Journal of Pharmacopuncture

ISSN 2093-6966 [Print], ISSN 2234-6856 [Online]
Journal of Pharmacopuncture 2016;19[2]:129-136
DOI: http://dx.doi.org/10.3831/KPI.2016.19.014

Original article

Inductions of Caspase-, MAPK- and ROS-dependent Apoptosis and Chemotherapeutic Effects Caused by an Ethanol Extract of *Scutellaria barbata* D. Don in Human Gastric Adenocarcinoma Cells

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Key Words

apoptosis, human gastric adenocarcinoma cell, proliferation, *Scutellaria barbata* D. Don

Abstract

Objectives: The crude extracts of *Scutellaria barbata* D. Don (SB) have traditionally demonstrated inhibitory effects on numerous human cancers both *in vitro* and *in vivo*. Gastric cancer is one of the most common types of cancer on world. The authors investigated the effects of an ethanol extract of *Scutellaria barbata* D. Don (ESB) on the growth and survival of MKN-45 cells (a human gastric adenocarcinoma cell line).

Methods: The MKN-45 cells were treated with different concentrations of ESB, and cell death was examined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Analyses of sub-G1 peaks, caspase-3 and -9 activities, and mitochondrial membrane depolarizations were conducted to determine the anti-cancer effects of SB on MKN-45 cells. Also, intracellular reactive oxygen species (ROS) generation was investigated.

Results: ESB inhibited the growth of MKN-45 cells, caused cell cycle arrest, and increased the sub-G1 population. In addition, ESB markedly increased mitochondrial membrane depolarization and the activities of caspase-3 and -9. ESB exerted anti-proliferative effects on MKN-45 cells by modulating the mitogen-activated protein kinase (MAPK) signaling pathway and by increasing the generation of ROS. Furthermore, combinations of anti-cancer drugs plus ESB suppressed cell growth more than treatments with an agent or ESB, and this was especially true for cisplatin, etoposide, and doxorubicin.

Conclusion: ESB has a dose-dependent cytotoxic effect on MKN-45 cells and this is closely associated with the induction of apoptosis. ESB-induced apoptosis is mediated by mitochondria- , caspase- and MAPK dependent pathways. In addition, ESB enhances ROS generation and increases the chemosensitivity of MKN-45 cells. These results suggest that treatment with ESB can inhibit the proliferation and promote the apoptosis of human gastric adenocarcinoma cells by modulating the caspase-, MAPK- and ROS-dependent pathway.

Received: Feb 15, 2015 **Reviewed:** Feb 29, 2015 **Accepted:** Mar 08, 2016

1. Introduction

Gastric cancer is one of the most common types of cancer and often spreads from the stomach to other parts of the body, particularly to the liver, lungs, bones, the abdominal lining, and lymph nodes [1]. During the last decade, considerable research has been conducted on the induction of apoptosis by traditional Chinese medicines (TCMs). In addition, TCM can be combined with modern medicine to improve symptoms, en-

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hance quality of life, prevent recurrence and metastasis, and prolong survival. However, comparably little is known of the mechanisms whereby TCMs affect cancer cells.

Scutellaria barbata D. Don is an important component of numerous medicinal formulas that have been traditionally used in China and Korea to treat various cancers [2]. *Scutellaria barbata* D. Don is known to induce the apoptosis of human colon carcinoma cells by activating mitochondria and the signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), and p38 signaling-dependent pathways [3, 4]. In tumor-bearing Lewis-Bearing C57BL/6 mice, *Scutellaria barbata* D. Don decreased interleukin (IL)-17, IL-10, forkhead box P3 (FOXP3), transforming growth factor beta (TGF-β1), RORγt, and IL-6 levels, but remarkably increased IL-2 and interferon gamma (IFN-γ) levels and inhibited tumor growth by regulating immune function [5]. *Scutellaria barbata* D. Don induced the apoptosis of human hepatocarcinoma MHCC97-H cells *via* the dose-dependent up-regulations of caspase-3 and -9 [6], and combined treatment with *Scutellaria barbata* D. Don and low dose 5-fluorouracil (5-FU) significantly inhibited tumor growth *in vitro* and *in vivo* possibly *via* apoptosis and regulating 5-FU metabolism [7]. Also, Zhang *et al* [8] investigated the anti-tumor effect of different solvent fractions of *Scutellaria barbata* D. Don and the potential underlying molecular mechanisms. They suggested that the chloroform fraction of *Scutellaria barbata* D. Don exhibited the most potent inhibitory effect on the growth of colon cancer cell lines and that SW620 cells exhibited the most sensitive response to the chloroform fraction of *Scutellaria barbata* D. Don treatment [8].

