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Antiproliferation effect of sulforaphene isolated from radish (*Raphanus sativus* L.) seeds on A549 cells

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Abstract

Sulforaphene (SFE), a major isothiocyanate in radish seeds, is a close chemical relative of sulforaphane (SFA) isolated from broccoli seeds and florets. The anti-proliferative mechanisms of SFA against cancer cells have been well investigated, but little is known about the potential anti-proliferative effects of SFE. In this study, we showed that SFE purified from radish seeds inhibited the growth of six cancer cell lines (A549, CHO, HeLa, Hepa1c1c7, HT-29, and LnCaP), with relative half maximal inhibitory concentration values ranging from 1.37 to 3.31 µg/mL. Among the six cancer cell lines, SFE showed the greatest growth inhibition against A549 lung cancer cells, where it induced apoptosis by changing the levels of poly(ADP-ribose) polymerase and caspase-3, -8, and -9. Our results indicate that SFE from radish seeds may have significant anti-proliferative potency against a broad range of human cancer cells via induction of apoptosis.

Keywords: Anti-proliferation, *Brassica* vegetables, Human disease, Isothiocyanate, MTT assay

Introduction

Glucosinolates (GSLs) are major secondary metabolites found in *Brassica* vegetables such as radish, broccoli, kale, and kimchi cabbage. GSLs are not bioactive, but their hydrolytic breakdown products are thought to protect against cancer [1]. GSLs are released from the vacuoles of myrosin cells in response to plant tissue damage and are hydrolyzed by cytosolic myrosinase, a thioglucoside glucohydrolase (EC 3.2.3.1) [2]. Isothiocyanates (ITCs), byproducts of the hydrolytic breakdown of GSLs, are thought to play an important protective role in plants against fungi, bacteria, viruses, and insects [3]. The early studies focused mainly on the ability of ITCs to inhibit the growth of cancer cells, recent studies showed that ITCs also suppress the survival and proliferation of existing cancer cells. Anticancer potency of ITCs on cancer cells via multiple mechanisms, including inhibition of carcinogen-activating enzymes, induction of carcinogen-detoxification enzymes, induction of apoptosis, and other mechanisms not yet well understood [4–8]. Apoptotic induction is of particular interest because it can be exploited for cancer prevention and treatment.

Sulforaphane (SFA) obtained from broccoli is the most well-known example of a plant ITC with anticancer properties [5]. SFA was isolated from broccoli extracts as a potent inducer of mammalian cytoprotective enzymes [5–7]. Broccoli seeds and sprouts are rich sources of glucoraphanin (GRA), which is the chemical precursor of SFA [9]. Broccoli has been used as a source of GRA or SFA in studies on human health. When fresh broccoli floret is consumed, the major degradation product of GRA is SFA nitrile, which has little anticancer activity [10, 11]. Radish seeds and sprouts are rich in sulforaphene (SFE), a major ITC with a chemical structure similar to that of SFA [12]. In radish, glucoraphenin (GRE), the GSL corresponding to GRA in broccoli, is broken down into SFE,

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rather than SFE nitrile, because radish seeds and sprouts lack the epithiospecifier protein (ESP), which promotes nitrile formation during the breakdown of GSLs by myrosinase [12]. The anticancer properties of SFA from broccoli have been well documented, and research on the potential biological activities of SFE from radish are continuously update for recent. It was reported that the potency of anticancer against human gastric cancer, thyroid cancer, and esophageal cancer cell lines and that SFE extracts in radish roots have a significant and specific anti-proliferative activity against HepG2 cells [8, 13–15]. The mechanism of action of SFE or its effect on apoptosis remains unknown in various spectrum against cancer cells. We investigated the biological activity of SFE obtained from radish seeds on anti-proliferation assay of six cancer cell lines. After then, we tried to imply the mechanisms of apoptosis in cancer cells which is considering the efficacy of anti-proliferation under above mentioned six cancer cell lines.

Materials and methods

Chemical reagents

'Taeback' radish seeds were obtained from Monsanto Korea (Anseong, Korea). All chemicals, antibiotics, and broths were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Becton Dickinson (San Jose, CA, USA). All solvents used for high-performance liquid chromatography (HPLC) analysis were purchased from J.T. Baker (Center Valley, PA, USA). SFE and SFA standards were obtained from LKT Laboratories Inc. (St. Paul, MN, USA). The six cancer cell lines, A549 (lung cancer), CHO (ovarian cancer), HeLa (cervical cancer), Hepa1c1c7 (lung cancer), HT-29 (colon cancer), and LnCaP (prostate cancer), were a generous donation from the Korean Cell Line Bank (Seoul, Korea; https://cellbank.snu.ac.kr/index.php). The above mentioned 5 cancer cell lines except A549 cell line had been studied to elucidate the anticancer properties of SFA [4, 8, 10, 12].

