Gomisin N isolated from *Schisandra chinensis* significantly induces anti-proliferative and pro-apoptotic effects in hepatic carcinoma

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Abstract. Lignans isolated from *Schisandria chinensis* have been prescribed as anti-cancer and anti-hepatitis treatments in Chinese medicine. To investigate the applications of lignans isolated from *Schisandria chinensis* in hepatic carcinoma therapy, their apoptotic ability was screened using a cell proliferation assay. Compared to the other lignans, gomisin N induced high apoptotic levels in hepatic carcinoma. Cell morphology and flow cytometric analysis demonstrated that this lignan induced cell death at high concentrations, but did not induce any changes at low concentrations. In addition, the expression levels of Bcl-2 and Bax proteins, which are involved in the apoptotic pathway, were markedly increased in only the 320 μM-treated group compared to the vehicle and other concentration groups, while the expression level of p53 protein remained unchanged in this group. These results suggest that gomisin N is an anti-cancer drug candidate capable of inhibiting the proliferation and inducing the apoptosis of human hepatic carcinomas.

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Introduction

Schisandra chinensis has a well-recognized history in traditional Chinese medicine. 'Chinese Magnolia', as it is commonly called, is widely distributed in the eastern regions of Siberia, China, Japan and Korea (1). Over the past 20 years, many active lignans have been isolated from this plant, including gomisin A, B, C, D, E, F, G, K3, N, J, schisandrol B, schisandrin and schisandrin C, (1,2).

Based on their therapeutic properties, these lignans have been classified into four major categories: adaptogenic action, hepatic protection, hepatic stabilization and hepatic regeneration. The first category, adaptogenic action, has specific action mechanisms, with lignans acting as energizers, stimulaters of the nervous system, oxygenators, immune-modulators, antioxidants and skin protectors (1,3-6). These lignans have also been found to reduce hyperlipidemia and to significantly inhibit the development of arteriosclerosis (7). The second category is hepatic protection. These lignans have been closely correlated with increases in the hepatic level of ascorbic acid, the inhibition of NADPH oxidization and lipid peroxydization, and the induction effect of the hepatic microsomal cytochrome P-450 enzyme. Hepatic protection lignans have also been reported to protect against cholestasis induced by toxic substances (8-11). Regarding the third category, hepatic stabilization, previous studies have shown that these compounds contribute to the hepatic regeneration process through the induction of hepatocyte growth factor mRNA transcription, the stimulation of hepatocyte proliferation, and increased blood flow to the liver. In addition, in order to improve regenerative capacity through reduced glutation and the synthesis of hepatic glucogen, these lignans induce an increase in the levels of mitochondrial hepatic glutation and the activity of mitochondrial reductase glutation (12,13). However, few functional studies have been conducted to fully investigate whether the lignans isolated from *Schisandra chinensis* have an effect on cell death in hepatic carcinoma *in vitro*.

The present study investigated the functions involved in the anti-cancer effects of several compounds isolated from *Schisandra chinensis*. Four lignans were screened using the MTT assay. Of these lignans, gomisin N significantly induced cell death in hepatic carcinoma. These results suggest that gomisin N has anti-cancer effects in hepatic carcinoma.

Materials and methods

Reagents. Minimum essential medium (MEM) powder, trypsin and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was supplied by Invitrogen (NY, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material. The fruits of *Schisandra chinensis* used in this study were collected from Moonkyeng-city in Korea in September of 2005. A voucher specimen (accession no. SC-PNUNPRL-1) was deposited in the Herbarium of Pusan National University.

