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Cytotoxic and apoptotic effects of prenylflavonoid artonin B in human acute lymphoblastic leukemia cells[1]

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Key words

artonin B; apoptosis; Bcl-2 family; caspase 3; CCRF-CEM leukemia cell

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Abstract

Aim: To investigate the anticancer effects and molecular mechanism of artonin B on the human acute lymphoblastic leukemia CCRF-CEM cells compared with other prenylflavonoid compounds. **Methods**: The effects of four prenylflavonoids on the growth of CCRF-CEM and HaCa cells were studied by 3-(4,5)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis were detected through Hoechst 33258 staining. The effect of artonin B on the cell cycle of CCRF-CEM cells were studied by propidium iodide method. The change in mitochondrial membrane potential was detected by rohdamine 123 staining. The cytochrome c release and caspase 3 activity were checked by immunoassay kits, respectively. The expression of Bcl-2 family proteins was detected by Western blot. **Results:** Our data revealed that artonin B strongly induced human CCRF-CEM leukemia cell death in a dose- and time-dependent manner by MTT assay, but not on normal epithelia cells (HaCa cells). Artonin B-induced cell death was considered to be apoptotic by observing the typical apoptotic morphological change by Hoechst 33258 staining. The induction of human CCRF-CEM leukemia cancer cell death was caused by an induction of apoptosis through mitochondrial membrane potential change, cytochrome c release, sub-G1 proportion increase, downregulation of Bcl-2 expression, upregulation of Bax and Bak expression and activation of caspase 3 pathways. **Conclusion:** These results clearly demonstrated that artonin B is able to inhibit proliferation by induction of hypoploid cells and cell apoptosis. Moreover, the anticancer effects of artonin B were related to mitochondrial pathway and caspase 3 activation in human CCRF-CEM leukemia cells.

Introduction

Natural products from plants or Chinese herbs have been used as traditional remedies in Asian countries for hundreds of years. The development of compounds with antitumor effects from natural products has become a very important topic. Flavonoids are commonly found in most plants and exert a remarkable spectrum of biological activities that affect basic cell functions, and several beneficial biological activities of flavonoids including antioxidant, antitumor, and anti-inflammation properties have been identified in several studies[1,2]. Flavonoids are also dietary pharmacological agents, which may block neoplastic inception or delay disease progression[3,4]. These data indicate that certain flavonoids may be used as possible chemopreventive or chemotherapeutic agents.

The great prevalence of flavonoids in the vegetal kingdom act, not only as the colored pigments of flowers, but also as enzyme inhibitors, precursors of toxic substance, and a defense against ultraviolet radiation exposure. Flavonoids were found to act on the growth of cancer cells, which means that it possesses potential anti-tumor activity. For example, baicalein, epigallocatechin (EGC) gallate and green tea extract were reported to inhibit tumor growth[5–7]. Furthermore, epidemiological studies indicated that diets containing linseed and soy (rich in isoflavonoids and lignans)

might protect against colon, breast, and prostate cancer[8]. Flavonoids are benso-r-pirone derivatives that can be grouped according to the presence of different substituents on the rings and to the degree of benso-r-pirone ring saturation. Artonin B is a prenylflavonoid that is obtained from the root bark of *Artocarpus heterophyllus* Lamk[9]. Artonin B is a derivative from heterophyllin and possesses the structure in C-10 position of isoprenoid moiety. However, the effects of artonin B on cancer cell growth have rarely been investigated in great detail.

Apoptosis is a cell suicide program, which is essential for the development and maintenance of tissue homeostasis and the elimination of unwanted or damaged cells from multicellular organisms[10,11]. Apoptosis is characterized by a series of morphological changes involving cell shrinkage, chromatin condensation and the formation of apoptotic bodies[12]. It can be triggered by various extracellular and intracellular stimuli that result in the coordinated activation of family proteases called caspases. The activation of caspase 3 pathways is an important downstream executioner in apoptosis[13].

Human leukemia is a commonly diagnosed neoplasm and the major leading cause of human death. CCRF-CEM cells are acute lymphoblastic leukemia (ALL) cells. ALL represents the clonal proliferation of malignantly transformed lymphoid progenitors in the bone marrow. The treatment of patients with recurrent cancer is usually unsuccessful, and the development of new potent treatments has become the focal point for cancer treatment. Therefore, we evaluated the effects and action mechanism of artonin B on human acute lymphoblastic leukemia CCRF-CEM cells. In this present study, the cell cytotoxicity of four prenylflavonoid compounds was examined. Furthermore, morphological nuclear fragmentation, apoptotic body formation, the change of mitochondrial membrane potential, cytochrome c release, cell cycle change, Bcl-2, Bax, and Bak protein expression, and caspase 3 activity in artonin B treated human CCRF-CEM leukemia cells were investigated.

Materials and methods

Materials RPMI-1640, fetal bovine serum (FBS), and antibiotics were purchased from Hyclone. Caspase 3 assays kits and caspase 3 inhibitor (z-DEVD-fmk) were obtained from Biovision. The cytochrome c was purchased from R&D Systems. Monoclonal antibodies of Bcl-2, Bax, Bak, and anti-rabbit IgGs were obtained from Cell Signaling. Hoechst 33258 and the rest of chemicals were purchased from Sigma.