Apoptosis is a form of programmed cell death that occurs in multicellular organisms and is associated with changes in cell morphology, such as, blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal deoxyribonucleic acid (DNA) fragmentation [9, 10]. However, the mechanisms whereby ethanol extracts of *Scutellaria barbata* D. Don exert anti-cancer activity in gastric cancer cells are poorly understood. In this study, we investigated the anti-cancer effect of *Scutellaria barbata* D. Don on gastric cancer by using the MKN-45 cell line (a human gastric adenocarcinoma cell line), a cell model of a human gastric carcinoma. Our results demonstrate that *Scutellaria barbata* D. Don triggers the apoptosis of MKN-45 cells by activating caspase-, mitogen-activated protein kinase (MAPK)- and reactive oxygen species (ROS)-dependent pathways.

2. Materials and Methods

Powdered ethanol extract of rhizomes of *Scutellaria barbata* (Catalog number: CA01-061 (2nd)) was obtained from the plant extract bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea). For the purification of this extract for the present study, it was immersed in ethanol, sonicated for 15 minutes, and extracted for 72 hours. The mixture obtained was then filtered through non-fluorescent cotton, evaporated under reduced pressure by using a rotary evaporator (N-1000SWD, Eyela, Japan) at 45°C, and lyophilized using a Modul Spin 40 dryer (Biotron Corporation, Calgary, Can-

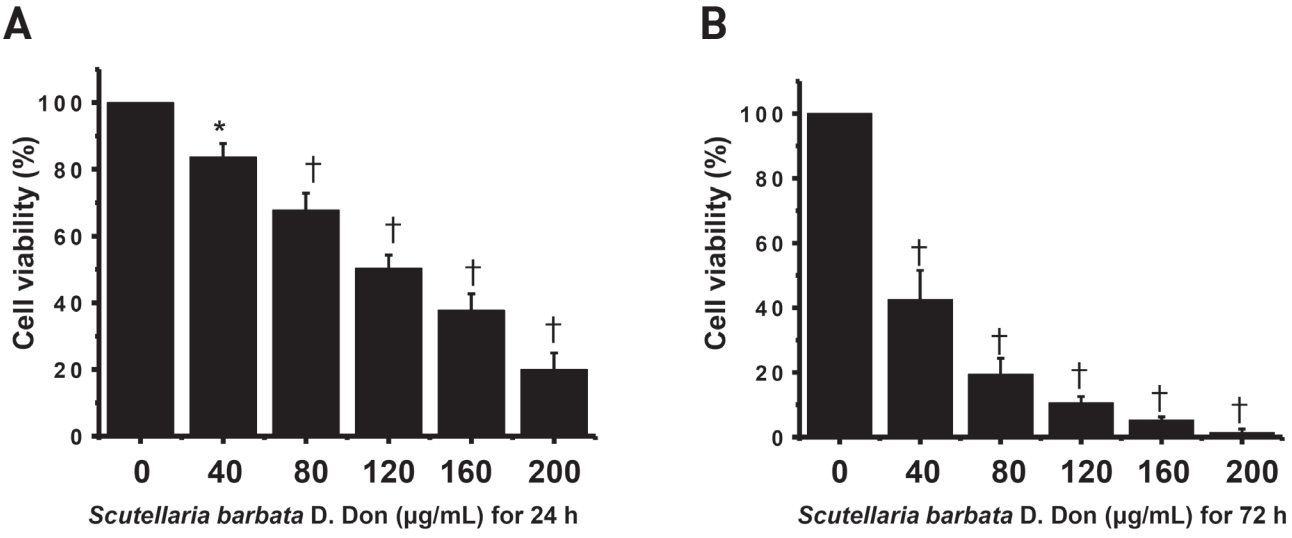

Figure 1 The cytotoxic effect of the ethanol ESB on MKN-45 cells. MKN-45 cells were incubated with ESB at the indicated doses (μg/mL), and cell viabilities were determined after (A) 24 hours or (B) 72 hours by using a MTT assay as described in Methods. Values are expressed as percentages (%) of controls. The figures in the columns are means ± SDs. $^{*}P < 0.05$, $^{\dagger}P < 0.01$.

ESB, extract of *Scutellaria barbata* D. Don; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDs, standard deviations.