Extraction and structure elucidation of the compound. The dried fruits of *Schisandra chinensis* (2.5 kg) were ground to a fine powder and successively extracted at room temperature with *n*-hexane, EtOAc and MeOH. The hexane extract (308 g) was evaporated under a vacuum and chromatographed on a silica gel (40 μm; J.T. Baker, NJ, USA) column (70 x 8.0 cm) with a step gradient of 0, 5, 10, 20 and 30% EtOAc in hexane (1 liter each) (14). Of these extracts, fraction 11 (3,476 mg) was separated on a silica gel column (100 x 3.0 cm) with 25% hexane in $CHCl_3$ to generate 5 fractions. Fraction 11IA, one of the five subfractions originating from fraction 11, was further purified by column chromatography on silical gel and eluted with $CHCl_3$-acetone (19:1) to produce gomisin N (774 mg). Fraction 8 (1,579 mg) was separated on a silica gel column (100 x 3.0 cm) with CH_2Cl_2 to produce schisandrin C (501 mg). Fraction 29 (1,992 mg) was separated on a silica gel column (100 x 3.0 cm) with 15% $CHCl_3$ in acetone to produce gomisin A (973 mg). Fractions 36, 37 and 38 (10,533 mg) were separated on a silica gel column (100 x 3.0 cm) with 5% CH_2Cl_2 in acetone to produce schisandrin (4,606 mg).

Pure gomisin A was identified by high performance liquid chromatography on a Phenomenex Luna C18 column (Phenomenex; 150 x 4.6 mm I.D., 5-μm particle size) (15). The chemical structure of all lignans used in this study was verified by liquid chromatography-mass spectrometry (LC-MS; Bruker BioApex FT Mass Spectrometer) and nuclear magnetic resonance (NMR) analysis (Varian Inova 500 Spectrometer). Optical rotations were recorded on a Jasco DIP-370 Digital Polarimeter. IR spectra were recorded on an AATI Mattson Genesis Series FTIR. NMR spectra (^1H, ^{13}C) were recorded in $CDCl_3$ on a Varian Inova 500 Spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C, running gradients and using residual solvent peaks as internal references. High-resolution mass spectra were recorded on a Bruker BioApex FT Mass Spectrometer.

Cell culture and treatment. HepG2, a human hepatoblastoma cell line, was purchased from the Korean Cell Line Bank (Seoul, Korea). This cell line was grown in monolayers in MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), incubated at 37˚C in a humidified incubator containing 5% CO_2 in air.

Cell proliferation assay. First, the wells of a 96-well plate were divided into four groups in preparation for gomisin N, schisandrin, schisandrin C and gomisin A treatment. These groups were further classified into 5 subgroups of different concentrations (vehicle, 40, 80, 160 and 320 μM). HepG2 cells were seeded at a density of $4x10^4$ cells/200 μl in a 96-well plate and grown for 24 h in an incubator at 37˚C. When the cells attained 70-80% confluence, various concentrations of gomisin N dissolved in dimethyl sulfoxide (DMSO) were added to each well and the cells were further incubated for another 24 h. Cell proliferation was evaluated using the tetrazolium compound MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma M2128). After the supernatant in the lignan- and vehicle-treated wells was discarded, 200 μl of fresh MEM solution and 50 μl of MTT solution (2 mg/ml in PBS) were added to each well. These cells were then incubated in an incubator at 37˚C. MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals. After 4 h, 220 μl of the MTT mixture was removed from each well, and MTT formazan precipitates were dissolved in 150 μl of DMSO. Finally, the absorbance of the sample in the well was directly read using a SoftMax Pro5 Spectrophotometer (Molecular Devices, USA). The optimal absorbance wavelength was set at 570 nm. Data involving cell number versus absorbance were analyzed, allowing for the quantification of changes in cell proliferation.

Flow cytometric analysis. The percentage of cells undergoing apoptosis and dead cells were detected using FITC Annexin V staining (BD Bioscience, USA). HepG2 cells were seeded at a density of $2x10^6$ cells in 100-mm^2 dishes and grown for 24-48 h in an incubator at 37˚C. When the cells attained 70-80% confluence, various concentrations of gomisin N solution were added to each culture dish, and the cells were further incubated for an additional 24 h. Each group of cells was harvested, washed twice with ice-cold PBS, and then resuspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. Subsequently, ~$1x10^5$ cells in 100 μl of solution were transferred to a round-bottom culture tube. FITC Annexin V (5 μl) was added to stain the cells. After a 15-min incubation at room temperature, 400 μl of 1X binding buffer was added to each tube, and the cells were analyzed by FACSCalibur (BD Biosciences) within 1 h.

