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Cimiside E Arrests Cell Cycle and Induces Cell Apoptosis in Gastric Cancer Cells

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Cimiside E was isolated from the *Cimicifuga heracleifolia* Komarov extract, which has been previously demonstrated to possess apoptotic action on gastric cancer cells. The IC_{50} value of cimiside E on gastric cancer cells for 24 h was 14.58 μM. The mechanism of apoptosis was further elucidated through western blot, RT-PCR, morphology, Annexin V-FITC/PI staining and cell cycle analysis. Cell cycle arrest was induced by cimiside E in S phase at a lower concentration (30 μM) and G2/M phase at higher concentrations (60 and 90 μM). Cimiside E mediated apoptosis through the induction of the caspase cascade for both the extrinsic and intrinsic pathways. These findings suggest that cimiside E may be an effective chemopreventive agent against cancer.

Key words: *Cimicifuga heracleifolia* Komarov, Cimiside E, Apoptosis, Gastric cancer cells

Selected by Editors

INTRODUCTION

Apoptosis is known to participate in various biological processes and is the basis for the meticulous elimination of potentially harmful cells, such as autoreactive immune cells that attack the body's own cells, or neurons that have failed to properly connect (Jacks and Weinberg, 2002). Apoptosis also defends against infection and virus-infected cells. In contrast to necrosis, apoptosis is a programmed cell death. The apoptotic program can be triggered by ionizing radiation (Belka et al., 2000), chemotherapeutic anticancer drugs (Engels et al., 2000) and by natural product-derived supplements (Wieder et al., 2001; Khan et al., 2007). The morphological characteristics include cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation. Failures in apoptotic pathways can lead to uncontrolled cancerous growth. Thus, the proteins preventing the death of cancer cells are common drug targets. One important pathway in the apoptotic mechanism is the Fas-Fas ligand-mediated pathway. The Fas ligand is a cell-surface protein expressed by activated natural killer cells and cytotoxic T lymphocytes; it acts through Fas, which is an important cell-surface death receptor. The Fas ligand has a single transmembrane domain and is activated when ligand binding brings three receptor molecules into close proximity. The trimeric receptor complex attracts a protein called the Fas-associated death domain (FADD), which is an adapter that recruits and activates caspase-8. Cleavage of procaspase-8 induces the cleavage and activation of execution caspase-3, leading to apoptosis of mitochondrial-independent cells (Peter and Krammer, 2003). Another important pathway is related to receive the death signals including the proapoptotic proteins (Bax and Bid) in the cytoplasm that bind to the outer membrane of the mitochondria to signal the release of cytochrome C with the assistance of Bak. Following its release, cytochrome C forms a complex in the cytoplasm with adenosine triphosphate (ATP) and Apaf-1. The complex

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activates caspase-9, which in turn activates caspase-3. The caspase-3 activation leads to apoptosis in a mitochondrial-dependent pathway (Prokop et al., 2000).

Traditional herbal medicines have been used for centuries, some of which have proven to be clinically effective in the treatment of many kinds of cancers. The mechanisms of action of extracts or compounds from herbal medicines have been elucidated in different ways. After many years of research, certain compounds which have anticancer effects have been identified, such as, saponins (Luo et al., 2008), polysaccharides (Ibrahim et al., 2008), terpenoids (Jayaprakasha et al., 2008), alkaloids (Magedov et al., 2008) and flavonoids (Topcu et al., 2008). These kinds of compounds have cytotoxic effects on cancer cells, inhibit DNA synthesis, arrest cell cycle, modulate apoptotic and anti-apoptotic genes, or inhibit invasion and metastasis. Recently, black cohosh, the extract of *C. racemosa,* which is closely related to *C. heracleifolia,* has become increasingly popular as a dietary supplement in the United States for the alleviation of symptoms related to menopause (Radowicki et al., 2006). The extract of *C. heracleifolia* has many uses, such as in apoptosis (Tian et al., 2007), as an anticancer agent (Tian et al., 2006) or an anti-coronavirus (Kim et al., 2008). It also has anti-allergic and estrogen-like activities (Kim et al., 2004). Ferulic acid and isoferulic acid, both from *C. heracleifolia,* were reported as having an anti-inflammatory effect (Sakai et al., 1997; Hirabayashi et al., 1995). To determine which component is responsible for the anti-cancer effect, several compounds isolated from *C. heracleifolia* were studied. Of these compounds, cimiside E showed a highly potential effect on apoptosis in AGS cells. In this study we focused on the apoptotic mechanism of cimiside E in AGS gastric cancer cells through cell cycle arrest.

