

Bigelovii A induces apoptosis of HL-60 human acute promyelocytic leukaemia cells

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Abstract. In the present study, the antitumor effects of the nor-Oleanane type triterpene saponin, Bigelovii A, isolated from *Salicornia bigelovii* Torr, were examined. Bigelovii A was demonstrated to inhibit HL-60 human acute promyelocytic leukaemia cell growth with an IC₅₀ value of 2.15 µg/ml. In addition, Bigelovii A promoted apoptosis in HL-60 cells, as shown by apoptotic morphological changes and the hypodiploid cell assay. Apoptotic induction by Bigelovii A was associated with the downregulation of Bcl-2, the upregulation of Bax and the activation of caspase-3, as demonstrated by RT-PCR and western blot analysis. In addition, a lactate dehydrogenase release test indicated that Bigelovii A may exhibit cytotoxic activity by the induction of cell membrane impairment. This study is the first to identify that Bigelovii A exhibits potential antitumor activity and induces marked apoptosis and membrane permeabilisation in HL-60 cells. Bigelovii A may be a novel candidate for future cancer therapy.

Introduction

Apoptosis or programmed cell death, is a physiological process responsible for the removal of cells that have completed their specific functions or are harmful to the organism. Apoptosis plays a key role in combatting cancer cells (1). Therefore, the induction of apoptosis in cancer cells is a strategy used in anticancer therapy. Over the past two decades a number of

studies have reported the apoptotic activities of members of the triterpene saponin group (2-4).

Nortriterpene saponin is an important triterpene saponin and numerous studies have reported the anti-inflammatory (5), anticancer (6), anticomplement (7), anti-HIV (8,9), insecticidal (10), melanogenesis inhibitory (11), gastroprotective, platelet aggregative (12), antioxidant (13), antiviral (14) and protein tyrosine phosphatase inhibitory (15) activities of nortriterpenoids. Although various bioactivity studies of nortriterpenoids have been performed, the molecular mechanism by which nortriterpene saponin acts on cancer cells remains unclear.

Previously, we reported the isolation and structural elucidation of a new nor-Oleanane type triterpene saponin (Bigelovii A; Fig. 1) from the dried herbs of *Salicornia bigelovii* Torr (16). In addition, Bigelovii A was shown to inhibit the proliferation of HL-60 (leukemia), MCF-7 (breast) and HepG2 (liver) cells, with the HL-60 cells being the most sensitive (16). The present study used the HL-60 cells to further investigate the cytotoxic mechanism of Bigelovii A and found that the mechanism may be dependent on, not only membrane permeabilisation, but also on the apoptosis-inducing effect of Bigelovii A, which was observed to be involved in Bcl-2 suppression and caspase-3 activation.

Materials and methods

Materials. Bigelovii A was isolated from the whole herbs of *Salicornia bigelovii* Torr and the structure is presented in Fig. 1. Bigelovii A was dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) to generate a stock solution which was stored at -20°C and diluted with medium prior to each experiment. The final DMSO concentration was ≤0.1% DMSO throughout the study (all control groups were composed of 0.1% DMSO). Iscove's modified Dulbecco's medium (IMDM) was purchased from Gibco-BRL (Carlsbad, CA, USA) and fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Penicillin, streptomycin and DMSO were purchased from Sunshine Biotechnology (Nanjing, China). RNase A and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were

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obtained from Amersco LLC (Solon, OH, USA). Propidium iodide (PI) was purchased from Sigma. Hoechst 33258 and the CaspACETM Assay System were purchased from KeyGen Biotechnology (Nanjing, China) and Promega Corporation (Madison, WI, USA), respectively. A lactate dehydrogenase (LDH) detection kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Primary antibodies against Bcl-2, Bax, caspase-3 and β -tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horse-radish peroxidase (HRP)-conjugated secondary antibody was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China) and the enhanced chemiluminescence (ECL) kit was obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture. The human leukocyte cancer cell line, HL-60, was obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in IMDM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. The media were changed every other day. All the cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The study was approved by the ethics committee of the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences

MTT assay. The effect of Bigelovii A on the viability of the tumor cells was assessed by a MTT assay, which was based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to form a purple formazan product. Briefly, the HL-60 cells were seeded at 5x10⁴ cells/well in a 96-well plate and treated with various concentrations of Bigelovii A or vehicle (control). Each of the treated or control groups contained 6 parallel wells. Following incubation for 24 h at 37°C in a humidified incubator, cell viability was determined. MTT [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for 4 h. Then, 100 μ l solubilization solution (10% SDS in 0.012 M HCl) was added to each well and the plate was left to stand overnight in the incubator. Absorbance was recorded on a microplate reader (Tecan US, Inc., Morrisville, NC, USA) at a wavelength of 570 nm (reference wavelength, 690 nm). The percentage cell proliferation was calculated as a ratio of the sample to control OD values. Experiments were performed under the same conditions at least three times. The cell inhibitory ratio was calculated using the following formula: inhibitory ratio (%) = (1 - average absorbance of treated group / average absorbance of control group) x 100. IC₅₀ was defined as the concentration that caused a 50% inhibition of cell viability.

LDH release assay. The HL-60 cells were seeded in 96-well round bottom plates at a density of 5x10⁴ cells/well in 100 μ l IMDM with 1% FBS (serum contains natural LDH activity) and containing 1-10 μ l/ml Bigelovii A. Following a 24-h incubation at 37°C, the plates were centrifuged at 250 x g (Eppendorf AG, Hamburg, Germany) for 10 min. Aliquots (100 μ l) of the supernatants were collected. A mixture of diaphorase/NAD⁺ and iodotetrazolium chloride (100 μ l) was added to each well. Following 30 min at room temperature in the dark, absorbance was recorded on a microplate reader

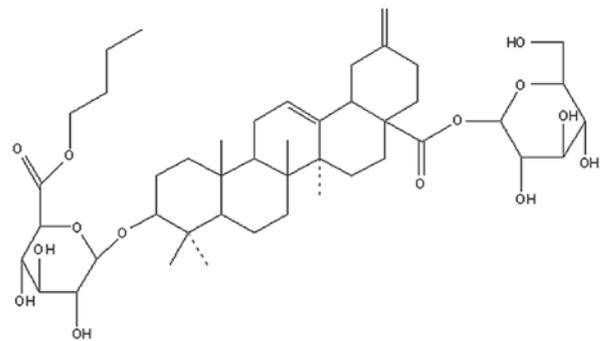


Figure 1. Chemical structure of Bigelovii A.

at a wavelength of 490 nm. Spontaneous LDH release from untreated normal cells (Lc) and high level maximum release by disrupting the cells with Triton X-100 (Hc) were analyzed. The percentage of LDH release compared with the control was calculated as: [(treated mean - Lc) / (Hc - Lc)] x 100.

Morphological analysis of apoptosis by Hoechst 33258 staining. Hoechst 33258 staining of the HL-60 cells was performed to evaluate the cell death pattern induced by increasing the Bigelovii A concentration. The morphology of the HL-60 cells treated with Bigelovii A for 24 h was observed under an inverted microscope. Hoechst 33258 staining was then used to determine apoptotic morphology. Briefly, the cells were seeded at a concentration of 1x10⁶ cells/ml in 6-well tissue culture plates and treated with the indicated concentration of Bigelovii A. The cells were harvested, washed twice with PBS, fixed with 4% formaldehyde for 10 min and stained with Hoechst 33258 staining solution according to the manufacturer's instructions. The stained nuclei were examined and immediately photographed under a fluorescence microscope (Olympus IX51, Tokyo, Japan) with a peak excitation wavelength of 340 nm.

Cell cycle analysis and sub-G₀/G₁ measurement. The HL-60 cells were seeded in a 6-well culture plate at a density of 1x10⁶ cells/ml and treated with Bigelovii A for 12 and 24 h. The cells were then collected, washed with PBS and fixed in 1 ml 70% ice-cold ethanol at 4°C. After being left to stand overnight, cell pellets were collected by centrifugation, resuspended in 100 μ l RNase (100 μ g/ml) and incubated at 37°C for 30 min. Then, 400 μ l PI solution (50 μ g/ml) was added and the mixture was allowed to stand on ice for 30 min. The fluorescence emitted from the PI-DNA complex was quantified following excitation of the fluorescent dye by FACSscan flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). The percentage of cells in the G₀/G₁, S, G₂/M and sub-G₁ phases was analysed using the CellQuest software program.