Western blot analyses. The HepG2 cells harvested from the 100-mm^2 culture dish were solubilized with 1% Nonidet P-40 in 150 mM NaCl, 10 mM Tris HCl (pH 7.5) and 1 mM EDTA, and supplemented with a protein inhibitor mixture (Roche, Germany). They were then centrifuged at 10,000 x g for 10 min at 4˚C. The homogenated proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels for 3 h and transferred to nitrocellulose membranes over 2 h at 40 V. The membranes were then incubated with primary antibodies [anti-Bcl-2 (SC-7382), anti-Bax (SC-493), anti-p53 (SC-6243) and anti-α-tubulin (Sigma)] to detect Bcl-2, Bax, p53 and

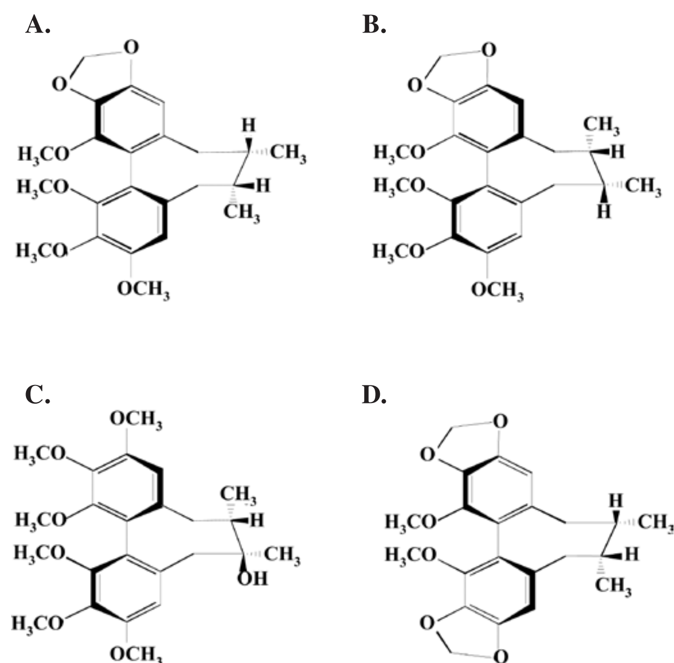

Figure 1. Chemical structure of four lignans isolated from *Schisandra chinensis*: (A) gomisin N, (B) gomisin A, (C) schisandrin and (D) schisandrin C.

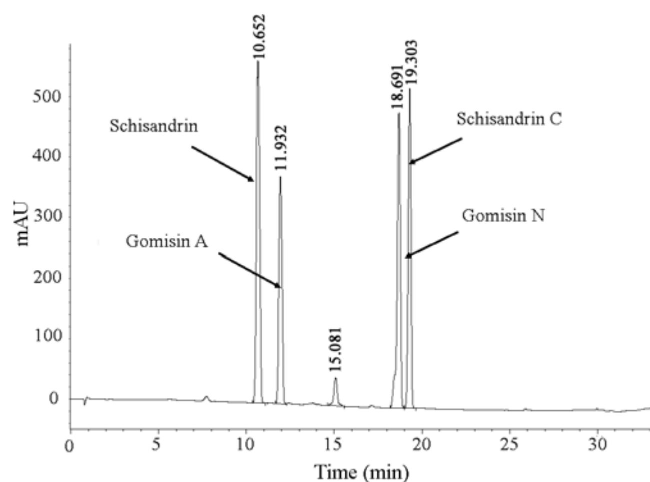

Figure 2. High performance liquid chromatography (HPLC) profiles of the lignans of *Schisandra chinensis*: gomisin N, gomisin A, schisandrin and schisandrin C. HPLC analysis showed that the isolated lignans were >96% pure.

anti-α-tubulin. Each antigen-antibody complex was visualized with a biotinylated secondary antibody, (goat anti-rabbit)-conjugated HRP streptavidin (Zymed, Histostain-Plus Kit), diluted 1:1,500 in 1X PBS.

Statistical analysis. Tests for the significance between vehicle-treated and compound-treated groups were performed using a one-way ANOVA test of variance (SPSS for Windows, release 10.10, standard version, Chicago, IL, USA). All values were reported as the mean ± standard deviation (SD). P-values <0.05 were considered significant.

