Induction of apoptosis by Bigelovii A through inhibition of NF-κB activity

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Received September 30, 2016; Accepted May 17, 2018

DOI: 10.3892/mmr.2018.9104

Abstract. Bigelovii A is a 30-nortriterpenoid glycoside, isolated from *Salicornia bigelovii* Torr. Until now, the effect of Bigelovii A on breast cancer treatment was unknown. The present research indicated that Bigelovii A significantly inhibited the proliferation of human breast cancer cells (MCF-7, MDA-MB-231 and MDA-MB-468) in a concentration-dependent manner. It was particularly effective in MCF7 cells, with an IC_{50} value of 4.10±1.19 μM. The anti-proliferative effect of Bigelovii A was ascribed to the induction of apoptosis, which was characterized by chromatin condensation, externalization of phosphatidylserine on the plasma membrane, hypodiploid DNA, activation of caspases and poly (ADP-ribose) polymerase cleavage. Furthermore, Bigelovii A reduced B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xl) expression and caused disruption of mitochondrial membrane potential, which are indicative features of mitochondria-dependent apoptotic signals. It was also identified that Bigelovii A downregulated the constitutive activation of nuclear factor (NF)-κB, as indicated by the electrophoretic mobility gel shift assay and immunocytochemistry. Furthermore, Bigelovii A suppressed constitutive IκBα phosphorylation via inhibition of IκB kinase activity. In addition to the effects on Bcl-2 and Bcl-xl, Bigelovii A also downregulated the expression of the NF-κB-regulated gene products, Cyclin D1 and cyclooxygenase-2. This led to the induction of apoptosis and arrest of cells at the G1 phase of the cell cycle.

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Key words: bigelovii A, apoptosis, NF-κB, Bcl-2, cyclin D1

Introduction

Breast cancer is the most common life-threatening cancer type among women worldwide (1). In spite of numerous improvements in early diagnosis, surgical treatment and endocrine therapy, ~30% of patients with breast cancer do not respond to these standard treatments (2). Depending on the subtype (2,3), the average survival time of these unresponsive patients is 1-4 years, which thus represents a significant unsolved medical challenge. The development of more effective therapeutic drugs is urgently needed. As a family of transcription factors, nuclear factor (NF)-κB plays critical roles in cell proliferation, differentiation and survival, and inflammation and immunity (4). The NF-κB subunits, including RelA (p65), RelB, c-Rel, NF-κB1, and NF-κB2, are held in the cytoplasm by the specific inhibitors IκBs (IκBα, IκBβ, and IκBγ). Inflammation in the tumor microenvironment can activate IκB kinases (IKKs), which phosphorylate and degrade IκB. As a result, NF-κB is released to the cell nucleus and may promote cancer invasion and metastasis. Many tumors, including breast cancer (5,6), have been observed to have aberrant NF-κB activation. Therefore, inhibition of the NF-κB pathway is expected to prevent and treat cancer.

As a traditional folk medicine, *Salicornia* plants have been used to treat hypertension, cephalalgia, scurvy and cancer (7). Bigelovii A is a 30-nortriterpenoid glycoside, isolated from *Salicornia bigelovii* Torr (8). Yan *et al* (9) reported that Bigelovii A could also mitigate LPS-induced acute lung injury by suppressing NF-κB and CCAAT/enhancer-binding protein δ pathways. Our previous studies indicated that Bigelovii A exhibited potential cytotoxicity against HL-60, MCF7, HepG2, A549, Lovo and LN229 cells (8,10,11). In particular in HL-60 cells, Bigelovii A decreased the expression of apoptosis regulator Bcl-2 (Bcl-2), activated caspase-3 and induced cell apoptosis (10). However, the anti-tumor mechanism of Bigelovii A in human breast cancer cells has not been examined. The current study initially compared the cytotoxicity of Bigelovii A in three human cancer cell types (MCF7, MDA-MB-231 and MDA-MB-468). Subsequently, the apoptotic effects mediated by IKK/NF-κB signaling pathways were investigated.