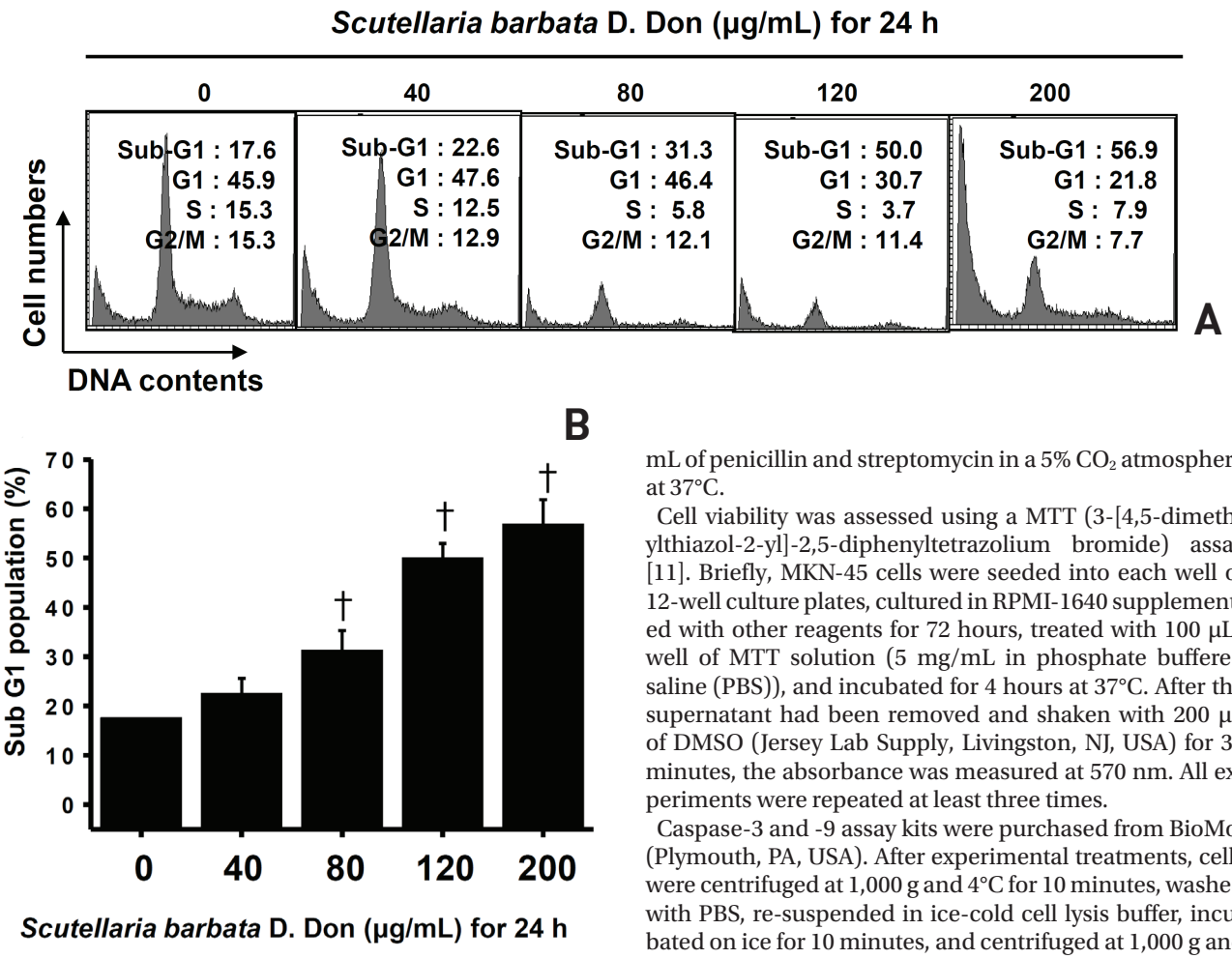

Figure 2 ESB-induced MKN-45 cell apoptosis. MKN-45 cells were incubated at the indicated ESB doses (μg/mL). (A) After 24 hours of treatment, sub-G1 peaks were measured using a FACScan as described in Methods. (B) Values are expressed as percentage (%) of the untreated control, and columns represent means ± SDs. $^{\dagger}P < 0.01$.

ESB, extract of *Scutellaria barbata* D. Don; FACS, fluorescence-activated cell sorter; SDs, standard deviations.

ada) for 24 hours. The yield of lyophilized powder obtained (extract of *Scutellaria barbata* D. Don (ESB)) was 12.3%. ESB was then dissolved in dimethyl sulfoxide (DMSO, Jersey Lab Supply, Livingston, NJ, USA) at a concentration of 100 mg/mL and stored at 4˚C. This stock solution was then diluted with medium to the desired concentrations prior to use. The final concentration of DMSO was always < 0.1% and did not affect the results.

MKN-45 cells were used in this study. This cell line was established at the Cancer Research Center, College of Medicine, Seoul National University, Korea. Cells were propagated in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum and 20 μg/mL of penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C.

Cell viability was assessed using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [11]. Briefly, MKN-45 cells were seeded into each well of 12-well culture plates, cultured in RPMI-1640 supplemented with other reagents for 72 hours, treated with 100 μL/well of MTT solution (5 mg/mL in phosphate buffered saline (PBS)), and incubated for 4 hours at 37°C. After the supernatant had been removed and shaken with 200 μL of DMSO (Jersey Lab Supply, Livingston, NJ, USA) for 30 minutes, the absorbance was measured at 570 nm. All experiments were repeated at least three times.