Results

Isolation of four lignans from Schisandra chinensis. In order to prepare lignans for the study of their anti-cancer effects, various compounds were extracted from *Schisandra chinensis* by *n*-hexane, EtOAc and MeOH. Subsequently, four lignans that were extracted in large quantities compared to the other lignans were harvested. The structure of these lignans was determined by LC-MS and NMR analysis, and the lignans were identified as gomisin N (16), schisandrin (17), schisandrin C (16) and gomisin A (18,19) (Fig. 1). Chromatography verified that the four lignans were pure enough to be used in experiments to determine their anti-cancer effects (Fig. 2). Thus, four lignans for testing anti-cancer effects were successfully isolated from *Schisandra chinensis* and used for further experimentation.

Effects of the four extracts on cell proliferation. To select the lignan with the highest apoptotic effect on hepatic carcinomas, the MTT assay was used to screen proliferation activity in each group receiving one of the four lignans at varying concentrations. Schisandrin C was found to induce cell proliferation rather than cell death in HepG2 cells at concentrations of 40-160 μM (Fig. 3D). Cell proliferation in the gomisin A-treated group remained at a constant level (Fig. 3B). However, two lignans, gomisin N and schisandrin, significantly induced cell death in comparison to the other lignans. Cell proliferation in the 40 μM gomisin-treated group was slightly increased compare to the vehicle, while cell proliferation rapidly decreased with concentrations of gomisin N ranging from 80 to 320 μM (Fig. 3A). Schisandrin also induced cell death at higher concentrations, but with a lower cell death ratio than gomisin N (Fig. 3C). These results indicate that gomisin N treatment was highly effective in inducing the death of HepG2 cells at high concentrations, but not at low concentrations. Gomisin N was therefore selected for further analysis as the most suitable candidate lignan, with proven anti-proliferation and pro-apoptosis activity.

Effects of gomisin N on cell morphology. In order to study whether the cell death effects observed using the MTT assay were concurrent with cell morphological changes, HepG2 cells were observed using a phase-contrast microscope after 24 h of treatment with various concentrations of gomisin N. In the 40 μM-treated group, the number and morphology of HepG2 cells were increased compared to the vehicle-treated group. HepG2 cells in the 80 μM-treated groups showed a pattern similar to the vehicle-treated group, while in the 160 μM-treated group a small number of dead cells was observed. In the groups treated with 320 μM, the number of cells was markedly increased (Fig. 4). Thus, the results of cell morphology analysis of gomisin N-treated cells corroborated the results of the MTT assay.

Effects of gomisin N on apoptosis. FITC Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis (20,21). To investigate the effect of gomisin N on apoptosis, HepG2 cells treated with various concentrations of gomisin N were stained with FITC Annexin V, and fluorescence was detected by flow cytometry. As shown in Fig. 5, gomisin N significantly induced a 15-98% increase in the number of cells undergoing

