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Thiosulfinates from *Allium tuberosum* L. induce apoptosis via caspase-dependent and -independent pathways in PC-3 human prostate cancer cells

So-Yeon Kim,[a] Kyoung-Wuk Park,[a] Jae-Yong Kim,[b] Il-Yun Jeong,[c] Myung-Woo Byun,[c] Jung-Eun Park,[d] Sung-Tae Yee,[d] Kee-Hong Kim,[e] Johng S. Rhim,[e] Koji Yamada[f] and Kwon-Il Seo[a,*]

[a]*Department of Food and Nutrition, Sunchon National University, 315 Maegok, Suncheon, Jeonnam 540-742, Republic of Korea*
[b]*Department of Food Science and Technology, Kyungpook National University, Deagu 750-701, Republic of Korea*
[c]*Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Republic of Korea*
[d]*Department of Biology, Sunchon National University, Suncheon 540-742, Republic of Korea*
[e]*Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of Health Sciences, Bethesda, MD 20814, USA*
[f]*Department of Bioscience and Biotechnology, Kyushu University, Fukuoka 812, Japan*

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Abstract—This study was aimed to evaluate the apoptotic effects of thiosulfinates purified from *Allium tuberosum* L. on PC-3 human prostate cancer cells, and to elucidate detailed apoptosis mechanisms. Thiosulfinates significantly decrease viable cell numbers in dose- and time-dependent manners by apoptotic cell death via DNA fragmentation, chromatin condensation, and an increased sub-G1 phase. Apoptosis induced by thiosulfinates is associated with the activation of initiator caspase-8 and -9, and the effector caspase-3. In this study, thiosulfinates stimulated Bid cleavage, indicating that the apoptotic action of caspase-8-mediated Bid cleavage leads to the activation of caspase-9. Thiosulfinates decreased the expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax. Thiosulfinates also increased the expression of AIF, a caspase-independent mitochondrial apoptosis factor, in PC-3 cells. These results indicate that thiosulfinates from *A. tuberosum* L. inhibit cell proliferation and induce apoptosis in PC-3 cells, which may be mediated via both caspase-dependent and -independent pathways.
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Prostate cancer is the most common cancer as well as the second leading cause of cancer-related deaths in men in Western countries.[1] One of nine men over 65 years of age is frequently diagnosed with prostate cancer in the United States.[1,2] Dietary pattern has been identified as one of the major factors for the difference in prostate cancer incidence between Western and Asian countries.[2–4] But prostate cancer becomes more frequently diagnosed disease in Asian countries, potentially attributable to the Westernized food habbit. At present, there is no effective therapy available for the treatment of androgen-independent stage of prostate cancer, which is usually after hormonal deprivation/ablation therapy.[5] PC-3 cells are epithelial cells derived from a human prostate adenocarcinoma whose growth is androgen-independent.[6] This androgen-insensitive cell line provides excellent model system not only for basic studies, but also for evaluation of clinical treatment and management of human prostatic carcinoma.[7] *Allium* species have been cooked to season foods in many countries such as American, European, Chinese, Japanese, and Korean, and used since ancient times as pharmaceuticals.[8] *Allium tuberosum* L., one of *Allium* species, contains thiosulfinates, which is used as folk medicine as well as to flavor foods. Thiosulfinates are known in the *Allium* species as unstable intermediates in the enzymatically initiated degradation of *S*-alk(en)yl-L-cysteine sulfoxide.[9] *A. tuberosum* L. is also the major ingredient for leek Kimchi in Korea, which has long been used as a medicinal food for the treatment of abdominal pain, diarrhea, hematemesis, snakebite, and asthma in folk

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* Corresponding author. Tel.: +82 61 750 3655; fax: +82 61 750 3608; e-mail: seoki@sunchon.ac.kr

