



Apoptosis induction of human leukemia U937 cells by gomisins N, a dibenzocyclooctadiene lignan, isolated from *Schizandra chinensis* Baill

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ABSTRACT

We compared the pro-apoptotic effect of two dibenzocyclooctadiene lignans, gomisins A and gomisins N, isolated from *Schizandra chinensis* Baill, in U937 human promyelocytic leukemia cells *in vitro*. Gomisins N, but not gomisins A, inhibited cell growth in a dose-dependent manner, which was associated with the induction of apoptosis. The increase in apoptosis that was induced by gomisins N was correlated with down-regulation of anti-apoptotic Bcl-2 expression, a decrease in the mitochondrial membrane potential (MMP) and a release of cytochrome *c* from the mitochondria into the cytosol. Furthermore, gomisins N induced the proteolytic activation of caspase-9 and -3 and a concomitant degradation of poly(ADP-ribose) polymerase. However, caspase-8 was not activated and cleavage of Bid was not observed in gomisins N-treated U937 cells. The cytotoxic effects and apoptotic characteristics induced by gomisins N were significantly inhibited by z-DEVD-fmk, a caspase-3 inhibitor, demonstrating the important role that caspase-3 plays in the process. We conclude that gomisins N induces the apoptosis of U937 cells through a signaling cascade of mitochondria-mediated intrinsic caspase pathways and gomisins N may be a useful chemotherapeutic agent.

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1. Introduction

Apoptosis, a programmed cell death, occurs during many important physiological conditions, such as embryonic development and tissue remodeling. Apoptotic cells are characterized by several unique features, including cell shrinkage, chromatin condensation, DNA fragmentation, cell surface expression of phosphatidylserine, and membrane blebbing (Okada and Mak, 2004; Han et al., 2008). In general, depending on the cell type or trigger, apoptosis can be initiated in two ways: by an extrinsic (death receptor-mediated) pathway or by an intrinsic (mitochondrial-mediated) pathway. In the former, plasma membrane death receptors are involved and the apoptosis signal is provided by the interaction be-

tween the ligand and the death receptor, which then activates caspase-8 and apoptotic cell death. However, changes in the mitochondrial integrity in response to a broad range of physical and chemical stimuli can trigger the intrinsic pathway of apoptosis (Jin and el-Deiry, 2005; Fulda and Debatin, 2006). It is believed that the release of cytochrome *c* from the mitochondria into the cytosol is a key event in the intrinsic pathway (Mow et al., 2001; Mohamad et al., 2005). Once in the cytosol, cytochrome *c* can activate caspase-9, which in turn cleaves and activates the executioner, caspase-3. After activation, several specific substrates for caspase-3 including poly(ADP-ribose) polymerase (PARP) are cleaved, which eventually leads to apoptosis (Lazebnik et al., 1994; Han et al., 2008). Consistent with the role that the mitochondria plays in the control of cell death, survival or apoptotic factors such as the Bcl-2 family act on the organelle to prevent or facilitate the release of apoptogenic factors (Yin, 2000; Zinkel et al., 2006; Jeong and Seol, 2008). Many recent studies have shown that many chemopreventive and/or chemotherapeutic agents can cause tumor cell death via the induction of apoptosis, the preferred method of

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managing cancer. However, most cancer cells block apoptosis, allowing them to survive despite undergoing genetic and morphologic transformations. Therefore, the induction of apoptotic cell death is an important mechanism in the anti-cancer properties of many drugs.

Leukemias are characterized by an abnormal proliferation of white blood cells and classified into lymphocytic leukemia and myelogenous leukemia (Gilliland, 2004), which are originated from lymphocytes in the bone marrow and mainly from granulocytes or monocytes, respectively (Abramson and Melton, 2000). Defects in the receptor- or mitochondria-mediated pathway may give important prognostic information in the risk stratification of acute myeloid leukemia patients (Del Poeta et al., 2003). To overcome these defects, novel treatment strategies that target key proteins in the mitochondrial, death receptor, and convergent pathways of caspase activation are urgently needed to improve the clinical management of this serious disease.

