resuspended at 5×10^5 cells/mL in 4% PBS buffered paraformaldehyde solution containing 10 μL/mL of 4,6-diamidino-2-phenylindole (Sigma, St. Louis, MO). Ten μL of this suspension was placed on a glass slide and covered with a coverslip. The cells were examined with a fluorescence microcope (Olympus Optical Co. Ltd., Japan) to determine nuclei fragmentation and chromatin condensation (*17*).

Cell Cycle Analysis and Sub-G1 DNA Measurement. The cells were seeded at a density of 1×10^6 cells in six-well plates and cultured for 24 h in RPMI-1640. After being cultured, the cells were treated with the indicated concentrations of thiosulfinates for 24 h. For growth inhibition analysis and the measurement of sub-G1 DNA content, the cells were collected and fixed in ice-cold 70% ethanol in media and stored at 4 °C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma, St. Louis, MO), 20 μL of propidium iodide (1 mg/mL) (Sigma, St. Louis, MO), and 500 mL of PBS at 37 °C for 30 min. After the cells were stained, flow cytometry was used to analyze the cell cycle phase and sub-G1 DNA content (*14*).

Antitumor Activity. The effects of the thiosulfinates on tumor host survival in the sarcoma-180 tumor cells of mice were estimated by evaluating the percentage increase survival times of the tumor hosts. On day 0, a group of nine mice was inoculated ip with 1×10^6sarcoma-180 cells/mouse. Thiosulfinate treatments of 10, 30, and 50 mg/kg were started 24 h after tumor cell inoculation and continued for seven consecutive days. The control group was treated with distilled water only. The antitumor activity of the thiosulfinates was estimated by the survival times of the tumor-bearing-mice for 1 month (*18*).

Statistical Analysis. The data were analyzed by Student's *t* test to evaluate significant differences. $P < 0.05$ was regarded as statistically significant.

RESULTS

Purification of *S*-Methyl Methanthiosulfinate and *S*-Methyl 2-Propene-1-thiosulfinate from *Allium tuberosum* L. Crude thiosulfinates (3.92 g), containing mostly *S*-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate, were isolated from AT (4 kg) using CH_2Cl_2; both compounds (1600 and 240 mg, repectively) were purified from the crude thiosulfinates using silica gel column chromatography. A strong IR absorption band at 1088 cm^{-1} suggested the presence of a thiosulfinate group in each compound. The mass spectral data were consistent with the molecular formula of $C_2H_6OS_2$ for **1** and $C_4H_8OS_2$ for **2**. In the prominent mass fragmentation pattern, *m*/*z* 95 (20%) for $[S(O)S - CH_3]^+$ was observed in **1**, while *m*/*z* 41 (100%) for $[C_3H_5]^+$ and *m*/*z* 95 (15%) for $[S(O)S - CH_3]^+$ were observed in **2**. The high-resolution mass spectral data for **2** showed molecular ion *m*/*z* at 136.0013, requiring a molecular formula of $C_4H_8OS_2$. An analysis of the ^1H–^1H COSY spectrum of *2* showed the H-1 methylene protons (3.80 ppm) linked to their neighboring H-2 (5.94 ppm), and the allylic proton was directly correlated with the H-3 (5.46 ppm) proton. Additionally, the mass spectrum ($M^+ - 41$) of **2** indicated the presence of an allyl group. The high-field chemical shift (2.68 ppm) of the methyl singlet of each compound was in agreement with the reported shift of a methyl group linked to the sulfursulfinyl in thiosulfinates. With these various spectroscopic data, these compounds were respectively identified as *S*-methyl methanethiosulfinate (**1** in **Figure 1**) and *S*-methyl 2-propene-1-thiosulfinate (**2** in **Figure 1**) (*10*).