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remedies.[10] Since the first detailed chemical report on thiosulfinates, many chemists and pharmacists have tried to separate and identify them by using GC–MS, LC–MS, and NMR spectroscopy due to their striking biological activity[11,12] and interesting organosulfur chemistry.[13] Although GC and GC–MS analyses have provided excellent resolution and identification, many of the compounds from the *Allium* species observed by GC may have been artifacts of the analysis due to the high reactivity associated with the weak S–S bond (a bond energy of 46 kcal mol^{-}1 or less).[14,15] Several weaknesses also exist with the HPLC method such as retention time variation and the present limitation of LC–MS in characterizing volatile compounds.[16] But we first reported the separation of two thiosulfinates (A and B) from *A. tuberosum* L. by simple chromatography in previous study.[17] In this paper, we report the anti-proliferation activities of thiosulfinates from *A. tuberosum* L. against human prostate cancer cells, the induction of apoptosis, and the detailed apoptotic mechanisms related with caspase activation in PC-3 cells.

Thiosulfinates, containing mostly *S*-methyl methanethiosulfinate and *S*-methyl 2-propene-1-thiosulfinate, were isolated from *A. tuberosum* L. using CH_2Cl_2. Both compounds 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.[17] The mass spectral data were consistent with the molecular formula of $C_2H_6OS_2$ for A and $C_4H_8OS_2$ for B. In the prominent mass fragmentation pattern, *m/z* 95 (20%) for $[S(O)S\text{-}CH_3]^+$ was observed in A, while *m/z* 41 (100%) for $[C_3H_5]^+$ and *m/z* 95 (15%) for $[S(O)S\text{-}CH_3]^+$ were observed in B. The high-resolution mass spectral data for B 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 B showed the H-1 methylene protons (3.80 ppm) linked to their neighboring H-2 (5.94 ppm), and allylic was directly correlated with the H-3 (5.46 ppm) proton. Additionally, the mass spectrum (M^+-41) of B indicated the presence of an allyl group. The high-field chemical shift (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. Putting these various spectroscopic data together, these compounds were, respectively, identified as *S*-methyl methanethiosulfinate (Fig. 1A) and *S*-methyl 2-propene-1-thiosulfinate (Fig. 1B).[17]

The prostate cancer cells such as RC58T/h/SA#4,[18] LNCap·FGC,[19] PC-3, and DU145 were treated with thiosulfinates for 24 h. 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 4.58, 7.90, 10.07, and 15.31 μg/mL, respectively. The values of *S*-methyl methanethiosulfinate were 10.56, 11.24, 18.22, and 20.20 μg/mL. The values of *S*-methyl 2-propene-1-thiosulfinate were 3.52, 4.04, 8.561, and 11.67 μg/mL in the RC58T/h/SA#4, LNCap·FGC, PC-3, and DU145 cells, respectively (Table 1). In order to show the time-dependent actions of the thiosulfinates in cancer cells, the PC-3 cells were treated with the various concentrations of thiosulfinates. Viability was assessed after 24, 48, and 72 h of incubation by both SRB assay and cell count using Trypan blue dye. The anti-proliferation of PC-3 cells treated with thiosulfinates is presented as time-dependent manner in Figure 2. While viable cell numbers decreased, cell death rates increased in dose-dependent manners in the PC-3 cells treated with thiosulfinates (Fig. 3). The results indicate that thiosulfinates from *A. tuberosum* L. inhibit the proliferation of the prostate cancer cells in concentration- and time-dependent manners and their activities were strong, in the order of *S*-methyl 2-propene-1-thiosulfinate, thiosulfinates, and *S*-methyl methanethiosulfinate.

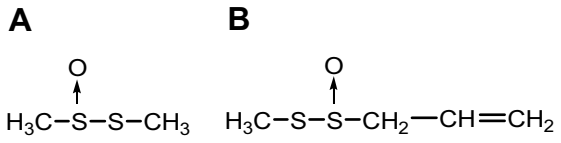

Figure 1. Structures of *S*-methyl methanethiosulfinate (A) and *S*-methyl 2-propene-1-thiosulfinate (B) purified from *Allium tuberosum* L.

