Research Article

6-Shogaol induces apoptosis in human colorectal carcinoma cells *via* ROS production, caspase activation, and GADD 153 expression

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Ginger, the rhizome of *Zingiber officinale*, is a traditional medicine with anti-inflammatory and anti-carcinogenic properties. This study examined the growth inhibitory effects of the structurally related compounds 6-gingerol and 6-shogaol on human cancer cells. 6-Shogaol [1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] inhibits the growth of human cancer cells and induces apoptosis in COLO 205 cells through modulation of mitochondrial functions regulated by reactive oxygen species (ROS). ROS generation occurs in the early stages of 6-shogaol-induced apoptosis, preceding cytochrome *c* release, caspase activation, and DNA fragmentation. Up-regulation of Bax, Fas, and FasL, as well as down-regulation of Bcl-2 and Bcl-X_L were observed in 6-shogaol-treated COLO 205 cells. *N*-acetylcysteine (NAC), but not by other antioxidants, suppress 6-shogaol-induced apoptosis. The growth arrest and DNA damage (GADD)-inducible transcription factor 153 (GADD153) mRNA and protein is markedly induced in a time- and concentration-dependent manner in response to 6-shogaol.

Keywords: 6-Shogaol / Apoptosis / GADD153 / Reactive oxygen species

Received: April 23, 2007; revised: August 11, 2007; accepted: September 13, 2007

1 Introduction

A large number of chemopreventive and therapeutic agents that induce apoptosis in malignant cells have shown promise in the fight against cancer [1, 2]. Phytochemicals such as curcumin, zerumbone, and tea polyphenols are known to induce apoptosis through down-regulation of apoptotic suppressor, Bcl-2 and Bcl-X_L, in several different tumor cell lines [3–5]. Ginger (*Zingiber officinale*) has been widely used as a spice, and as a medicinal herb in traditional herbal medicine. It contains several pungent constituents such as gingerols, shogaols, paradols, and gingerdiols. Those pungent phenolic substances have been reported to have pronounced antioxidative, anti-inflammatory, and anticarcinogenic activities [6, 7]. In the fresh ginger rhizome, the gingerols were identified as the major active components, and 6-gingerol is the most abundant constituent [8]. A recent report has shown that ginger could be useful in preventing the development of colorectal cancer [7], but Dias *et al*. [9] reported that ginger meal failed to reduce chemical-induced colon carcinoma in rats. However, at the cellular level, it is unclear which constituents of ginger work as cancer chemopreventive agents.

Apoptosis is defined as a type of cell death, involving the concerted action of a number of intracellular signaling pathways, including members of the caspases family of cysteine proteases, stored in most cells as zymogens or procaspases [10]. The two main apoptotic pathways, the death

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Abbreviations: ALL, allopurinol; **CAT,** catalase; **DCFH-DA,** dichlorodihydrofluorescein diacetate; **DFF,** DNA fragmentation factor; **DPI,** diphenylene iodonium; **GADD153,** growth arrest and DNA damage-inducible gene 153; **NAC,** *N*-acetylcysteine; **PARP,** poly(ADP-ribose) polymerase; **PDTC,** pyrrolidine dithiocarbamate; **ROS,** reactive oxygen species; **SOD,** superoxide dismutase

receptor (extrinsic) and mitochondrial (intrinsic) pathways, are activated by caspase-8 and caspase-9, respectively. Recent studies of the ER as a third subcellular compartment were implicated in apoptotic execution induced by ER stress [11 – 13]. The ER stress-induced cell death modulator is a CCAAT/enhancer-binding protein (CEBP) homology protein (CHOP)/growth arrest and DNA damage-inducible gene 153 (GADD153), known as CHOP, is a member of the CEBP family of transcription factors [14]. Expressed at low levels in proliferating cells, it is strongly induced in response to stresses that result in growth arrest or cellular death, including oxidative injury [15], DNA damaging agents such as peroxynitrite [16], UV radiation [17], anticancer chemotherapy [18], and ER stress [14, 19]. Recent studies suggest that overexpression of GADD153 plays a central role in the apoptosis [10], including the dephosphorylation of the proapoptotic protein Bad [20] and down-regulation Bcl-2 expression [19].

We have evaluated gingerols, shogaols, and related compounds isolated from ginger for their anticancer properties in various human cancer cells. On the basis of our studies, 6-shogaol is far more potent than 6-gingerol in inhibiting the proliferation and inducing apoptosis in COLO 205 cells (data not shown). In the present study, the mechanism of apoptosis for 6-shogaol was investigated. This included studies on the effects of 6-shogaol on the activation of reactive oxygen species (ROS) production and caspase cascade, expression of Bax, Bad, Bcl-2, Bcl-X_L, and Bid, as well as changes in expression of Fas, Fas ligand, and GADD153 gene in COLO 205 cells.

2 Materials and methods

2.1 Cell culture and chemicals

The cell line COLO 205 (CCL-222; American Type Culture Collection) is developed from a poorly differentiated human colon adenocarcinoma. COLO cells were grown in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY), 100 U/mL of penicillin, 100 μg/mL of streptomycin), 2 mM L-glutamine (Gibco BRL), and were kept at 37°C in a humidified 5% CO_2 incubator. The inhibitor of broad caspase (Z-Val-Ala-Asp-fluoromethyl ketone, Z-VAD-FMK) was purchased from Calbiochem (La Jolla, CA). Propidium iodide was obtained from Sigma Chemical (St. Louis, MO).

2.2 Isolation of 6-gingerol and 6-shogaol from ginger extract

Crude ginger extract was a gift from Sabinsa Corporation (Piscataway, NJ). It was subjected to solvent extraction prior to column chromatography. The methanolic extract of ginger (50 g) was dissolved in ethyl acetate and partitioned with water. The ethyl acetate portion was collected, evaporated, and the residue dissolved in methanol. This was further partitioned with hexane and the methanolic portion evaporated and used for the isolation of gingerols and shogaols.