Thiosulfinates Induced Cell Death. The MCF-7, SW480, HepG2, and LNCaP.FGC cells were treated with thiosulfinates for 24 h as indicated. Cell viability, which was assayed by SRB, decreased with increasing thiosulfinate concentration (data not shown), and the IC_{50} values of the thiosulfinate treatments were 14.23, 10.52, 13.84, and 7.90 μg/mL, respectively. The values of *S*-methyl methanthiosulfinate were 17.05 (155.1 μM), 16.28 (147.97 μM), 20.2 (183.8 μM), and 11.24 (102.25 μM) μg/mL. The values of *S*-methyl 2-propene-1-thiosulfinate were 7.51 (51.09 μM), 7.17 (48.78 μM), 11.81 (148.39 μM), and 4.04 (27.48 μM) μg /mL in the MCF-7, SW480, HepG2, and LNCaP.FGC cells, respectively (**Table 1**). In order to show the time-dependent actions of the thiosulfinates in cancer cells, the MCF-7 cells were treated with the various indicated concentrations of thiosulfinates and viability was assessed after 24, 48, and 72 h. The antiproliferation caused by the thiosulfinates in the MCF cells was in a treatment-time-dependent manner (**Figure 2**).

Also, as shown in **Figure 3**, cell numbers decreased and cell death rates increased, in dose-dependent manners, in the MCF-7 cells treated with thiosulfinates.

These results indicate that thiosulfinates, and *S*-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate from AT, inhibit the proliferation of cancer cells in concentration- and time-dependent manners and that their activities were strong, in the order of *S*-methyl 2-propene-1-thiosulfinate, thiosulfinates, and *S*-methyl methanthiosulfinate.

Apoptosis Induction by Thiosulfinates. While the untreated MCF-7 cells were well-spread with flattened morphologies, many apoptotic bodies were noticed in the cells treated with 20 and 30 μg/mL thiosulfinates for 24 h (**Figure 4**). Similarly, the nuclei were of normal size in the untreated cells, whereas

Table 1. IC_{50} Values of Thiosulfinates from AT for 24 h on Human Cancer Cells[a]

cell lines	IC_{50} (μg/mL) crude thiosulfinates	*S*-methyl methanthiosulfinate	*S*-methyl 2-propene-1-thiosulfinate
MCF-7	14.23 ± 0.72	17.05 ± 0.34 (155.0 μM)	7.51 ± 1.34 (55.22 μM)
SW480	10.52 ± 1.10	16.28 ± 0.55 (148.0 μM)	7.17 ± 0.71 (52.72 μM)
HepG2	13.84 ± 0.38	20.2 ± 1.45 (183.64 μM)	11.81 ± 0.74 (86.83 μM)
LNCap.FGC	7.90 ± 7.93	11.24 ± 0.23 (102.18 μM)	4.04 ± 0.33 (29.71 μM)

[a] IC_{50} is the concentration that inhibited 50% of the growth. Data values are expressed as the mean ± SD of triplicate determinations. An amount of 10 μg of crude thiosulfinates, *S*-methyl methantiosulfinate, and *S*-methyl 2-propene-1-thiosulfinate is equivalent to 10.2, 25, and 166.7 mg of AT, respectively.

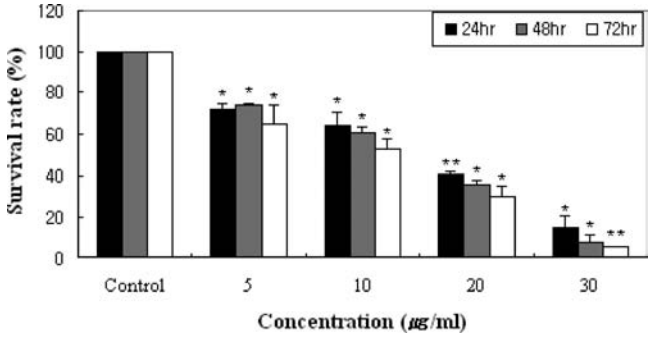

Figure 2. Cell growth inhibition effects in MCF-7 cells treated with crude thiosulfinates from AT for 24, 48, and 72 h by SRB assay.The MCF-7 cells were treated with thiosulfinates from AT at 0–30 μg/mL for 72 h and then fixed and stained with SRB for cellular protein content analysis. Data values are expressed as the mean ± SD of triplicate determinations. Significant differences were compared with the control at (∗) $p < 0.05$ and (∗∗) $p < 0.01$ by Student's *t* tests.