Semi-quantitative RT-PCR. The effect of Bigelovii A treatment on the expression of Bcl-2 and Bax was determined by RT-PCR analysis. Following 24 h incubation, total RNA was isolated using an RNA extraction kit according to the manufacturer's instructions (Takara Biotechnology, Dalian, China). The RNA concentration was determined by the absorption at 260 nm.

cDNA was synthesized by extension of oligo (dT) primers with 10 units AMV reverse transcriptase in a mixture containing 1 μ g total RNA. Amplification of the cDNA was performed using the PCR kit. The oligonucleotide primer sequences were as follows: GADPH, 5'-GGTCCGAGTCAACGGATTTGGTTCG-3' (sense) and 5'-CCTCCGACGCCTGCTTACCAC-3' (antisense); Bcl-2, 5'-TGCCACGGTGGTGGAGGAGC-3' (sense) and 5'-GCATGTTGACTTCACTTGTGGC-3' (antisense); and Bax, 5'-TCCACCAAGAAGCTGAGCGAG-3' (sense) and 5'-GTCCAGCCCATGATGGTTCT-3' (antisense). The samples for Bcl-2 were inactivated for 3 min at 94°C prior to hotstart amplification. The amplification cycle was performed at 94°C for 30 sec, 65°C for 1 min and 72°C for 2 min for 35 cycles. The samples for Bax were inactivated for 7 min at 97°C prior to hotstart amplification. This amplification cycle was performed at 94°C for 1 min, 60°C for 45 sec and 72°C for 45 sec for 35 cycles. The PCR products were separated by electrophoresis in 1% agarose gels and visualized by ethidium bromide staining. For quantitation, images of the agarose gels were scanned and the bands were densitometrically analyzed with Scan Analysis software (Tanon Science and Technology, Shanghai, China).

Western blot analysis. The HL-60 cells were treated for 24 h with various concentrations of Bigelovii A (1, 2 and 4 μ g/ml) and then collected. The cells were lysed in lysis buffer [50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% (m/v) NP-40, 0.2 mM PMSF, 0.1 mM NaF and 1.0 mM DTT] and the lysates were clarified by centrifugation at 4°C for 15 min at 13,000 x g. The protein concentration was measured by the Bradford assay. Following this, equal concentrations of protein were separated by SDS-PAGE and transferred onto the PVDF membranes (Millipore, Billerica, MA, USA). The blots were incubated with specific antibodies against the indicated primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibody for 1 h at 37°C. Immunoreactive proteins were detected with the ECL kit. All blots were stripped and reprobed with polyclonal anti-tubulin antibody to ascertain equal loading of the proteins.

Measurement of caspase-3 activity. The activation of caspase-3 was determined using a caspase-3 assay kit. This assay is based on the spectrophotometric detection of chromophore p-nitroanilide (pNA) released following cleavage of the labeled substrate, DEVD-pNA. The absorbance of pNA from an apoptotic sample was compared with that of an uninduced control. For the inhibited apoptosis samples, the inhibitor, Z-VAD-FMK, was added to the cells at the same time as Bigelovii A. Briefly, the HL-60 cells (1×10^6) were harvested by centrifugation. The cell pellets were resuspended in cold lysis buffer and placed on ice for 15 min and then the resuspension was centrifuged at 15,000 x g for 20 min. The supernatant was collected as cell lysate and incubated with Z-VAD-FMK according to the manufacturer's instructions for 4 h at 37°C. The release of pNA was measured at 405 nm using a 96-well microplate reader. The quantification of viable cells was determined by a cytometer.

Statistical analysis. Experiments were repeated at least three times. Results are presented as mean \pm SD. Statistically

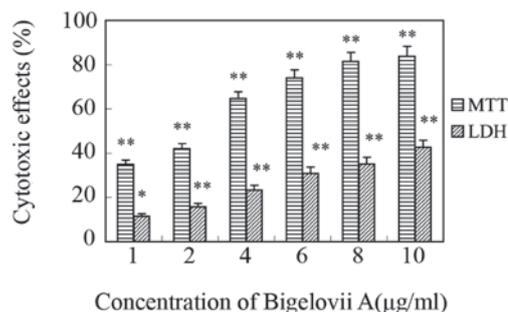


Figure 2. MTT and LDH assays. Dose-response plot for HL-60 cells following 24-h treatment with Bigelovii A. * $P < 0.05$ and ** $P < 0.01$, vs. control. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase.