MATERIALS AND METHODS

Extraction and isolation of compounds

The roots of *C. heracleifolia* were purchased and identified by Professor Je Hyun Lee, Dongkuk University in Kyungjoo, Korea. The dried roots of Cimicifuga Rhizome (10.35 kg) were percolated with methanol (MeOH) three times and concentrated in vacuo. The residue (1.43 kg) was suspended in H_2O and partitioned successively with hexane, CH_2Cl_2, ethylacetate (EtOAc), and butanol (BuOH), yielding hexane (101.09 g), CH_2Cl_2 (232.07 g), EtOAc, (115.93 g) and BuOH (112.24 g) soluble fractions, respectively. A portion of the EtOAc fraction (100 g) was subjected to a silica gel column chromatography eluted with a stepwise gradient of CH_2Cl_2 : MeOH to yield ten fractions (E1~E10) ordered by their polarities. Fraction E7 was chromatographed again on a silica gel column and eluted with gradient mixtures of CH_2Cl_2 and MeOH to give three subfractions (E7.1~E7.3). Then, E7.3 was chromatographed again on an RP-18 column eluted with a gradient mixture of MeOH and H_2O to give two subfractions (E7.3.1~E7.3.2). E.7.3.2 was recrystallized with MeOH, resulting in pure cimiside E. The structure of cimiside E was verified by the comparison of NMR data with those in the literature (Fig. 1) (Li et al., 1994). The purity of the compound was assessed 98% based on the HPLC analysis. The HPLC analysis of cimiside E was accomplished on a Waters 2695 instrument (Milford, MA, USA) equipped with a refractive index detector (Waters 2414). An Alltech-Grom C_{18} column (150 × 4.6 mm, 5 μm particle size, Rottenburg-Hailfingen, Germany) was used at 30°C. Isocratic elution was carried out using acetonitrile : water (80:20) at a flow rate of 0.8 mL/min.

Cells, reagents and antibodies

AGS cells (human gastric adenocarcinoma cells) were obtained from the American Type Culture Collection. RPMI 1640 medium, Dulbecco's phosphate buffer saline (D-PBS) and dimethyl sulfoxide were acquired from Sigma Chemical Co. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories. The nitrocellulose membrane (NC membrane) was obtained from Whatman GmbH. An enhanced chemiluminescence (ECL) detection kit was purchased from LabFrontier. Other chemicals and solvents were obtained from Aldrich Chemical Co. The primary antibodies for procaspase 3, Bax, Bcl-2, and β-actin and the secondary antibody were acquired from Santa Cruz Biotechnology (Santa Cruz). All of the samples, solutions and buffers were prepared in deionized water.

Cell culture and cell viability assay

AGS cells were incubated in RPMI 1640 medium at

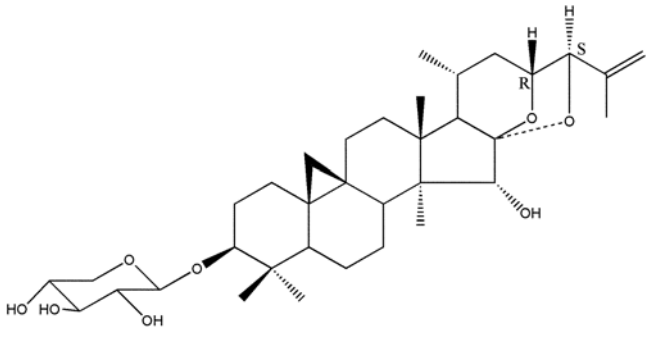

Fig. 1. Chemical structure of cimiside E

37°C under 5% CO_2 in humidified air. The cells were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to adhere for 24 h, also at 37°C under 5% CO_2. After incubation, cells were treated with compounds for 12, 24, and 48 h, and then 10 μL of CCK-8 solution was added to each well, followed by incubation for 2 h at 37°C. The resulting color was assayed at 450 nm using an Emax microplate reader (Molecular Devices).