Materials and methods

Reagents. Bigelovii A (Fig. 1) was isolated from *Salicornia bigelovii* Torr. and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to a 100 mM stock solution. The final DMSO concentration in the medium did not exceed 0.1% (v/v) throughout the study. Fetal bovine serum and modified Eagle's medium (MEM) were provided by Gibco™ (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Antibiotics, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and RIPA lysis buffer were purchased from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). An Annexin V-PE apoptosis kit was provided by BD Biosciences (Franklin Lakes, NJ, USA). Primary antibodies against NF-κB pathway sampler kit (cat. no. 9936), Cyclin D1 (cat. no. 2978), COX-2 (cat. no. 4482), p-Bcl-2 (cat. no. 2827), Bcl-2 (cat. no. 2870), B-cell lymphoma-extra large (Bcl-xl; cat. no. 2764), cleaved caspase-3 (cat. no. 9936), cleaved caspase-7 (cat. no. 8438), cleaved caspase-9 (cat. no. 9505), cleaved PARP (cat. no. 5625), cleaved caspase-8 (cat. no. 9496) and β-actin (cat. no. 4970), horseradish peroxidase (HRP)-conjugated secondary antibody and an enhanced chemiluminescence (ECL) kit were supplied by Cell Signaling Technology, Inc. (Danvers, MA, USA). NE-PER™ Nuclear and Cytoplasmic Extraction Reagents, Halt™ Protease Inhibitor Cocktail (EDTA-Free) and a LightShift Chemiluminescent EMSA kit were purchased from Thermo Fisher Scientific, Inc. Hoechst 33258 and a Cellular NF-κB Translocation kit were obtained from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture. Three human breast cancer cell lines (MCF7, MDA-MB-231 and MDA-MB-468) were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). They were propagated using the methods provided. MCF7 cells were cultured in MEM supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin, while MDA-MB-231 and MDA-MB-468 cells were cultured without insulin.

Cell viability assays. The effect of Bigelovii A on cell viability was determined by MTT assay. Cells were seeded at a density of $1x10^4$ cells/well in 96-well plates and treated with Bigelovii A at various concentrations. Following incubation for the indicated time, 5 mg/ml MTT was added to each well and incubated in the CO_2 incubator for 4 h. The formazan crystals were dissolved in DMSO and absorbance was recorded on an ELISA reader (Tecan Group, Ltd., Mannedorf, Switzerland) at a test wavelength of 570 nm and a reference wavelength of 690 nm. Inhibition rate was calculated by the following formula: Inhibition rate (%)=$(1-OD_t/OD_c)x100\%$. OD_t and OD_c denoted the average absorbance of treated groups and control groups, respectively. IC_{50} was considered to be the concentration that caused 50% inhibition rate.

Annexin V-PE/7-AAD analysis and Hoechst 33258 staining for cell apoptosis. MCF7 cells were plated at a density of $4x10^5$ cells/well. Following treatment with 5, 10 or 20 μM Bigelovii A for 24 h, cells were washed twice with PBS, resuspended in binding buffer, then incubated with 7-AAD and Annexin V-PE in the dark for 15 min. The samples were analyzed using an Accuri C6 flow cytometer (BD Biosciences) for 1 h. Then, the apoptotic morphology was studied using the Hoechst 33258 dye. Cells ($1x10^6$/ml) were seeded on glass slides in 6-well tissue culture plates. After treatment with 20 μM Bigelovii A for 24 h, cells were fixed with 4% formaldehyde for 10 min, washed with PBS twice and stained with Hoechst 33258 dye for 5 min. Stained nuclei were observed at magnification, x100 under an IX51 fluorescence microscope.

