Apoptotic effects of alisol B 23-acetate on gastric cancer cells

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Abstract. Alisol B 23-acetate (AB23A) is a natural triterpenoid isolated from *Alismatis rhizoma*, which exhibits a number of pharmacological activities. In the present study, AB23A-induced anticancer efficacy was examined in AGS gastric cancer cells. Cell viability assay, cell cycle analysis, caspase activity assay, western blotting and reactive oxygen species (ROS) assay were used to investigate the anticancer effects of AB23A on AGS cells. AB23A reduced the viability of AGS cells, increased the sub-G1 cell fraction and depolarized the mitochondrial membrane. Notably, AB23A-induced cell death was associated with downregulation of the B-cell lymphoma 2 and survivin proteins, and upregulation of the Bax protein. In addition, AB23A increased caspase-3 and -9 activities, and regulated the activation of mitogen-activated protein kinases (MAPK). Moreover, AB23A increased the production of reactive oxygen species. These results suggested that AB23A may induce apoptosis through cell cycle arrest and the mitochondrial pathway, accompanied by the caspase and MAPK signaling cascades. In conclusion, AB23A may have potential as a novel anticancer drug for the treatment of gastric cancer.

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Introduction

Gastric cancer accounts for a large percentage of cancer-related deaths (1). Recent advances in diagnostic techniques, including radiation tests, endoscopy, and biopsy, as well as the heightened public awareness of cancer have steadily improved survival rates and facilitated the early detection of gastric cancer over time (2). In general, surgery, chemotherapy, and radiation therapy are regularly employed as treatment methods, and many anticancer drugs have recently been developed and utilized to complement chemotherapy according to postoperative treatment, but the resulting survival rates remain unsatisfactory (3). As a result, many studies have been conducted to identify effective anticancer drugs; however, many of these therapeutics displayed negative side effects in clinical trials (4). Therefore, it is necessary to develop a new anticancer drug that has no side effects on normal cells.

Natural products can be used in combination therapies against various diseases as a mixture of different ingredients that interact with each other (5). Because it affects multiple targets *in vivo*, new medicines are being developed using natural components as medicines or by synthesizing individual components or derivatives (5). Naturally derived anticancer drugs are expected to continue to be developed for the current pharmaceutical market (6). Therefore, the development of novel anticancer drugs will likely focus on the utilization of existing natural products, the special mechanism of which will be revealed afterwards. Most chemotherapies are closely linked to apoptosis (7) and in the field of anticancer research targeting apoptosis, a variety of drugs are being developed, especially via chemical and radiotherapeutic mechanisms, and many studies are currently being conducted on anticancer supplements (8). The study of anticancer drugs related to these natural products mainly focuses on factors involved in signal transduction related to apoptosis (8).

In recent years, molecules separated from natural substances have been found to be useful in various diseases treatment as alternative medicines. Alisol B 23-acetate (AB23A) is a major ingredient isolated from *Alismatis rhizome* (9); it has been reported to have various pharmacological activities, including anti-hepatic (10), antibacterial (11), diuretic (12),

hyperlipidemic (13), hepatoprotective (14) and anti-inflammatory effects (15). Furthermore, it is known to have a cell death effect on some cancer cells, including SGC7901 stomach cancer cells (16), HEY ovarian cancer cells (17), HCT116 colon cancer cells (18), A549 lung cancer cells (19,20) and HepG2 or SK-HEP-1 hepatoma cells (21,22). However, the detailed anticancer efficacy mechanisms of AB23A remain largely unknown in gastric cancer cells. Therefore, we investigated the anticancer mechanisms of AB23A in AGS gastric cancer cells.

Materials and methods

Cell proliferation. The gastric cancer cell line AGS were obtained from the American Type Culture Collection (ATCC). AGS cells were cultured in RPMI-1640 medium (Gibco-BRL; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) containing 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C and seeded onto 12-well plates at a density of $3x10^4$ cells/well. Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay for 24, 48 and 72 h. Also, to identify the effects of AB23A on the mitogen-activated protein kinase (MAPK) pathways, PD98059 (p42/44 MAPK inhibitor; 10 μM), SB203580 (p38 MAPK inhibitor; 10 μM), or SP600125 (JNK inhibitor; 10 μM) was used with the MTT assay for 24 h.

Cell cycle measurement. After 24 h of treatment with AB23A, AGS cells were treated with ethyl alcohol (3 ml; 100%) and vortexed prior to overnight incubation at 4˚C. Samples were centrifuged for 5 min and the supernatant was discarded. Cell pellets were resuspended in propidium iodine (PI) staining solution (5 mg/ml; 2 μl) containing RNase (2 μl), spun at 2,0000 x g for 10 sec and incubated for 40 min in the dark at room temperature. Samples were analyzed using a fluorescence-activated cell sorter (FACScan; Becton-Dickinson).