Morphology observation

AGS cells (1×10^5 cells/well) were seeded in 6-well plates. After 24 h incubation, cells were treated with cimiside E (30, 60, and 90 μM) for 24 h. Then cells were washed with D-PBS, fixed in 70% ethanol for 15 min, washed with D-PBS and stained with DAPI (1 μg/mL) for 15 min. The cells were then washed with D-PBS again. Cells were viewed under an inverted Leica fluorescence 40 × 10 microscope.

DNA fragmentation analysis

AGS cells (1×10^6 cells/well) were treated with cimiside E (30, 60, and 90 μM) for 6, 12 and 24 h. Adherent and floating cells were collected and washed with cold D-PBS. The cells were lysed with Triton X-100 lysis buffer (40 mL of 0.5 M EDTA; 5 mL of 1 M TrisCl buffer; pH 8.0; 5 mL of 100% Triton X-100; 50 mL of H_2O), and incubated for 20 min on ice before being centrifuged. The supernatant was transferred into a new 1.5 mL Eppendorf tube and then extracted with a 1:1 mixture of phenol:chloroform (gentle agitation for 5 min followed by centrifugation) and precipitated in two equivalences of cold ethanol and one-tenth equivalence of sodium acetate. The precipitates were centrifuged, decanted and resuspended in 30 μL of deionized water-RNase solution (0.4 mL of water + 5 μL of RNase) and 5 μL of loading buffer for 30 min at 37°C. All of the DNA was loaded on a 1.5% agarose gel containing ethidium bromide. After electrophoresis, the gel was visualized under a ultraviolet transilluminator.

Cell cycle analysis

The effect of cimiside E on AGS cell cycle phase distribution was assessed using flow cytometry. Briefly, cells (1×10^6 cells/well) were seeded in 6-well plates and incubated for 24 h. After treatment with cimiside E (30, 60 and 90 μM) for 3, 6, 24 h, cells were collected and washed with D-PBS. Then cells were centrifuged and the pellet was resuspended in 50 μL cold PBS, followed by an addition of 1 mL of D-PBS at room temperature. The full volume of resuspended cells was transferred to 4 mL of absolute ethanol at -20°C by pipetting the suspension slowly into the ethanol while vortexing at top speed. The cells were restored in ethanol at -20°C overnight. The cells were then collected by centrifugation. The pellet was washed with cold D-PBS, suspended in 500 μL PBS and incubated with 5 μL RNAase (20 μg/mL) at room temperature for 30 min. The cells were stained with PI (50 μg/mL) for 15 min and then analyzed by flow cytometry.

Annexin V/PI analysis

The Annexin V-FITC/PI apoptosis detection kit (BD Bioscience) was used to detect the effects of cimiside E. AGS cells (1×10^6 cells/well) were seeded in 6-well plates and incubated for 24 h. After treatment of cells with cimiside E (30, 60 and 90 μM) for 24 h, adherent and floating cells were collected and washed with D-PBS, then cells were centrifuged. The pellet was suspended with 1 × binding buffer (100 μL). The cells were stained with Annexin V (5 μL) and PI (5 μL), and incubated for 15 min at room temperature in the dark. After incubation, cells received 400 μL 1 × binding buffer and then were analyzed with FACS Vantage SE (Becton Dickinson) using CellQuest Software, which can determine the percentage of apoptotic cells. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm.

Western blot analysis

AGS cells (1×10^6 cells/well) were treated with compound for 3, 6, 12, and 24 h. After incubation, total cytoplasmic extracts were lysed as described previously (Zhou et al., 2007) and SDS-PAGE was performed. Proteins were transferred onto NC membranes. After blocking with 5% skim milk, the membranes were incubated with primary and secondary antibodies, in series. Finally, the blot was developed using WEST-SAVE Up luminal-based ECL reagent (LabFrontier). The intensity of each band was quantitatively determined using UN-SCAN-IT™ software (Silk Scientific), and the density ratio showed the relative intensity of each band compared to the controls in each experiment.