The four prenylflavonoid compounds that were extracted from *Artocarpus* species (Moraceae) were obtained from Dr Chun-nan LIN (School of Pharmacy, Kaohsiung Medical University, Taiwan, China). Artocarpanone (Figure 1A) was an isoprenoid-flavone[14]. Artonin A and artonin B (Figure 1B and 1C) were extracted from the root bark of *Artocarpus heterophyllus* Lamk (9). And, the new prenylflavonoid-Artocammunols CE (Figure 1D) was obtained from the *Artocarpus communis*[15]. These four prenylflavonoid compounds were dissolved in dimethyl sulfoxide (DMSO) as stock solution. The final concentration of DMSO in each experiment was less than 0.1%.

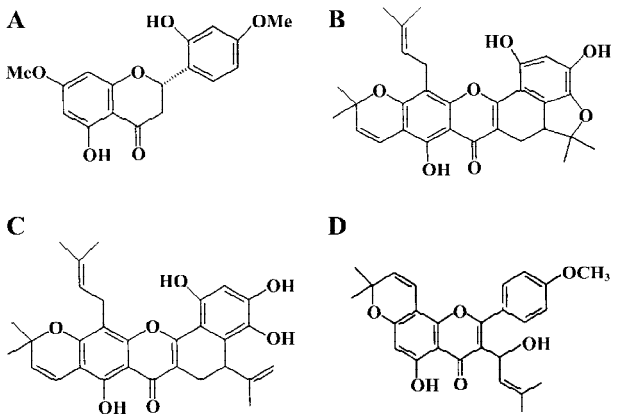

Figure 1. Chemical structures of four prenylflavonoid compounds. A: Artocarpanone; B: Artonin A; C: Artonin B; D: Artocammunols CE.

Cell culture Human acute lymphoblastic leukemia cell line (CCRF-CEM) was purchased from the Culture Collection and Research Center (Taiwan). CCRF-CEM cells were maintained in RPMI-1640 medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 100 μg/mL penicillin, 100 μg/mL streptomycin and 100 μg/mL amphotericin B (Hyclone). The cells were grown in a humidified incubator at 37 ºC under a 5% CO_2/95% air atmosphere. For each experiment, 3×10^5 cells were seeded in each well in a 24-well plate containing 1 mL of fresh medium and incubated with or without chemical treatment for the indicated time. For toxicity study, the cells were treated with drugs during the exponential phase of cell growth.

Cytotoxicity assay The cytotoxic effect of drugs was determined using the MTT method[16]. In brief, 100 μL MTT solution (0.5 mg/mL in phosphate-buffer saline or PBS) was added to each well at the end of each experiment. After 1–2 h incubation at 37 ºC, 10 μL Triton X-100 (10%) was added and mixed well. Once the cells were completely dissolved, the absorbance difference at 550 nm was measured using a

microplate reader, with the RPMI medium as a blank.

Microscopic observation of morphology and nuclear fragmentation After artonin B treatment, cells were harvested by centrifugation, washed with PBS, and fixed with 1% glutaraldehyde in 100 μL of PBS at room temperature for 1 h. Fixed cells were washed with PBS and then stained with 200 μmol/L Hoechst 33258 in 20 μL PBS for 30 min at room temperature. Five hundred stained cells from each treatment group were examined and counted under an Olympus fluorescence microscope.

DNA content and cell cycle analysis Human CCRF-CEM cells were collected and rinsed with PBS, after being cultured with 0, 1, 5, or 10 μmol/L artonin B for 24 h, and suspended in 75% ethanol at -20 ºC overnight. Fixed cells were centrifuged at 1200×*g* and washed with PBS twice. To detect DNA content, cells were contained in the dark with Propidium Iodide (PI) 50 mg/L and 0.1% RNase A in 400 μL PBS at 25 ºC for 30 min. Stained cells were analyzed on FACSort (Becton Dickinson). The percentage of apoptotic cells was determined using the CellQuest software program.

Assay of mitochondrial membrane potential Initially, 1×10^6 human CCRF-CEM cells/mL were incubated with 2 mmol/L rhodamine 123 for 10 min at 37 °C. After the incorporation of a fluorescent probe, the cells were incubated for up to 4 h with or without 10 μmol/L artonin B. At the end of incubation, the cells were washed twice with PBS, harvested by centrifugation, and then resuspended in 1.5 mL PBS. The fluorescent intensity of each cell suspension was measured at an excitation wavelength 480 nm and an emission wavelength 530 nm in a Perkin-Elmer Victor 3 fluorescent microplate reader. The fluorescence intensity was used as an arbitrary unit representing the mitochondrial transmembrane potential.