Propidium iodide (PI) staining for cell cycle analysis. MCF7 cells ($1x10^6$/well) were treated with different doses of Bigelovii A and incubated for 24 h. Floating and adherent cells were harvested and washed with cold PBS. The cell pellet was fixed in 70% ethanol overnight at 4˚C, incubated with 100 μg/ml RNaseA for 30 min at 37˚C and stained with PI (50 μg/ml) for 30 min at 4˚C. Cell cycle distribution was analyzed in an Accuri C6 flow cytometer (BD Biosciences).

Mitochondrial membrane potential. Following treatment with Bigelovii A (0, 5, 10 and 20 μM) for 24 h, MCF7 cells were washed twice with cold PBS and incubated with Rh123 in a CO_2 incubator for 30 min. The stained cells were collected by centrifugation, washed twice with PBS, then analyzed by flow cytometry.

Isolation of total cellular proteins, cytosolic and nuclear proteins. MCF7 cells were cultured to 80% confluence and treated with various concentrations of Bigelovii A for the indicated times. Total cellular proteins were isolated with RIPA lysis buffer, with the lysates centrifuged for 15 min at 12,000 x g and 4˚C. Cytosolic and nuclear proteins were separated according to the manufacturer's recommended protocol. After washing, cells were trypsinized and centrifuged at 500 x g for 5 min. Cells were lysed with ice-cold CER I, vigorously vortexed on the highest setting for 15 sec, then incubated on ice for 10 min. Ice-cold CER II was added to the tube, then the tube was vortexed for 5 sec on the highest setting, and centrifuged for 5 min at 16,000 x g. The supernatants were saved as the cytoplasmic fractions and the nuclear pellets were resuspended in ice-cold NER. The tubes were vortexed on the highest setting for 15 sec, then placed on ice. Vortexing was continued for 15 sec every 10 min, for a total of 40 min. The tube was then centrifuged at 16,000 x g for 10 min and the nuclear supernatant was immediately transferred to a clean, pre-chilled tube.

Western blot analysis. The concentrations of total cellular proteins, cytosolic and nuclear proteins were measured by the Bradford assay. Equal amounts of proteins (50 μg) were fractionated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% milk, the blots were probed with specific primary antibodies at 4˚C overnight, followed by HRP-labeled secondary antibody at 37˚C for 1 h. Immunoreactive proteins were visualized with the enhanced chemiluminescence reagents. Anti-β-actin antibody was used to ascertain equal loading of proteins.