Reverse transcriptase polymerase chain reaction (RT-PCR)

AGS cells were incubated with compound for 1, 3, and 6 h. Total RNA was extracted using the Easy-BLUE™ Total RNA Extraction Kit (Intron Biotechnology). The RT-PCR was performed using the ONE-STEP RT-PCR PreMix kit™ (Intron Biotechnology) for 20 cycles, with each cycle consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The primers used for β-actin amplification were: sense 5'-AATCTG-

GCACCACACCTTCTACA-3' and antisense 5'-CGACGTAGCACAGCTTCTCCTTA-3', the primers for FasL were: sense 5'-CAACTCAAGGTCCATGCCTC-3' and antisense 5'-AGATTCCTCAAAATTGACCAG-3', the primers for Fas receptor were: sense 5'-GACAAAGCCCATTTTTCTTCC-3' and antisense 5'-ATTTATTGCCACTGTTTCAGG-3'. The RT-PCR products were separated by electrophoresis using a 1.5% agarose gel stained with ethidium bromide, and the gels were viewed under a UV transilluminator.

Statistical analysis

The results were expressed as means ± S.E.M. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) and Student's *t*-test. For the septic shock assay, we used the log-rank test. Statistical significance was assessed as $p < 0.05$ [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$].

RESULTS

Anti-proliferative effect of cimiside E on AGS cells

Cimiside E has a strong cytotoxicity on AGS cells. When cells were treated with cimiside E (30, 60 and 90 μM) for 12, 24 and 48 h, the IC_{50} values were 28.7, 14.6 and 8.1 μM, respectively. The effect of cimiside E on cell viability is shown in Fig. 2.

Apoptotic effect of cimiside E on morphologic changes in AGS cells

AGS cells treated with cimiside E (30 μM) for 24 h showed clearly condensed chromatin, and cells treated with cimiside E (60 and 90 μM) for 24 h showed chromatin condensation and nuclear fragmentation (Fig. 3).

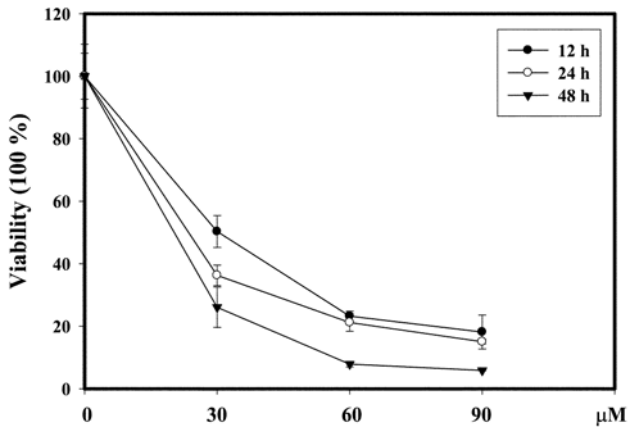

Fig. 2. Cytotoxic effect of cimiside E on AGS cells. Cells were treated with cimiside E (30, 60 and 90 μM) for 12, 24 and 48 h

Apoptotic effect of cimiside E on DNA fragmentation in AGS cells

As shown in Fig. 4, cells treated with cimiside E for 6 h at the concentration of 30 μM showed an unobvious DNA fragment ladder, and at 60 μM showed an obvious DNA ladder. Furthermore, cells treated with