Cytochrome c release Human CCRF-CEM cells were seeded in 2 mL fresh medium at an initial density of 1×10^6 cells/mL and incubated for up to 4 h with or without 10 μmol/L artonin B. After the incubation, the cells were harvested by centrifugation and washed twice with PBS. The cells were suspended in 200 mL lysis buffer (195 mmol/L mannitol; 65 mmol/L sucrose; 2 mmol/L HEPES, pH 7.4; 0.05 mmol/L EGTA; 0.01 mmol/L $MgCl_2$; 0.5 g/mL BSA) and lysed by the addition of 0.01% digitonin. The cytosolic fraction was obtained from 10 000×*g* centrifugation for 10 min and was collected for cyt c assay in 1×RD5P calibrator diluent (cytochrome c Immunoassay Kit; R&D Systems, MN, USA). After reacting with cyt c antibody and substrate, the absorbance was measured at 450 nm (reference wavelength is 540 nm).

Western blot analysis After being exposed to the indicated concentration of artonin B, human CCRF-CEM cells were washed with cold PBS. Whole cell extracts were prepared by incubating the cells with cold lysis buffer (20 mmol/L Tris-HCl; pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mg/mL leupeptin, and 1 mmol/L PMSF). The protein content of the lysates was determined using the DC protein assay kit (Bio-Rad). The cell lysates (25 μg protein/lane) were electro-phoresized on 12% SDS-polyacrylamide gels. The cellular proteins were then transferred to PVDF membranes by electroblotting for 2 h and Western blot analysis was carried out as previously described[17]. The protein levels were visualized with an enhanced chemiluminescence detection kit (Amersham).

Preparation of cytosolic extract and measurement of caspase 3 activity After the treatment with indicated agents, cells were harvested and washed with PBS by centrifugation at 750× *g* for 5 min at 4 ºC. The cell pellets were resuspended in lysis buffer (caspase colorimetric assay kits; Biovision) and left on ice for 30 min. The lysates were centrifuged at 10 000×*g* for 10 min and the supernatant (20 μL) was used for caspase-3 activity assay in the lysis buffer containing DEVD-pNA, a specific substrate to caspase-3. The concentration of pNA, as the product from enzymatic converting of DEVD-pNA by caspase-3, was measured at 405 nm and used as an indication of caspase-3 activity.

Assessment of cell necrosis The necrotic cell death was measured by the release of lactate dehydrogenase (LDH) into the culture medium, which indicates the loss of membrane integrity and cell necrosis. LDH activity was measured using a commercial assay kit (Cytotoxicity assay kit, Promega), where the released LDH in culture supernatants is measured with a coupled enzymatic assay, which results in the conversion of a tetrazolium salt into a red formazan product. The necrotic percentage was expressed as (sample value/maximal release)×100%, where the maximal release was obtained following the treatment of control cells with 0.5% Triton X-100 for 10 min at room temperature.

Statistic analysis For each experiment involving assessment of cell survival, apoptotic cell, and caspase-3 activity are presented as the mean and standard error (SEM) for four to five experiments. The statistical analysis of data was performed by one-way ANOVA, followed by the Schefft test, and *P*-values less than 0.05 were considered significant.

Results

Effects of prenylflavonoid compounds on cytotoxicity in human CCRF-CEM leukemia cells The cytotoxic effects of four prenylflavonoid compounds were examined in human CCRF-CEM leukemia cells. When CCRF-CEM leukemia cells

were incubated with 10 μmol/L of four prenylflavonoids, the data showed that artonin B had a more potent cytotoxicity than the other compounds (Figure 2). In addition, the cell survival rate decreased in a dose-dependent manner (Figure 3A). Under the same treatment, artonin B did not cause any cell loss in HaCa cells (Figure 3B). Artonin B elicited a significant decrease of cell survival rate in human CCRF-CEM leukemia cells, which also exhibited a time-dependent manner (Figure 4). After an exposure time of 6 h, the cytotoxic effects were noticed at a concentration of 5 μmol/L and 10 μmol/L of artonin B, with a the survival rate decreasing to 64% at 5 μmol/L and 22% at 10 μmol/L. Accordingly, after 24 h exposure cytotoxic effects started to become apparent at a concentration of approximately 3–10 μmol/L, cell viability declined considerably at 3 μmol/L and was approximately 10% at 10 μmol/L of artonin B (Figure 4). The IC_{50} value of artonin B was 3.45±0.50 μmol/L.

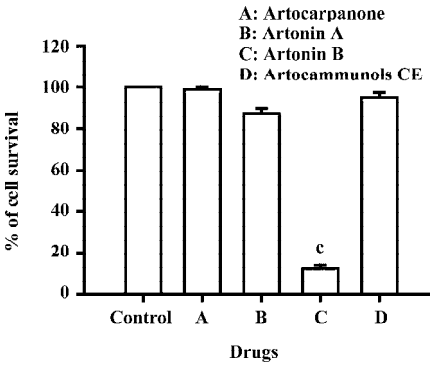

Figure 2. Cytotoxicity of four prenylflavonoids on human CCRF-CEM leukemia cells. CCRF-CEM leukemia cells were incubated with 10 μmol/L of four prenylflavonoids for 24 h. At the end of incubation, the cell survival rate was determined by MTT methods as described in the Materials and Methods. The cytotoxicity is expressed as the percentage of cell survival rate compared with the control. Controls were exposed to the solvent only (0.1%, *v*/*v*). The data are expressed as mean±SEM of 4–5 determinations. $^{c}P<0.01$ *vs* control group.