Mitochondrial membrane depolarization assay. After 24 h of treatment with AB23A, AGS cells were treated with 50 nM tetramethylrhodamine methyl ester (TMRM; Sigma-Aldrich; Merck KGaA) for 30 min. The fluorescence intensities were measured using a BD FACSCanto II (BD Biosciences) at the excitation and emission wavelengths of 510 and 580 nm, respectively.

Western blot analysis. The Bradford method (Bio-Rad Laboratories) was used to extract the total protein. The protein samples were separated via 8 or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with specific antibodies. Antibodies against survivin (cat. no. 2808), extracellular signal-regulated kinase (ERK; cat. no. 9102), phosphorylated (p) ERK (cat. no. 9106), c-Jun N-terminal kinase (JNK; cat. no. 9252), pJNK (cat. no. 9251), p38 (cat. no. 9212), and pp38 (cat. no. 9216) were purchased from Cell Signaling Technology, and antibodies against B cell lymphoma 2 (Bcl-2; cat. no. sc-783), Bax (cat. no. sc-493), caspase-3 (cat. no. sc-7148), caspase-9 (cat. no. sc-7885), poly (ADP-ribose) polymerase (PARP; cat. no. sc-7150), β-actin (cat. no. sc-47778) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. sc-32233) were procured from Santa Cruz Biotechnology. After 24 h of treatment with AB23A, survivin, Bcl-2, Bax, caspase-3, caspase-9 and PARP experiments were conducted. In case of ERK, p-ERK, JNK, p-JNK, p38 and pp38, experiments were conducted after 0.5, 1, 2 and 4 h of treatment.

Caspase assay. Caspase-3 and -9 assay kits (Cellular Activity Assay kit Plus; BioMol Research Laboratories, Inc.) were used. After 24 h of treatment with AB23A, caspase experiments were conducted. After resuspending the cells in ice-cold cell lysis buffer, the supernatant was removed. Supernatant samples were incubated with caspase substrate (400-lM Ac-DEVD-pNA; 50 μl) at 37˚C and then, samples were read at 405 nm.

Reactive oxygen species (ROS) measurement. After 24 h of treatment with AB23A, AGS cells were treated with 20 μl using DCF-DA (2',7'-dichlorodihydrofluorescein diacetate: Molecular Probes) at 37˚C for 30 m in and washed with PBS. Fluorescence was measured using FACS (Becton-Dickinson), at excitation/emission wavelengths of 488/525 nm, respectively (23).

Statistical analysis. Two-way analysis of variance (ANOVA) or one-way ANOVA with Tukey's post hoc comparison method were used for multiple comparisons. The analysis was performed using the Prism 6.0 (GraphPad Software, Inc.) and Origin 8.0 (OriginLab Corporation) software. Data are expressed as the mean ± standard error of the mean (SEM), and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of AB23A on AGS gastric cancer cell viability. The MTT method was used to evaluate the effects of AB23A on cell viability in AGS cells. AB23A (10, 20, 30, 40 or 50 μM) reduced the cell viability by 99.3±1.1, 82.8±1.6% ($P<0.01$), 48.3±0.2% ($P<0.01$), 36.6±3.6% ($P<0.01$) and 27.9±1.3% ($P<0.01$), respectively, at 24 h (Fig. 1A), by 84.1±2.9% ($P<0.01$), 77.5±1.3% ($P<0.01$), 39.3±2.2% ($P<0.01$), 29.5±0.6% ($P<0.01$) and 10.1±0.8% ($P<0.01$) at 48 h (Fig. 1B) and by 77.7±1.2% ($P<0.01$), 58.7±4.7% ($P<0.01$), 29.1±1.0% ($P<0.01$), 9.7±0.6% ($P<0.01$) and 5.1±0.1% ($P<0.01$) at 72 h (Fig. 1C) as determined by MTT assay. In addition, cell cycle analysis and mitochondrial membrane depolarization experiments were conducted to assess the apoptotic effects of AB23A. The sub-G1 phase ratios were increased by 7.8±1.2% at 10 μM, 11.3±1.4% ($P<0.01$) at 20 μM, 19.6±1.1% ($P<0.01$) at 30 μM, 28.8±2.0% ($P<0.01$) at 40 μM, and 36.8±2.9% ($P<0.01$) at 50 μM for 24 h (Fig. 2A and B). Mitochondrial membrane depolarization was examined via TMRM staining, and the mitochondrial membrane was indeed depolarized by AB23A (Fig. 2C). The TMRM fluorescence level was decreased by 104.8±3.0% at 10 μM, 99.0±2.1% at 20 μM, 66.9±1.6% ($P<0.01$) at 30 μM, 19.8±2.6% ($P<0.01$) at 40 μM, and 2.6±0.2% ($P<0.01$) at 50 μM for 24 h (Fig. 2D). These results suggest that AB23A inhibits