Caspase-3 and -9 assay kits were purchased from BioMol (Plymouth, PA, USA). After experimental treatments, cells were centrifuged at 1,000 g and 4°C for 10 minutes, washed with PBS, re-suspended in ice-cold cell lysis buffer, incubated on ice for 10 minutes, and centrifuged at 1,000 g and 4°C for 10 minutes. Supernatants (10 μL) were incubated with 50 μL of substrate (400-μM Ac-DEVD-pNA) in 40 μL of assay buffer at 37°C, and the absorbance at 405 nm was read at different times. The pNA concentrations in samples were read off standard pNA concentration/absorbance plots.

A flow cytometric analysis with propidium iodine (PI) staining was used to determine the cell-cycle distributions [12, 13]. MKN-45 cells (1×10^6) were placed in an e-tube, and ice-cold fixation buffer (ethyl alcohol: 700 μL) was then slowly added with vortexing. Tubes were sealed with parafilm, incubated at 4°C overnight, and spun for 3 minutes at 106 g and 4°C. After the supernatants had been discarded, cell pellets were re-suspended in 200 μL of PI staining solution (2 μL of PI at 5 mg/mL, and 2 μL of RNase in 196 μL of PBS) at 20,817 g for 5 seconds and left for 30 minutes in the dark at room temperature. Samples were analyzed using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson, Mountain View, CA, USA) at λ= 488 nm by using Cell-Quest software (Becton-Dickinson). The DNA content distributions of normal cells were characterized using the two peaks corresponding to the G1/G0 and the G2/M phases. The G1/G0 phase represents normal function and the resting state of the cell cycle with greatest diploid DNA content while the G2/M phase more than diploid DNA content. With respect to the cell cycle, cells in the sub-G1 phase have the least DNA content and are

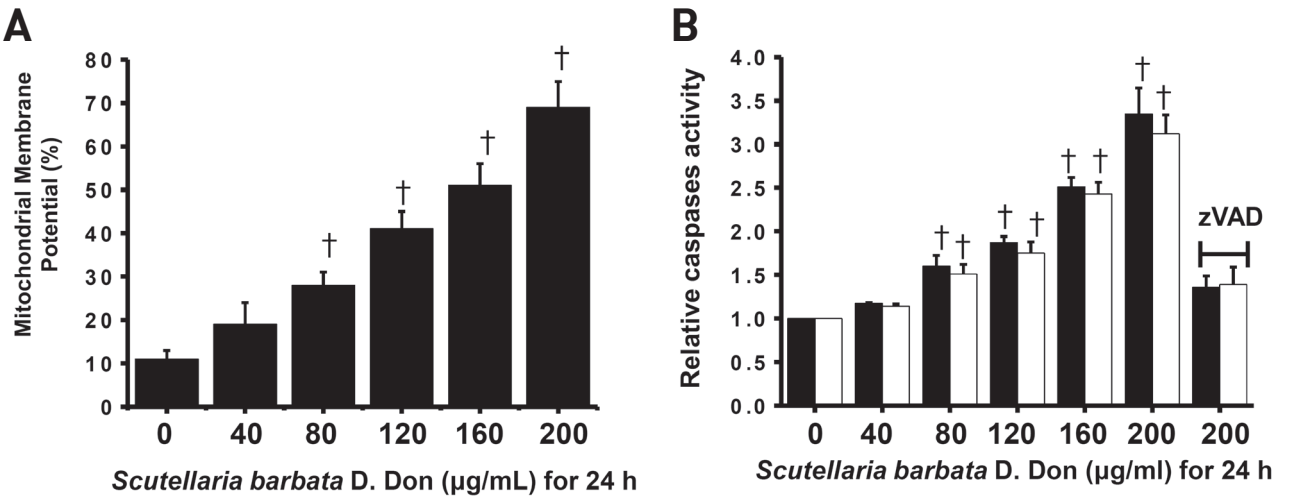

Figure 3 ESB increased mitochondrial membrane depolarization and the activities of caspase-3 and -9 in MKN-45 cells. MKN-45 cells were incubated with ESB at the indicated doses (μg/mL). (A) After 24 hours of treatment, mitochondrial membrane depolarization was measured using a FACScan as described in Methods. (B) Caspase-3 and -9 activities were measured using enzyme assays, which were conducted in quadruplicate. Values are percentages (%) of the untreated control, and columns represent means ± SDs. $^{\dagger}P < 0.01$.

ESB, extract of *Scutellaria barbata* D. Don; FACS, fluorescence-activated cell sorter; SDs, standard deviations.

described as hypodiploid, which reflects DNA fragmentation [14].