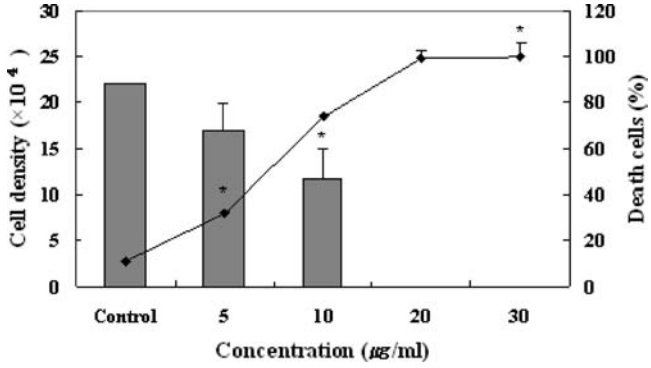

Figure 3. Cytotoxicities determined via cell count in MCF-7 cells treated with thiosulfinates from AT for 24 h. The bars and line are the mean values of the cell densities and cell deaths, respectively. The MCF-7 cells were detached by trypsinization and harversted by resuspension in medium for immediate analysis of the cell number using a hemacytometer. Data values are expressed as the mean ± SD of triplicate determinations. Significant differences were compared with the control at (∗) $p < 0.05$ and (∗∗) $p < 0.01$ by Student's *t* tests.

condensation of nuclei and apoptotic bodies was observed in the cells treated with 20 and 30 μg/mL thiosulfinates for 24 h (**Figure 4**). These results suggest that crude thiosulfinates induced apoptosis.

To determine the correlation between the crude thiosulfinate-induced inhibition of proliferation and cell cycle blockage, the effects of the thiosulfinates on cell cycle distribution were determined, and the results are summarized in **Figure 5**. The proportion of the sub-G1 peak was negligible in the control MCF-7 cells without thiosulfinates, whereas 24 h of exposure of the MCF-7 cells to 5, 10, 20, and 30 μg/mL of thiosulfinates resulted in cell accumulation in the sub-G1 phase in a dose-dependant manner (**Figure 5**).

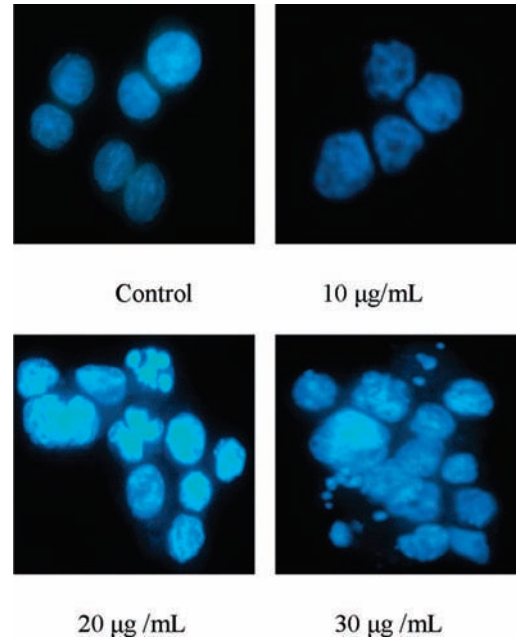

Figure 4. Nuclear fragmentation was induced by treatment with crude thiosulfinates from AT in MCF-7 cells. The MCF-7 cells were treated with thiosulfinates from AT at 0–30 μg/mL for 24 h, washed once with PBS and 0.1% Triton X 100, fixed in 4% parafomaldehyde, stained with 10 μL/mL DAPI, and finally, examined by a fluorescence microscope.

Effects of Thiosulfinates on the Survival Times of Mice with Sarcoma-180 Ascitic Tumors. The in vivo anticancer test was carried out in sarcoma-180 tumor-bearing mice. The mice treated with vehicle survived only for 16.8 days (**Table 2**). However, the survival times of mice treated for 7 consecutive days with 10, 30, and 50 mg/kg crude thiosulfinates containing mostly *S*-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate were extended to 22.8, 26.2, and 29.0 days, respectively. These thiosulfinates increased survival times in the tumor-bearing mice by 35.7%, 56.0%, and 72.6%, respectively.

DISCUSSION

AT is a principal vegetable in Asia. In the case of South Korea, various foods containing *Kimchi* with AT have been used for years and are regarded as medicinal foods because of the thiosulfinates.

Fresh AT has been reported to contain up to 9% *S*-methyl 2-propene-1-thiosulfinate, 5% menthanesulfinothionic acid *S*-(*Z*,*E*)-1-propenyl ester, 13% methanesulfinothionic acid *S*-2-propenyl ester, and 72% *S*-methyl methanthiosulfinate. It is interesting to point out that *S*-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate were the only major compounds isolated by simple column chromatography, while the other thiosulfinates could not be isolated.