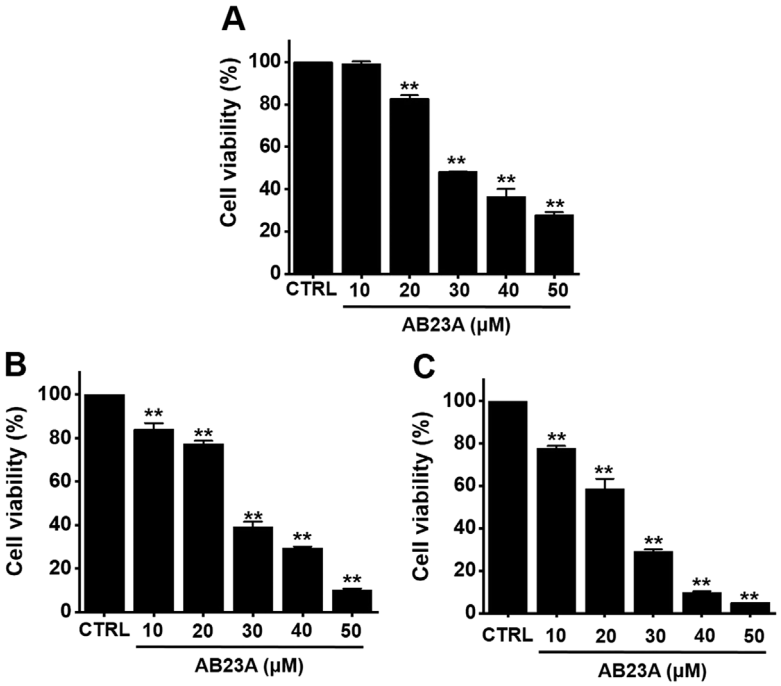

Figure 1. Effects of AB23A on AGS gastric cancer cell viability. AB23A dose-dependently reduced cell viability in AGS cells (observed using the MTT assay) at (A) 24 h, (B) 48 h and (C) 72 h. The results are presented as the means ± SEM. **P<0.01 vs. CTRL. AB23A, alisol B 23-acetate; CTRL, control; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SEM, standard error of the mean.

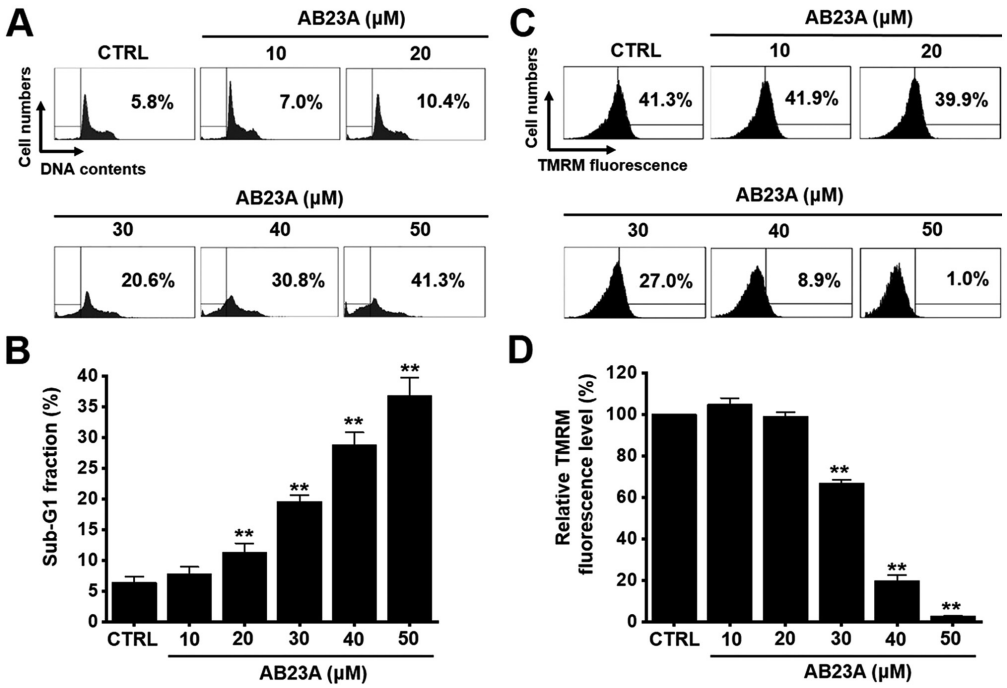

Figure 2. AB23A increased the sub-G1 ratio and mitochondrial damage. (A) Using flow cytometry, cell cycle analysis was performed. (B) The sub-G1 fractions are expressed as percentages. (C) Using FACS analysis, fluorescence for the mitochondrial membrane depolarization assay was conducted. (D) The relative mitochondrial TMRM fluorescence levels were calculated. The results are presented as the means ± SEM. **P<0.01 vs. CTRL. AB23A, alisol B 23-acetate; CTRL, control; FACS, fluorescence-activated cell sorter; SEM, standard error of the mean; TMRM, tetramethylrhodamine methyl ester.

the proliferation of AGS cells and that these effects are related to apoptosis.