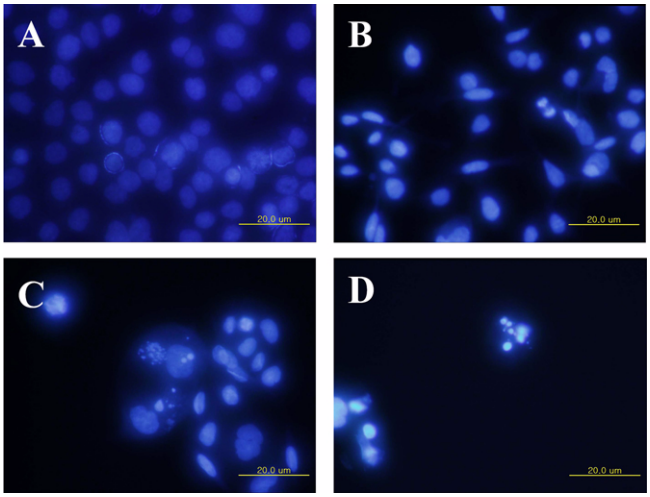

Fig. 3. Cimiside E induced apoptosis in AGS cells, as assessed by fluorescence microscopy. Cells (1×10^5 cells/well) were seeded in 6-well plates. After 24 h incubation, cells were treated with cimiside E (30 and 60 μM) for 24 h. Then cells were washed with D-PBS before fixing with 70% ethanol for 15 min. After the fixed cells were washed with D-PBS, they were stained with DAPI (1 μg/mL) for 15 min. Then cells were washed with D-PBS again. Cells were viewed under an inverted Leica fluorescence 40 × 10 microscope. (**A**) Cells were treated with vehicle for 24 h. (**B**) Cells were treated with cimiside E (30 μM) for 24 h. (**C**) Cells were treated with cimiside E (60 μM) for 24 h. (**D**) Cells were treated with cimiside E (90 μM) for 24 h.

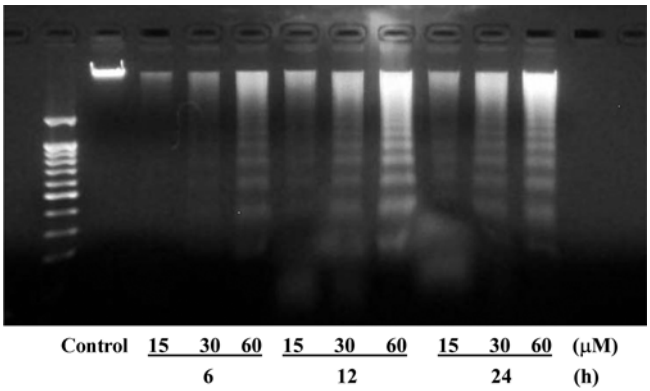

Fig. 4. Cimiside E induced DNA fragmentation of AGS cells. Cells were treated with cimiside E (15, 30 and 60 μM) for 6, 12 and 24 h. To extract DNA, AGS cells were lysed with Triton X-100 lysis buffer. Then total DNA was loaded on a 1.5% agarose gel with ethidium bromide staining. After electrophoresis, the gel was visualized under a ultraviolet transilluminator.

cimiside E over 6 h at each concentration of 15, 30 and 60 μM showed significant DNA ladders, which indicated that many of the cells were dying due to apoptosis.

Effect of cimiside E on AGS cell cycle phase distribution

The cells with DNA content in sub-G1 phase were apoptotic cells. As shown in Fig. 5A, the percentage of sub-G1 cells was 1.87, 3.97, 17.05 and 43.37% at 0, 30,