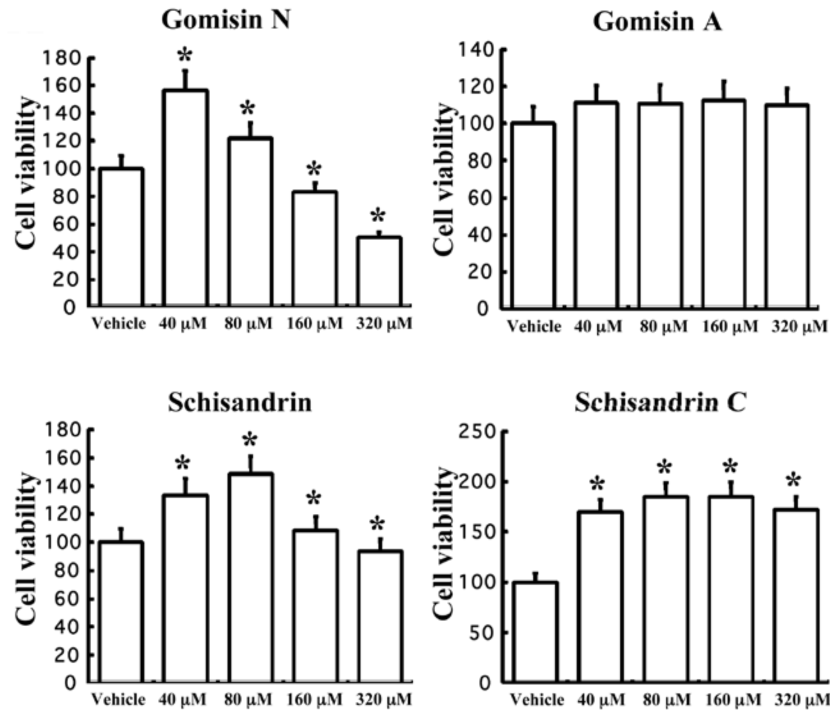

Figure 3. Anti-proliferative effect of the four *Schisandra chinensis* compounds on the HepG2 cell line. Cells were cultured with one of the four compounds in DMSO at various concentrations for 24 h. The vehicle was treated with DMSO only. Cell proliferation was measured using the MTT assay. Data represent as the mean ± SD of three experiments. *P<0.05 compared to the vehicle-treated group, set as the level of significance.

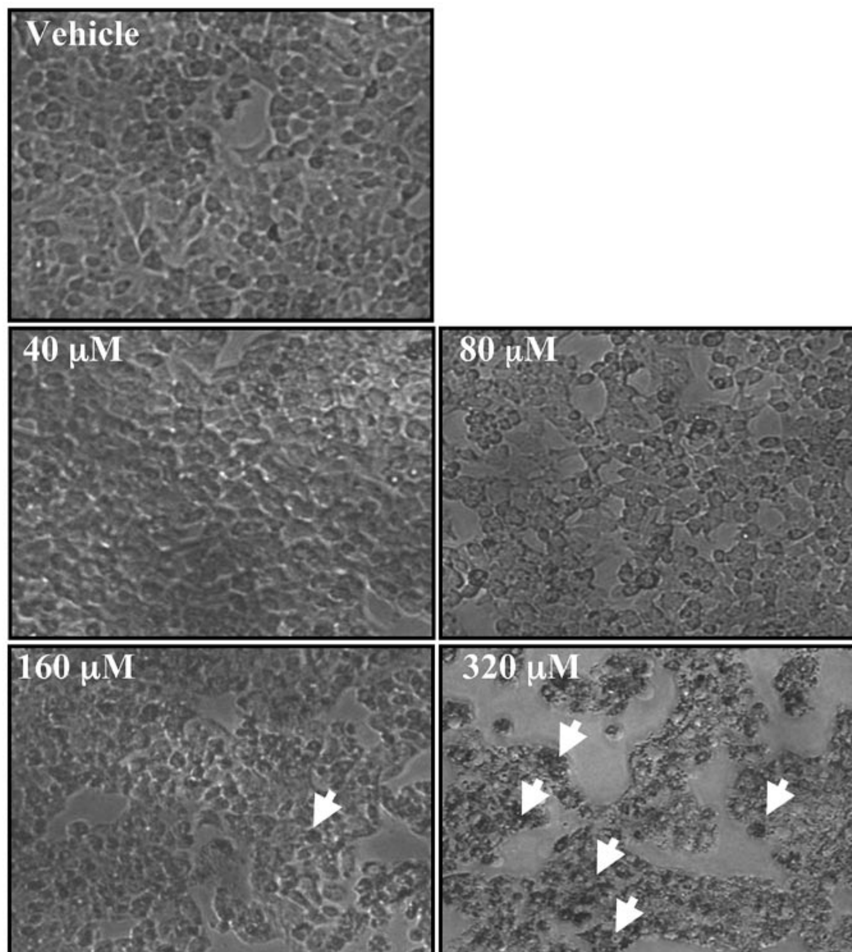

Figure 4. Microscopic images of HepG2 cells after 24 h of treatment with gomisin N at various concentrations. Vehicle-treated cells were treated with DMSO using the dissolving agents of the four *Schisandra chinensis* compounds. Cellular morphology was viewed at x20 magnification.