Assessment of artonin B-induced cell apoptosis and intracellular events To determine whether the artonin B-induced cytotoxicity was to undergo the apoptotic cell pathway, human CCRF-CEM leukemia cells were incubated in presence of 1–10 μmol/L artonin B for 24 h. The morphological examination reveled that artonin B-treated cells showed typical apoptotic morphological changes, such as cell shrinkage, nuclear fragmentation, and apoptotic body formation (Figure 5). Artonin B-treatment significantly increased the number of cells with apoptotic body formation (Figure 5D,5E), while the control cells and 1–3 μmol/L of

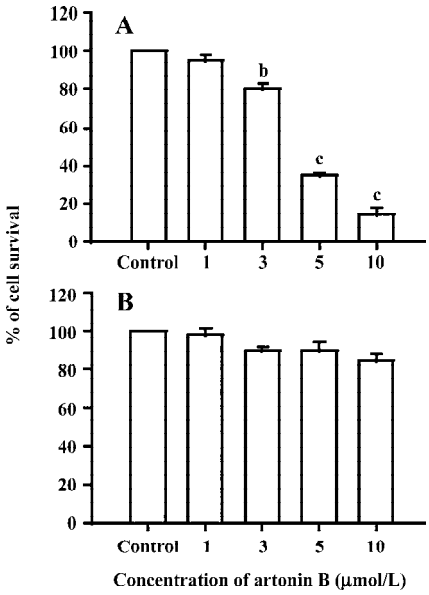

Figure 3. Cytotoxicity of artonin B on human acute lymphoblastic leukemia CCRF-CEM cells. CCRF-CEM leukemia cells were incubated with 0, 1, 3, 5, or 10 μmol/L of artonin B (Panel A) for 24 h. HaCa cells were also incubated with 0, 1, 3, 5, or 10 μmol/L of artonin B (Panel B) for 24 h. At the end of incubation, the cell survival rate was determined by MTT methods as described in the Materials and Methods. The cytotoxicity is expressed as the percentage of cell survival rate compared with the control. Controls were exposed to the solvent only (0.1 %, *v*/*v*). The data are expressed as mean±SEM of 4–5 determinations. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group.

artonin B-treated cells showed seldom apoptotic body formation (Figure 5A–5C). The number of apoptotic cells, which carry fragmented nuclear particles, were significantly increased in artonin B-treated cells in a dose-dependent manner (Figure 5F).

The necrotic indication of cellular lactate dehydrogenase (LDH) release was also examined after artonin B treatment (Figure 6). The data showed that 5 μmol/L and 10 μmol/L artonin B induced an increase of LDH release in only 20.59%±2.12% and 28.23%±0.81%, respectively, while the cell survival rates were 35.33%±0.9% and 14.76%±3.56%, respectively. These data indicated necrotic cytotoxicity was less involved in artonin B action. Thus, artonin B induces leukemia cell death employing an apoptotic pathway.

Cell cycle analysis Figure 7 illustrates the changes of DNA content distribution treated with artonin B 0, 1, 5, or 10 μmol/L for 24 h. We examined these cells for DNA degradation characteristic of apoptosis, indicated by hypoploid DNA content using hypotonic PI staining. Exposure of human CCRF-CEM leukemia cells to 1 μmol/L artonin B promoted approximately the same percentage of hypoploid cells ob-

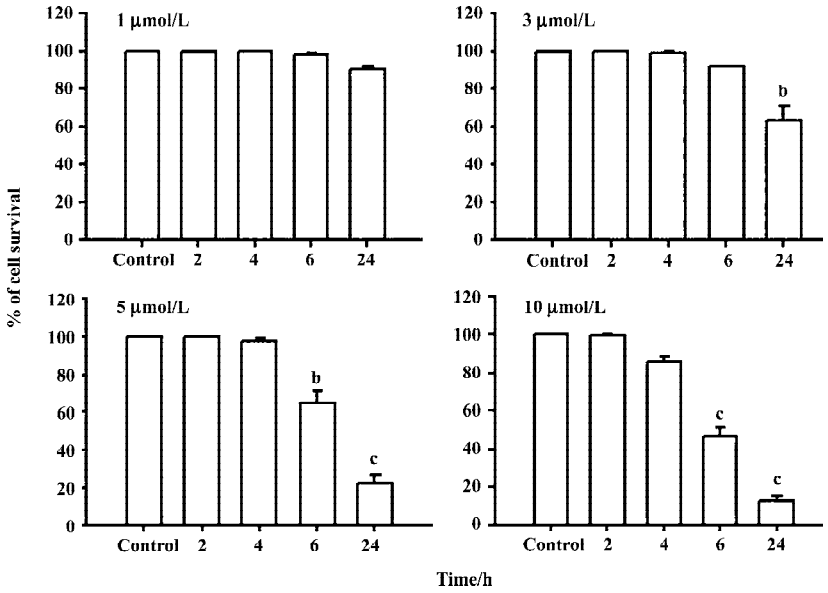

Figure 4. Time course of cytotoxicity of artonin B in human CCRF-CEM leukemia cells. CCRF-CEM leukemia cells were incubated with artonin B (0, 1, 3, 5, or 10 μmol/L) for different period of time. At the end of incubation, the cell survival rate was determined by MTT methods as described in the Materials and Methods. The cytotoxicity is expressed as the percentage of cell survival rate compared with the control. Controls were exposed to the solvent only (0.1%, *v*/*v*). The data are expressed as mean±SEM of 4–5 determinations. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group.