Mitochondrial membrane depolarization was evaluated using a 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescence probe (Molecular Probes, Eugene, OR) according to the manufacturer's instructions [15]. Briefly, MKN-45 cells were labeled with 2 μM of JC-1 for 30 minutes at 37°C and then analyzed using flow cytometry at an excitation wavelength of 488-nm with 530/30 or 585/42 nm bypass emission filters. A lack of red fluorescence was regarded as a manifestation of mitochondrial membrane depolarization.

Intracellular ROS generation was determined using carboxy-H2DCFDA (5-(and-6)-carboxy-20,70-dichlorodihydrofluorescein diacetate; Molecular Probes, Eugene, OR) [16]. Briefly, after treatments, cells were treated with 100-μM carboxy-H2DCFDA in culture medium, incubated at 37°C for 30 minutes, and washed with PBS. Fluorescence was measured using a FACScan (Becton-Dickinson, Mountain View, CA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Results are presented as means ± standard deviations (SDs). The significances of the differences were evaluated using the Student's *t*-test. Statistical significance was accepted for *P* values < 0.05.

The drugs used in the experiments, namely, PD 98059 (ERK inhibitor), SB203580 (p38 kinase inhibitor), and LY294002, were purchased from Sigma-Aldrich (St. Louis, MO, USA). JNK inhibitor II (SP600125) was purchased from Calbiochem (San Diego, CA, USA). The chemotherapeutic agents, namely, paclitaxel, 5-fluorouracil, cisplatin, etoposide, doxorubicin, and docetaxel, were purchased from Tocris Bioscience (Bristol, UK). All other agents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Results

Treatment of MKN-45 cells with ESB (40 – 200 μg/mL) for 24 hours reduced cell viability by 83.7% ± 4.1% at 40 μg/mL, 67.8% ± 5.2% at 80 μg/mL, 50.3% ± 4.3% at 120 μg/mL, 37.7% ± 5.5% at 160 μg/mL and 20.0% ± 5.2% at 200 μg/mL (Fig. 1A). The half maximal inhibitory concentration (IC50) of ESB for 24 hours is 105.6 μg/mL. In addition, treatment with ESB for 72 hours reduced viability by 42.6% ± 9.3% at 40 μg/mL, 19.4% ± 5.2% at 80 μg/mL, 10.6% ± 2.4% at 120 μg/mL, 5.3% ± 1.2% at 160 μg/mL and 1.5% ± 1.1% at 200 μg/mL (Fig. 1B). IC50 of ESB for 72 hours is 31.5 μg/mL. These findings indicate that ESB has a dose-dependent cytotoxic effect on MKN-45 cells.

After treatment with ESB for 24 hours, MKN-45 cells were stained with PI, and cell-cycle progression was assessed by using flow cytometry. ESB increased cell numbers in the sub-G1 peak in a dose-dependent manner, which was consistent with cell-death results (Fig. 2). Treatment with ESB for 24 hours resulted in accumulations in the G1 phase of 22.6% ± 3.1% at 40 μg/mL, 31.3% ± 4.3% at 80 μg/mL, 50.0% ± 3.3% at 120 μg/mL, and 56.9% ± 5.0% at 200 μg/mL as compared with 17.6% for vehicle control cells, and the corresponding accumulations after treatment with ESB for 24 hours were 47.6% at 40 μg/mL, 46.4% at 80 μg/mL, 30.7% at 120 μg/mL, and 21.8% at 200 μg/mL. In addition, treat-