To assess whether or not the thiosulfinates-induced apoptosis of the PC-3 cells, we performed Hoechst 33258 staining, a DNA fragmentation by ELISA kit, and detection of PARP cleavage by using Western blotting. After thiosulfinate treatments of 5, 10, and 20 μg/mL for 24 h, many PC-3 cells shrank and adhered together, detached condensed nuclei or apoptotic bodies were clearly seen in the cells after staining with Hoechst 33258 (Fig. 4). Another distinct feature of apoptosis, DNA fragmentation activity, was seen in PC-3 cells after thiosulfinate treatment. The relative apoptotic levels in the cells treated with 5, 10, and 20 μg/ mL of thiosulfinates were increased 1.3-, 1.7-, and 1.8-fold that of control cells, respectively (Fig. 5).

To assess correlation between thiosulfinate-induced proliferation inhibition and cell cycle blockage, the effects of the thiosulfinates on cell cycle distribution were determined. The proportion of the sub-G1 peak was negligible in the control PC-3 cells without thiosulfinates, whereas 24 h of exposure of the PC-3 cells to 5, 10, and 20 μg/mL of thiosulfinates resulted in cell accumulation in the sub-G1 phase in a dose-dependent manner (Fig. 6).

For the cleavage of PARP, the anti-PARP antibody recognized a 116-kDa band, which corresponds to the intact PARP in the thiosulfinate-treated and untreated PC-3 cells (Fig. 7). This 116-kDa band was intense in the control cells, however, it became faint in cells after 24 h of thiosulfinate treatment. In addition, this antibody also detected a band running about 85-kDa, which may correspond to the 85-kDa fragmentation of PARP in PC-3 cells after 24 h of treatment. This band was not seen in the untreated cells.

Caspases, a family of cysteine proteases, are activated during the execution phase of the apoptotic process.

Table 1. IC_{50} value of thiosulfinates from *Allium tuberosum* L. on the prostate cancer cells

Cell lines	IC_{50} value (μg/mL)		
	Thiosulfinate	*S*-Methyl Methanethiosulfinate	*S*-Methyl 2-propene-1-thiosulfinate
RC58T/h/SA#4	4.58 ± 5.19	10.56 ± 2.34(96.06 μM)	3.52 ± 1.71(25.28 μM)
LNcap·FGC	7.90 ± 7.93	11.24 ± 0.23(102.18 μM)	4.04 ± 0.33(29.70 μM)
PC-3	10.07 ± 2.24	18.22 ± 2.08(165.63 μM)	8.56 ± 2.16(62.94 μM)
DU145	15.31 ± 1.8	20.2 ± 1.45(183.63 μM)	11.67 ± 1.22(85.80 μM)

Data values were expressed as mean ± SD of triplicate determination.

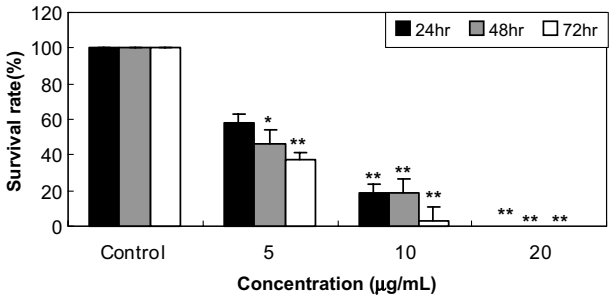

Figure 2. Cell growth inhibition effects in the prostate cancer cells (PC-3) treated with thiosulfinates from *Allium tuberosum* L. for 24, 48, and 72 h by SRB assay. The PC-3 cells were treated with the thiosulfinates at 0–20 μg/mL for 72 h, and then fixed and stained with SRB for cellular protein content analysis. Data values were expressed as 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.

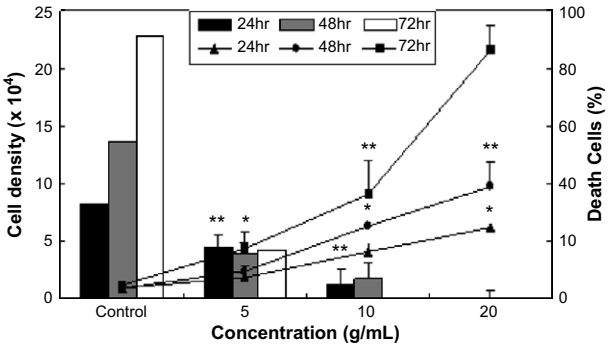

Figure 3. Cell growth inhibition effects in the prostate cancer cells (PC-3) treated with thiosulfinates from *Allium tuberosum* L. for 24, 48, and 72 h by trypan blue dye. Results were expressed as the percentage of control. The bars and line are means of the cell densities and cell deaths, respectively. The PC-3 cells were detached by trypsinization and harvested by resuspension in medium for immediate analysis of the cell number using a hemacytometer. Data values were expressed as mean ± SD (standard deviation) of triplicate determinations. Significant differences were compared with the control at $^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's *t*-tests.