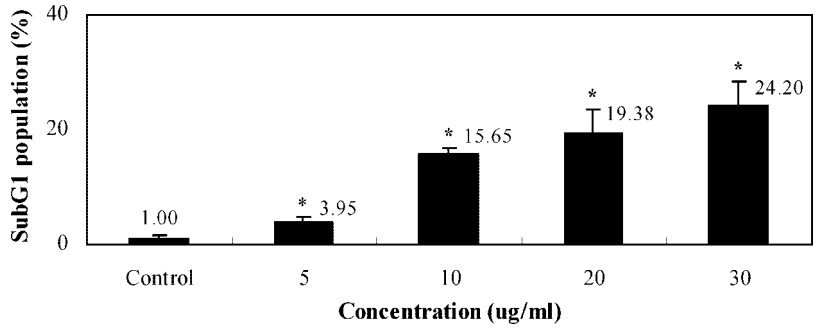

Figure 5. Sub-G1 population of MCF-7 cells treated with crude thiosulfinates from AT for 24 h. The MCF-7 cells were cultured with different concentrations of thiosulfinates from AT for 24 h, fixed, and stained with PI, and then DNA content was analyzed by flow cytometry. Data values are expressed as the mean ± SD of triplicate determinations.

Table 2. Effects of Crude Thiosulfinates from AT on the Survival Times of Sarcoma-180 Ascitic Tumors in Mice[a]

treatment	dose (mg/kg)	median survival time (days)	increase survival time (%)
control		16.8 ± 5.02	
crude	10	22.8 ± 6.91**	35.7
crude	30	26.2 ± 5.2*	56.0
thiosulfinates	50	29.0 ± 2.2*	72.6

[a] Mice were inoculated ip with 0.1 mL of ascitic tumors (1×10^6 cells/mL) on day 0. The mice were administered compounds at 10, 30, and 50 mg/kg, ip, for 7 consecutive days, starting 24 h after implantation of the tumor cells. The survival times were then measured. Significant differences were compared with the control at (*) $p < 0.05$ and (**) $p < 0.01$ by Student's *t*-tests. An amount of 10 μg of crude thiosufinates is equivalent to 10.2 g of AT.

The results of this study clearly demonstrate the cytotoxic activities of thiosulfinates in human cancer cells, and the activities were strong, in the order of *S*-methyl 2-propene-1-thiosulfinate and then *S*-methyl methanthiosulfinate. Similar to the cytotoxic activity, in our previous studies, the antibacterial activity of *S*-methyl 2-propene-1-thiosulfinate was also slightly stronger than that of *S*-methyl methanthiosulfinate (*13*).

For the in vivo anticancer test, the survival times of the sarcoma-180 tumor-bearing mice treated with thiosulfinates were extended compared to the mice treated with vehicle.

The proportion of the sub-G1 peak was negligible in the control MCF-7 cells without thiosulfinate treatment, whereas the MCF-7 cells treated with thiosulfinates had accumulations of cells in the sub-G1 phase in a dose-dependant manner.

While the MCF-7 cells not treated with thiosulfinates were well-spread with flattened morphologies, many apoptotic bodies were exhibited in the cells treated with thiosulfinates. Similarly, nuclei were of normal size in the untreated cells, whereas condensation of the nuclei and apoptotic bodies featuring characteristics of apoptosis were observed in the cells treated with thiosulfinates.

In previous studies, we first purified *S*-methyl methanthiosulfinate, thiosulfinates, and *S*-methyl 2-propene-1-thiosulfinate from AT by simple column chromatograpy. In this research, our results indicate that crude thiosulfinates, containing mostly S-methyl methanthiosulfinate and *S*-methyl 2-propene-1-thiosulfinate, have in vivo antitumor activity, as well as inhibit in vitro proliferation of cancer cells through apoptosis. We have attempted to find results on the anticancer effects of thiosulfinates isolated from AT, but no reports were found. We are also continuously studying to better understand the various mechanisms regulating antiproliferative effects as well as apoptosis induced by thiosulfinates from AT.

Supporting Information Available: Figure showing reversibility of cell-cycle arrest by thiosulfinates from AT. This material is available free of charge via the Internet at http://pubs.acs.org.

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