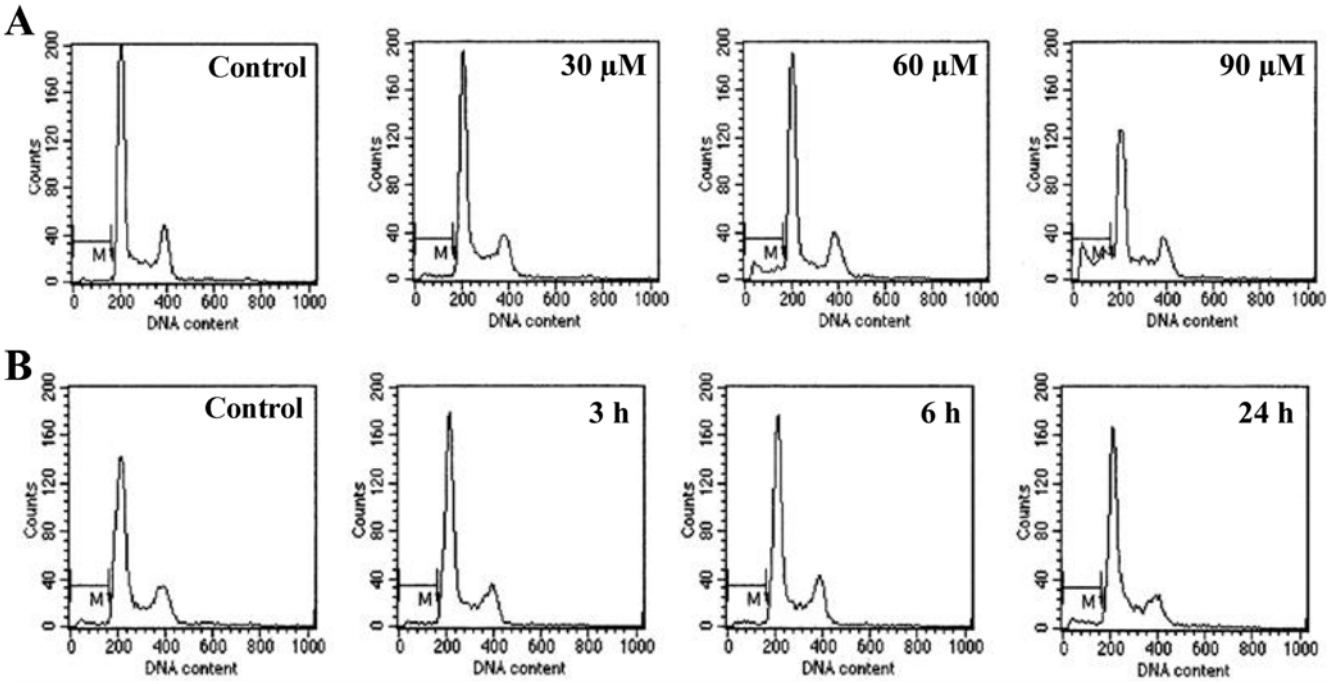

Fig. 5. Effect of cimiside E on AGS cell cycle phase distribution. A: Treatment of cells (1×10^6 cells/well) with cimiside E (30, 60 and 90 μM) for 24 h. B: Treatment of cells with cimiside E (30 μM) for 3, 6 and 24 h. Then cells were collected and resuspended in 50 μL cold PBS, and 1 mL of D-PBS was added at room temperature. The full volume of resuspended cells was transferred to 4 mL of absolute ethanol at -20°C by pipetting the cell suspension slowly into the ethanol while vortexing at top speed. After the cells were restored in ethanol at -20°C overnight, they were collected by centrifugation. The pellet was washed with cold D-PBS, suspended in 500 μL PBS and incubated with 5 μL RNAase (20 μg/mL) at room temperature for 30 min. The cells were stained with PI (50 μg/mL) for 15 min and then analyzed by flow cytometry.