The methanolic extract obtained by the above solvent extraction step was loaded onto a Sephadex column and eluted with 95% ethanol to yield gingerols, shogaols, and related compounds. These extracts were analyzed by TLC (using a 2:1 hexane/ethyl acetate solvent system) and similar fractions were combined. The extracts were then subjected to Diaon HP-20 column chromatography and eluted with 30 – 70% ethanol. Similar fractions were combined to give rise to gingerol mixture and shogaol mixture. The gingerol mixture was combined, evaporated to dryness under vacuum and the residue subjected to normal phase column chromatography using hexane, ethyl acetate as the solvent system to isolate 6-gingerol. For isolation of 6-shogaol, the shogaol mixture was subjected to RP C18 column chromatography, and eluted with methanol – water solvent system.

The purity and identity of the isolated 6-gingerol and 6-shogaol were confirmed using HPLC and NMR.

2.3 DNA extraction and electrophoretic analysis

Both attached and detached cells were harvested, washed twice with ice-cold PBS, and DNA was isolated as described in our previous study [21]. Samples were electrophoresed in a 2% w/v agarose gel and DNA was visualized by ethidium bromide staining.

2.4 Acridine orange staining assay

Cells (5×10^5) were seeded into 60-mm Petri dishes in RPMI-1640 medium and incubated at 37°C for 24 h. The cells were then harvested, and 5 μL of suspended cells was mixed on a glass coverslip with an equal volume of acridine orange solution (10 μg/mL in PBS). Apoptotic cells were distinguished from viable cells by showing homogeneously bright staining in nuclei, however, with chromatin condensation indicative of apoptosis.

2.5 Flow cytometry

The cells were harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of 100% ethanol at – 20°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37°C for 30 min. Next, 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand crushed water ice for 30 min. Fluorescence emitted from the propidium iodide – DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA). Quantitation of the fraction of each cell cycle

stage was performed with ModFit LT for Mac 3.0 software (Becton Dickinson).

2.6 Determination of ROS production and intracellular GSH depletion

ROS production was monitored by flow cytometry using dichlorodihydrofluorescein diacetate (DCFH-DA). This dye is a stable, nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield the DCFH, trapped within the cells. Hydrogen peroxide, or low molecular weight peroxides, produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of hydrogen peroxide produced by the cells. Dihydroethidium (DHE) was used as a probe, recognizing mainly the oxygen species super oxide anion. The thio-reactive fluorescent dye 5-chloromethylfluorescein diacetate (CMFDA) was assayed for GSH determination. CMFDA, a nonfluorescence compound, can enter the intracellular compartment and react with esterase to allow CMF trapped inside the cell. The interaction of CMF with thio group of GSH will result in the generation of fluorescence [22]. Cells were treated with 6-shogaol (60 μM) for different time periods, and CMFDA (30 μM), DCFH-DA (20 μM), and DHE (20 μM), respectively, was added to the medium for a further 30 min at 37°C. Histograms were analyzed using Cell Quest software and were compared with histograms of untreated control cells.

2.7 Analysis of mitochondrial trans-membrane potential

The change of the mitochondrial trans-membrane potential was monitored by flow cytometry. Briefly, COLO 205 cells were treated with 6-shogaol (60 μM) for different time periods, and the mitochondrial trans-membrane potential was measured directly using 40 nM 3,3′-dihexyloxacarbocyanine (DiOC6(3)) (Molecular Probes, Eugene, OR). Fluorescence was measured after staining the cells for 30 min at 37°C. Histograms were analyzed using Cell Quest software and were compared with histograms of untreated, control cells.

2.8 Preparation of subcellular fraction

Subcellular fraction was performed as described previously [23]. Briefly, cells were trypsinized and washed once with ice-cold PBS following lysed in lysis buffer and homogenized (20 strokes with a B-pestle Dounce homogenizer). The lysate was centrifuged ($750 \times g$ for 5 min) to remove nuclei and unlysed cells and centrifuged again ($10\,000 \times g$ for 10 min) to obtain the mitochondria fraction (pellet). The supernatant was centrifuged at $14\,000 \times g$ for 1 h to obtain the cytosolic fraction.

2.9 Western blotting

For the determination of the expression of Bcl-2 family, Bid, Fas, and FasL in COLO 205 cells, the nuclear and cytosolic proteins were isolated from COLO 205 cells in Petri dishes with a cell scraper after treatment with 60 μM 6-shogaol for 0, 1, 2, 3, 6, 9, 12, and 24 h. Western blotting analysis was performed by the method described previously [21]. Briefly, cell lysates were prepared, electrotransferred, immunoblotted with antibodies, and then visualized by measuring the chemiluminescence of the blotting agent (ECL, Amersham, Arlington Heights, IL). The mitochondria and cytosolic fractions isolated from the cells were used for immunoblot analysis of cytochrome *c* as previously described [24]. The cytochrome *c* protein was detected using an anticytochrome *c* antibody (Research Diagnostic, Flanders, NJ).

2.10 Activity of caspases

After 6-shogaol treatment, cells were collected and washed with PBS and suspended in 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothione, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin after treatment. Cell lysates were clarified by centrifugation at $12\,000 \times g$ for 20 min at 4°C. Caspase activity in the supernatant was determined as described previously [22].

2.11 Assessment of DNA damage

The DNA damage level caused by 6-shogaol was measured as previously described [25]. Briefly, COLO 205 cells were pretreated with NAC for 2 h, prior to exposure to 6-shogaol (60 μM); or treated with H_2O_2 (200 μM), cells were then suspended in agarose gel, and the suspension was pipetted onto glass slides. The DNA of the nucleoids was stained with ethidium bromide to permit visualization using a fluorescence microscope at 100×, with an exciting filter of 515 – 560 nm and a barrier filter of 590 nm.