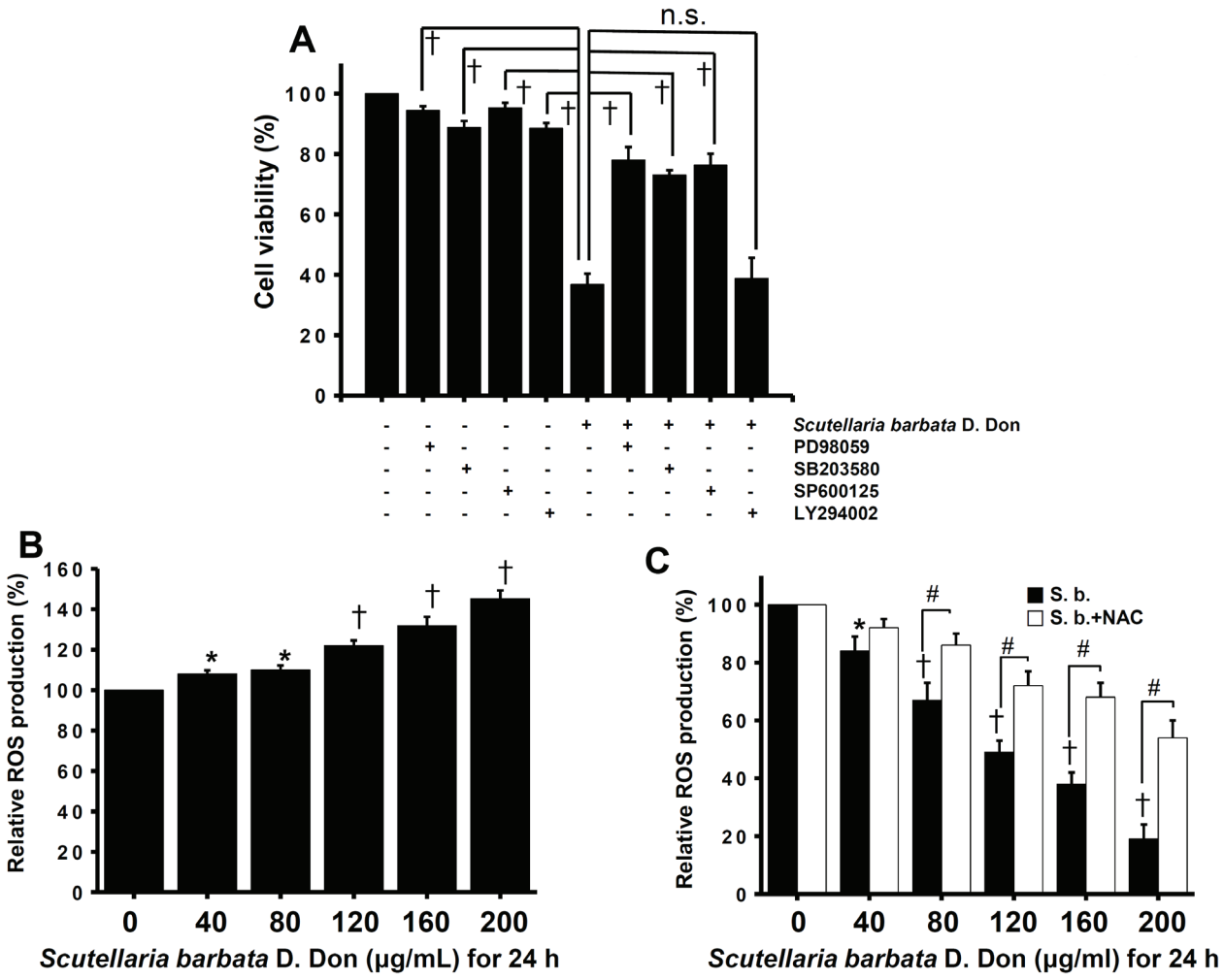

Figure 4 Effects of MAPK and Akt inhibition on ESB-induced MKN-45 cell death and ROS production. (A) Cells were pre-treated with the indicated MAPK inhibitors (SB203580 (20 μM), SP600125 (20 μM), PD98059 (50 μM)) or the Akt inhibitor (LY294002 (20 μM) for 1 hour and then treated with ESBG for 24 hours. (B) Intracellular ROS levels were assessed in MKN-45 cells after treatment with the indicated concentrations of ESB for 24 hours. ROS production levels are expressed as percentages of levels in untreated cells. (C) Cells were co-treated with ESB and NAC for 24 hours. Cell viabilities were determined using a MTT assay. Values are percentages (%) of control, and columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.

MAPK, mitogen-activated protein kinase; ESB, extract of *Scutellaria barbata* D. Don; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDs, standard deviations; n.s., not significant versus ESB-treated cells.

ment with ESB for 24 hours resulted in accumulations in the G2/M phase of 12.9% at 40 μg/mL, 12.1% at 80 μg/mL, 11.4% at 120 μg/mL, and 7.7% at 200 μg/mL, which were lower than that observed in vehicle control cells (15.3%) (Fig. 2). These results suggest that ESB has an anti-cancer effect and that this is closely associated with the induction of apoptosis in MKN-45 cells.

Mitochondrial membrane depolarization (an early event of intrinsic apoptosis signaling) was elevated by using ESB. Flow cytometry showed that the mitochondrial membrane depolarization caused by 24 hours of treatment with ESB was markedly increased by 19.1% ± 5.1% at 40 μg/mL, 28.2% ± 3.3% at 80 μg/mL, 41.4% ± 4.2% at 120 μg/mL, 51.4% ± 5.4% at 160 μg/mL, and 69.4% ± 6.4% at 200 μg/mL (n = 6, Fig. 3A). Also, because caspase activation is required for apoptosis, caspase activity assays were used to observe the activities of caspase-3 and -9 in MKN-45 cells. Caspase activities were found to be dose-dependently elevated after cells had been treated with ESB at doses from 40 to 200 μg/mL for 24 hours, and these activities were found to be repressed by pre-treating cells with zVAD-fmk, a pan-caspase inhibitor (Fig. 3B). These results suggest that