Once activated, caspases activate downstream caspases, leading to apoptosis.[20] Thiosulfinates significantly stimulated the activities of caspase-8, caspase-9, and caspase-3 in the PC-3 cells by 1.9-, 2.1-, and 1.7-fold at 10 μg/mL concentration, respectively (Fig. 8). To determine whether or not the apoptosis induced by thiosulfinates occurs via the caspase-dependent pathway, Z-VAD-fmk, a universal caspase inhibitor,[21] was added with thiosulfinates. Although the caspase inhibitor reduced apoptosis by the thiosulfinates, cell death via thiosulfinates was significantly elevated in a dose-dependent manner (Fig. 9).

To investigate the mitochondrial apoptotic events involved in thiosulfinate-induced apoptosis, we analyzed changes in the levels of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 by Western blotting. As shown in Figure 10A, the thiosulfinates dose-dependently increased expression of the pro-apoptotic protein Bax, decreased expression of the anti-apoptotic protein Bcl-2, in the PC-3 cells.

Since thiosulfinate-mediated apoptosis involved initiation of death receptor and mitochondrial signaling, it was possible that thiosulfinates activated the mitochondrial apoptotic pathway through caspase-8-mediated Bid cleavage, which then resulted in caspase-9 activation.[22] To test this idea, we checked the status of the Bid protein during thiosulfinate-induced apoptosis. Figure 10B shows that a full size Bid (22 kDa) protein was cleaved to yield a 15 kDa fragment following cell treatment with thiosulfinates, and this closely matched the appearance of capase-8 activation.

AIF is known to be involved in induction of apoptotic cell death via a caspase-independent pathway. Mitochondrial AIF is released in response to death stimuli, and subsequently translocates into the nucleus and causes nuclear condensation.[23] We determined whether or not AIF plays a role in thiosulfinate-induced apoptotic cell death by analyzing changes in the levels of AIF by Western blot (Fig. 11A), as well as AIF translocation into the nucleus by immunostaining (Fig. 11B). The results suggest that AIF translocation into the nucleus is required for the thiosulfinate-induced cell death of PC-3 cells.

Allium tuberosum L. has been used for years and is regarded as medicinal foods because of the thiosulfinates. In our previous study, *S*-methyl methanethiosulfinate and *S*-methyl 2-propene-1-thiosulfinate were the only major compounds isolated by simple column chromatography.[17]

In the present study, we elucidated the cytotoxic activities of thiosulfinates in both androgen-positive and androgen-negative human prostate cancer cells and apoptotic mechanism in PC-3 cells. Since cancer is a disorder of deregulated cell proliferation and cell sur-