significant differences compared with untreated controls were calculated using the Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxic effect of Bigelovii A on the HL-60 cells. To evaluate the effect of Bigelovii A on cell growth, proliferation assays were performed on the HL-60 cells using increasing drug concentrations (1-10 μ g/ml) and cell viability was determined using the MTT assay. As demonstrated in Fig. 2, incubation of the cells for 24 h with Bigelovii A was found to significantly decrease cell survival in a dose-dependent manner. A reduction in cell viability by 50% in comparison with the control was achieved at a dose of 2.15 μ g/ml. In contrast with the Bigelovii A treated cells, the cells treated with DMSO showed limited or no cytotoxicity. Since the ability to interact with cell membranes is a characteristic of saponins, using the LDH release test we investigated whether Bigelovii A altered the cellular membrane. The test must be performed with a low level of serum in the medium. The results indicated that the release of LDH was concentration-dependent. Collectively, the results of the MTT assay and LDH release test indicate that cell death caused by Bigelovii A may not only be due to membrane permeabilization.

Bigelovii A-induced apoptotic cell death in the HL-60 cells. To determine whether the HL-60 cells treated with Bigelovii A underwent apoptosis, two apoptotic assays, including Hoechst 33258 staining and a hypodiploid DNA assay, were performed. Marked apoptosis was observed by both assays. Hoechst 33258 dye selectively bound the DNA and enabled monitoring of nuclear morphological changes under a fluorescence microscope. The nuclei of the untreated cells stained blue with Hoechst were observed to exhibit loose chromatin and be distributed throughout the cell. Bigelovii A at 2 and 4 μ g/ml induced significant nuclear fragmentation and condensation in the HL-60 cells following 24 h of treatment (Fig. 3). The majority of the cells decreased in size and had a relatively small size compared with the untreated cells. To investigate the effects of Bigelovii A on cell cycle status, the HL-60 cells were treated with various concentrations of Bigelovii A for 12 and 24 h and then analyzed for alterations in the cell cycle by flow cytometry. Bigelovii A caused dose- and time-dependent

Table I. Effect of 24 h Bigelovii A treatment on caspase-3 activity in HL-60 cells.

Parameters	N	A	I
OD ₄₀₅	0.0452±0.0035	0.1084±0.0041 ^a	0.0267±0.0016 ^b
Cell viability (%)	100±2.7	51.4±3.2 ^a	100±4.1 ^b

N, untreated cells; A, 4 µg/ml Bigelovii A; and I, 4 µg/ml Bigelovii A+10 µmol/l Z-VAD-FMK. ^aP<0.05, vs. N. ^bP<0.01, vs. A.

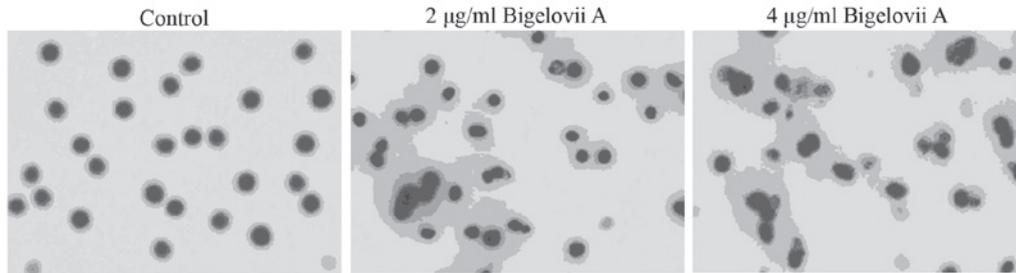
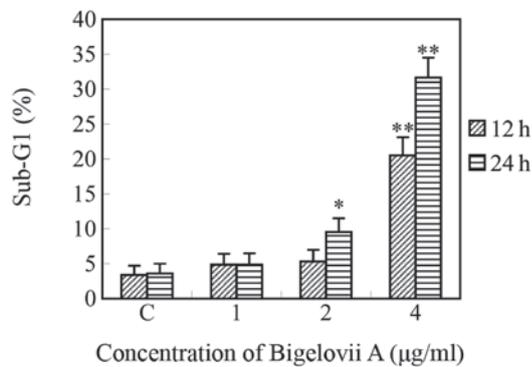


Figure 3. Hoechst 33258 staining to determine morphological changes in HL-60 cells induced by Bigelovii A (magnification, x400).