2.12 Isolation of RNA and RT-PCR

Total cellular RNA was isolated with a TRIzol Reagent kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. Changes in the steady-state concentration of mRNA in GADD153 were assessed by reverse-transcription PCR (RT-PCR). Total RNA (2 μg) was converted to cDNA in a series of standard 10 μL reverse transcription reactions. DNA amplification was carried out in "Ready To Go" PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ). The amplification cycles were 95°C for 30 s, 65°C for 45 s, and 72°C for 2 min. The PCR products were separated by electrophoresis on a 2% agarose after 30 cycles (422-bp GADD153 fragment; 294 bp β-actin fragment) and visualized by ethidium bromide

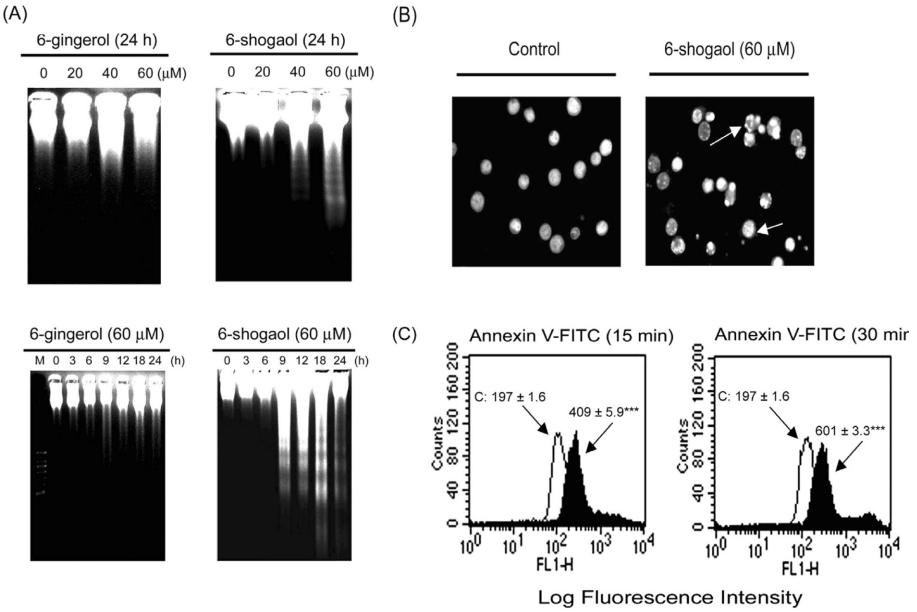

Figure 1. 6-Shogaol induced apoptosis in human colorectal carcinoma COLO 205 cells. (A) COLO 205 cells treated with increasing dose of 6-gingerol and 6-shogaol for 24 h, or treated with 60 μM for indicated time, and internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. M: 100 bp DNA ladder size marker. (B) COLO 205 cells were treated with 60 μM 6-shogaol for 24 h, the morphological changes was determined by fluorescence microscopy (200 ×). (C) Determination of apoptosis in 6-shogaol treated COLO 205 cells by annexin V-FITC staining was quantities by flow cytometry. Cells were treated with 60 μM 6-shogaol for 15 and 30 min, data are presented as log fluorescence intensity. Cell also treated with DMSO (0.05%) as a control, C. The values shown are means ± SE (***$p < 0.001$; Student's *t*-test).

staining. Amplification of β-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisence primer, respectively; GADD153; 5′-GCACCTCCCAGAGCCCT-CACTCTCC-3′ and 5′-GTCTACTCCAAGCCTTCCCC-CTGCG-3′, and 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

2.13 Statistical analysis

Data are presented as means ± SE for the indicated number of independently performed experiments. Significant differences from the respective controls for each experimental test condition were assayed using Student's *t*-test for each paired experiment. A *p*-value of < 0.05 was considered statistically significant.

3 Results

3.1 6-Shogaol is a more effective apoptotic inducer than 6-gingerol in human colorectal carcinoma cells

To investigate potential mechanism of the cytotoxic effects, COLO 205 cells were treated with 6-shogaol or 6-gingerol for 24 h followed by DNA fragmentation analyses. As shown in Fig. 1A, significant DNA ladders were observed in COLO 205 cells treated for 24 h and this effect was dose-dependent. In contrast, no evidence of apoptotic induction was observed with 6-gingerol. DNA ladder formation with 6-shogaol (60 μM), but not 6-gingerol, was time-dependent and DNA ladder formation was observed as early as 9 h after 6-shogaol treatment (Fig. 1A, lower panel). The cell death induced by 6-shogaol was characterized by examining the nuclear morphology of dying cells using a fluorescent DNA-binding agent, acridine orange. Within 24 h of treatment with 60 μM 6-shogaol, cells clearly exhibited significant morphological changes and chromosomal condensation, indicative of apoptotic cell death (Fig. 1B). The early translocation of phosphatidyserine (PS) from the internal to external leaflet in COLO 205 cells in the presence of 6-shogaol treatment for various times (Fig. 1C) was examined. Log fluorescence intensity clearly exhibited a significant increase, from 197 ± 1.6 to 409 ± 5.9 and 601 ± 3.3, as detected by annexin V-FITC, during 6-shogaol-treatment of COLO 205 cells at 15 and 30 min, respectively (Fig. 1C).

3.2 6-Shogaol induces caspase cleavage and activation

Activation of caspase-3 has been reported in 6-shogaol-treated Mahlavu cells [26]; however the roles of caspase-9, and -3 in 6-shogaol-induced apoptosis in COLO 205 cells is still unclear. As illustrated in Fig. 2, 6-shogaol time-