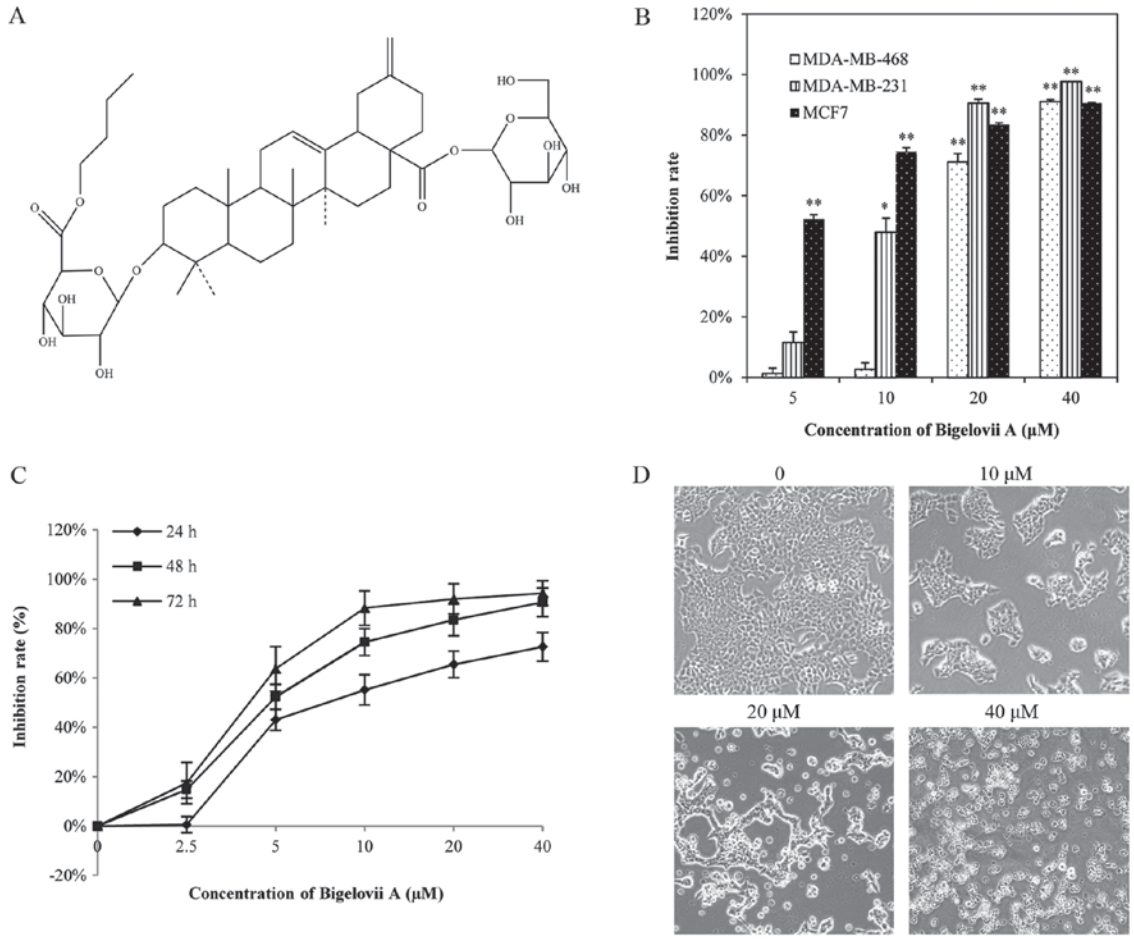

Figure 1. Effects of Bigelovii A on cell viability of breast cancer cells. (A) Chemical structure of Bigelovii A. (B) MDA-MB-468, MDA-MB-231 and MCF7 were treated with Bigelovii A for 48 h. (C) MCF7 cells were treated with Bigelovii A for 24, 48 and 72 h. Then, cell viability was evaluated by MTT assay. (D) Following treatment with Bigelovii A for 24 h, morphological changes in MCF7 cells were visualized at x100 magnification under a IX51 microscope. *P<0.05, **P<0.01 vs. 0 μM.

Electrophoretic mobility shift assay (EMSA). EMSA was performed to investigate the inhibitory effect of Bigelovii A on NF-κB activation. The nuclear extracts, isolated as described above, were incubated with biotin-labeled oligonucleotide probes (5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCA ACTCCCCTGAAAGGGTCCG-5') for 20 min at room temperature. Unlabeled oligonucleotide was used to confirm the specificity of binding. The formed DNA-protein complex was electrophoresed on 6% non-denaturing polyacrylamide gel, transferred onto a nylon membrane, and cross-linked for 10-15 min under 312 nm bulbs. The nylon membrane was visualized using the Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc.).