Extraction and qualitative identification of SFE from radish seeds

The procedure for SFE extraction from radish seeds was adopted from a previous report [16]. Radish seeds (100 g) were combined with 1 L water and homogenized for 3 min at 20,000 rpm on ice. The homogenates were incubated for 3 h at 37 °C to allow the hydrolysis of GRE by myrosinase. The hydrolysed seed meal was centrifuged for 20 min at $6000 \times g$. The supernatant was extracted with 1 L dichloromethane (MC) three times. The MC layer was passed through anhydrous sodium sulphate to remove water. The MC layer was evaporated to 30 mL using a rotary evaporator (N-1001

series, Eyela Co., Tokyo, Japan) at 35 °C. To remove contaminants, MC layer was applied to a Sep-Pak silica cartridge (Waters, Milford, MA, USA) equilibrated by MC. The cartridge was washed with ethyl acetate and SFE extract eluted with ethanol. Extracted SFE was subjected to a recycling preparative-LC system (LC-9104, JAI, Tokyo, Japan) equipped with a Bondapak C18 HPLC column (500×3.9 mm, Waters, Milford, MA, USA) fitted with a C18 guard column. The mobile phase consisted of chloroform/methanol/water (8:1:1, v/v/v). The flow rate was 3.0 mL/min and the absorbance was monitored at 254 nm. Purified SFE from radish seeds was compared to authentic SFE standard using GC–MS. Mass spectra were obtained by electron ionization at 70 eV over a mass range of 40–500 m/*z* and compared with the mass spectrum of a commercial SFE standard (Additional file 1: Figure S1). SFE standard were used for quantification of extracted SFE.

Cell culture conditions and strains

Six cancer cell lines were evaluated for their sensitivities to SFE. SFA from broccoli was used as a control. Cell lines seeded at 2×10^5/mL were maintained under 5% CO_2, 100% relative humidity, and 37 °C. The cancer cell lines were cultured as monolayers in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1 mM antibiotic at 37 °C in humidified air containing 5% CO_2.

MTT assay

Cells were seeded at 1×10^5/mL in a 96-well plate and incubated for 24 h in 5% CO_2 and 100% humidity at 37 °C. Solutions of 0.5–16 μg/mL SFE or SFA were added to each well and gently mixed with 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) reagent using a multi-channel pipette. Dimethyl sulfoxide (DMSO) was used as a control. The mixtures were incubated for 24 h until a purple precipitate was visible. The MTT reaction was stopped by adding detergent (100 μL). The mixtures were incubated at room temperature in the dark for 2 h. The number of living cells in the wells was determined by measuring the absorbance at 570 nm using a microplate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as relative half maximal inhibitory concentration (IC_{50}) and plotted against SFE and SFA concentrations. Each experiment was conducted in triplicate, and the results were expressed as means and standard deviations.

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Antiproliferation assay

SFE was removed from the culture, and proliferation was assessed again by MTT assay after 24, 30, 36 h in A549 cells in order to confirm the survival ability of the cell.

Cell viability (%) = Mean optical desity in treatment well × 100/Mean optical density in control well at same time.

Observation of morphological changes in cells by Hoechst and PI staining

The procedure was conducted by followed a previous report [14, 15]. Cells were cultured in six-well plates with various concentrations (0.5–8.0 µg/mL) of semi-purified SFE for 24 h at 37 °C. At 24 h after treatment, SFE was removed from the culture, and treated cells were incubated an additional 24 h without SFE. After incubation, photomicrographs were taken using a phase-contrast microscope. Apoptotic and necrotic cells were distinguished using propidium iodide (PI) staining. After incubation, cells were washed with phosphate-buffered saline (PBS) solution, fixed in ethanol for 30 min at 4 °C, rehydrated with PBS, and incubated with 100 µL PI at 37 °C for 5 min. Photomicrographs were taken using a fluorescence microscope (BX51; Olympus Co., Tokyo, Japan).