Schizandra chinensis (TURCZ.) Baill. (Schisandraceae) has been regarded as a useful material in the regulation of various pathological conditions over the last several decades in Korea, China, and Japan (Hancke et al., 1999). In traditional Oriental medicine, the fruit of *S. chinensis*, Schizandra berry, has been used to treat a variety of diseases. Previous reports suggest that the major bioactive constituents of Schizandra berry are lignans belong to the dibenzocyclooctadiene type, and more than 40 lignans have been isolated from this fruit (Ikeya et al., 1979; Lee et al., 1990; Opletal et al., 2004) and intensively studied from pharmacological and phytochemical points of view. Previous studies have shown that several lignans, including gomisin N and gomisin A, protect the liver from hepatotoxic chemicals (Maeda et al., 1985; Yamada et al., 1993). Pharmacological studies of lignans isolated from Schizandra berry have also revealed anti-cancer properties, anti-hepatocarcinogenesis, and antioxidant and anti-inflammatory activities (Yasukawa et al., 1992; Lu and Liu, 1992; Ohtaki et al., 1996; Wu et al., 2003; Choi et al., 2006). In particular, some lignans such as gomisin A, schisandrin A and B, and schisantherin A restored cytotoxic activities of anti-cancer agents in multidrug-resistant human cancer cells, and schisandrin B enhanced cytotoxic and pro-apoptotic effects of doxorubicin (Pan et al., 2006; Wan et al., 2006; Li et al., 2006). In addition, other lignans including gomisin N, schisantherin C and G and propinquin E were selectively cytotoxic in human cancer cells (Xu et al., 2006; Choi et al., 2006; Min et al., 2008), suggesting the anti-cancer and cancer chemopreventive potential of lignans. Although some lignans have previously been reported to induce apoptosis in human cancer cell lines including leukemia cells (Wu et al., 2004; Matsumoto et al., 2006; Lin et al., 2008; Min et al., 2008), the mechanisms underlying anti-proliferative effects of lignans in human cancer cells have not been thoroughly studied.

As part of an ongoing study to isolate chemopreventive or therapeutic compounds from medicinal plants, we have previously isolated several lignans from the fruit of *S. chinensis* and compared their anti-cancer properties (Choi et al., 2006, 2008). In the present study, the lignans gomisin A and gomisin N are compared as potential pro-apoptotic agents in human leukemia U937 cells. We found that gomisin N, but not gomisin A, strongly suppresses the proliferation of U937 cells by induction of apoptosis through activation of the mitochondrial-mediated intrinsic caspase pathway.

2. Materials and methods

2.1. Cell culture and viability assay

U937 cells were obtained from the American Type Culture Collection (Rockville, MD), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Gaithersburg, MD) at 37° and 5% CO₂. Gomisin A and

gomisin N isolated from dried fruits of *S. chinensis* were prepared as described previously (Fig. 1A, Choi et al., 2006, 2008), dissolved in dimethyl sulfoxide (DMSO) as a stock solution at 100 mM concentration, and stored in aliquots at –20 °C. Cell number and cell viability were determined by trypan blue exclusion assay and MTT assay, respectively.

2.2. Nuclear staining with DAPI

After treating U937 cells with gomisin A and gomisin N, the cells were harvested, washed in ice-cold phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 10 min at room temperature. The fixed cells were washed with PBS and stained with a 4,6-diamidino-2-phenylindole (DAPI, Sigma) solution for 10 min at room temperature. The nuclear morphology of the cells was examined by fluorescent microscopy (Carl Zeiss, Germany).

2.3. DNA fragmentation assay

The cells were treated with different concentrations of gomisin A and gomisin N and lysed on ice in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min. The lysates were vortexed and cleared by centrifugation at 10,000g for 20 min. The fragmented DNA in the supernatant was extracted using an equal volume of neutral phenol: chloroform: isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on 1.0% agarose gel containing ethidium bromide (EtBr, Sigma).

2.4. Flow cytometry analysis for measurement of sub-G1 phase

The cells were harvested and washed once with PBS, fixed in ice-cold 70% ethanol and stored at 4 °C. Prior to analysis, the cells were washed once again with PBS, suspended in 1 mL of a cold propidium iodide (PI, Sigma) solution containing 100 µg/mL RNase A, 50 µg/mL PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) NP-40, and further incubated on ice for 30 min in the dark. Flow cytometric analyses were carried out using a flow cytometer (FACS Caliber, Becton Dickinson, San Jose, CA) and CellQuest software was used to determine the relative DNA content based on the presence of a red fluorescence.