Effects of AB23A on the mitochondria-dependent pathway in AGS gastric cancer cells. We investigated whether the Bcl-2 (anti-apoptotic) and the Bax (pro-apoptotic) proteins were involved in the apoptosis induced by AB23A. Using the western blot method, it was observed that the Bcl-2 level was decreased by 105.3±2.5% at 10 μM, 86.2±3.1% (P<0.01) at 30 μM, and 51.3±4.2% (P<0.01) at 50 μM for 24 h (Fig. 3A and B), whereas

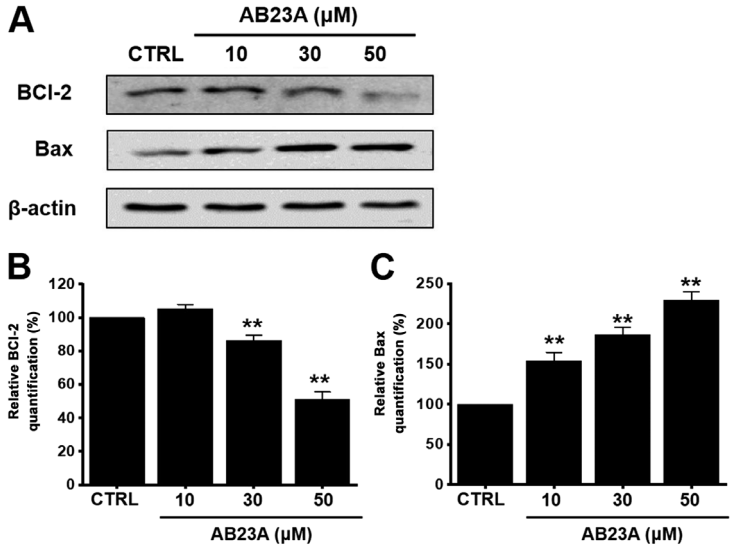

Figure 3. Effects of AB23A on the Bcl-2 and Bax proteins in AGS cells. (A) Bcl-2 expression was downregulated, whereas Bax expression was upregulated (observed using the western blot method). (B) Bcl-2 and (C) Bax protein expression levels were normalized to those of β-actin. The results are presented as the means ± SEM. **P<0.01 vs. CTRL. β-actin was used as the loading control. AB23A, alisol B 23-acetate; Bcl-2, B cell lymphoma 2; CTRL, control; SEM, standard error of the mean.