Western blot analysis of apoptotic proteins

A549 cells grown on culture plates were treated with SFE or SFA. After treatment, detached cells were collected and washed twice with cold PBS. Proteins were extracted from the cells using radioimmune precipitation assay buffer (50 mM Tris–HCl pH 8.0, 150 mM sodium chloride, 1.0% IGEPAL CA-630/NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease and phosphatase inhibitors). Protein concentrations were determined using the Bradford protein assay [14, 15]. Extracted proteins (50–100 µg) were subjected to electrophoresis on 10% polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane. Electrophoresis and blotting were performed using the PowerPac200 Electrophoresis System (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat milk for 1 h, membranes were incubated overnight at 48 °C with primary antibodies and subsequently probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibodies for 1 h. Protein bands were detected using the In Vivo Imaging System FX (Kodak Co., New Haven, CT, USA).

Statistical analysis

All experiments were conducted three replications and results were expressed as means ± standard deviation. The one-way ANOVA and post-statistical analysis were conducted using SPSS ver. 22.0 (IBM, Armonk, NY, USA).

Results

Inhibition of cancer cell growth by SFE

The DMSO control exerted no cytotoxicity against any of the six cancer cell lines. The viabilities of all cancer cell lines after SFE or SFA treatment were dependent on the SFE/SFA concentration and ranged from 9.1 to 53.7% relative to the control at high doses (Fig. 1). Significant inhibitory activity was observed in A549, CHO, HeLa, Hepa1c1c7, and HT-29 cells, with strong inhibition at concentrations > 4.0 µg/mL (Fig. 1a–e); growth inhibition by SFE was not significant in LnCaP cells (Fig. 1f). The relative SFE and SFA IC_{50} values are presented in Additional file 1: Table S1. The highest IC_{50} of SFE was observed in A549 as 1.50 µg/mL. The IC_{50} of SFE in HeLa cells was on 1.37 mg/mL (Fig. 1c), but at the highest SFE concentration (16 µg/mL) it was still 30% of the control-group viability. In all cancer cell lines except HeLa and LnCaP, the inhibitory effect of SFE on cell growth was slightly greater than that of SFA (Fig. 1; Additional file 1: Table S1). SFE had 1.25- and 1.31-fold greater inhibitory effect (IC_{50} value) on A549 and HT-29 cell growth compared with that of SFA.

Anti-proliferative effect of SFE in A549 cancer cells

The reduced cell viability induced by SFE was maintained even after SFE removal (Fig. 2), particularly in treatments with 4.0 µg/mL SFA or SFE. Thus, SFE effectively inhibited A549 cell proliferation. Morphological changes in A549 cells indicative of cell death were observed in SFE-treated cells compared with SFA-treated and control cells (Fig. 3). Cells became detached from the plates at higher SFA and SFE concentrations. The number of detached, rounded, and condensed dead cells increased sharply at concentrations > 2.0 µg/mL SFA or SFE, with almost all cells exhibiting this phenotype at 8.0 µg/mL (Fig. 3). The number of detached cells was greater after 2.0 µg/mL SFE than after 2.0 µg/mL SFA treatment.