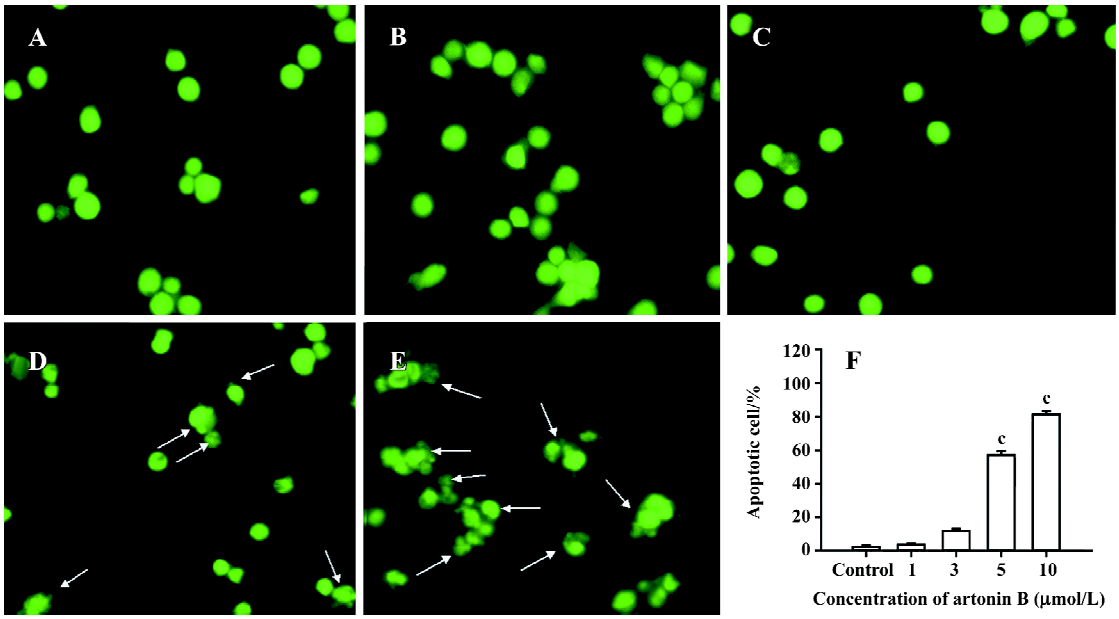

Figure 5. Apoptotic morphology and cell counts of human CCRF-CEM leukemia cells treated with artonin B. The morphology of human CCRF-CEM cells incubated with different concentration of artonin B for 24 h. (A) Control; (B) 1 μmol/L; (C) 3 μmol/L; (D) 5 μmol/L; (E) 10 μmol/L of artonin B. After the incubation, human CCRF-CEM leukemia cells were collected by centrifugation, washed with PBS twice, and stained with Hoechst 33258. The number of cells carrying fragmented nuclear particles (apoptotic bodies pointed by arrows) was counted and is represented in Panel F as the percentage of apoptotic cell counts. The data are expressed as mean±SEM of 4–5 determinations. $^{c}P<0.01$ *vs* control group. Controls were exposed to the solvent only (0.1%, *v*/*v*).

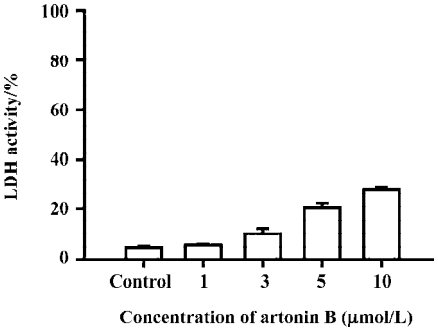

Figure 6. Lactose dehydrogenase (LDH) release in artonin B treated human CCRF-CEM leukemia cells. CCRF-CEM leukemia cells were incubated with 0, 1, 3, 5, or 10 μmol/L of artonin B for 24 h. At the end of incubation, the LDH activity was determined by LDH kits from Promega as described in the Materials and Methods. The data are expressed as mean±SEM of 4–5 determinations. Controls were exposed to the solvent only (0.1%, *v*/*v*).

served in the DMSO treated control. As the treatment dose increased the percentage of cells in the hypoploid (sub-G_1) phase increased accordingly. Treated with 5 or 10 μmol/L artonin B for 24 h, the rate of sub-G_1 phase cells were increased by 11.43% or 18.56%, respectively.