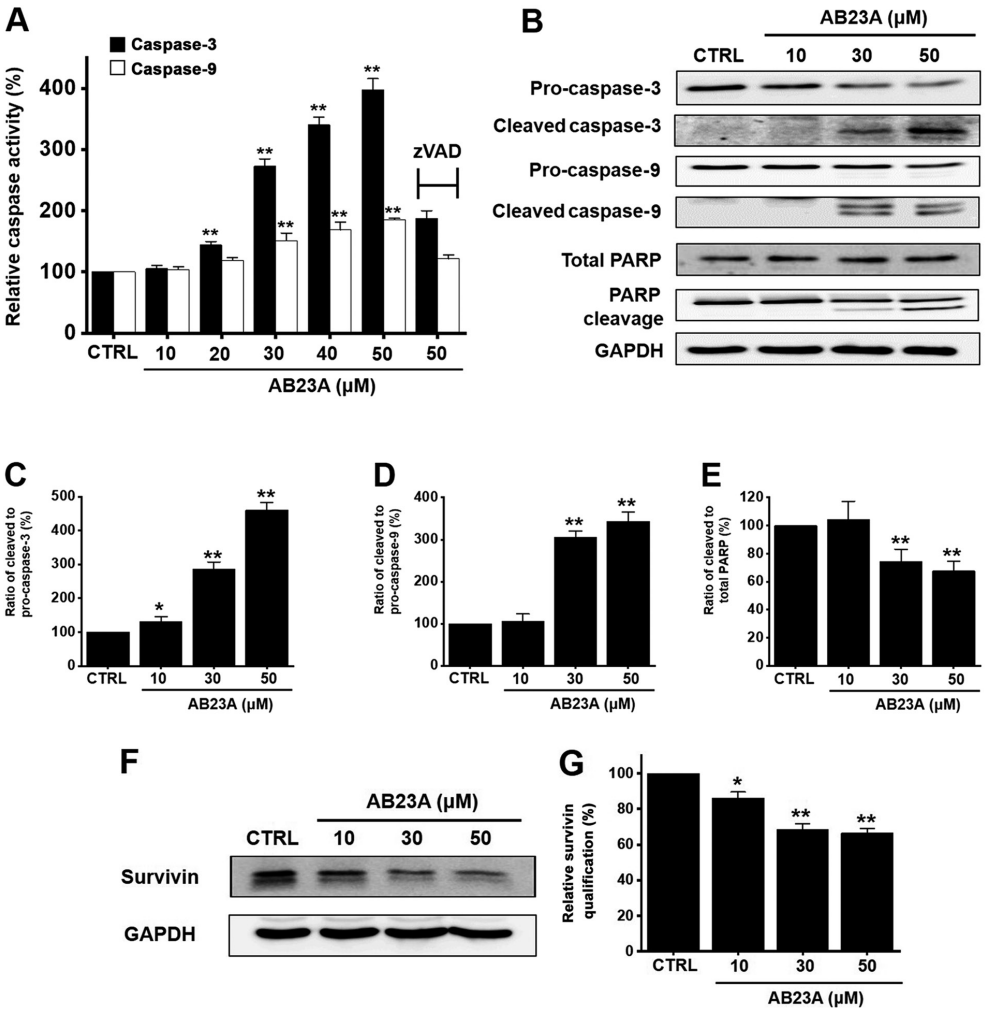

Figure 4. Effects of AB23A on apoptosis regulatory proteins in AGS cells. (A) AB23A increased the activities of caspase-3 and -9. (B) Various apoptosis regulatory proteins were examined following AB23A treatment. Membranes were probed with the indicated antibodies. (C) The ratio of cleaved caspase-3 to pro-caspase-3 was calculated. (D) The ratio of cleaved caspase-9 to pro-caspase-9 was calculated. (E) The ratio of cleaved PARP to total PARP was calculated. (F) Survivin expression was downregulated by AB23A. (G) The survivin protein expression level was normalized to that of GAPDH. The results are presented as the means ± SEM. *P<0.05 and **P<0.01 vs. CTRL. GAPDH was used as the loading control. AB23A, alisol B 23-acetate; CTRL, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean.

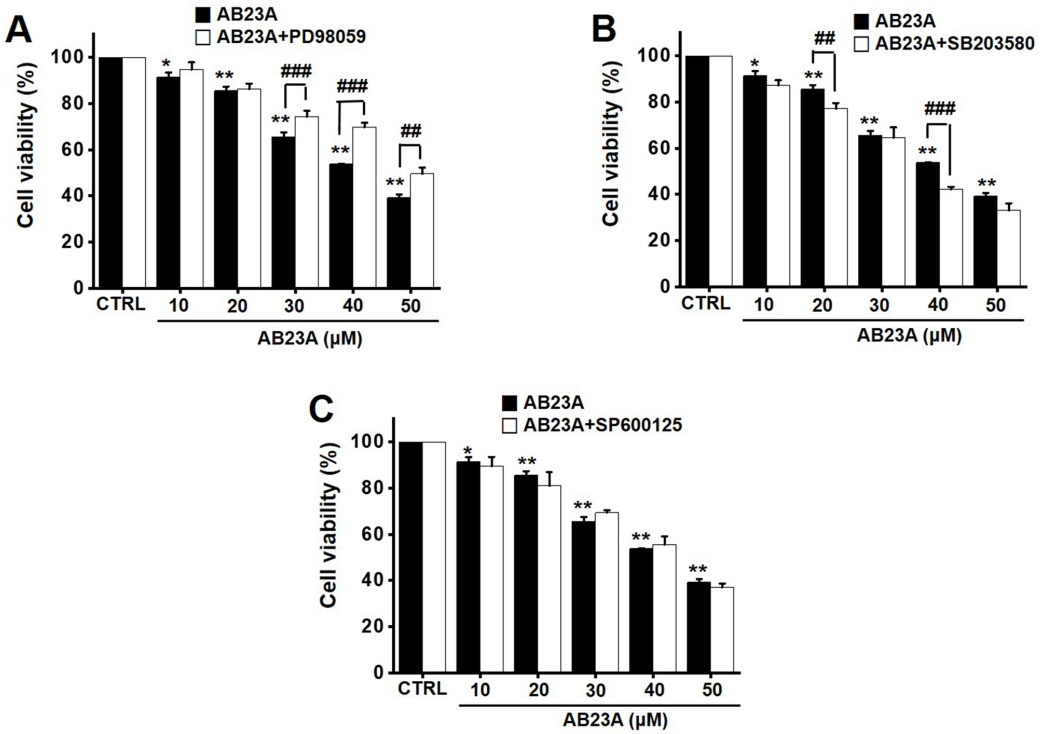

Figure 5. Effects of AB23A on MAPK pathway inhibitors in AGS cells. Cell viabilities were determined after co-treating the cells with AB23A plus (A) PD98059, (B) SB203580, or (C) SP600125 at 24 h. The change after 24 h with 100% of control (0 h) was organized. The results are presented as the means ± SEM. *P<0.05, **P<0.01 vs. CTRL.; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, as indicated. AB23A, alisol B 23-acetate; CTRL, control; MAPK, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; SEM, standard error of the mean.

the Bax level was increased by 154.1±10.2% (P<0.01) at 10 μM, 186.3±9.3% (P<0.01) at 30 μM, and 229.5±10.1% (P<0.01) at 50 μM for 24 h (Fig. 3A and C). These results suggest that the AB23A-induced apoptosis in AGS cells is related to the mitochondria-dependent pathway.