To confirm and distinguish between apoptotic and necrotic cells induced by SFA and SFE, detached cells were stained with Hoechst and PI after SFA/SFE treatments (Fig. 4). Treatment with both compounds resulted in nuclear changes in a concentration-dependent manner. Hoechst-positive and PI-negative cells with bright or condensed blue nuclei (Fig. 4, indicated by circles) displayed morphological features characteristic of apoptosis. Hoechst-negative (or weakly negative) and PI-positive cells, stained red, were necrotic (Fig. 4, indicated by arrows). The SFA and SFE treatments produced detached A549 cells with highly condensed
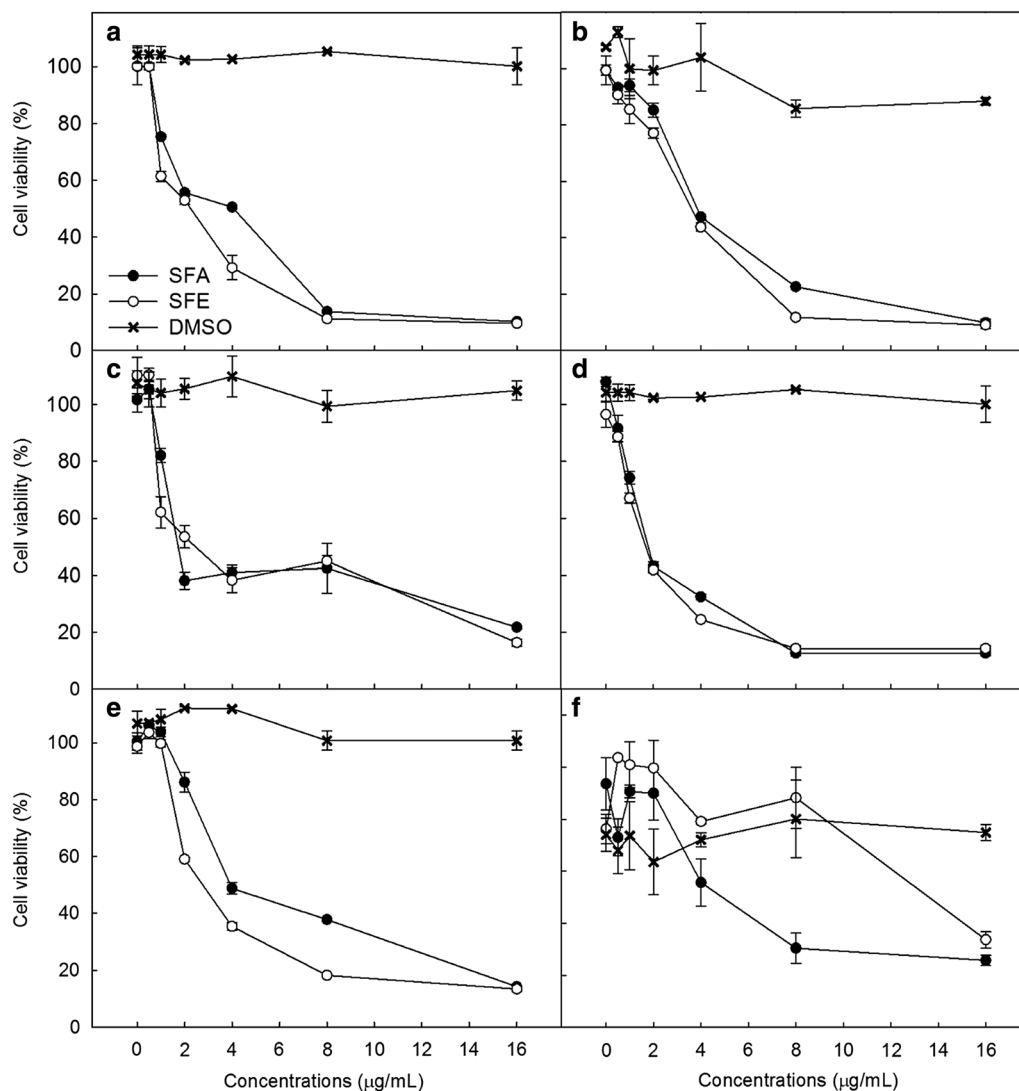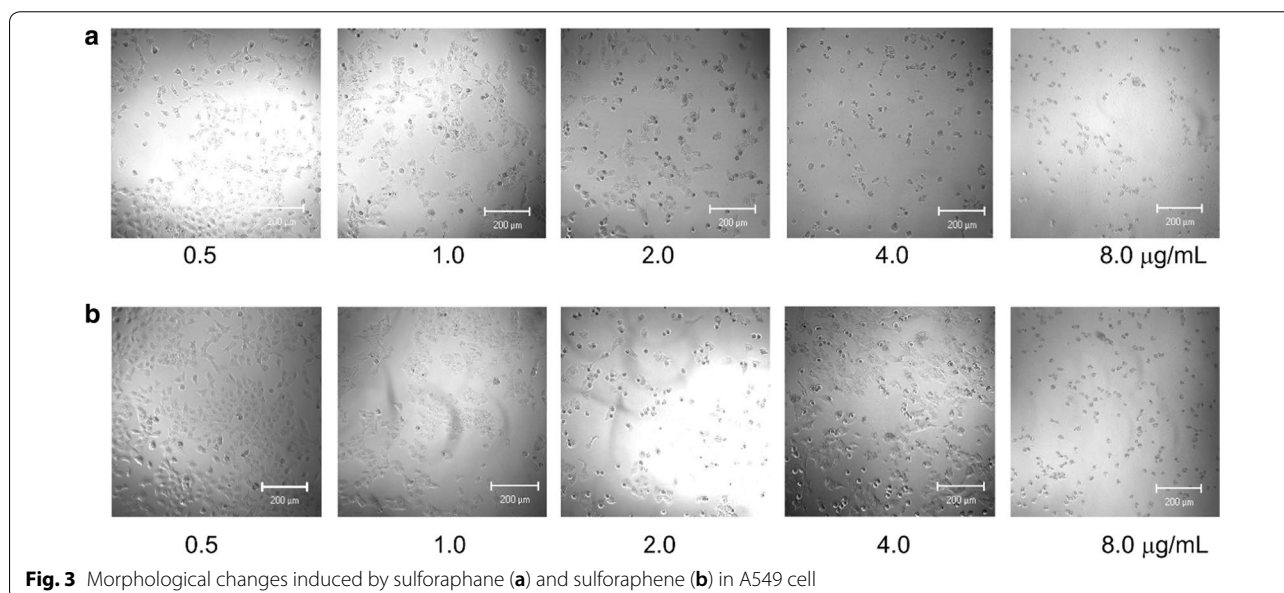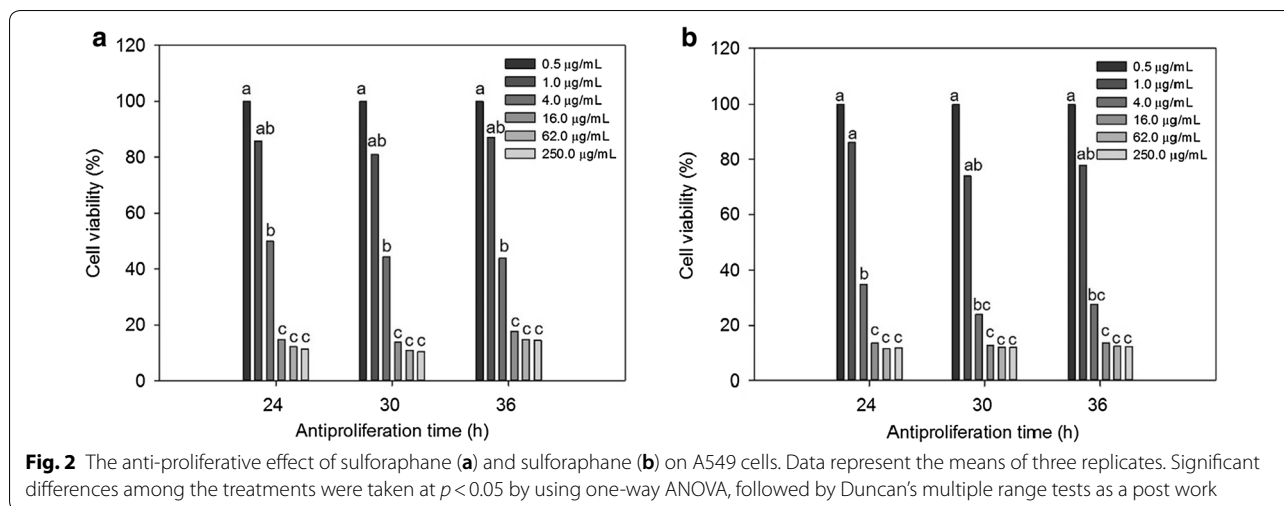
Fig. 1 The effects of sulforaphane (SFA) and sulforaphene (SFE) on the viability of six cancer cell lines: A549 (**a**), CHO (**b**), HeLa (**c**), Hepa1c1c7 (**d**), HT-29 (**e**), and LnCaP (**f**). Data represent the means ± standard deviations of six replicates. Cell viability was defined as the percentage of mean optical density in treatment well/mean optical density in control well at 24 h after inoculation