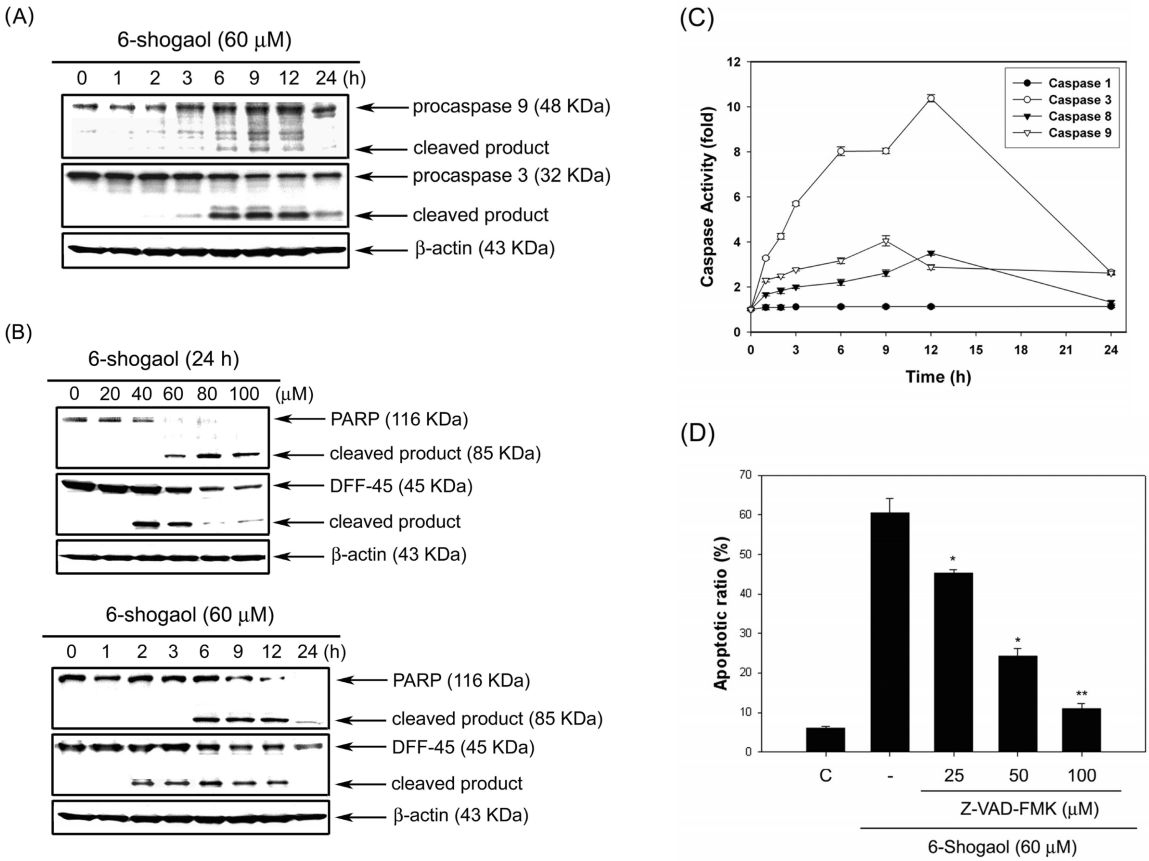

Figure 2. Induction of caspase activities, PARP cleavage, and DFF-45 degradation in 6-shogaol-induced apoptosis. (A) 6-Shogaol induced caspase-9 processing and further caused caspase-3 activation. Total cell lysates were prepared from COLO 205 cells treated with 60 μM 6-shogaol in a time-dependent manner and analyzed by western blotting. (B) Dose- and time-dependent increases in cleavage of PARP and DFF-45 during 6-shogaol-induced apoptosis. (C) Enzymatic activity of caspase-1, caspase-3, caspapase-8, caspase-9 were determined by incubation of 50 μg of total protein with fluorogenic substrate receptively, for 1 h at 30°C. The release of AMC was monitored spectorfluometrically (excitation = 360 nm; emission = 460 nm). (D) COLO 205 cells were pretreated with inhibitor Z-VAD-FMK (25, 50, 100 μM, respectively) for 1 h followed by 6-shogaol for another 24 h. The apoptotic ratio (%) was detected by flow cytometry. Each experiment was independently performed three times and expressed as mean ± SE. Asterisk denotes a statistically significant decrease compared with values of positive control (*$p < 0.05$, **$p < 0.01$).

dependently induced the processing of caspase-9 and -3 and its downstream substrates, poly-(ADP-ribose)-polymerase (PARP) and DNA fragmentation factor (DFF)-45 proteins. The cleaved fragments of caspase-9 and -3 were observed by Western blotting in presence of 6-shogaol (60 μM) for different times (Fig. 2A). To monitor the enzymatic activity of caspase-1, -3, -8, and -9, the caspase activity was measured following treatment of COLO 205 cells with 60 μM 6-shogaol for various times. As shown in Fig. 2C, 6-shogaol induced a dramatic increase in caspase-3 activity to approximately 10.5-fold after 12 h of treatment. Additionally, both caspase-8 and -9 were time dependently activated by 6-shogaol, but the data showed a very low level of caspase-1 activity following 6-shogaol treatment, whereas z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), a universal caspase inhibitor, dose-dependently suppressed 6-shogaol-induced apoptosis (Fig. 2D). This indicates that activation of caspase-9, -3, and -8 participates in the induction of apoptosis by 6-shogaol.

3.3 Involvement of ROS production, GSH depletion, mitochondrial dysfunction, and release of cytochrome *c* from mitochondria to cytosol in 6-shogaol-induced apoptosis

We further investigated if ROS production is involved in 6-shogaol-induced apoptosis. Results of flow cytometry analysis using DCFH-DA and DHE as fluorescent ROS, H_2O_2, and O_2^-.indicators demonstrate an increase in intracellular ROS levels in 6-shogaol-treated COLO 205 cells (Fig. 3A). We further studied the possibility that 6-shogaol could deplete intracellular GSH contents using the fluorescent probe CMFDA, the decrease in fluorescence reflects the depletion of the availability of GSH. Treatment with