Effects of AB23A on the caspase pathway in AGS gastric cancer cells. Apoptosis typically takes place via the extrinsic or intrinsic apoptotic pathway (24). Caspases represent some of the important genes that regulate apoptosis to maintain homeostasis via the intrinsic and extrinsic apoptotic pathways (25). AB23A increased caspase-3 activation by 105.7±4.9% at 10 μM, 144.3±5.3% (P<0.01) at 20 μM, 272.5±12.1% (P<0.01) at 30 μM, 340.1±12.9% (P<0.01) at 40 μM, and 397.1±18.7% (P<0.01) at 50 μM for 24 h (Fig. 4A) as well as caspase-9 activation by 104.2±4.6% at 10 μM, 119.2±4.4% at 20 μM, 151.2±12.1% (P<0.01) at 30 μM, 168.9±12.6% (P<0.01) at 40 μM, and 185.0±2.8% (P<0.01) at 50 μM for 24 h (Fig. 4A). In addition, Z-VAD-FMK inhibited this activation by 187.4±12.7% for caspase-3 and 121.7±6.3% for caspase-9 at 50 μM for 24 h (Fig. 4A). Using the western blot method, it was observed that the expression levels of pro-caspase-3 and -9 were reduced by AB23A, and the expression levels of the active forms were increased. PARP cleavage levels were also increased for 24 h (Fig. 4B). The ratio of cleaved caspase-3 to pro-caspase-3 was increased for 24 h (Fig. 4C) and the ratio of cleaved caspase-9 to pro-caspase-9 was also increased for 24 h (Fig. 4D). However, the ratio of cleaved PARP to total PARP was decreased for 24 h (Fig. 4E). In addition, survivin, an inhibitor of the apoptosis protein, was decreased by 86.1±3.5% (P<0.05) at 10 μM, 68.3±3.4% (P<0.01) at 30 μM, and 66.4±2.6% (P<0.01) at 50 μM for 24 h (Fig. 4F and G). These results suggest that the AB23A-induced apoptosis is related to caspase activation in AGS cells.

Effects of AB23A on the MAPK pathways in AGS gastric cancer cells. To identify the effects of AB23A on the MAPK pathways in AGS cells, PD98059, SB203580, or SP600125 was applied along with AB23A using the MTT assay to investigate the effects on cell viability. Co-treatment with AB23A (10, 20, 30, 40, or 50 μM) and PD98059 reduced cell viability by 94.8±3.1, 86.4±2.3, 74.4±2.5% (P<0.001), 69.8±1.9% (P<0.001) and 49.6±2.7% (P<0.01), respectively, for 24 h (Fig. 5A), and co-treatment with AB23A and SB203580 reduced cell viability by 87.3±2.2, 77.1±2.6% (P<0.01), 64.6±4.4, 42.4±1.0% (P<0.001) and 33.1±3.1%, respectively, for 24 h (Fig. 5B). In addition, co-treatment with AB23A and SP600125 reduced cell viability by 89.7±3.9, 80.9±6.0, 69.4±1.1, 55.6±3.6 and 37.1±1.6%, respectively, for 24 h (Fig. 5C). To find out more about the efficacy of AB23A on the MAPK pathways, we investigated the AB23A-induced phosphorylation of MAPK proteins (ERK, JNK and p38) using the western blot. The phosphorylation of these proteins increased with AB23A treatment for 0.5, 1, 2 or 4 h (Fig. 6A). The ratio of phosphorylated ERK to ERK was increased at 0.5, 1, 2 or 4 h (Fig. 6B-a) and the ratio of phosphorylated JNK to JNK was also increased at 0.5, 1, 2 or 4 h (Fig. 6B-b). In addition, the ratio of phosphorylated p38 to p38 was increased at 0.5, 1, 2 or 4 h (Fig. 6B-c). These results suggest that AB23A induces apoptosis by regulating the MAPK signaling pathways in AGS cells.