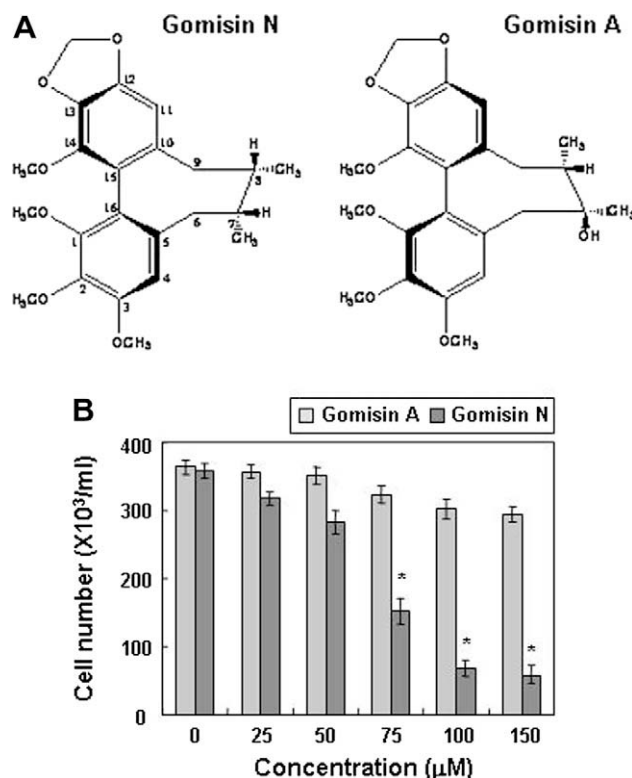


Fig. 1. Growth inhibition in response to gomisin N treatment of human leukemia U937 cells. (A) Chemical structures of gomisin A and gomisin N. (B) U937 cells were plated at a concentration of 1×10^5 cells per 60-mm plate and then incubated for 24 h, after which they were treated with various concentrations of gomisin A and gomisin N for 48 h. Cell number was determined by hemocytometer counts of trypan blue-excluding cells. Each point represents the mean $\pm$ SD of three independent experiments. The significance was determined by a Student's *t*-test (**p* < 0.05, compared with control).

2.5. Protein extraction and Western blotting

Cells were treated with gomisin A and gomisin N for 48 h and harvested with ice-cold PBS. The mitochondrial and cytosolic fractions were then isolated using a mitochondrial fractionation kit (Activemotif, Carlsbad, CA). For the isolation of total cellular protein, cells were gently lysed for 20 min in ice-cold lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μ M Tris–Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μ g/ml pepstatin A, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin). The protein concentrations were measured using a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA) according to the manufacturer's instructions. For Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS–polyacrylamide gel and transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were probed with the desired antibodies for 1 h, incubated with the diluted enzyme-linked secondary antibody and visualized by enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp., Arlington Heights, IL). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Cell Signaling Technology, Inc. (Boston, MA). The peroxidase-labeled donkey anti-rabbit immunoglobulin and peroxidase-labeled sheep anti-mouse immunoglobulin were purchased from Amersham Corp.

2.6. Assay of caspase-3, -8 and -9 activity

The enzymatic activity of the caspases induced by gomisin A and gomisin N was assayed using a colorimetric assay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Briefly, the cells were lysed in a lysis buffer for 30 min in an ice bath. The lysed cells were centrifuged at 12,000g for 10 min, and 100 μ g of the protein was incubated with 50 μ l of a reaction buffer and 5 μ l of the colorimetric tetrapeptides, Asp–Glu–Val–Asp (DEVD)-p-nitroaniline (pNA) for caspase-3, Ile–Glu–Thr–Asp (IETD)-pNA for caspase-8 and Leu–Glu–His–Asp (LEHD)-pNA for caspase-9, respectively, at 37 °C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at a wavelength of 405 nm.

2.7. Mitochondrial membrane potential assay

The mitochondrial membrane potential (MMP, $\Delta\Psi_m$) of intact cells was measured by flow cytometry with the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-V-tetraethyl-imidacarbocyanine iodide (JC-1, Sigma). JC-1 is a

radiometric, dual-emission fluorescent dye that is internalized and concentrated by respiring mitochondria and can reflect changes in MMP in living cells. There are two excitation wavelengths, 527 nm (green) for the monomer form and 590 nm (red) for the J-aggregate form. With normal mitochondrial function, MMP is high and the red fluorescence is predominant. However, when there is mitochondrial injury, MMP is reduced, leading to an increase in green fluorescence (Salvioli et al., 1997). Quantitation of red and green fluorescent signals measures mitochondrial damage. For this study, the cells were trypsinized and the cell pellets were resuspended in 500 μ l of PBS, incubated with 10 μ M JC-1 for 20 min at 37 °C. The cells were subsequently washed once with cold PBS, suspended in a total volume of 500 μ l and analyzed using flow cytometry.

2.8. Statistical analysis

All data are presented as means $\pm$ SD. Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of $p < 0.05$ was accepted as being statistically significant. All the figures shown in this article were obtained from at least two or three independent experiments.

3. Results

3.1. Growth inhibition by streptochlorin in U937 cells

In order to determine if gomisin A and gomisin N decrease cell growth, U937 cells were stimulated with various concentrations of gomisin A and gomisin N for 48 h and the cell number was then measured by the trypan blue exclusion method. As shown in Fig. 1B, treatment with gomisin N decreased the number of U937 cells in a concentration-dependent manner, while gomisin A did not inhibit the growth of U937 cells under the same conditions. Under the inverted microscope, gomisin N-treated cells, but not gomisin A, exhibited granulated morphology, and eventually degraded after treatment with 75 μ M gomisin N (data not shown).