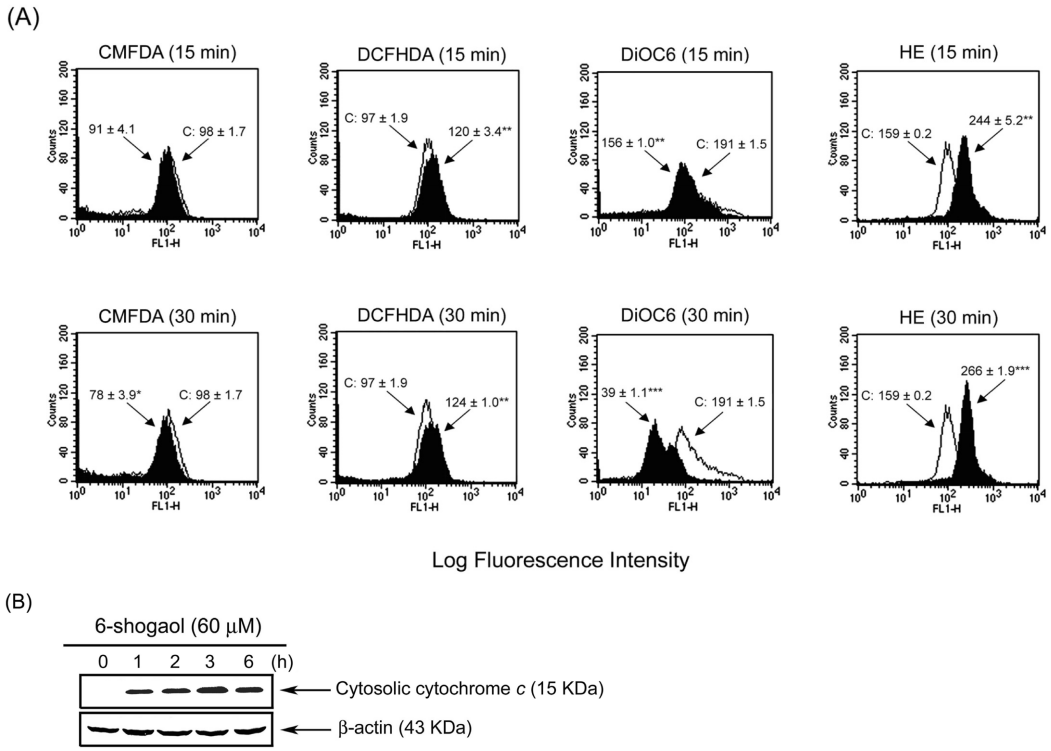

Figure 3. Induction of ROS generation, GSH depletion, mitochondrial dysfunction, and cytochrome *c* release in 6-shogaol-induced apoptosis. (A) COLO 205 cells were treated with 60 μM 6-shogaol for indicated times and were then incubated with CMFDA (20 μM), DCFH-DA (20 μM), 3, 3′-dihexyloxacarbocyanine (40 nM), and DHE (20 μM), respectively, and analyzed by flow cytometry. Data are presented as log fluorescence intensity; C, control. The values shown are means ± SE (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Student's *t*-test). (B) COLO 205 cells were treated with 60 μM 6-shogaol for different times (0, 1, 2, 3, and 6 h). Subcellular fractions were prepared as described in Section 2, and cytosolic cytochrome *c* was detected by cytochrome *c* antibody. These experiments were performed at least three times, and a representative experiment is presented.

6-shogaol resulted in decreases in CMF fluorescence from 98 ± 1.7 to 91 ± 4.1 and 77 ± 3.9 at 15 and 30 min, respectively (Fig. 3A). Figure 3A also compares COLO 205 cells exposed to 6-shogaol and control cells, the DiOC6(3) fluorescence intensity shifted to the left from 191 ± 1.5 to 156 ± 0.9 and 39 ± 1.1 in 6-shogaol-induced apoptotic COLO 205 cells at 15 and 30 min, respectively. These findings show that 6-shogaol has an effect on mitochondrial function and accumulation of ROS. As shown in Fig. 3B, the release of mitochondrial cytochrome *c* into the cytosol was detected at 1 h in 6-shogaol-treated COLO 205 cells. Therefore, these results suggest that mitochondrial dysfunction caused cytochrome *c* to be the cascade between caspase-9 and -3.

3.4 Effect of 6-shogaol on the expression of Bcl-2 family, Fas, Fas ligand (FasL), and Bid protein in COLO 205 cells

The expression of the Bad, Bax, Bcl-X_L, and Bcl-2 at different time points in 6-shogaol-treated cells was investigated. As shown in Fig. 4A, there are a similar pattern of Bax, Bcl-X_L, and Bcl-2 levels to the loading control after 6-shogaol treatment. The apparent reduction in Bax, Bcl-X_L, and Bcl-2 protein expression and the increase in Bad expression relative to β-actin at 24 h. Immunoblot analysis of the subcellular fractions showed that 6-shogaol treatment led to translocation of Bcl-2 protein to the cytosol from the mitochondria and Bad protein to the mitochondria from the cytosol (Fig. 4B) in a time-dependent manner. This phenomenon also found in previous studies that acrolein-induced translocation of antiapoptotic protein Bcl-2 from mitochondria to the cytosol [27]. These results suggest a potential involvement of mitochondria pathway in 6-shogaol-induced apoptosis in COLO 205 cells. To assess whether 6-shogaol promoted apoptosis *via* receptor-mediated pathway, the Fas and FasL protein were determined by Western blotting. The result showed that 6-shogaol could stimulate the expression of Fas and FasL (Fig. 4C). To verify whether the activation of caspase-8 was associated with Fas and FasL production in response to 6-shogaol treatment, the activation of caspase-8 was detected after treatment of COLO 205 cells with 6-shogaol at the indicated time points. As shown in Fig. 4C, the level of procaspase-8 was gradual decrease and associ-

ated with activation of caspase-8 (Fig. 2C). We next examined the proapoptotic protein, Bid, which upon cleavage by caspase-8, produced the truncated Bid fragment. The results showed that Bid cleavage occurred at 3 h (Fig. 4C). These data suggested that the cleavage of Bid by active caspase-8 may be one of the mechanisms that contributed to the activation of mitochondrial pathway during 6-shogaol-induced apoptosis.

3.5 Effect of free radical scavengers or antioxidants on 6-shogaol-induced apoptosis

Growing evidence has indicated that ROS plays an important role in the induction of apoptosis [28]. As shown in Fig. 5A, antioxidants such as *N*-acetylcysteine (NAC), superoxide dismutase (SOD), diphenylene iodonium (DPI), pyrrolidine dithiocarbamate (PDTC), allopurinol (ALL), and catalase (CAT) were used in the present study to examine whether ROS production is an essential event for 6-shogaol-induced apoptosis of COLO 205 cells. Results of the flow cytometry assay showed that pretreatment with NAC, but not SOD, DPI, PDTC, ALL, and CAT, protects COLO 205 cells from 6-shogaol-induced apoptosis. Additionally, 6-shogaol induced the processing of PARP was dose dependently prevented by the addition of NAC (Fig. 5B).