Figure 4. Percentage of HL-60 cells treated with Bigelovii A for 12 and 24 h in the sub-G₁ phase. *P<0.05 and **P<0.01, vs. control. C, control.

increases in the hypodiploid sub-G₀/G₁ DNA fraction (<2n DNA), indicating apoptosis due to DNA fragmentation (Fig. 4). The sub-G₀/G₁ fraction was 3.6% in untreated cells, which gradually increased to 31.7% following 4 µg/ml Bigelovii A treatment for 24 h. Bigelovii A treatment had almost no effect on the G₂/M fraction, indicating that the compound does not produce a mitotic block or delay in the cell cycle.

Intrinsic pathway involvement in Bigelovii A-induced apoptosis. Bcl-2 family proteins, including anti-apoptotic members (e.g., Bcl-2) and pro-apoptotic members (e.g., Bax), play a pivotal role in apoptosis (17). Bcl-2 has been identified to form a heterodimeric complex with the proapoptotic member Bax, neutralizing its proapoptotic effects. Therefore, the Bcl-2/Bax ratio is a decisive factor and plays a significant role in determining whether cells are likely to undergo death or survival. To elucidate the mechanisms underlying Bigelovii A-induced apoptosis, the effect of Bigelovii A treatment on the gene and protein expression levels of Bcl-2 and Bax was examined. The HL-60 cells were treated for 24 h and the total RNA was

isolated, reverse-transcribed and amplified using Bcl-2 and Bax specific primers. As demonstrated in Fig. 5, treatment with Bigelovii A decreased the Bcl-2 expression and increased the Bax mRNA expression in a dose-dependent manner, leading to a decrease in the Bcl-2/Bax ratio. In addition, further western blot analysis was used to evaluate the protein expression levels of the Bcl-2 family members and caspase-3. The Bcl-2 protein levels were decreased and caspase-3 was activated, however, the Bax protein levels were not markedly changed following treatment with Bigelovii A for 24 h (Fig. 6). Overall, the reduction of Bcl-2 expression, together with the activation of caspase-3, indicated that Bigelovii A-induced apoptotic cell death was mediated through the intrinsic pathway.

Caspase activation involved in Bigelovii A-induced apoptosis. Activation of the caspase family is a crucial mechanism for the induction of death signals in apoptosis. The caspases are a family of cysteine proteases in mammalian cells that cleave proteins following an aspartic acid residue. Caspases may be divided into initiators and effectors. Caspase-3, an effector caspase, is central to the mediation of various apoptotic responses. In addition to being observed by western blot analysis, activated caspase-3 was also observed by colorimetric assay in the present study (Table I). To determine whether the activation of caspase-3 was required for the induction of cell death by Bigelovii A, the HL-60 cells were pretreated with the pan-caspase inhibitor, Z-DEVD-FMK, which completely reversed Bigelovii A-induced growth inhibition. These results indicate that caspase-3 is activated in response to apoptosis induced by Bigelovii A.

Discussion

Acute leukaemia, as with other types of cancer, is a progressive clonal disorder driven by mutation. Leukaemic cells proliferate primarily in the bone marrow and lymphoid

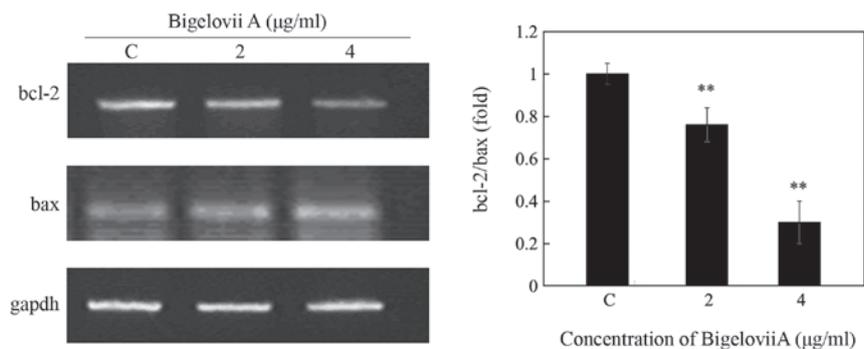


Figure 5. RT-PCR analysis of the effect of Bigelovii A for 24 h on the expression of Bcl-2 and Bax in HL-60 cells. **P<0.01, vs. control. C, control.