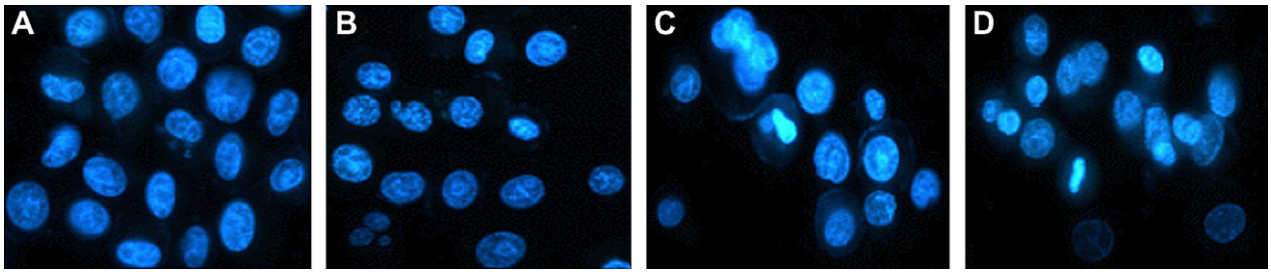

Figure 4. Nuclear fragmentation induced in the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. for 24 h. (A) Non-treated. (B) PC-3 cells treated with 5 μg/mL of thiosulfinates for 24 h. (C) PC-3 cells treated with 10 μg/mL of thiosulfinates for 24 h. (D) PC-3 cells treated with 20 μg/mL of thiosulfinates for 24 h. The PC-3 cells were treated with thiosulfinates from AT at 0–20 μg/mL for 24 h, washed once with PBS and stained with 10 μL/mL of Hoechst 33258, and finally, examined by a fluorescence

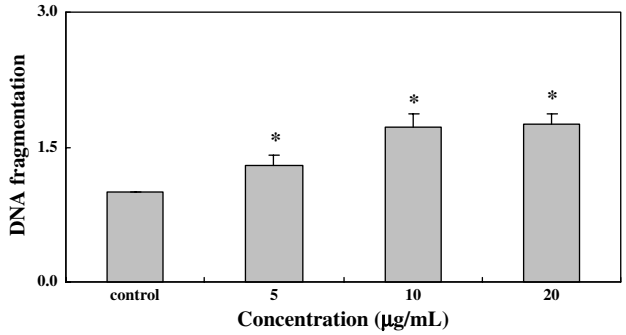

Figure 5. DNA fragmentation in the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. for 24 h. The PC-3 cells were treated with the thiosulfinates at 0–20 μg/mL for 24 h, and then DNA fragmentation was measured by ELISA kit.

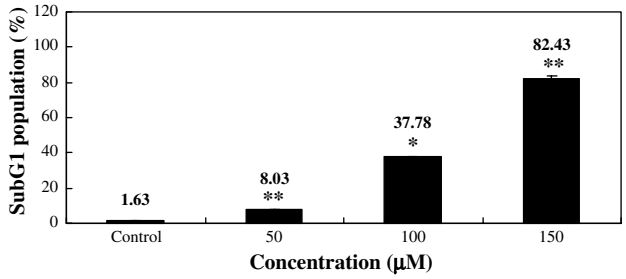

Figure 6. Sub-G1 population of the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. for 24 h. The PC-3 cells were cultured with different concentrations of the thiosulfinates for 24 h, fixed and stained with PI, and then DNA content was analyzed by flow cytometry. Data values were expressed as 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.

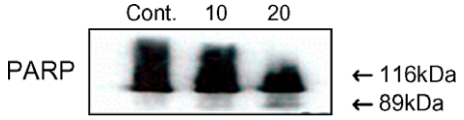

Figure 7. Apoptotic PARP cleaved fragmentation in the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. for 24 h by Western blot.

vival,[24] inhibiting cell proliferation and increasing apoptosis in tumors are effective strategy for preventing tumor growth.[25]