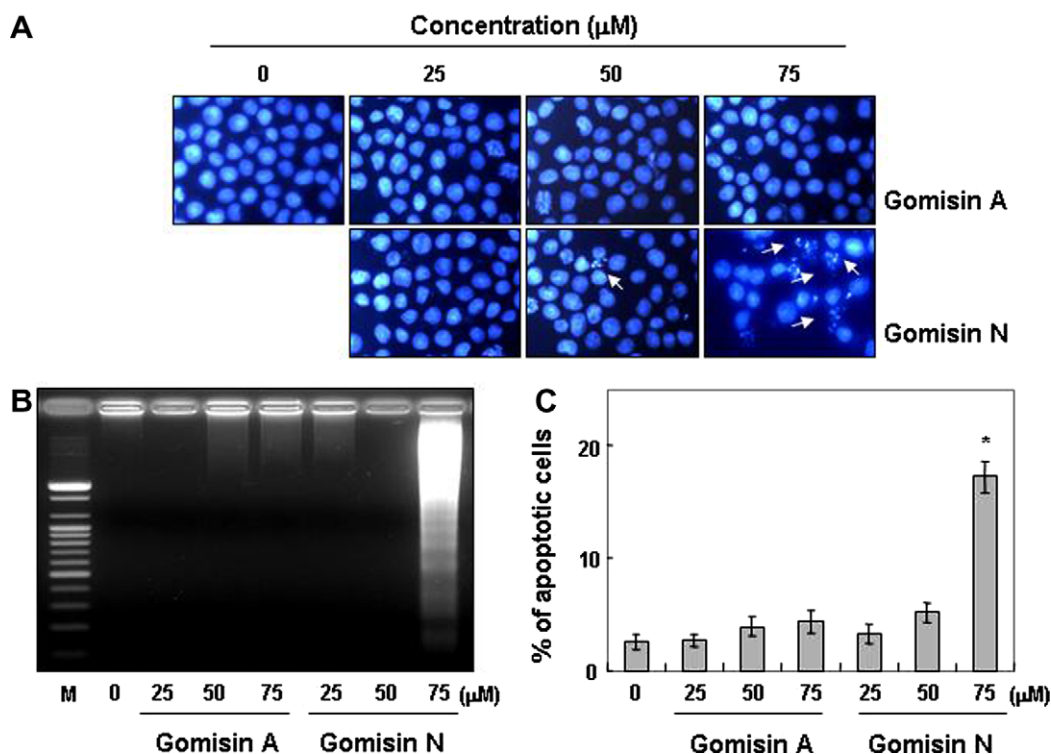


Fig. 2. Induction of apoptosis by gomisin N treatment in U937 cells. After being treated with gomisin A and gomisin N for 48 h, the cells were fixed and stained with DAPI. (A) Stained nuclei with DAPI solution were then photographed with a fluorescent microscope using a blue filter (original magnification 400 $\times$). (B) To analyze the DNA fragmentation, the genomic DNA was extracted, electrophoresed in a 1.0% agarose gel and then visualized by EtBr staining. (C) To quantify the degree of apoptosis induced by gomisin A and gomisin N, the cells were evaluated for sub-G1 DNA content, which represents the fractions undergoing apoptotic DNA degradation, using a flow cytometer. Each point represents the mean of representative experiments performed at least three times. Significance was determined by Student's *t*-test (* $p < 0.05$ vs. untreated control).

3.2. Induction of apoptosis by gomisin N in U937 cells

Further experiments were conducted to determine if the inhibitory effect of gomisin N on cell growth was the result of apoptotic cell death. DAPI staining revealed that nuclei with chromatin condensation and apoptotic bodies were formed in cells that were cultured with gomisin N, and that this phenomenon occurred in a concentration-dependent manner; however, these features were present in only a few control and gomisin A-treated cells (Fig. 2A). We also analyzed whether DNA fragmentation, another hallmark of apoptosis, was induced by gomisin N treatment of U937 cells. Following agarose gel electrophoresis of U937 cells treated with 75 μ M gomisin N for 48 h (but not gomisin A), a typical ladder pattern of internucleosomal fragmentation was observed (Fig. 2B). The degree of apoptosis was determined by analyzing the amount of sub-G1 DNA in U937 cells that were treated with gomisin N using flow cytometry. As shown in Fig. 2C, the treatment of U937 cells with gomisin N resulted in the increased accumulation of cells in the sub-G1 phase suggesting that cells undergo apoptosis after exposure to gomisin N, but not gomisin A.