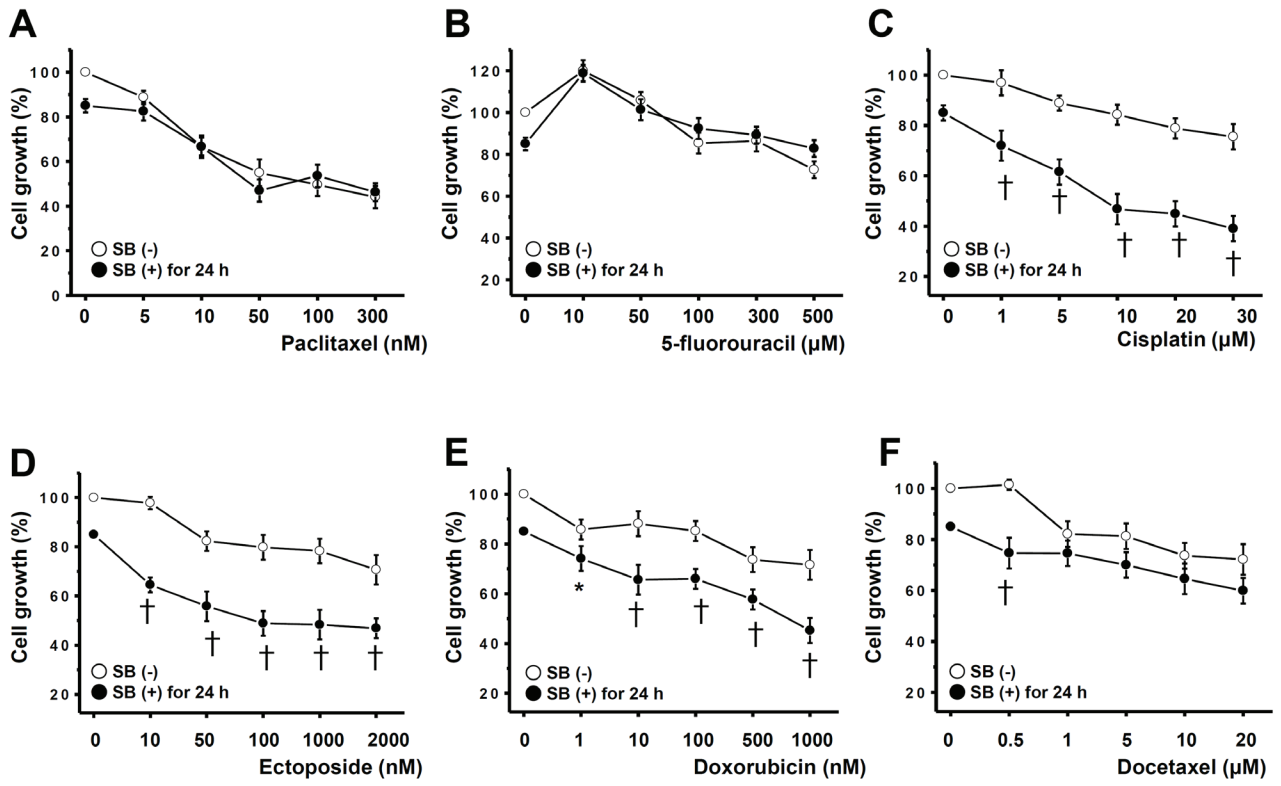

Figure 5 ESB increased the chemosensitivity of MKN-45 cells for 24 hours. Cells were co-treated with ESB (40 μg/mL) and a chemotherapeutic agent, such as paclitaxel, 5-fluorouracil, cisplatin, etoposide, doxorubicin, or docetaxel at the indicated concentrations and then subjected to MTT assays. Values are expressed as percentages (%) of control, and columns represent means ± SDs. $^{*}P < 0.05$. $^{\dagger}P < 0.01$.

ESB, extract of *Scutellaria barbata* D. Don; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SDs, standard deviations.

ESB-induced apoptosis is mediated by mitochondria- and caspase-dependent pathways in MKN-45 cells.

To investigate the relationship between the regulation of MAPK pathways and the inhibition of cancer-cell proliferation by ESB, we pretreated the MKN-45 cells with the MAPK inhibitors PD98059 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor) and then treated them with ESB (160 μg/mL) for 24 hours (Fig. 4A). All three MAPK inhibitors significantly diminished the cell death induced by ESB: PD98059 reduced cell death by 41.3% ± 2.1%, SB203580 by 36.3% ± 1.3%, and SP600125 by 39.5% ± 2.3% as compared with the vehicle control. In contrast, ESB-induced cell death was not attenuated by LY294002. Taken together, these data suggest that the anti-proliferative effect of ESB on MKN-45 cells is due to modulation of MAPK signaling pathways, which results in apoptosis. In addition, because intracellular ROS play important roles in apoptosis, we examined whether ESB was capable of generating ROS in MKN-45 cells. Cells were treated with different concentrations of ESB from 40 to 200 μg/mL for 24 hours, and the levels of ROS generation were measured by using flow cytometry. As shown in Fig. 4B, treatment with ESB significantly and dose-dependently increased ROS generation by almost 1.4-fold versus the vehicle control. We also measured cell viabilities after co-treating cells with ESB (40 – 200 μg/mL) and N-acetyl-L-cysteine (NAC; a ROS scavenger) for 24 hours. As shown in Fig. 4C, NAC remarkably reduced ESB-induced cell death. Thus, these results indicate that ESB enhances ROS generation.