Immunocytochemistry for NF-κB p65 localization. The effect of Bigelovii A on NF-κB nuclear translocation was examined by immunofluorescence confocal microscopy according to the kit's protocol. MCF7 cells were plated on glass coverslips in 6-well plates and treated with 10 μM Bigelovii A for 24 h. Following washing, cells were incubated with a blocking buffer at room temperature for 1 h and the primary NF-κB p65 antibody at 4˚C overnight. The slides were washed again in washing buffer and incubated with Cy3-labeled secondary antibody for 1 h. The nuclei were counterstained with DAPI for 5 min. The stained slides were observed using a laser confocal microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of mRNA levels. RT-qPCR was performed as described in previous reports (12,13). Briefly, total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. First-strand cDNA was synthesized using HiScript II Q RT SuperMix for qPCR (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer's protocol. The primers were designed using the software Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and are listed in Table I. The quantified expression levels of the tested genes were normalized against the housekeeping gene β-actin. qPCR was performed using SYBR-Green Master mix (Vazyme Biotech Co., Ltd.) and run on a qTOWER2.2 Real-Time PCR system (Analytik Jena AG, Jena, Germany). The conditions for quantitative analysis were as follows: 95˚C for 5 min; 40 cycles of 95˚C for 15 sec, 56˚C for 15 sec, and 72˚C for 20 sec; 72˚C for 5 min. The specificity of each PCR reaction was determined by melting curve analysis. Data for each sample were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