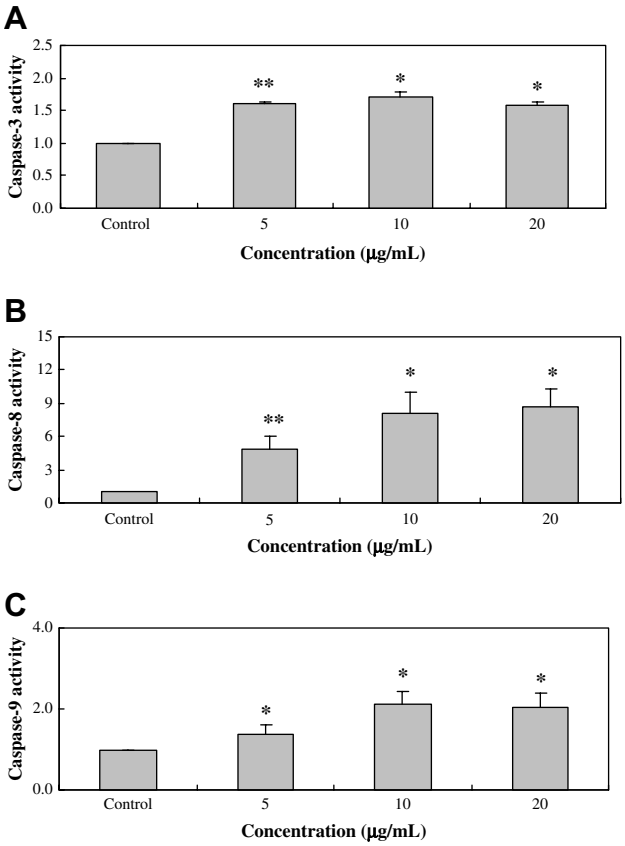

Figure 8. Caspase activities in the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. (A) Caspase-3 activity. (B) Caspase-8 activity. (C) Caspase-9 activity. Data values were expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at $^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's *t*-tests.

Our current results show that thiosulfinates inhibited PC-3 cell proliferation and induced apoptosis. Important features of apoptotic cell death are cell shrinkage, chromatin condensation, DNA fragmentation, and apoptotic bodies.[26] Another distinct feature of apoptosis, relative apoptotic levels in the thiosulfinate-treated cells was increased. Interneucleosomal DNA fragmentation is a significant feature of apoptotic cell death, and this phenomenon is caused by caspase-activated DNAse and endonuclease G, which cleave DNA between nucleosome.[27] The thiosulfinate-treated PC-3 cells had accu-

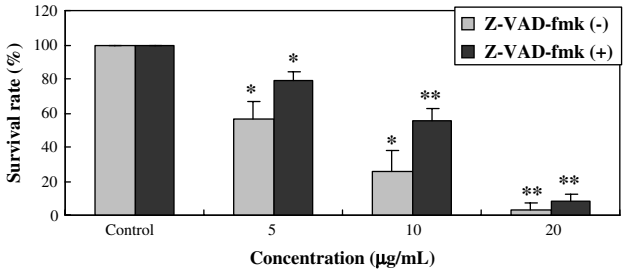

Figure 9. Effect of a caspase inhibitor (Z-VAD-fmk) on cell death induced by thiosulfinates from *Allium tuberosum* L. PC-3 cells were pre-incubated with 10 μM of Z-VAD-fmk for 2 h and then treated with various concentrations of the thiosulfinates. Data values were expressed as mean ± SD of triplicate determinations. Significant differences were compared with the control at $^{*}p < 0.05$ and $^{**}p < 0.01$ by Student's *t*-tests.

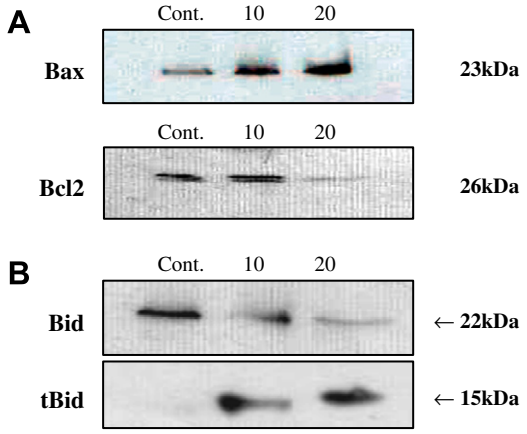

Figure 10. Thiosulfinates from *Allium tuberosum*-induced apoptosis in the mitochondrial pathway. (A) Expression level of Bcl-2 family protein in the PC-3 cells treated with the thiosulfinates. (B) Cleaved Bid in the thiosulfinate-treated PC-3 cells. Western blot analyses were assessed for protein expression.

mulations of cells in the sub-G1 phase in a dose-dependent manner, which is a common indication of the presence of apoptotic cells.

Meanwhile, PARP, a 116-kDa nuclear enzyme, seems to be involved in DNA repair by catalyzing the synthesis of PARP from nicotinamide adenine dinucleotide.[28] Because PARP is important for cells to maintain their viability, the cleavage of PARP helps cellular disassembly and serves as a marker of cells undergoing apoptosis.[29] In this study, a PARP 116-kDa band became faint with respect to increasing thiosulfinate, and an 85-kDa band became dark, in cells after 48 h of thiosulfinate treatment. PARP is one of the key cleavage targets of caspase-3.[30] In this study, thiosulfinates significantly increased caspase-3 activity at 5, 10, and 20 μg/mL concentrations. DNA fragment and nuclear chromatin condensation have been demonstrated in PC-3 cells by thiosulfinates. Moreover, the observation of caspase-3 activation and PARP cleavage also confirms that the promotion of apoptosis by thiosulfinates involves a caspase-dependent pathway.