3.3. Effects of gomisin A and gomisin N on the levels of Bcl-2 family members in U937 cells

To investigate the apoptotic pathways activated by gomisin N, we used Western blotting to measure the expression of the Bcl-2 family members. As shown in Fig. 3, Western blot analyses revealed that the levels of pro-apoptotic Bax and Bad remained virtually unchanged in response to gomisin N, whereas the levels of anti-apoptotic Bcl-2, but not Bcl-xL, were markedly inhibited by gomisin N treatment in a concentration-dependent manner (Fig. 3). Under these conditions, the pro-apoptotic protein Bid, a BH3-only pro-apoptotic member of the Bcl-2 family, was not truncated and remained unchanged. In addition, the expression levels of death receptor-related proteins such as TRAIL, DR4, DR5, Fas and FasL were relatively unchanged in response to gomisin N treatment (data not shown). As expected, the expression levels of Bcl-2 family proteins were not significantly changed in gomisin A-treated U937 cells. These data suggest that the intrinsic pathway is involved in gomisin N-induced apoptosis in U937 cells.

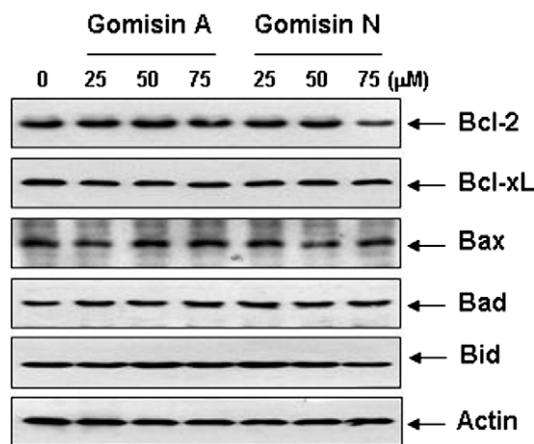


Fig. 3. Effects of gomisin A and gomisin N on the levels of the Bcl-2 family members in U937 cells. After incubation with gomisin A and gomisin N for 48 h, the cells were lysed and the cellular proteins (30 μ g) were then separated in SDS-polyacrylamide gels, after which they were transferred onto nitrocellulose membranes. The membranes were then probed with the indicated antibodies and the proteins were visualized using an ECL detection system. Actin was used as the internal control.

3.4. Loss of MMP and release of cytochrome c by gomisin N

To determine the role of the mitochondria in gomisin N-induced apoptosis of U937 cells, we further investigated the levels of cytosolic and mitochondrial cytochrome c, as well as MMP ($\Delta\psi_m$). As shown in Fig. 3A, exposure of U937 cells to gomisin N led to a significant increase in the level of cytosolic release of the mitochondrial pro-apoptotic protein cytochrome c. We also found that gomisin N treatment attenuated the MMP level, and this decrease occurred in a dose-dependent manner (Fig. 4B). However, gomisin A did not significantly induce the translocation of cytochrome c from mitochondria to cytosol; nor did gomisin A induce MMP loss. These results suggest a direct role of the mitochondria in gomisin N-induced apoptosis of U937 cells.

3.5. Activation of caspase-3 and -9 by gomisin N

To further investigate the apoptotic cascades involved in the effects of gomisin N, we measured expression levels of caspases (-3, -8, and -9) and the *in vitro* activities of these proteins. As shown in Fig. 5A, gomisin N treatment decreased the expression of pro-caspase-3 and -9 proteins in a concentration-dependent manner but the expression levels of pro-caspase-8 remained unchanged. To further quantify the proteolytic activation of the caspases, the con-

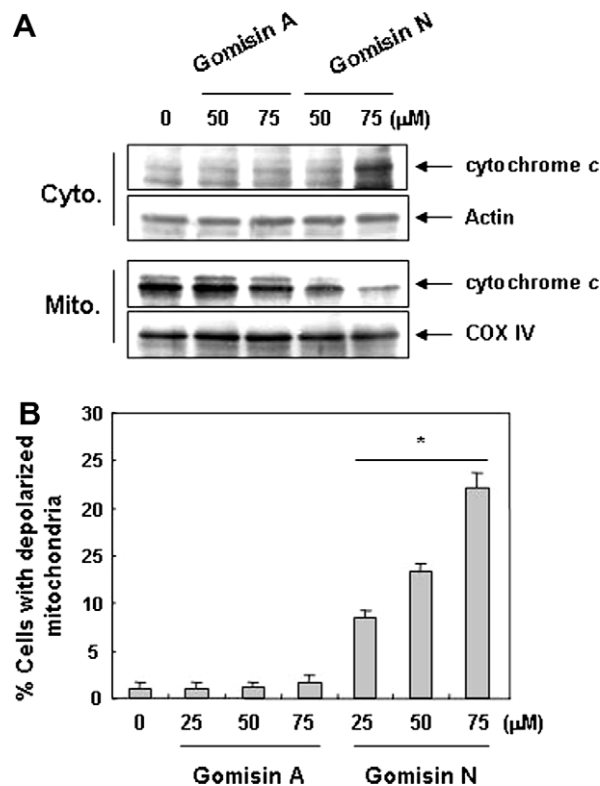


Fig. 4. Loss of MMP and release of cytochrome c by gomisin N treatment of U937 cells. (A) After incubation with gomisin A and gomisin N for 48 h, equal amounts of cytosolic and mitochondrial proteins were extracted, separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the anti-cytochrome c antibody. The proteins were visualized using an ECL detection system. Actin, a marker of cytoskeletal proteins, was included as a control for protein loading. A mitochondrial marker was detected by cytochrome oxidase subunit IV (COX IV). (B) U937 cells grown under the same conditions as (A) were stained with JC-1 and then incubated at 37 $^{\circ}$ C for 20 min, after which the mean JC-1 fluorescence intensity was detected using a flow cytometer. Data represent the mean $\pm$ the SD of representative experiments performed at least three times. The significance was determined by a Student's *t*-test ($p < 0.05$, compared with control).