3.6 Effect of NAC on 6-shogaol-induced DNA damage in COLO 205 cells

To determine if exposing COLO 205 cells to 6-shogaol induces DNA damage as an indicator of oxidative stress, cells were exposed to 60 μM shogaol for 3 h. The Comet assay was then performed to detect any single-strand breaks in the DNA (Fig. 6). As can be seen in the representative photos from fluorescence microscopy, the nucleoids of control cells (Fig. 6a) are uniformly spherical in shape, reflecting the absence of DNA damage. In contrast, the nucleoids of 6-shogaol-treated cells appeared as "comets", reflecting the presence of significant DNA damage. The typical "comet tail" is illustrative that DNA single-strands breaks exist and is evidence of DNA damage with H_2O_2 (200 μM) as the positive (Fig. 6c). We speculated that the intracellular generation of ROS could be an important factor in 6-shogaol-induced DNA damage. To verify this, we performed a test to determine whether antioxidants NAC could inhibit 6-shogaol-induced DNA damage. As shown in Figs. 6d–f, pretreatment with NAC significantly inhibited 6-shogaol-induced DNA damage. Moreover, NAC by itself did not affect DNA damage (Fig. 6b).

3.7 Induction of GADD153 mRNA and protein expression by 6-shogaol in COLO 205 cells

Recently, several studies suggested that GADD153 triggers the critical early events leading to the initiation of apoptosis

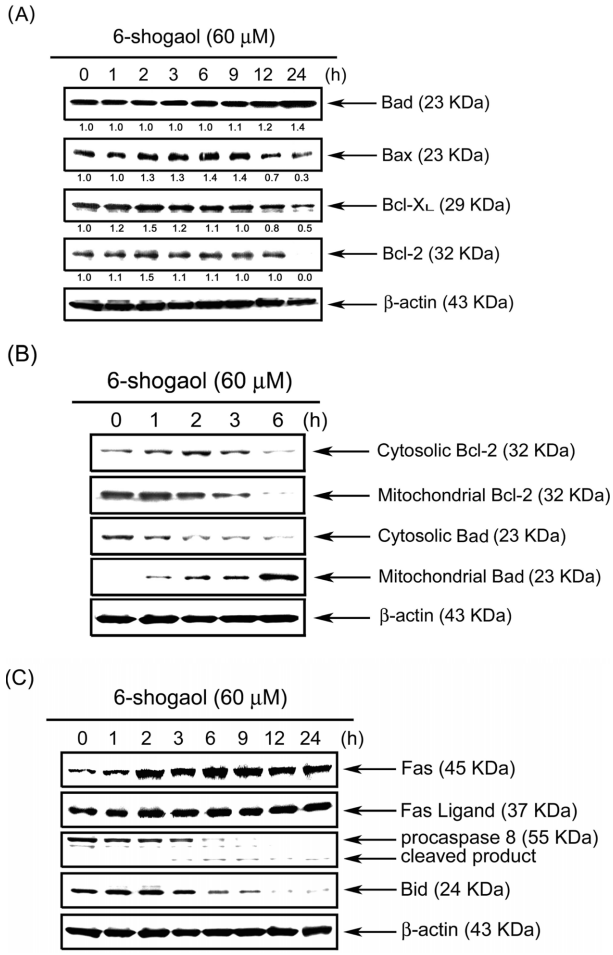

Figure 4. Effect of 6-shogaol on Bcl-2 protein family, Fas, Fas L, and Bid expression in 6-shogaol-treated COLO 205 cells. (A) COLO 205 cells were treated with 60 μM 6-shogaol for indicated amounts of time. Western blot analyses of Bad, Bax, Bcl-X_L, and Bcl-2, and Bid expression in COLO 205 cells. The values below the fig represent change in Bcl-2 family protein expression of the bands normalized to β-actin. (B) In subcellular fractions, 6-shogaol treatment led to translocation of Bcl-2 and Bad protein to the cytosol from mitochondria and to mitochondria from cytosol, respectively. (C) The expression of Fas, FasL, procaspase-8, and Bid were analyzed by Western blotting as described in Section 2. These experiments were performed at least in triplicate, and a representative experiment is presented.

[15–18]. Thus, the effect of 6-shogaol on the GADD153 mRNA level in COLO 205 cells was measured by RT-PCR analysis using gene-specific primers (Fig. 7A). After cells were exposed to 6-shogaol, GADD153 mRNA were up-regulated in a concentration (Fig. 7A, upper panels) and time dependent manner (Fig. 7A, lower panels) and increased noticeably for 1 h in 6-shogaol-treated cells. NAC, which is known to increase intracellular concentrations of the antioxidant glutathione [29], was able to prevent the increased GADD153 mRNA expression caused by