Changes of mitochondrial membrane potential and release of cytochrome c from mitochondria In the present study, the mitochondrial membrane potential and cytochrome c release were analyzed spectrophotometrically. As shown in Figure 8, artonin B-induced a time-dependent mitochondrial transmembrane depolarization, represented as the decrease of mitochondrial membrane potential (Figure 8A). Concomitantly, a time-dependent artonin B-induced cytochrome c release was also observed in human leukemia CCRF-CEM cells, representing a significant increase of cytosolic cytochrome c concentration (Figure 8B). These data suggest that loss of mitochondrial membrane potential may be required for artonin B-induced cytochrome c release into cytosol, that later triggered the cleavage and activation of mitochondrial downstream caspases and onset of apoptosis.

Regulation of Bcl-2 family proteins in artonin B-treated human leukemia CCRF-CEM cells To determine whether Bcl-2 family proteins were modulated in artonin B-induced apoptosis in human leukemia CCRF-CEM cells, the expression of several members of Bcl-2 family proteins was examined by Western blot analysis. As shown in Figure 9, the exposure of human leukemia CCRF-CEM cells to 1–10 μmol/L artonin B resulted in a marked decrease of Bcl-2 protein expression, but a drastic increase of Bax and Bak protein expression.

Determination of the involvement of caspase 3 activation Artonin B-induced nuclear fragmentation may thus be an apoptotic event provoked along with endonuclease activation via caspase 3 protein. We also examined the caspase 3 activity under the 1–10 μmol/L artonin B treatment. The present study has demonstrated that artonin B treatment increased caspase 3 activities in human CCRF-CEM leukemia cells in a dose-dependent manner (Figure 10). Human leukemia CCRF-CEM cells were pretreated with 50 μmol/L caspase 3 inhibitor (z-DEVD-fmk) for 2 h, and then induced to undergo apoptosis by treatment with artonin B. The results clearly showed that the administration of caspase 3 inhibitor alone did not affect the caspase 3 activation, apoptotic cell formation, and cell viability (Figure 11). However, z-DEVD-fmk (a specific caspase 3 inhibitor) significantly inhibited artonin B-induced caspase 3 activation, apoptotic cells formation, and cell death in human acute lymphoblastic leukemia cells.

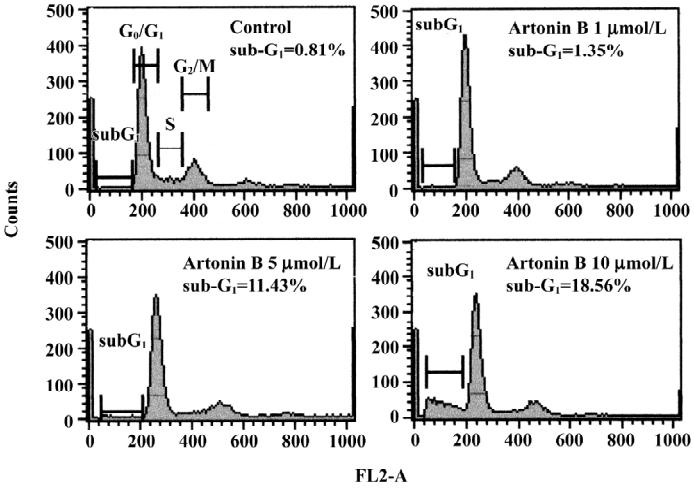

Figure 7. Evaluation of artonin B-induced apoptosis in human leukemia CCRF-CEM cells. Human CCRF-CEM cells were treated with the indicated concentration of artonin B (1, 5, or 10 μmol/L) or an equal volume of the vehicle DMSO (control) and examined after 24 h for DNA degradation (% of hypoploid cells) characteristic of apoptosis using hypotonic PI staining. The hypoploid cells have less cellular DNA content than cell typically found in the G_0/G_1, S or G_2/M phases of the cell cycle, which causes them to retain less PI, a fluorescent DNA stain. The decreased retention of PI by the hypoploid cells shifts their fluorescence intensity leftward on the linear X-axis of the representative histograms.

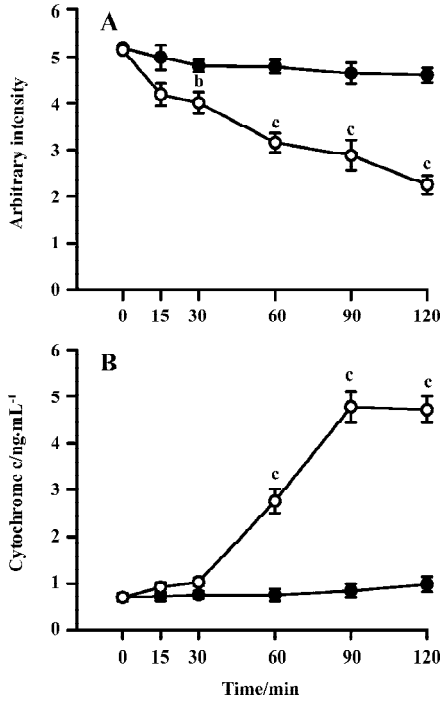

Figure 8. Measurement of mitochondrial membrane potential and cytoplasmic cytochrome c release in human acute lymphoblastic leukemia CCRF-CEM cells during the artonin B treatment. Panel A. Human leukemia CCRF-CEM cells were treated with 10 μmol/L Rhodamine 123 for 10 min prior to the artonin B incubation and then cells were incubated without (●) or with (○) 10 μmol/L artonin B for a different period of time. At the end of incubation, the mitochondrial membrane potential was measured as described in the Materials and Methods. Panel B. Human leukemia CCRF-CEM cells were incubated without (●) or with (○) 10 μmol/L of artonin B for the indicated time. Cytoplasmic cytochrome c release was determined by immunoassay as described in the Materials and methods. The data are expressed as mean±SEM of 4–5 determinations. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group.