chromatin nuclei in a concentration-dependent manner. SFA treatment markedly induced cell necrosis at 2.0–8.0 μg/mL (Fig. 4a), whereas SFE treatment resulted in no or few necrotic cells at the same concentrations (Fig. 4b). At a high SFA or SFE concentration (8.0 μg/mL), no apoptotic cells were observed. At 2.0 μg/mL, SFA generated a higher proportion of cells with condensed chromatin among the detached cells (24%), indicating more apoptotic cells, compared with SFE (8%; Fig. 5). At 4.0 μg/mL, SFE induced little necrosis in A549 cells, but SFA caused a significantly higher degree of necrosis (55% of detached cells). At 8.0 μg/mL

of SFE, most A549 cells were dead, as indicated by the absence of normal blue- and red-stained cells (Fig. 5).

Induction of apoptosis by SFE in A549 cancer cells

Inactivated poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3, -8, and -9 are markers of activation of different apoptotic pathways. A549 cell lysates were subjected to western blot analysis using antibodies targeting these markers. SFE and SFA elevated the levels of these proteins, consistent with apoptotic activation (Fig. 6). At 8.0 μg/mL SFE, the PARP level was decreased, and the cleaved form of the protein was evident. Cleaved

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Fig. 2 The anti-proliferative effect of sulforaphane (**a**) and sulforaphane (**b**) on A549 cells. Data represent the means of three replicates. Significant differences among the treatments were taken at $p < 0.05$ by using one-way ANOVA, followed by Duncan's multiple range tests as a post work

Fig. 3 Morphological changes induced by sulforaphane (**a**) and sulforaphene (**b**) in A549 cell

caspase-3 was upregulated at lower SFE and SFA concentrations (2.0 and 4.0 µg/mL), whereas cleaved caspase-8 and -9 were upregulated at higher SFE and SFA concentrations (4.0 and 8.0 µg/mL). The levels of all three cleaved caspases were increased more by SFE than by SFA. At high SFE and SFA concentrations (16.0 µg/mL), most cells were necrotic (data not shown); thus, we did not evaluate these cells by western blot analysis.

Discussion

SFE exhibited remarkably effective IC_{50} values (1.37–3.68 µg/mL = 7.8–18.9 µM) (Fig. 1; Additional file 1: Table S1). Based on our findings, SFE can be classified as a bioactive compound due to its low IC_{50} (below