In the present study, we investigated whether ESB was able to enhance the sensitivity of MKN-45 cells to chemotherapeutic agents *in vitro*. Combinations of ESB and several chemotherapeutic agents, used individually, suppressed cell growth more than each agent in isolation did (Fig. 5). In particular, cisplatin, etoposide, or doxorubicin, when administered with ESB, markedly suppressed cell growth. These results suggest that ESB increases the chemosensitivity of MKN-45 cells.

4. Discussion

Scutellaria barbata D. Don has been shown to possess

various anti-cancer effects in liver, colorectal, lung, and breast cancer [17-20], but little is known of its effect on gastric cancer. Therefore, in the present study, we investigated the anti-cancer effect of *Scutellaria barbata* D. Don on gastric cancer and the mechanism involved by using an ethanol extract of SB and MKN-45 cells. Our results show ESB dose-dependently reduces the viability of MKN-45 cells (Fig. 1), increases cell numbers in the sub-G1 peak, which is consistent with our cell viability findings, and reduces cell numbers in the G2/M phase (Fig. 2). In addition, flow cytometry showed that mitochondrial membrane depolarization was markedly increased by ESB, and that the activities of caspase-3 and -9 were dose-dependently elevated at the molecular level by ESB (Fig. 3). To investigate the mechanisms underlying apoptotic pathways, we pretreated the MKN-45 cells with the MAPK inhibitors PD98059 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor), and all three were found to diminish ESB-induced cell death significantly (Fig. 4A). However, LY294002 did not diminish ESB-induced cell death (Fig. 4A). In addition, ESB dose-dependently induced ROS production (Fig. 4B), and NAC (a ROS scavenger) (Fig. 4C) remarkably reduced ESB-induced cell death. Furthermore, co-treatment with ESB plus anti-cancer agents, one at a time, suppressed cell growth more so than using these agents in isolation did; this effect was most obvious for cisplatin, etoposide, and doxorubicin (Fig. 5).

Several research groups are investigating the anti-cancer effects of *Scutellaria barbata* D. Don on various cancer cells. *Scutellaria barbata* D. Don has been reported to have anti-tumour effects in mice transplanted with human hepatocellular carcinoma (HepG2) cells [17], and to induce the deaths of LoVo cells (a human colon cancer cell line) [18] and A549 cells (a human lung cancer cell line) [19]. and on metastatic breast cancer [20]. In addition, *Scutellaria barbata* D. Don induced the apoptosis of human colon carcinoma cells [3, 4] and human hepatocarcinoma MHCC97-H cells [6].

Mechanistically, TCM have been reported to inhibit the growth and survival of gastric adenocarcinoma cells *via* transient receptor potential melastatin 7 (TRPM7) ion channels [21-23]. Therefore, in the future we intend to determine the involvements of TRPM7 channels in the anti-cancer effects of ESB. Furthermore, *Sophorae radix* [21], *Orostachys japonicus* [22], *Ulmi Pumilae Cortex* [24], *Buxus Microphylla var. Koreana Nakai* Extract [25] and *Flos carthami* [26] have been reported to inhibit the growth and survival of gastric cancer cells by blockading TRPM7 channel and MAPK signaling, and thus, they have been considered a starting point for the development of agents against gastric cancer [27]. According to results of the present study, pretreating MKN-45 cells with PD98059 (an ERK1/2 MAPK inhibitor), SB203580 (a p38 inhibitor), or SP600125 (a JNK inhibitor) attenuated the suppressive effect of ESB on MKN-45 cells, but AKT pretreatment had no such effect. These results support our hypothesis that ESB act as chemopreventive agent in gastric cancer cells by blockading MAPK pathways, and that targeting of the MAPK signaling pathways by ESB offers a strategy for the development of a therapy for gastric cancer.

5. Conclusion

ESB was found to reduce MKN-45 cell proliferation and induce apoptosis, which was confirmed by accumulation of cells in the sub-G1 phase. Furthermore, ESB-induced apoptosis was found to be associated with activations of caspases and mitochondrial dysfunction, and inhibition of the MAPK pathway inhibited ESB-induced MKN-45 cell apoptosis. These findings suggest that ESB should be considered a potential agent for the treatment of human gastric adenocarcinoma.

Acknowledgment

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant no. NRF-2014R1A1A2054469).

Conflict of interest

The authors declare that there are no conflict of interest.

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