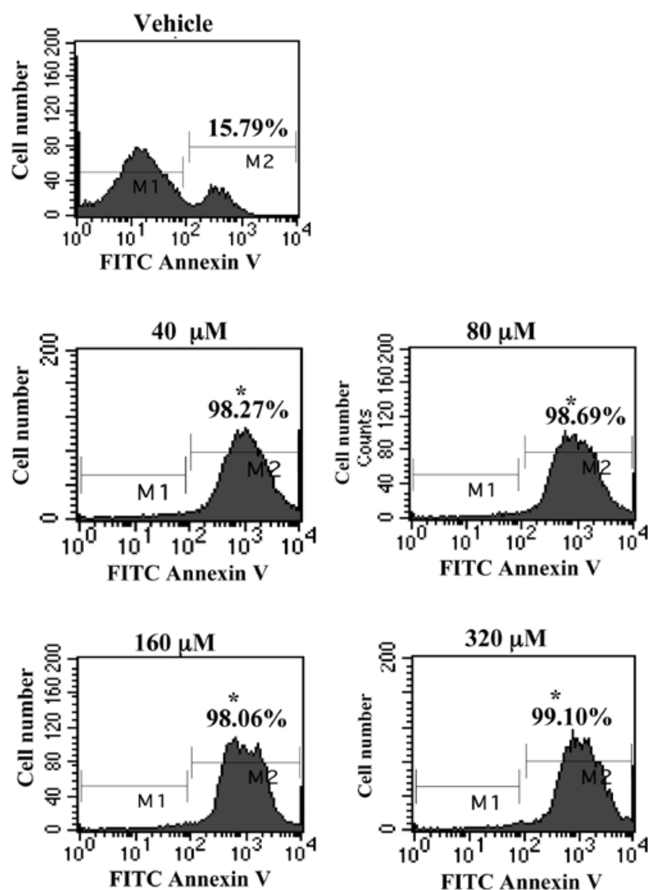

Figure 5. Identification of apoptotic cells. HepG2 cells were incubated with gomisin N at various concentrations for 24 h and stained with FITC Annexin V in order to detect apoptotic cells. The fluoresence emitted by these cells was analyzed using a flow cytometer. M1 indicates live cells; M2, cells undergoing apoptosis or cells that had already died. Data represent the mean ± SD of three experiments. *P<0.05 compared to the vehicle-treated group, set as the level of significance.