centration of protein in the lysates of cells treated with gomisin N was normalized and then assayed for their caspase-3, -8 and -9 activity using fluorogenic substrates. Gomisin N treatment markedly increased the activity of caspase-3 and -9 in a concentration-dependent manner; caspase-8 was not activated (Fig. 5B–D). Subsequent Western blot analysis revealed that progressive proteolytic cleavage products of PARP, which are downstream targets of the activated caspase-3 (Lazebnik et al., 1994), occurred in U937 cells that were treated with gomisin N (Fig. 5A). Furthermore, the levels of IAP family proteins such as XIAP and cIAP-1 were down-regulated by gomisin N treatment (Fig. 5A). However, these changes were not observed in gomisin A-treated U937 cells.

3.6. Inhibition of gomisin N-induced apoptosis by a caspase-3 inhibitor

Caspase-3 is one of the key proteases responsible for the cleavage of PARP and subsequent apoptosis. In order to show that the activation of caspase-3 is a key step in gomisin N-induced apoptosis, cells were pretreated with z-DEVD-fmk (50 μ M), a caspase-3 specific inhibitor, for 1 h, followed by treatment with 75 μ M gomisin N for 48 h. As shown in Fig. 6A and B, pre-treatment with z-DEVD-fmk attenuated chromatin condensation and formation of apoptotic bodies, and restored cell viability. These results indicate that activation of caspase-3 plays an important role in gomisin N-induced apoptosis in U937 cells.

4. Discussion

Recent studies have reported that dibenzocyclooctadiene lignans isolated from the fruit of *S. chinensis* have various pharmacological and biological activities including anti-cancer effects (Ohtaki et al., 1996; Xu et al., 2006; Choi et al., 2006; Min et al., 2008). More than 50 lignans have been isolated from *S. chinensis*

and authenticated by modern analytical techniques. From the structural point of view, Gomisin N had a similar structure with gomisin A except for the orientation of hydroxy group at C-7, configuration, and chirality. Gomisin A with *R*-biphenyl configuration possessed a hydroxy group at C-7, but not in gomisin A with *S*-biphenyl configuration (Ikeya et al., 1979). Our findings confirmed that gomisin N exhibited strong anti-proliferative activity, however, gomisin A was inactive in apoptosis induction of human leukemia U937 cells. Min et al. (2008) suggested that the substitution groups and configuration of phenyl ring and the hydroxy group at C-7 might have influence on the anti-proliferative activity. Especially, the lignans with *R*-biphenyl configuration is less potent than one with *S*-biphenyl configuration. From these results, we suggesting that the increase of apoptosis induction in human leukemia U937 cells by gomisin N with *S*-biphenyl is in good agreement with those achieved on a human cancer cells (Min et al., 2008) compared to gomisin A with *R*-biphenyl configuration. While some cell-killing effects of these extracts have been suggested (Wu et al., 2004; Matsumoto et al., 2006; Lin et al., 2008; Min et al., 2008), little is known about their effects on the growth of human cancer cells. In the present study, we found that gomisin N, but not gomisin A, causes apoptosis in human leukemia U937 cells, which appears to account for its anti-cancer activity. The present study provides the first evidence that gomisin N induces growth inhibition associated with induction of apoptosis through the mitochondrial-mediated intrinsic pathway. Furthermore, we showed that gomisin N treatment induced mitochondrial damage and a caspase-3 inhibitor significantly attenuated the gomisin N-induced apoptosis.

It has recently been proposed that mitochondria represent a possible target for anti-cancer drug induced apoptosis in the death receptor (extrinsic) pathway as well as the mitochondrial (intrinsic) pathway (Mow et al., 2001; Mohamad et al., 2005). Since the discovery of Bcl-2, several mechanisms for the anti-apoptotic prop-