the defined upper limit of 20 µg/mL) [17]. SFE isolated from radish seeds was slightly more effective against the five cancer cell lines than was commercial SFA. SFE has strong anti-proliferative effects on cancer cell lines [14, 15, 18]. SFA clearly exhibits anticancer mechanisms on LnCaP cells, such as cell cycle arrest [19]. The viability of LnCaP cells was decreased by SFA treatment at 8.0 µg/mL, but not by SFE treatment even at high doses (Fig. 1f; Additional file 1: Table S1). SFE exerts a different spectrum of anticancer properties on various cancer cell lines compared with SFA. The different structures of ITCs isolated from *Brassica* crops are responsible for their differential anticancer effects [4–7]. Bioactive ITC concentrations of 5–50 µM, which are effective for
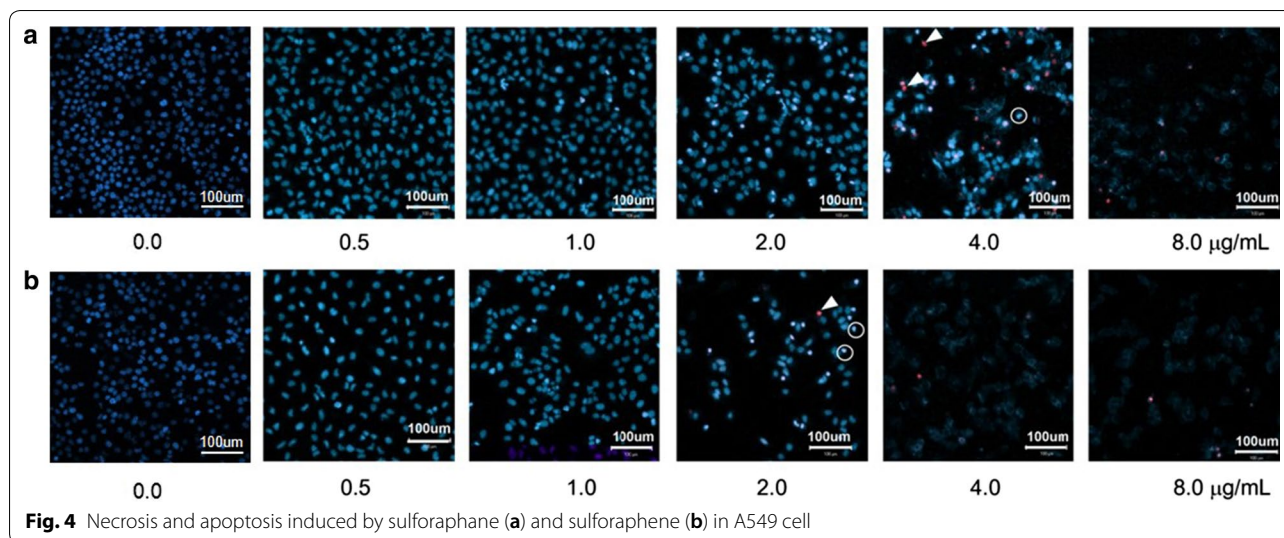
Fig. 4 Necrosis and apoptosis induced by sulforaphane (**a**) and sulforaphene (**b**) in A549 cell
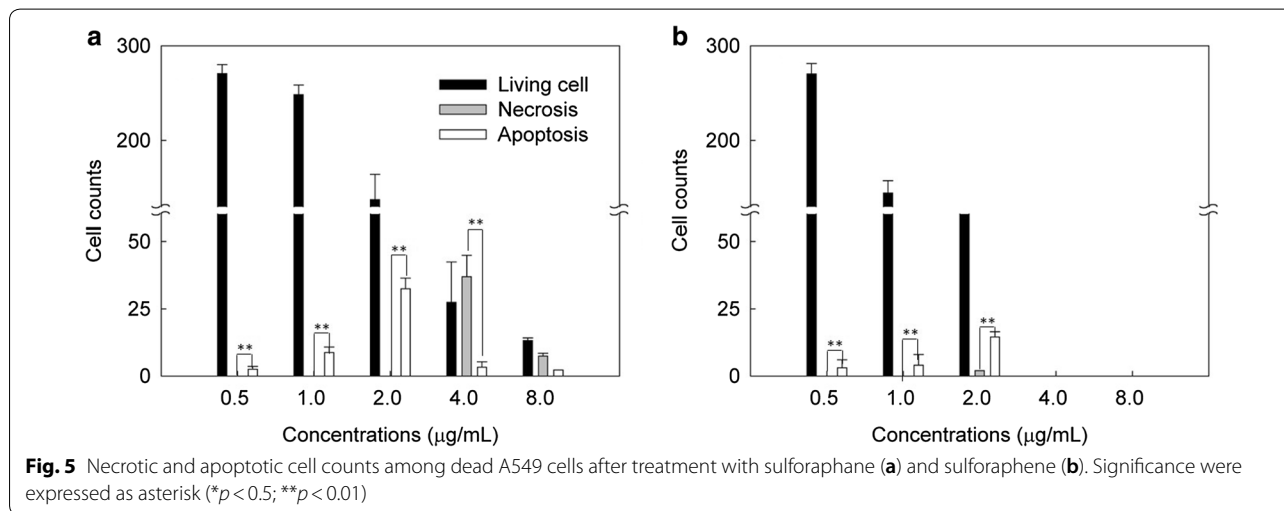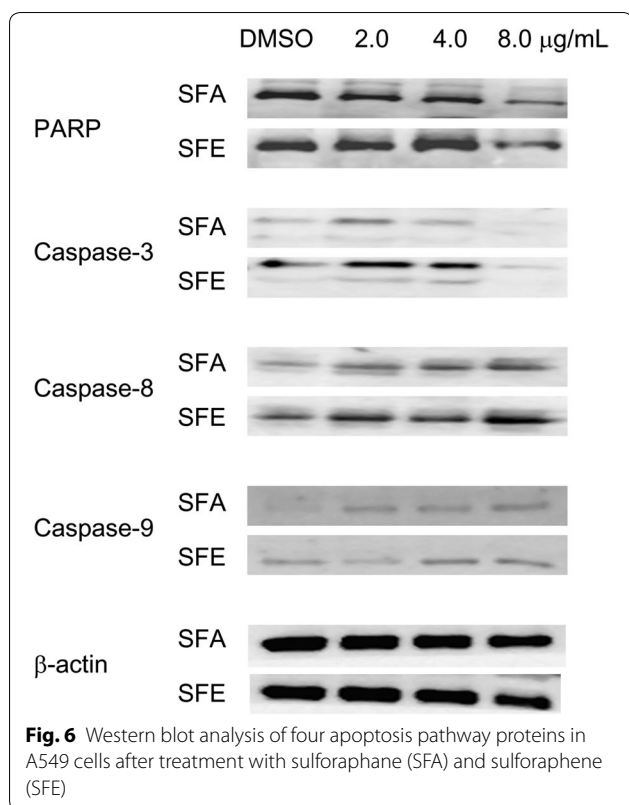

Fig. 5 Necrotic and apoptotic cell counts among dead A549 cells after treatment with sulforaphane (**a**) and sulforaphene (**b**). Significance were expressed as asterisk (*$p < 0.5$; **$p < 0.01$)

the prevention of lung, bladder, and colon cancers, can be achieved by consuming 200–400 g cruciferous vegetables per day [3]. We analyzed GRE, the precursor of SFE, in different plant organs of 'Taeback' radish (Fig. S2) and detected its highest concentrations (7.8 mM/g dry weight) in mature seeds. The consumption of only a few radish seeds per day would be required to achieve the bioactive ITC concentration.