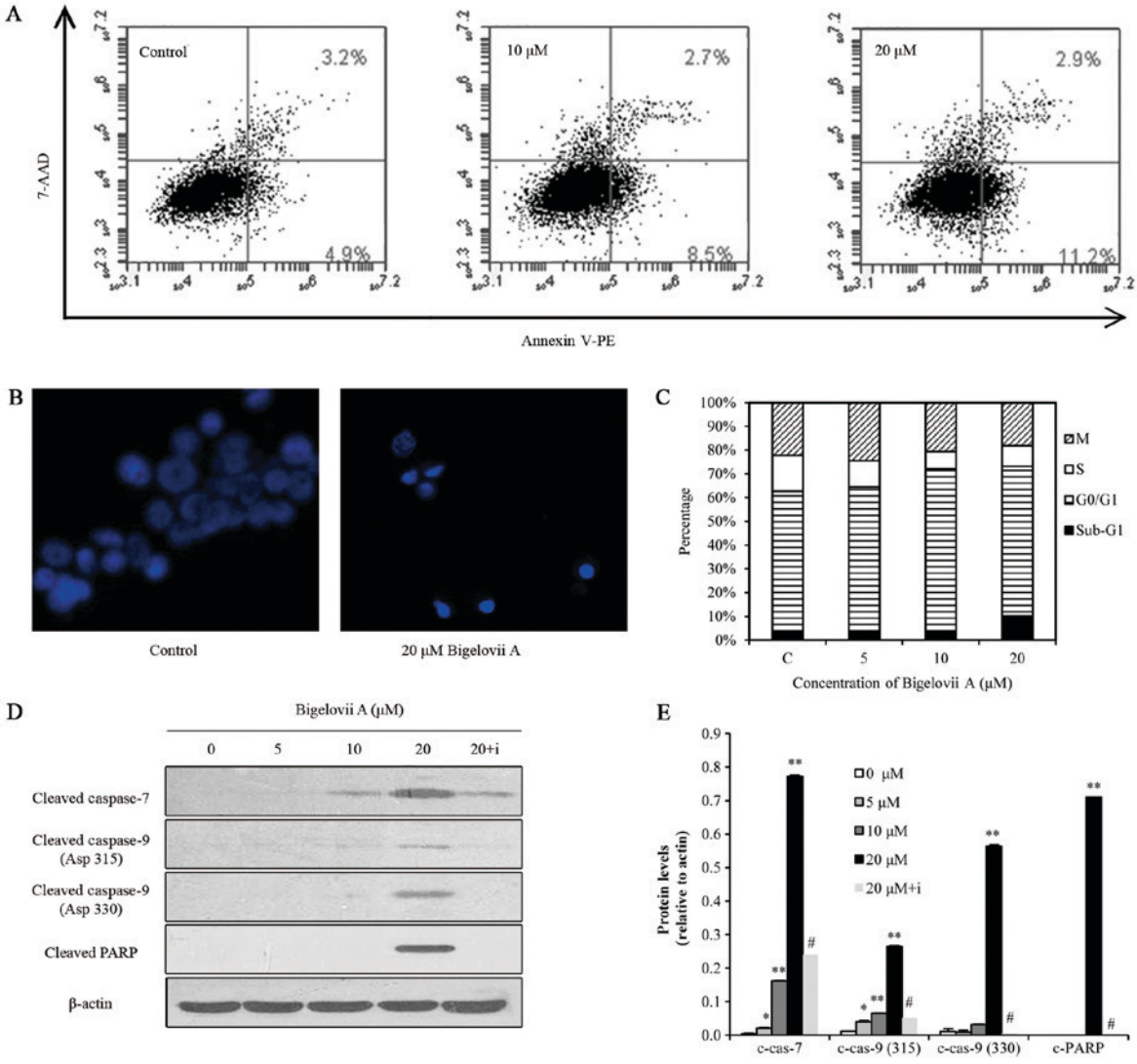

Figure 2. Bigelovii A induced apoptosis in MCF7 cells. (A) Cells were treated with 10 or 20 μM Bigelovii A for 24 h and Annexin V-PE/7-AAD double staining was performed to measure apoptotic and necrotic cells. (B) Morphological changes in MCF7 cells induced by 20 μM of Bigelovii A for 24 h were detected by Hoechst 33258 staining. (magnification, x400). (C) The ratio of cells at sub-G1 phase was detected by flow cytometry. (D) Western blotting was performed to evaluate protein levels of cleaved caspase-7, cleaved caspase-9 and PARP. (E) The protein levels of cleaved-caspase-7, cleaved-caspase -9, and cleaved-PARP were quantified using Image J. *P<0.05, **P<0.01 vs. 0 μM; $^{\#}$P<0.01 vs. 20 μM. i, 10 μM Z-VAD-FMK.

Statistical analysis. Data are presented as the mean ± standard deviation from triplicate experiments. The statistical significance was evaluated using Student's t-test to compare the difference between two groups, and one-way analysis of variance followed by a Newman-Keuls post-hoc test was used to perform multiple comparisons (GraphPad Software, Inc., San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Bigelovii A inhibits the proliferation of human breast cancer cells. To measure the cytotoxic effect of Bigelovii A, three human breast cancer cell lines (MCF-7, MDA-MB-231 and MDA-MB-468) were treated with 5, 10, 20 and 40 μM of Bigelovii A for 48 h. As presented in Fig. 1B, the inhibitory effect of Bigelovii A occurred in a concentration-dependent manner, particularly in MCF7 cells (IC_{50}: 4.10±1.19 μM). The inhibitory effect on MCF7 cells was also in a time-dependent manner (Fig. 1C). Microscopy observations indicated that MCF7 cells with treatment with Bigelovii A became round and floated, while untreated cells exhibited a epithelial and adherent cytoskeleton (Fig. 1D).

Bigelovii A induces apoptosis in MCF7 cells. To evaluate whether Bigelovii A induced apoptosis in MCF7 cells, Annexin V-FITC, Hoechst 33258 staining, sub-G1 DNA content, caspase and PARP cleavage assays were conducted (Fig. 2). Flow cytometric analysis of Annexin V/7-AAD staining suggested that Bigelovii A increased the percentage of Annexin V-positive MCF7 cells in a dose-dependent manner (Fig. 2A), indicating the activation of apoptosis. Hoechst 33258 staining (Fig. 2B) indicated that Bigelovii A at 20 μM induced chromatin fragmentation and condensation in MCF7 cells following 24 h of treatment. Cell cycle analysis revealed that treatment with this compound at 5 and 10 μM for 24 h resulted in cell cycle arrest at G1 phase, while treatment at 20 μM induced the hypodiploid sub-G1 phase, confirming an apoptotic effect (Fig. 2C).

Table I. Sequences of primers used for PCR and RT-qPCR.