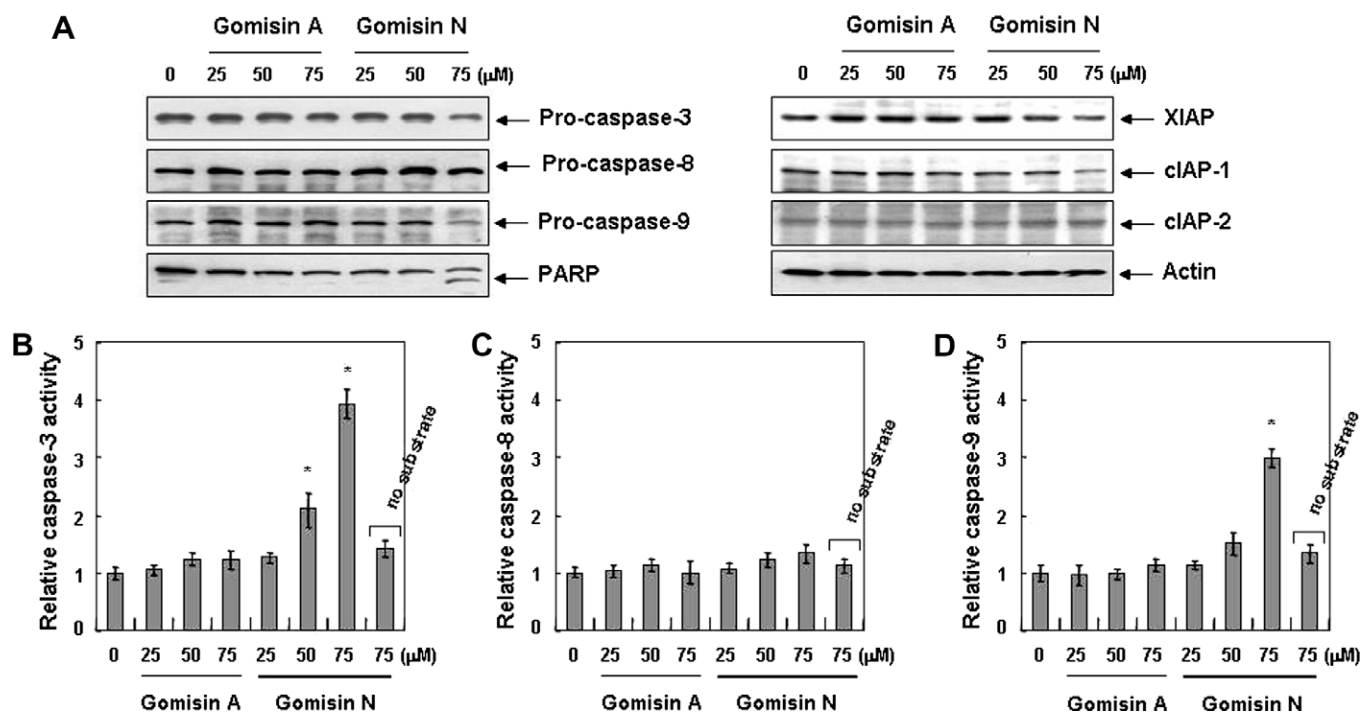


Fig. 5. Activation of caspases by gomisin N treatment of U937 cells. (A) After being treated with gomisin A and gomisin N for 48 h, equal amounts of the cell lysates (30 μ g) were resolved by SDS-polyacrylamide gels, transferred to nitrocellulose membranes and then probed with the indicated antibodies. The proteins were then visualized using an ECL detection system. Actin was used as the internal control. (B–D) The cell lysates obtained from cells grown under the same conditions as (A) were assayed for the *in vitro* caspase-3, -8 and -9 activity using DEVD-pNA, IETD-pNA and LEHD-pNA, respectively, as substrates. The concentrations of the fluorescent products released were then measured. The results are expressed as the mean $\pm$ SD of three independent experiments. The significance was determined by a Student's *t*-test ($p < 0.05$, compared with control).