Our analysis of cell morphology (Fig. 3) clearly indicated that SFE inhibits cell spreading and elongation. Hoechst and PI staining showed distinctive morphological features characteristic of apoptosis-related cell death in dead cells produced after SFE treatment (Fig. 4). No necrosis was observed in SFE-treated cancer cells at any concentration evaluated. The extent of apoptosis induced by SFE was similar to that induced by SFA (Fig. 5).

Previous reports found anti-proliferative effects of radish extracts on HeLa, hepatoma, and colon cancer cell lines by cell staining [13, 20].

Apoptosis-related protein expression was evaluated to show that SFE strongly induces apoptosis in A549 cells (Fig. 6). PARP, which is responsible for DNA repair, was found in its cleaved inactive form after SFE and SFA treatments. Inactivation of PARP implies that SFE induces programmed cell death in lung cancer cells by inhibiting the cellular DNA repair systems. In general, the cleavage of caspase-3, -8, and -9 plays an important role in the execution phase of intrinsic and extrinsic apoptosis through PARP inactivation [21]. SFE in A549 cells induced the cleavage of caspase-3, -8, and -9 involved in apoptosis mechanism (Fig. 6). The cleaved caspase levels varied depending on the SFE/SFA concentration. Apoptotic

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Fig. 6 Western blot analysis of four apoptosis pathway proteins in A549 cells after treatment with sulforaphane (SFA) and sulforaphene (SFE)

present research suggest that the consumption of radish seeds may help with the prevention of cancer. Further, biotechnological and agricultural departments will focus on breeding radish seeds or their edible parts with optimal SFE contents for cancer prevention.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13765-020-00561-7.

Additional file 1: Table S1. Effects of sulforaphane (SFA) and sulforaphene (SFE) on the viability of six cancer cell lines. **Figure S1.** GC-MS spectra of sulforaphene (SFE). (A) A commercially obtained SFE standard and (B) SFE isolated from radish seeds. **Figure S2.** Glucoraphenin (GRE) concentrations in different organs of the 'Taeback' radish plant.All data are represented as the means ± standard deviations. N. D.; not detected. Individual glucosinolates were calculated to millimolar per gram dry weight. All data are represented as the mean ± standard deviation. Immature seeds were harvested at 20 days after pollination. Mature seeds were harvested at 40 days after pollination.

Authors' contributions
SL and JK designed the study. SL performed the experiments. SL, JCA, and EJL interpreted the data and wrote the manuscript. All authors commented on the results and the manuscript. All authors read and approved the final manuscript.

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activation following caspase-3 cleavage resulted in inactivation of PARP. The induction of caspase-8 and -9 by 4.0 µg/mL SFE suggests that SFE upregulates the intrinsic and extrinsic apoptosis pathways in A549 cells. The elevated levels of these apoptotic marker proteins indicate that multiple apoptosis pathways are involved in the cell death elicited by SFE.

Compared with broccoli, kale, and other cruciferous vegetables, radish is a better source of ITC SFE because it lacks ESP, the inhibitor of ITC formation. When fresh broccoli containing active ESP is consumed, the major hydrolysis product of GRA is predominantly SFA nitrile, which is an inactive form of SFA [12]. The major ITCs in radish are SFE and raphasatin (RH), which are abundant in the seeds and sprouts, respectively. Previous studies have demonstrated the potential anticancer properties of SFE and RH [13, 20]. However, given its rapid degradation, RH has only a weak effect in fighting cancer cells. SFE can remain stable for 1 day, whereas RH is degraded within 28 min after exposure to ambient temperatures [13]. We predict that SFE has greater potential as an anticancer agent than does RH, although we have not compared the anticancer activities of the two ITCs.

Our results contribute to the knowledge of the antiproliferative properties of SFE and demonstrate that it is a powerful stimulator of apoptosis. The results of the

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