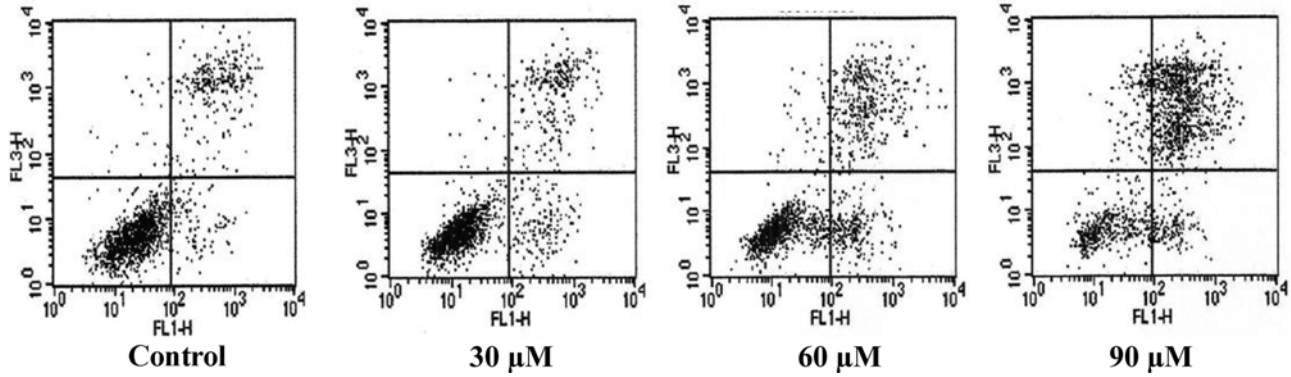

Fig. 6. Annexin V/PI analysis on cimiside E-induced apoptosis in AGS cells. The Annexin V-FITC/PI apoptosis detection kit (BD Bioscience Clontech) was used for detection of the effects of cimiside E. Cells (1×10^6 cells/well) were seeded in 6-well plates and incubated for 24 h. After treatment of cells with cimiside E (30, 60 and 90 μM) for 24 h, adherent and floating cells were collected and washed with D-PBS. After cells were centrifuged, the pellet was suspended by 1×binding buffer (100 μL). Then the cells were stained with annexin V (5 μL) and PI (5 μL), respectively, and incubated for 15 min at room temperature in the dark. After incubation, 400 μL of 1 × binding buffer was added and the cells were analyzed with FACS Vantage SE (Becton Dickinson), using CellQuest Software, which can determine the percentage of apoptotic cells. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm.

60 and 90 μM for 24 h, respectively. The G2/M phase distribution was 14.46, 13.94, 18.50 and 20.31%, at 0, 30, 60 and 90 μM for 24 h, respectively. Cimiside E treatment induced G2/M phase arrest at 60 and 90 μM. However, the cells treated with cimiside E at 30 μM for 24 h had a different result. The data showed that it induced S phase arrest not G2/M phase arrest, and the S phase distribution was 27.48 and 33.00% at 0 μM and 30 μM for 24 h. To further confirm it (Fig. 5B), AGS cells treated with cimiside E at 30 μM for 0, 3, 6 and 24 h were analyzed. The results showed that the S phase distribution was 24.49, 24.72, 26.82 and 35.53%, respectively. Therefore, cimiside E induced S phase arrest at a low concentration (30 μM) and induced G2/M phase arrest at high concentrations (60 and 90 μM) in AGS cells.

Annexin V/PI analysis on apoptotic effect of cimiside E in AGS cells

Annexin V specifically binds to phosphatidylserine and was employed for determination of apoptotic cells. When PI is excluded from viable cells binds to DNA, it can stain the late apoptotic and necrotic cells. After AGS cells were treated by cimiside E (0, 30, 60 and 90 μM) for 24 h, cells were stained with annexin V/PI and examined under a fluorescence microscope. Early and late apoptosis and necrotic cells were distinguished. The corresponding quantities of total cell apoptosis and early apoptosis were 21.26 and 6.10%; 24.10 and 9.26%; 35.68 and 13.10%; 65.34 and 9.50%, respectively. The data demonstrated that apoptotic cells were found to be increased in a dose-dependent manner (Fig. 6).

Effect of cimiside E on the expression of Fas and FasL in AGS cells

RT-PCR analysis indicated that cimiside E (30 and 60 μM) leads to the active expression of FasL at 3 h and Fas from 1 h (Fig. 7).

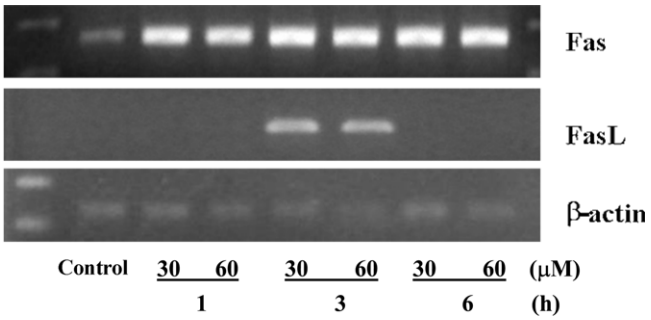

Fig. 7. Effects of cimiside E on Fas and FasL expression in AGS cells. Cells (1×10^6 cells/well) were treated with cimiside E (30 and 60 μM) for 1, 3 and 6 h. The equal loading was confirmed by stripping the immunoblots and reprobing them for β-actin.