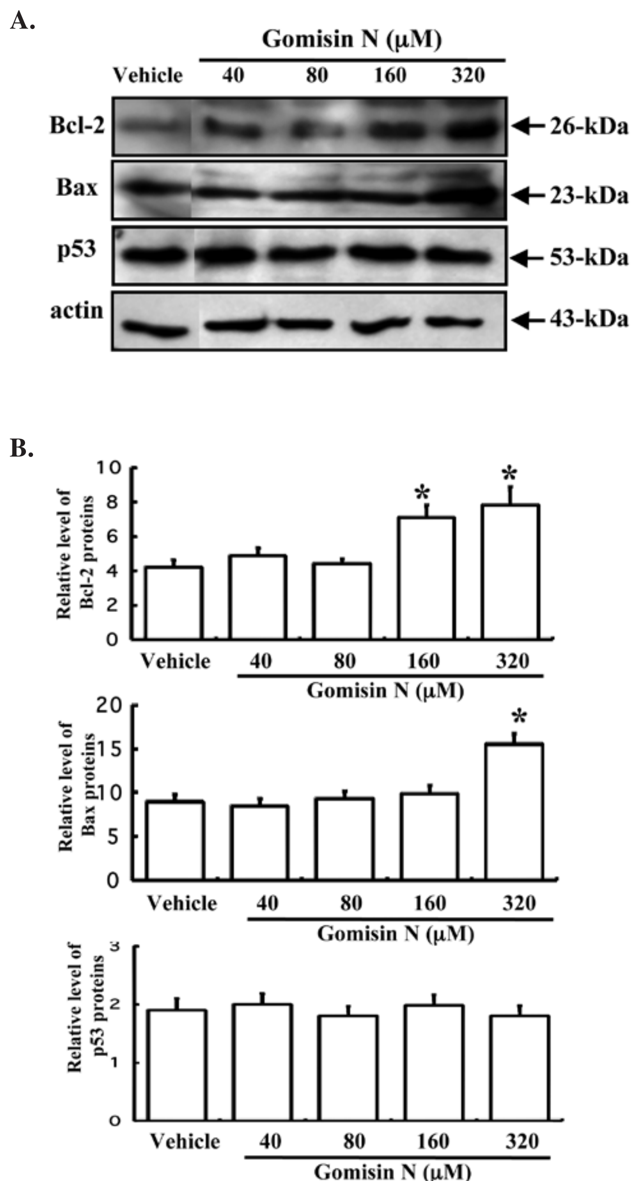

Figure 6. Effects of gomisin N on the apoptotic pathway and the expression levels of tumor suppressor gene. (A) Expression levels of Bcl-2, Bax and p53 proteins were analyzed using Western blot analysis. The membranes were incubated with antibodies for Bcl-2, Bax, p53 and β-actin proteins from the HepG2 cells treated with various concentrations of gomisin N. (B) Expression levels were quantified using an imaging densitometer. The sizes of each product are shown. Data represent the mean ± SD of three experiments. *P<0.05 compared to the vehicle-treated group, set as the level of significance.

apoptosis at 24 h. However, this reaction was induced at even low concentrations of gomisin N, and the level of induction remained at a constant level for higher concentrations. These results suggest that gomisin N dose-independently induces the apoptosis of HepG2 cells. Specifically, gomisin N induced the loss of plasma membrane asymmetry, one of the early events in the apoptotic process, for most of the cells treated at a concentration of 40 μM.

Effects of gomisin N on the apoptic pathway. Bcl-2 belongs to a family of proteins that includes both pro- and anti-apoptotic members. Of these members, Bcl-2 protein stimulates the anti-apoptotic process, while Bax protein significantly inhibits its anti-apoptotic actions (22,23). In order to study the effects of gomisin N treatment on the proteins associated with the apoptotic signaling pathway, the expression levels of Bcl-2 and Bax were determined in the vehicle- and gomisin N-treated groups using Western blot analysis. The expression level of Bcl-2 protein did not change in the low concentration range compared to the vehicle. However, the high concentration 160 and 320 μM-treated groups showed a higher expression level of Bcl-2 protein than the groups in the low concentration range. The expression level of Bax protein was markedly increased in only the 320 μM-treated group compared to the vehicle and to the other concentration groups. To examine whether the tumor suppressor gene in the HepG2 cells was affected by gomisin N, the expression level of p53 protein was detected in the vehicle- and gomisin N-treated groups. The expression level of p53 protein remained unchanged in the four treatment groups and the vehicle (Fig. 6). These results suggest that gomisin N simultaneously induces an increase in the proteins associated with the anti-apoptotic and pro-apoptotic processes, but does not affect the expression level of the tumor suppressor protein p53.

Discussion

Hepatocellular carcinoma is a primary malignancy of the hepatocytes, generally leading to death within 6-20 months. This disease is the fifth most common cancer in men and the eighth most common cancer in women worldwide (24). Cirrhosis of any etiology is known to be a major risk factor for hepatocellular carcinoma (25). To date, approximately 80% of patients with newly diagnosed hepatocellular carcinoma have pre-existing cirrhosis of the liver, caused mainly by excessive alcohol use, or by hepatitis C or B infection (26). Many therapeutic strategies are used for the medical treatment of hepatocellular carcinoma, including surgical resection and liver transplantation, although available treatment options depend on the characteristics of the tumor (27,28). In this study, we investigated the anti-tumor functions of lignans newly isolated from *Schisandra chinensis*. Using a cell proliferation assay, our study demonstrated that gomisin N significantly induced cell death in hepatic carcinoma.