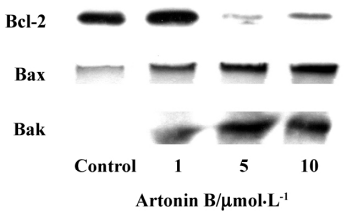

Figure 9. Expression of Bcl-2 family proteins in artonin B-treated human leukemia CCRF-CEM cells. Human leukemia cells were treated with 1–10 μmol/L atonin B for 24 h. After the treatment, cell lysates were extracted, and the levels of Bcl-2 family proteins were analyzed by Western blot analysis.

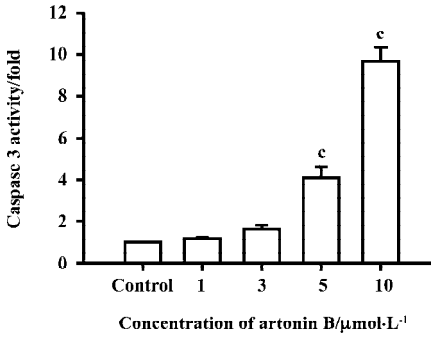

Figure 10. Caspase 3 activation by artonin B in human CCRF-CEM leukemia cells. CCRF-CEM leukemia cells were incubated with 0, 1, 3, 5, or 10 μmol/L of artonin B for 24 h. At the end of incubation, the caspase 3 activity was determined as described in the Materials and Methods. The data are expressed as mean±SEM of 4–5 determinations, $^{c}P<0.01$ *vs* control group. Controls were exposed to the solvent only (0.1%, *v*/*v*).

Discussion

In the present work, we demonstrated that artonin B, one of prenylflavonoids, strongly inhibited the growth of human acute lymphoblastic leukemia CCRF-CEM cells, whereas artonin A, artocarpanone, and artocammunols prenylflavonoid compounds had no effect on the growth of CCRF-CEM cells. This is a pioneer study of artonin B-induced cell cytotoxicity in human leukemia CCRF-CEM cells. Prenylflavonoids exist extensively in plants; however, the structure-activity relationship of their effect is still unknown. These results indicate that heterophyllin structure would be needed in the growth inhibitory effect of prenylflavonoid compounds. Importantly, we observed that artonin B had no effect on the growth of HaCa cells; this is in agreement with data showing that artonin B had a growth inhibitory effect on cancer cells, but not on normal cells. Therefore, artonin B could be a good candidate for acute lymphoblastic leukemia cells therapy without toxicity for normal cells.

Our results revealed that human CCRF-CEM cells treated with artonin B exhibited characteristic morphological features of apoptosis, such as membrane shrinkage chromosomal condensation. The notion that artonin-B treated cells undergo apoptosis rather than necrosis is further supported by the results from cell cycle analysis. The proportion of hypoploid cells (sub-G_1) was dramatically increased after artonin-B treatment. These results support the finding that artonin-B induces cell death through apoptotic pathway.

The several mechanisms of activation of apoptosis in different physiological or pathological conditions in cells

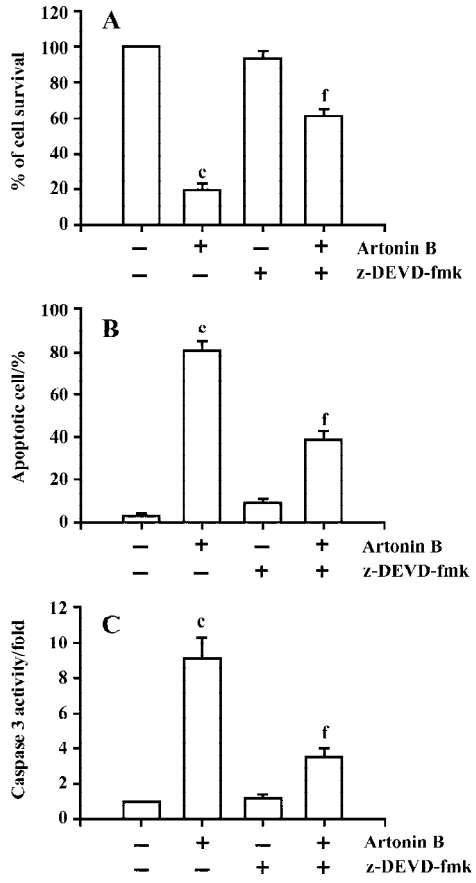

Figure 11. Inhibition of caspase 3 activity and attenuation of artonin B-induced apoptotic cells and cell death by caspase 3 inhibitor (z-DEVD-fmk). Human CCRF-CEM leukemia cells were treated with 50 μmol/L specific caspase 3 inhibitor (z-DEVD-fmk) 2 h prior to 24 h of 10 μmol/L artonin B treatment. After incubation, cell survival rate (A), apoptotic cells (B) and caspase 3 activity (C) were examined as described in the Materials and methods. All values are mean±SEM of 4–5 determinations, $^{c}P<0.01$ *vs* the respective artonin B and z-DEVD-fmk free control and $^{f}P<0.01$ comparison between the absence and presence of z-DEVD-fmk in the same artonin B treatment group.