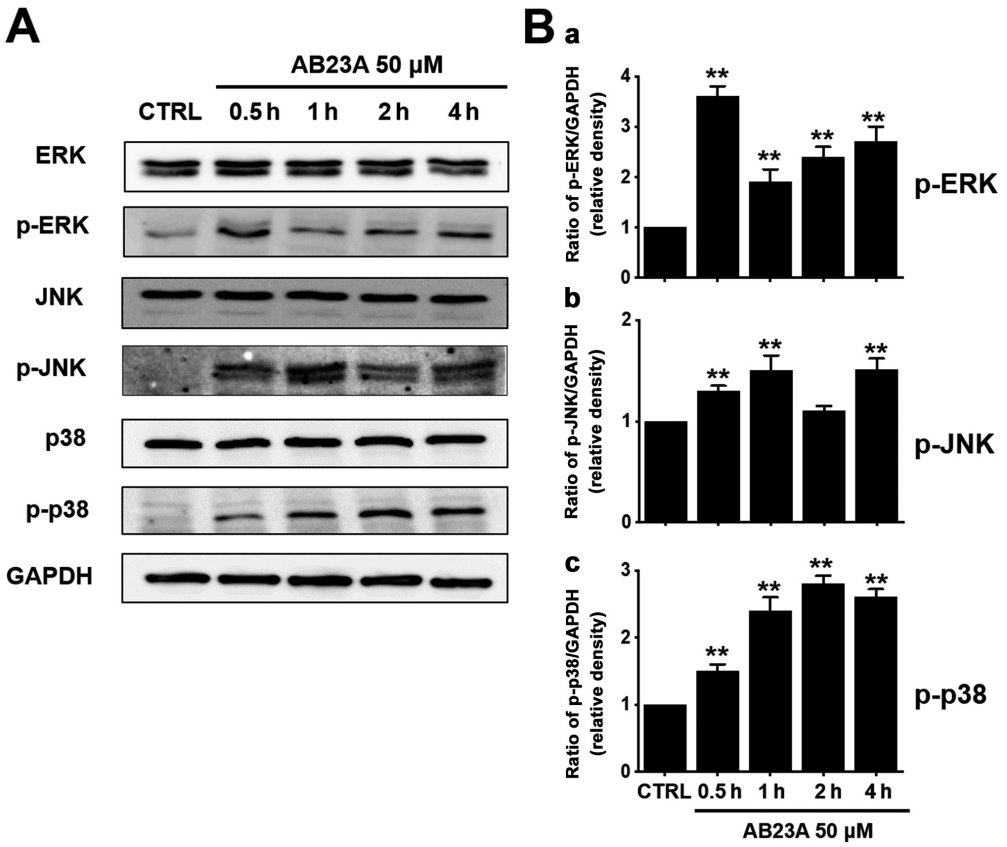

Figure 6. Effects of AB23A on ERK, JNK, and p38 MAPK pathway activation in AGS cells. (A) The phosphorylation of ERK, JNK and p38 was confirmed following AB23A treatment using the western blot method. (B) Phosphorylated levels of these proteins [(a) ERK, (b) JNK and (c) p38] are indicated as band densities relative to that of GAPDH. The results are presented as the means ± SEM. **P<0.01 vs. CTRL. AB23A, alisol B 23-acetate; CTRL, control; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; SEM, standard error of the mean.

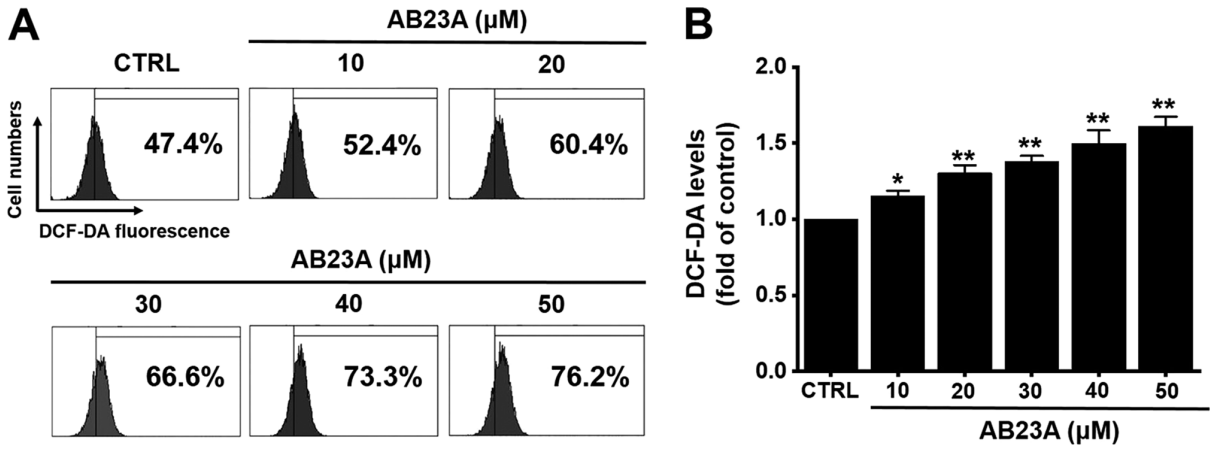

Figure 7. AB23A increased the levels of ROS in AGS cells. (A) Intracellular ROS levels were measured using DCF-DA. (B) ROS levels are expressed as percentages of untreated controls. The results are presented as the means ± SEM. *P<0.05, **P<0.01 vs. CTRL. AB23A, alisol B 23-acetate; CTRL, control; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; SEM, standard error of the mean.

Effects of AB23A on ROS generation in AGS gastric cancer cells. Many reports have suggested that ROS also plays a key role in apoptosis (26). Therefore, we investigated whether DCF-DA levels was increased by AB23A. AB23A increased the DCF-DA levels by the flow cytometry method for 24 h (Fig. 7). These results suggest that AB23A may induce apoptosis via ROS generation in AGS cells.

Discussion

Cancer is one of the major causes of death around the world, and the incidence of cancer is expected to increase further in the future owing to environmental problems and higher life expectancy (27). Research is being conducted on effective anticancer drugs, but since anticancer drugs are equally

applied to normal cells as well as cancer cells, damage to normal tissues, toxicity and side effects are inevitable when these drugs are administered (28). Therefore, efforts are being made to identify and isolate cancer-preventing substances from natural products to develop effective anticancer drugs with minimal side effects.