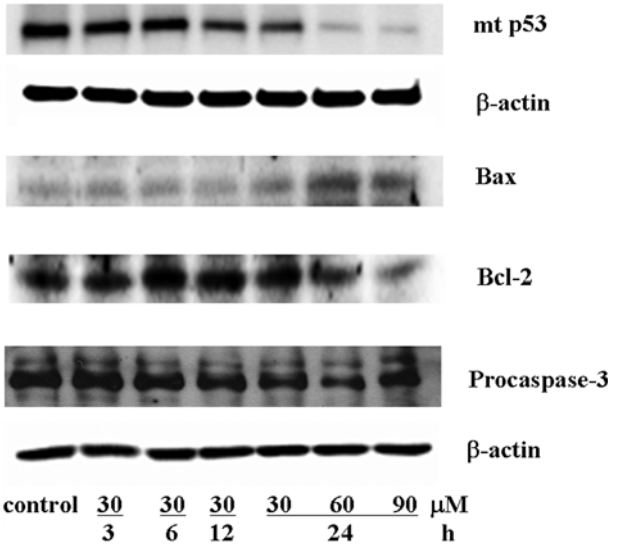

Fig. 8. Effects of cimiside E on mt p53, procaspase 3, Bax and Bcl-2 protein expression in AGS cells. Cells (1×10^6 cells/well) were treated with cimiside E (30, 60 and 90 μM) for 3, 6, 12 and 24 h. The equal loading was confirmed by stripping the immunoblots and reprobing them for β-actin.

Effect of cimiside E on the expression of p53, Bax, Bcl-2 and procaspase-3 in AGS cells

Western blot analysis determined that the ratio of Bax/Bcl-2 expression is increased from 60 μM. The expression of mutant type (mt) p53 decreased from 12 h at 30 μM. The expression of procaspase 3 significantly decreased in a dose-dependent manner from when treated more than 30 μM (Fig. 8).

DISCUSSION

Cell proliferation is governed by a cell cycle, which is a target of many anti-cancer agents. Based on the result of viability assay, cimiside E has a strong cytotoxicity in AGS cells. When cells were treated with cimiside E (30, 60 and 90 μM) for 12, 24 and 48 h, the cell viability curve was like a log scale with dose at each time point, in accordance with characteristics of cell cycle specific chemotherapeutic drugs (Patrick, 2005). The data from cell cycle analysis showed that AGS cells were arrested in S phase by cimiside E at a lower concentration (30 μM) and G2/M phase at higher concentrations (60 and 90 μM). Therefore, cimiside E is considered to be a cell cycle specific inhibitor. From the western blot analysis, the expression of mutant type p53 significantly decreased from 12 h at 30 μM, which can slow down the proliferation of AGS cells (Duan et al., 2008). In addition, the ratio of Bax/Bcl-2 increased significantly at 24 h after treatment with 60

and 90 μM cimiside E. Therefore, cimiside E has an apoptotic effect on AGS cancer cells through mitochondrial-dependent and mitochondrial-independent pathways of cell cycle arrest. We propose the mechanism in which cimiside E inhibits AGS proliferation by cell cycle arrest in S phase and induces apoptosis through activation of the caspase cascade in both extrinsic and intrinsic pathways, including upregulation of Fas and FasL expression and increasing the ratio of Bax/Bcl-2 in AGS cells. Apoptosis and cell cycle arrest could be attributed to its anti-proliferative effects. These results suggest that cimicide E might be a promising anti-cancer agent.

In conclusion, clarification of the mechanisms of cimiside E on AGS cells and capturing the time-dependency of dose-response curves are helpful for potential clinical extrapolation and it is thought to decrease the damage to normal cells in treating less than 15 μM for inhibiting cancer cell proliferation.

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