have been proposed and studied intensively[18]. Numerous factors, such as cytosolic cytochrome c release, the expression of Bcl-2 family proteins, and caspase 3 activation have been suggested to play an essential role in the apoptotic process in cancer cells. Our study demonstrated that a progressive decrease of the mitochondrial membrane potential and release of cytochrome c into the cytosol were observed in artonin-B treated human CCRF-CEM leukemia cells. It has been noticed in many *in vitro* systems that apoptosis was associated with a loss of mitochondrial membrane potential, which may correspond to the opening of an outer membrane permeability transition pore. Thus, this event has been suggested to be responsible for cytochrome c release into cytosol from mitochondria[19]. In our present study, the cytosolic cytochrome c accumulation in artonin B-induced human CCRF-CEM cells is probably the consequence of the loss of mitochondrial membrane potential, which finally leads to cell death.

The Bcl-2 family is composed of a number of genes that play critical roles in the control of mitochondrial integrity. Several studies have shown that overexpression of Bcl-2 prevents the mitochondrial release of cytochrome c, thereby inhibiting the activation of caspases cascade and apoptosis[20–23]. In the present study, artonin B-induced apoptosis in human CCRF-CEM leukemia cells was accompanied by upregulation of Bax and Bak and downregulation of Bcl-2. Other studies have demonstrated that Bcl-2, Bax, and Bak can act as channel proteins within the mitochondrial membrane[21,24,25]. It is conceivable that the channel property of Bax and Bak may control the mitochondrial permeability transition and other early mitochondrial perturbation. Thus, Bax and Bak may facilitate the passage of some important proteins, such as cytochrome c or other apoptosis-inducing factors that trigger the activation of caspases cascade and apoptosis. Previous reports have also documented that the ratio of pro-and anti-apoptotic proteins determines, at least in part, the susceptibility of cells to a death signal[21,26,27]. Our results showed that expression of Bcl-2 family proteins Bcl-2, Bax, and Bak can be regulated differently by artonin B, suggesting that the artonin B-induced apoptosis is controlled by a balanced expression between those apoptosis-inducing and apoptosis-suppressing molecules.

Apoptosis is a type of cell death, and agents with the ability to induce apoptosis in tumors have the potential to be used for antitumor therapy. The apoptotic mechanism has been extensively studied, and activation of caspase 3 has been shown to occur in the common apoptotic pathway[28]. The activation of caspases plays a pivotal role in the execution of cell apoptosis[29]. Recent studies have demonstrated that the caspase 3 is a major caspase, which is activated in response to distinct stimuli [30–33]. Moreover, human acute lymphoblastic leukemia CCRF-CEM cells were preincubated with specific caspase 3 inhibitor (z-DEVD-fmk) before treatment of artonin B, and the caspase 3 activity, apoptotic cells and cell viability were analyzed by spectrophotometry analysis, Hoechst 33258 staining and MTT assay, respectively. Results showed that pre-incubation of cells with z-DEVD-fmk effectively inhibited artonin B-induced caspase 3 activity, apoptotic cell formation and cell death. Our data reveled that artonin B-induced nuclear fragmentation may be an apoptotic event provoked along with endonuclease

activation via a caspase protein[34,35]. We also examined the possibility that caspase 3 was involved in the morphological changes in artonin B-treated cells by measuring the caspase 3 activity with or without its inhibitor, z-DEVD-fmk. Theses data demonstrated that artonin B activated caspase 3 activity and consequent cell death.

However, the development of effective chemopreventive approaches must take into consideration the selective and differential effects manifested by different bioactive substances. Target specific agents that are capable of inducing selective apoptosis of cancer cells, but are harmless to normal cells are receiving considerable attention in the fields of cancer prevention and therapy[36]. Artonin B-induced cell cytotoxicity on human leukemia CCRF-CEM cells, but not on normal cells. Therefore, artonin B is a candidate for development as a chemopreventive agent.

In summary, our results demonstrate that cell cytotoxicity induced by artonin B in human CCRF-CEM leukemia cells is possibly mediated through apoptotic cell formation, mitochondrial pathways, Bcl-2 family protein expression, and the activation of caspase 3. However, artonin A, artocarpanone, and artocammunols CE have no effect on the cell survival rate of human CCRF-CEM leukemia cells. By analyzing the structure, more effective compounds might be reconstructured and new strategies for cancer therapy can be explored.

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