To elucidate the apoptotic mechanism of PC-3 cells that is induced by thiosulfinates, we evaluated their effects on caspase-8 and caspase-9 activities. The results show that caspase-8 and caspase-9 activities significantly increased in the PC-3 cells, after 24 h of incubation with thiosulfinate. Activation of the caspase cascade is a key element in the apoptotic process. Caspase-8 and -9 are involved in the death receptor pathway and mitochondrial pathway, respectively. However, caspase-8 can activate caspase-9 through the proteolysis of Bid, a member of the Bcl-2 family.[22]

We observed that thiosulfinates enhanced Bid cleavage in the PC-3 cells. Thus, the induction of apoptosis by thiosulfinates might have been mediated through the death receptor pathway. Furthermore, although the inhibition of cell growth by the thiosulfinates decreased in the PC-3 cells treated with the caspase family inhibitor, cell death was significantly elevated in a dose-dependent manner. This result suggests that thiosulfinates might induce apoptosis via both capase-dependent and -independent pathways.

We found that thiosulfinates increased the expression of pro-apoptotic protein Bax and decreased the expression of anti-apoptotic Bcl-2 in PC-3 cells, and subsequently induced the release of cytochrome *c* from the mitochondria into the cytoplasm and then activated caspase-9, which in turn activates caspase-3. These findings suggest that apoptosis induction in thiosulfinate-treated PC-3 cells could be associated with a caspase-dependent cascade that involves activation of the mitochondrial pathway, initiated by the inhibition of Bcl-2 and activation of Bax.

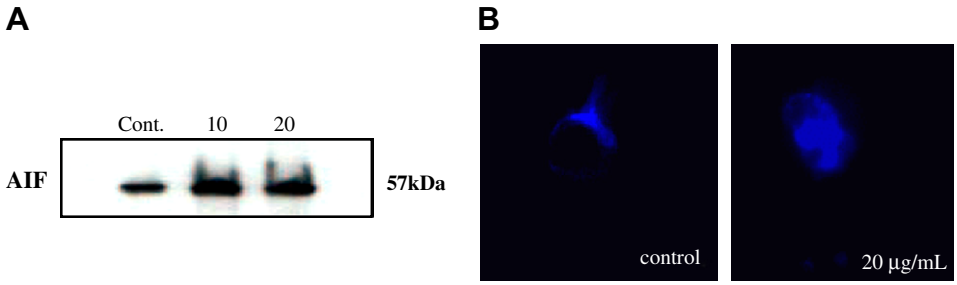

Figure 11. Induction of apoptosis through caspase-independent pathway in the PC-3 cells treated with thiosulfinates from *Allium tuberosum* L. (A) The expression level of AIF protein in the thiosulfinates-treated PC-3 cells for 24 h. Western blotting analysis was assessed for protein expression. (B) Representative images for translocation of AIF to the nucleus in the thiosulfinates-treated PC-3 cells.

On the other hand, thiosulfinate elevated AIF protein expression and AIF translocation into the nucleus. AIF is a mitochondrial apoptosis-inducing factor implicated in apoptosis, and a mitochondrial flavoprotein that translocates to the nucleus following apoptotic stimuli. In nucleus, AIF induces partial DNA fragmentation and chromatin condensation. AIF appears to promote apoptosis independent of caspase, although it likely acts in a cooperative manner with other factors to promote nuclear apoptosis.

In conclusion, the current study showed that thiosulfinates from *A. tuberosum* L. exert PC-3 prostate cancer cell growth inhibition by apoptosis induction. We suggest that the apoptotic mechanism of thiosulfinates may be mediated by caspase-dependent and -independent mitochondrial pathways. These results indicate that thiosulfinates from *A. tuberosum* L. can be potential anti-prostate cancer agent, therefore, further in vivo study is warranted.

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