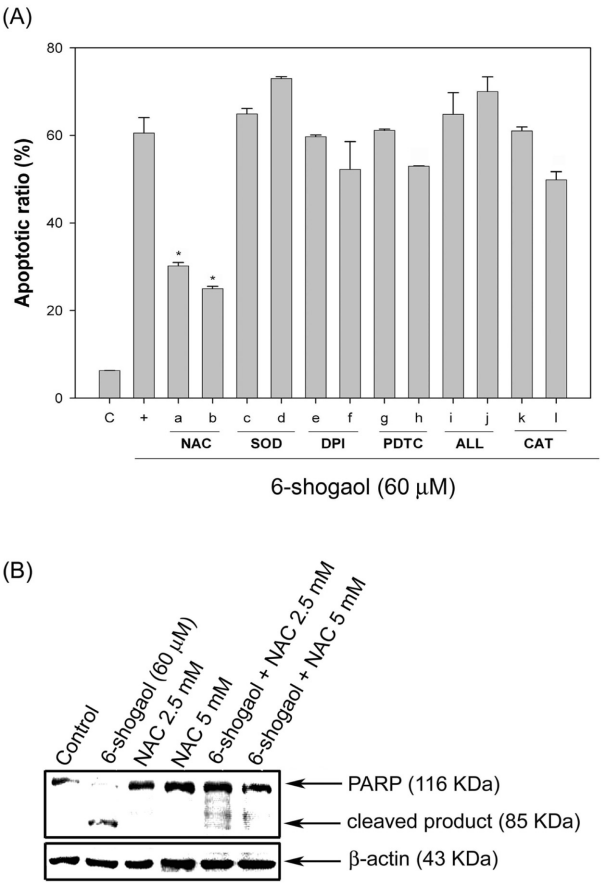

Figure 5. NAC addition inhibits apoptosis and PARP cleavage in 6-shogaol-treated COLO 205 cells. (A) COLO 205 cells were treated with different concentrations of NAC, SOD, DPI, PDTC, ALL, and CAT for 1 h followed by 6-shogaol (60 μM) treatment for another 24 h. The cell apoptotic ratio (%) was determined by flow cytometry. NAC (a: 2.5 mM, b: 5 mM), SOD (c: 100 μg/mL, d: 200 μg/mL), DPI (e: 10 μM, f: 20 μM), PDTC (g: 20 μM, h: 40 μM), ALL (i: 50 μM, j: 100 μM) and CAT (k: 200 U/mL, l: 400 U/mL). *$p < 0.01$ indicates a significant difference from the 6-shogaol treatment, as analyzed by Student's *t*-test. (B) The addition of NAC inhibited 6-shogaol-induced cleavage of the PARP protein. The cleavage of PARP was examined by Western blotting using specific antibodies. The experiment was repeated three times with similar results.

6-shogaol in COLO 205 cells (Fig. 7B). Moreover, 6-gingerol (60 M) did not affect basal expression of GADD153 mRNA (Fig. 7B, lane 2). Changes in mRNA expression do not always translate to change in protein. To determine whether the changes in GADD153 mRNA levels produced corresponding increases in GADD153 protein levels, Western blot analysis was first performed on 6-shogaol-treated COLO 205 cells. As can be seen, GADD153 protein expression was not detectable in untreated control cells. But, after being treated with 6-shogaol, the maximal level of GADD153 protein expression at 3 – 9 h, but apparent reduction at 12 h after treatment and increased considerably in a concentration-dependently manner (Fig. 7C). Furthermore,

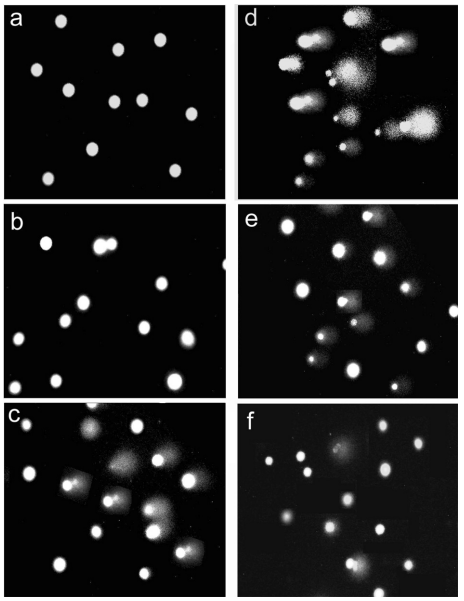

Figure 6. NAC addition inhibits DNA damage in 6-shogaol-treated COLO 205 cells. Assay of DNA damage with comet assay. Greater single strand breaks in DNA of COLO 205 cells exposed to 60 μM of 6-shogaol for 3 h. The Comet assay was then performed as described under Section 2 to assess to extent of DNA damage. a, control; b, NAC (5 mM); c, H_2O_2 (200 μM); d, 6-shogaol (60 μM); e, NAC 2.5 mM + 6-shogaol; f, NAC 5 mM + 6-shogaol. Similar results were produced by two additional different experiments.

NAC concentration-dependently inhibited 6-shogaol-induced GADD153 protein expression (Fig. 7D). However, NAC by itself did not affect basal expression of GADD153 protein.

4 Discussion

In this study, we demonstrated the molecular mechanism by which 6-shogaol triggered COLO 205 cells undergoing apoptosis. As shown in Fig. 1, 6-shogaol was the strong inducer of apoptosis than 6-gingerol, concurrent with DNA ladder, apoptotic appearance, and chromatin condensation. Indeed, treatment with 6-shogaol caused the activation of caspase-9, -3, and -8, but not caspase-1, associated with the degradation of PARP and DFF-45/ICAD, which preceded the onset of apoptosis.

ROS are a family of active molecules including superoxide (O_2^-), peroxyl ($ROO^\bullet$), hydroxyl ($OH^\bullet$), and nitric oxide ($NO^\bullet$) that are involved in the modulation of biological cell functions. However, a large amount or sustained levels of ROS can result in the oxidation of biomolecules, resulting in cell damage leading to growth arrest, senescence, or death [30, 31]. Herein, COLO 205 cells showed increasing ROS production, GSH depletion, and reducing mitochondria membrane potential within 15 min, caused by 6-sho-