Lignans are the most common constituents of *Schisandra chinensis*. Major lignans in European seeds include deoxyschisandrin (0.07-1.09%), gomisin N (0.24-1.49%), schisandrin (0.75-1.86%), wuweizisu C (0.01-0.34%), and gomisin A (0.13-0.90%) (29). In previous studies, several C18 dibenzocyclooctadiene lignans isolated from *Schisandra chinensis* have exhibited various therapeutic activities, including anti-cancer (30,31), anti-hepatocarcinogenetic (32,33), anti-hepatotoxic (34), anti-HIV (35), anti-oxidant (14,36), anti-inflammatory (31) and vascular relaxation (19) effects. Our research group previously discovered a hexane extract with cancer inhibitory abilities (14) in the fruits of *Schisandra chinensis* (Schisandraceae), which is indigenous to Korea, Japan and China. However, the literature is limited on the pharmacological mechanism of action of *Schisandra chinensis*.

The most active and abundant dibenzocyclooctadiene derivative isolated from *Schisandra chinensis* is schisandrin B (Sch B) (37). This derivative has been used for the treatment of viral and chemical hepatitis. Additionally, several studies have shown that Sch B protects against free radical-mediated hepatocellular damage and enhances hepatic glutathione antioxidant status in the mitochondria (38,39). It has been reported that schisandrin B has enantiomers [(+)Sch B and (-)Sch B] (40), which exhibit differential functions. (+)Sch B induces proliferation and apoptosis. Specifically, this compound was found to significantly enhance doxorubin-induced apoptosis in a human hepatic carcinoma cell line and in human breast cancer cells (41). In contrast, the structure of (-)Sch B is very similar to that of gomisin N. Pre-treatment with this compound significantly protected against hypoxia/reoxygenation-induced apoptosis in H9c2 cells (42). However, no studies have aimed to determine whether gomisin N affects cell proliferation and apoptosis. Gomisin N achieved the highest activity level among the four compounds screened in this study: gomisin N, schisandrin, schisandrin C and gomisin A. Specifically, gomisin N significantly inhibited cell proliferation by approximately 50% at high concentrations, though it did not contribute to the cell death process at low concentrations.

Apoptosis is characterized by various morphological features involving loss of plasma membrane asymmetry and attachment, fragmentation and condensation of the nucleus and formation of cytoplasm vesicles (43,44). The loss of the plasma membrane is one of the earliest features noted in apoptosis, and is detected by Annexin V-conjugated FITC (45,46). In our study, the above technique revealed that gomisin N markedly induced the loss of plasma membrane asymmetry at low concentrations. These effects remained unchanged at other concentration levels.

Apoptosis or programmed cell death plays a critical role in a variety of physiological processes during fetal development and in adult life. Defects in the apoptotic process lead to the development of many diseases involving progressive cell accumulation and, in most cases, cancer. Apoptosis involves many families of proteins. Of these proteins, Bcl-2 is one of the key molecules involved in induction as part of the anti-apoptotic process (47). Previous studies suggest that this protein is overexpressed in many solid tumors and contributes to chemotherapy resistance and radiation-induced apoptosis (47,48). Unlike many other known human oncogenes, bcl-2 exerts its influence by enhancing cell survival rather than stimulating cell division (48). In this study, we investigated whether the expression level of the Bcl-2 protein affects gomisin N treatment in hepatic carcinoma. As shown in Fig. 6, the expression level of Bcl-2 protein remained at a consistent level at low concentrations, while high-concentration gomisin N treatment significantly increased Bcl-2 protein expression levels.

The Bax protein, another member of the Bcl-2 family, inhibits the anti-apoptotic activity of Bcl-2. However, various reports suggest that bax acts as a classic tumor suppressor gene *in vivo*. Several human tumors had loss of function mutations for this protein, and knockout of the bax gene induced tumorigenesis in mice (49-51). When we examined the effects of gomisin N treatment on the expression level of the Bax protein using Western blot analysis, the expression level of Bax was significantly increased only in the 320 μM group. These results suggest that the increase in Bax expression upon gomisin N treatment contributes to cell death by inhibiting anti-apoptotic activity.

Collectively, the results suggest that gomisin N is a novel candidate for the therapeutic treatment of hepatic carcinoma. However, intensive research is still required to define the role of this compound in inducing cell death in hepatocellular carcinomas for future clinical applications.

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