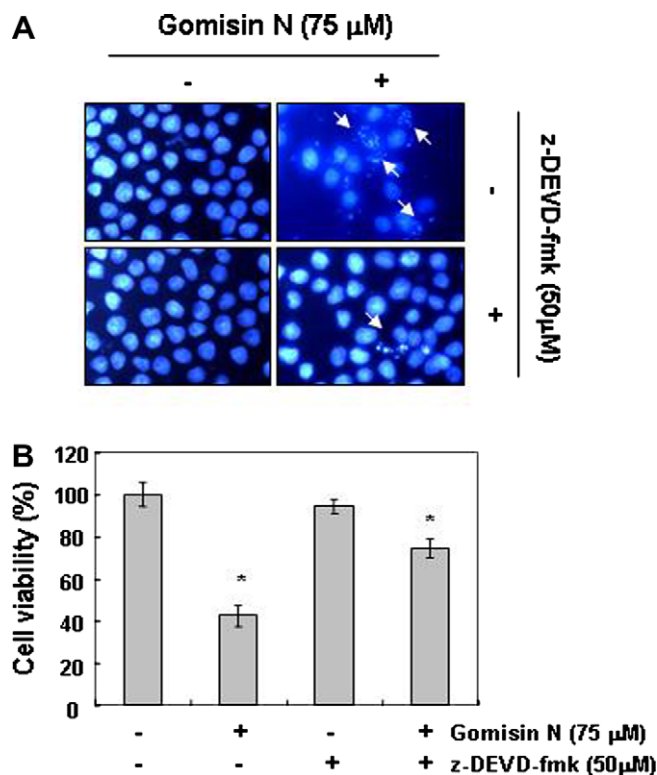


Fig. 6. Inhibition of gomisin N-induced apoptosis of U937 cells by a caspase-3 inhibitor. (A) U937 cells were treated with z-DEVD-fmk (50 μ M) for 1 h prior to being challenged with 75 μ M gomisin N for 48 h. The cells were stained with DAPI for 10 min and photographed with a fluorescent microscope using a blue filter (original magnification 400 $\times$). (B) In a parallel experiment, the degree of growth inhibition was determined by MTT assay. Each point represents the mean $\pm$ SD of three independent experiments. Significance was determined by Student's *t*-test (**p* < 0.05 vs. untreated control).

erties of this protein have been proposed (Murph et al., 1996; Kluck et al., 1997). The anti-apoptotic function of Bcl-2 against pro-apoptotic Bax may be explained by its ability to control several key steps involved in death signaling. The Bcl-2 family significantly regulates apoptosis either as an activator (e.g., Bax or Bad) or as an inhibitor (e.g., Bcl-2, Bcl-xL); therefore, it has been suggested that the Bax/Bcl-2 ratio is a key factor in regulation of the apoptotic process (Thees et al., 2005; Zinkel et al., 2006). The present study shows that gomisin N treatment, but not gomisin A treatment, results in significant inhibition of cell growth and the induction of apoptosis in U937 cells that is confirmed by characteristic morphological changes, DNA fragmentation and increases in the number of sub-G1 cells. We consequently evaluated the effects of gomisin N on the levels of proteins in the Bcl-2 family in U937 cells to test the mechanism by which gomisin N-induced apoptosis occurs. Our data revealed that gomisin N-induced apoptosis was related to down-regulation of Bcl-2, as well as to the loss of MMP; gomisin N increased the Bax/Bcl-2 ratio and induced mitochondrial dysfunction, leading to apoptosis in U937 cells.

The results of many studies have suggested that reduction of Bcl-2 and/or induction of Bax leads to the release of cytochrome *c* from the mitochondria as well as an increase in the activity of caspases. The caspase family plays an important role in driving apoptosis in both the extrinsic and intrinsic apoptosis pathways, and key components of the biochemical pathways of caspase activation have recently been elucidated (Stennicke and Salvesen, 1998; Jeong and Seol, 2008). They are synthesized initially as single polypeptide chains representing latent precursors that undergo proteolytic processing at specific residues to produce subunits that form the active heterotetrameric protease. On the other hand, cell

surface Fas/FasL and TRAIL/DRs systems are key signaling transduction pathways of the extrinsic pathway of apoptosis in cells. Binding FasL to Fas receptors or TRAIL to DRs lead to receptor oligomerization and the formation of the death-inducing signaling complex, followed by the activation of caspase-8, and then cleavage of Bid (tBid) which then translocates into the mitochondria. This relocation induces the release of cytochrome *c* and formation of the apoptosome, which leads to activation of executioner caspases such as caspase-3 (Yin, 2000; Baskin-Bey and Gores, 2005). Caspase-3 is either partially or totally responsible for the proteolytic cleavage of many key proteins including PARP, which is important for cell viability and serves as a marker of apoptosis when cleaved (Lazebnik et al., 1994). Further studies have shown that exposure of U937 cells to gomisin N induced the translocation of cytochrome *c* from the mitochondria into the cytosol, activated caspase-9 and -3, caused a concomitant cleavage of PARP without activation of caspase-8, a cleavage of Bid and modulation of death-receptor genes expression. The results suggest that gomisin N may increase mitochondrial dysfunction, which, in turn, results in the activation of caspase-9, leading to the activation of caspase-3 and the degradation of caspase-3 target proteins. Additionally, treatment with gomisin N in the presence of a caspase-3 inhibitor was found to prevent apoptosis and growth inhibition. Therefore, the present data indicate that the apoptotic effects of gomisin N on U937 cells are associated with the activation of the intrinsic pathway. These data also suggest that gomisin N-induced apoptosis is caused by caspase-3-dependent cell death in U937 cells.

In summary, our results indicate that gomisin N, a dibenzocyclooctadiene lignan, strongly suppresses the proliferation of U937 cells by induction of apoptosis through activation of the mitochondrial-mediated intrinsic caspase pathway. Although further studies are needed, the present work suggests that mitochondrial dysfunction may be a good surrogate biomarker for assessing the anti-tumor activity of gomisin N in human leukemia cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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