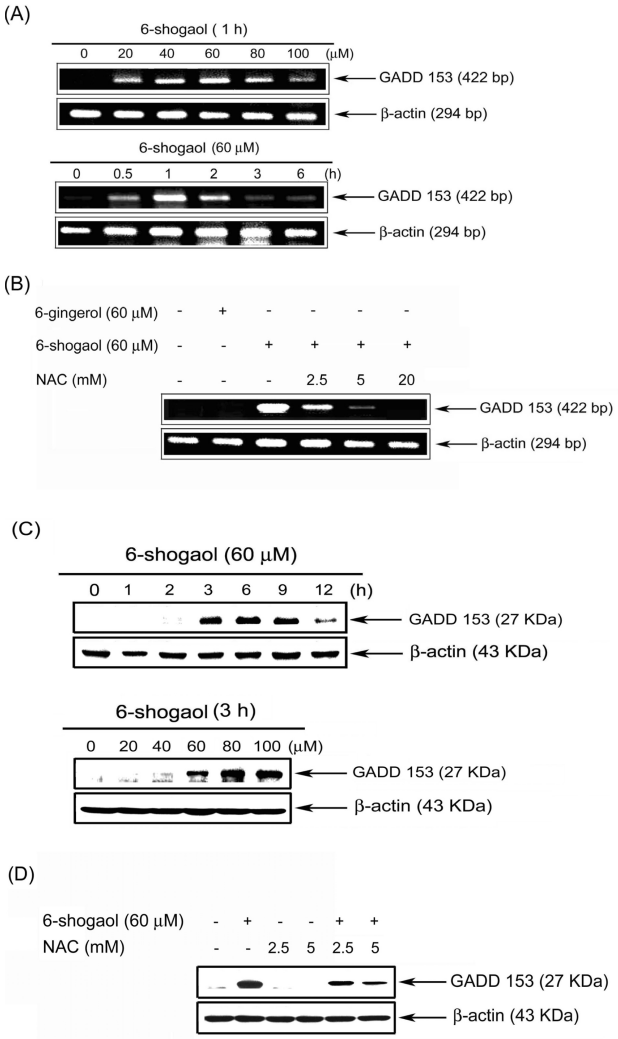

Figure 7. Effect of NAC on 6-shogaol-induced up-regulation of GADD153 mRNA and protein expression in COLO 205 cells. (A) COLO 205 cells were incubated with 0 – 100 μM 6-shogaol for 1 h or 60 μM 6-shogaol for 0 – 6 h. (B) In testing free radical scavenger, cell were pretreated with 2.5, 5, and 20 mM NAC for 1 h prior to exposing cell to 6-shogaol (60 μM) for 1 h. After incubation, total RNA was isolated for subsequent multiplex relative RT-RCR analysis using gene specific primers for the target gene, GADD153, and also the internal control gene, β-actin. (C) Cells were incubated with 60 μM 6-shogaol for 0 – 12 h or 0 – 100 μM 6-shogaol for 3 h. Whole cell lysates were prepared for subsequence Western blotting analysis of GADD153 protein and β-actin. (D) The addition of NAC inhibited 6-shogaol-induced GADD 153 expression. Cells were pretreated with 2.5 or 5 mM NAC for 1 h, prior to exposure to 6-shogaol for 6 h. This experiment was repeated three times with similar results.

gaol. Thus, the results suggest that 6-shogaol could induce apoptosis in COLO 205 cells through ROS production and intracellular GSH depletion. Our present results agree with the report of Chen *et al*. [26] that 6-shogaol-induced GSH depletion through the interaction of NO and GSH in Mahlavu cells.

However, 6-shogaol is presence of an α,β-unsaturated ketone moiety in the side chain, but not in 6-gingerol [32]. Previous studies suggest that α,β-undaturated carbonyls are very susceptible to nucleophilic addition reactions with thiols such as glutathione; the most abundant nonprotein thiol *in vivo* [33]. It is clear that α,β-unsaturated ketone moiety is essential for exerting cytotoxic activity *via* depletion of intracellular GSH [34]. Recent studies indicated that intracellular GSH depletion could result in mitochondria dysfunction [35, 36]. Moreover, oxidative damage to the mitochondria membrane due to increased generation of ROS has been shown to play an important role in apoptosis. Herein, we demonstrated that 6-shogaol could disrupt the functions of mitochondria at the early stages of apoptosis and subsequently coordinate caspase-9 activation, but not caspase-1, through the release of cytochrome *c*. Therefore, we speculated that intracellular generation of ROS could be an important factor in 6-shogaol-induced apoptosis. As the significant novel finding in the present study, 6-shogaol increased expression of the GADD153 gene, which has been acknowledged as a pro-apoptosis gene [37, 38]. Because the GADD153 gene is typically induced in response to cellular DNA damage, oxidative stress, and ER stress, it is suggested that 6-shogaol-induced up-regulation of GADD153 mRNA could be an early transcriptional response to 6-shogaol. Furthermore, increased GADD153 expression was essentially prevented by NAC, which is known to markedly increase the intracellular concentration of reduced glutathione [29], suggesting that a redox mechanism was involved in promoting GADD153 mRNA expression. The intracellular GSH/GSSG ratio is known to influence the expression of redox sensitive genes [39]. In addition to 6-shogaol, various cancer preventive agents – such as curcumin, resveratrol, and EGCG – induced apoptosis of cancer cells seemed to have created a cellular environment characterized by increased oxidative stress and perturbed glutathione homeostasis that were conducive for GADD153 gene activation [40 – 42]. Although the full significance of up-regulation of GADD153 gene expression in 6-shogaol-treated COLO 205 cells is not known, the effect of 6-shogaol on GADD153, in particular, might contribute to the capacity of 6-shogaol-induced apoptosis. Thus, the sum of these results suggests that the GADD153 gene may be an important target gene for apoptotic cell death induced by 6-shogaol. These findings suggest that GADD153 expression may modulate the sensitivity of COLO 205 cells to apoptosis triggered by 6-shogaol-induced DNA damage. Further studies will be required to elucidate how 6-shogaol modulates expression of the GADD153 protein. We do not rule out the possible mechanism that 6-shogaol could penetrate into cells, directly target the ER, leading to an increase in ER stress, and then cause mitochondrial increases in membrane permeability and the accompanying decreased $\Delta\Psi m$ that accompanies enhanced ROS production. Normal p53 function acts as tumor suppressor including both

growth arrest and apoptosis. p53 activates the Fas gene in response to DNA damage by anticancer drugs [43]. Treatment of COLO 205 cells with 6-shogaol results in an increase the level of Fas protein (Fig. 4C).

Taken collectively, the current findings can be interpreted to propose a temporal sequence of events related to the effects of 6-shogaol on COLO 205 cells. The initial event induced by 6-shogaol likely causes increased induction of ROS production, Fas activation, and coordinative modulation of GADD153 gene expression. This is followed by GADD153 protein promotion of mitochondrial dysfunction (loss of mitochondrial membrane potential), resulting in cytochrome *c* release, caspases activation, and apoptotic death. However, other genes may also be involved in the cellular responses to 6-shogaol exposure, an involvement that may eventually lead to the cells undergoing apoptosis. Further studies are needed to determine whether DNA damage and the GADD153 protein directly initiate apoptosis in COLO 205 cells exposed to 6-shogaol. During recent years, some clinical agents from natural product have been tested as anticancer agents [44 – 46]. Because induction of apoptosis is considered an important mechanism of prevention treatment of cancers by chemopreventive agent(s), further elucidation of the mechanism during 6-shogaol-mediated apoptosis would provide useful information concerning the basis for its use as a potential therapeutic and chemopreventive agent.

This study was supported by the National Science Council NSC 95-2321-B-022-001 and NSC 95-2313-B-022-003-MY3.

The authors have declared no conflict of interest.

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