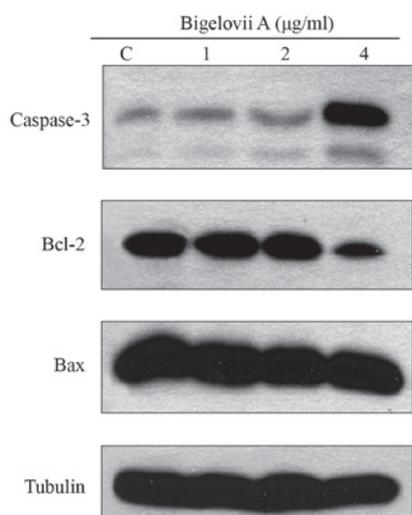


Figure 6. Western blot analysis of the effect of Bigelovii A for 24 h on the expression of caspase-3, Bcl-2 and Bax in HL-60 cells. C, control.

tissues, where they interrupt normal hematopoiesis and immunity. Regimens for the induction of remission, consolidation therapy and bone-marrow transplantation have improved the outlook for patients with acute leukaemia (18). However, there remains a considerable number of incidences of relapse and unsatisfactory results in patients with extramedullary disease (19). Therefore, the research and development of new and safe drugs for leukaemia is required by the pharmaceutical industry. The present study evaluated the chemopreventive potential of Bigelovii A in HL-60 human acute promyelocytic leukaemia cells, as well as their cytotoxic mechanism.

There are several assays currently available for the assessment of cell cytotoxicity, including the MTT reduction assay and the LDH release assay. A colorimetric MTT assay is available for measuring the mitochondrial-related reduction capacity and LDH release assays are used to evaluate cell membrane damage (20). In the present study, the novel 30-nortriterpenoid glycoside, Bigelovii A, was shown to exhibit marked cytotoxic effects on HL-60 cells by MTT and LDH assays. MTT assay suggested that the growth inhibitory effects were dose-dependent: for example, 34.9, 42.1 and 64.7% cytotoxicity at 1, 2 and 4 µg/ml, respectively, with an IC₅₀ value of 2.15 µg/ml. Also, LDH release was slowly

increased following treatment with Bigelovii A, indicating cell membrane impairment.

Apoptosis is an evolutionarily conserved process that eliminates irreversibly damaged or potentially harmful cells in order to protect the organism. In the present study, we found that treatment with Bigelovii A rapidly induced HL-60 cell death. Bigelovii A-induced cell death in these cells exhibited the typical characteristics of apoptotic cells, including membrane blebbing and cell and chromatin condensation. Flow cytometry confirmed that the treatment with Bigelovii A increased the fraction of cells with hypodiploid DNA and that this effect was concentration- and time-dependent. Bigelovii A induced apoptosis in a similar manner to macranthoside B in HepG2 (21) and in HL-60 cells (22).

Extrinsic and intrinsic-mediated pathways lead to apoptosis. The intrinsic or mitochondria-mediated pathways may be activated directly without being triggered by a death receptor. The Bcl-2 protein family are the most important regulators of the intrinsic apoptotic processes and this family includes proapoptotic members, such as Bax, and anti-apoptotic members, such as Bcl-2. Bcl-2 functions as a repressor of apoptosis by blocking the release of cytochrome c. Bcl-2 is also important for the resistance to chemotherapy and radiotherapy (23,24). However, Bax has been demonstrated to play a key role in initiating mitochondrial dysfunction (25). Therefore, the apoptotic process is regulated by the ratio of Bax and Bcl-2, which is recognized to initiate caspase signaling (26,27). In the present study, treatment with Bigelovii A decreased Bcl-2 expression while increasing Bax mRNA expression in a dose-dependent manner. The Bcl-2/Bax ratio was evidently lower than that of the control at 4 µg/ml Bigelovii A. The Bcl-2 protein levels were also decreased, however, the Bax levels were not markedly changed.

The caspases may be structurally divided on the basis of the presence or absence of an N-terminal prodomain. The caspases containing long prodomains are the first to be activated in response to apoptotic stimuli. The activated caspases destroy the cellular architecture and ultimately result in cell death (28). Caspase-3 is the most important member of the caspase family. Western blot analysis and the colorimetric assay indicated that 4 µg/ml Bigelovii A activated caspase-3. Taken together, these observations reveal that the downregulation of Bcl-2 may also lead to the activation of caspase-3 and the induction of apoptosis in Bigelovii A-mediated HL-60 cells.

In the present study, Bigelovii A was demonstrated for the first time to exhibit potent anticancer activity *in vitro* and induce apoptosis in HL-60 cells, indicating that Bigelovii A may be a promising novel anticancer drug.

Acknowledgements

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