AB23A, isolated from *A. rhizome* (9), has various pharmacological activities (10-15). It also has anticancer effects on various cancer cells. AB23A causes apoptosis via mitochondria and phosphatidylinositol 3-kinase (PI3K)/Akt mechanisms in SGC7901 gastric cancer cells (16) and SK-HEP-1 hepatocellular carcinoma (22) while blocking the G1 phase in HEY ovarian cancer cells, resulting in a decrease in related proteins and thus inhibiting cell growth (17). It also generates ROS and activates JNK to cause apoptosis in HCT116 human colon cancer cells (18) while promoting apoptosis in human lung cancer cells through intrinsic mechanisms associated with the mitochondria (20) or PIK/AKT/mTOR signaling (19). In addition, AB23A blocks the G1 phase in HepG2 hepatoma cells, again causing apoptosis (21). Furthermore, the present study demonstrates that AB23A induces apoptosis of AGS gastric cancer cells.

Factors belonging to the Bcl-2 family act as important modulating factors in the intrinsic apoptosis pathway associated with mitochondrial dysfunction (29). If the expression of the pro-apoptotic protein Bax increases relative to that of the anti-apoptotic protein Bcl-2, Bax moves to the mitochondria, inducing the loss of matrix metalloproteinase and the movement of cytochrome *c* to the cytoplasm, thus activating the intrinsic apoptosis pathway (30). In addition, the inhibitor of apoptosis protein (IAP) family is known to suppress caspase activity, thus inhibiting the induction of apoptosis (31). In the present study, Bcl-2 decreased and Bax increased following AB23A treatment (Fig. 3A-C). Among the IAP family proteins treated with AB23A, the expression of survivin reduced significantly (Fig. 4C and D). These observations indicate that mitochondrial damage due to the change in the Bcl-2 family protein expression is involved in the AB23A-induced apoptosis, which suggests that the intrinsic apoptosis pathway is activated rather than the extrinsic pathway.

Apoptosis is induced via the extrinsic and intrinsic pathways in which caspase activation plays a key role (24). The extrinsic pathway is initiated via the activation of caspase-8 by the death receptor present in the cell membrane, through the activity of caspase-3 and -7 (25). In contrast, in the intrinsic pathway, the cytochrome *c* protein of the mitochondria promotes caspase-9, -3, and -7 activity, causing apoptosis (25). According to the results shown in Fig. 4A, the activity of caspase-3 and -9 increased with increased concentrations of AB23A, and the activity expression of caspase-9 and -3 decreased with decreased concentrations of AB23A (Fig. 4B). Therefore, it can be seen that the activation of the intrinsic pathway is involved in the AB23A induced apoptosis.

Ion channels are engaged in the mechanism of killing cancer cells (32). Various ion channels such as TRPM7, TRPM2, and TRPC6 are involved in the killing of gastric cancer cells (33-35). The TRPM7 ion channel is involved in AGS cell survival (34), and the TRPM2 ion channel is involved in gastric cancer cell penetration (35). Furthermore, the TRPC6 ion channel is involved in gastric cancer formation (33). However, no studies have assessed the effects of AB23A on these ion channels. Therefore, in the future, it is necessary to study whether ion channels are related with the apoptosis of AGS gastric cancer cells induced by AB23A.

MAPKs mediate intracellular signal transmission in response to external stimuli (36). MAPKs have previously been known to be involved in various physiological mechanisms such as cell growth and differentiation (36). MAPK activation also involves apoptosis via three main mechanisms: ERK, JNK and p38 kinase (37). In the present study, we found that AB23A co-treatment with PD98059 increases cell viability (Fig. 5) and that AB23A activates the ERK, p38 and JNK pathways (Fig. 6). Thus, it appears that the activation of the MAPK mechanism by AB23A plays a role in preventing the growth of AGS cells.

Apoptosis can be caused by various stimuli, including ROS, reactive nitrogen species and hormones (38). In the present study, we found that AB23A increased DCF-DA levels (Fig. 7).

Therefore, ROS generation may be also involved in the apoptosis induced by AB23A. ROS accumulation can cause chronic cell damage and has been associated with apoptosis in cancer cells (38). High levels of ROS can also be generated abruptly as part of the immune response to pathogens and several enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase are involved in ROS detoxification (39). AB-induced ROS generation can be clearly determined by administering the catalase or antioxidant with AB23A and checking that apoptosis is suppressed.

In conclusion, AB23A inhibits AGS cell proliferation. It increased the sub-G1 proportion and depolarized the mitochondrial membrane. In addition, the AB23A-induced apoptosis was related to the downregulation of Bcl-2 and survivin as well as the upregulation of Bax. It activated caspase-3 and -9 and the MAPK cascades. AB23A also increased ROS generation. Thus, it is hoped that various natural products like AB23A will be developed into novel treatments for gastric cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BJK and JHN designed the research. MJK and JNK conducted the experiments. MJK, JNK, MJL, WKK, JHN and BJK analyzed the data. BJK and JHN wrote the manuscript. BJK

and JHN confirm the authenticity of all the raw data. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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