Gene	Primer sequence (5' to 3')
Bcl-2	
Forward	GGTCATGTGTGTGGAGAGCG
Reverse	CAGGGTGATGCAAGCTCCCA
Cyclin D1	
Forward	TGAACCTGAGGAGCCCCAAC
Reverse	GCCTTGGGGTCCATGTTCTGCT
COX-2	
Forward	GCAGTACAGAAAGTATCACAGGC
Reverse	CGATGTCACCATAGAGTGCTTCC
Bcl-xl	
Forward	ATGGGGTAAACTGGGGTCGC
Reverse	GCATTGTTCCCATAGAGTTCCAC
β-actin	
Forward	CCGACAGGATGCAGAAGGAG
Reverse	CTCGTCATACTCCTGCTTGCTG

RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Bcl-xl, B-cell lymphoma extra large; COX-2, cyclooxygenase-2.

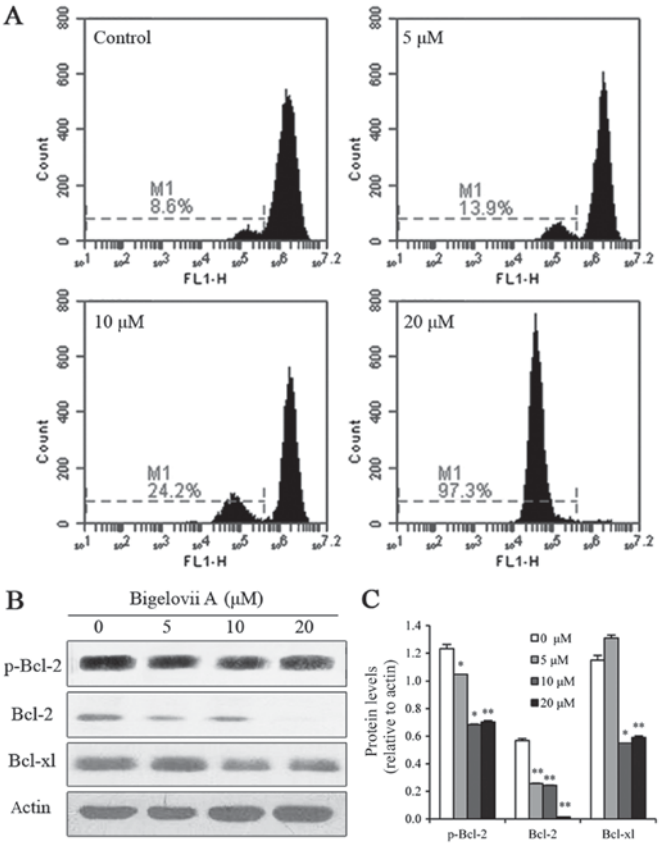

Figure 3. Bigelovii A induced apoptosis via mitochondrial pathways. (A) Following treatment with Bigelovii A for 24 h, mitochondrial membrane potential was measured by flow cytometry. (B and C) Protein levels of p-Bcl-2, Bcl-2 and Bcl-xl were analyzed by (B) western blotting and (C) quantified by Image J. *P<0.05, **P<0.01 vs. 0 μM. Bcl-xl, B-cell lymphoma-extra large.

As indicated in Fig. 2D, treatment of MCF7 cells with Bigelovii A (5, 10 or 20 μM) led to activation of caspase-7 and caspase-9, and cleavage of PARP. Z-VAD-FMK, a pan caspase inhibitor, reversed Bigelovii A-induced caspase and PARP activation. These data indicated that Bigelovii A killed MCF7 cells by inducing apoptosis. However, activation of caspase-3 and caspase-8 was not detected. Thangaiyan and Sipra (15) reported that MCF-7 cells do not express caspase-3. Bigelovii A activated caspase-9 instead of caspase-8, indicating mitochondrion-mediated apoptosis.

Bigelovii A induces apoptosis via mitochondrial pathways. To confirm whether the mitochondrial pathway contributed to the induction of apoptosis by Bigelovii A, mitochondrial membrane potential was examined in MCF7 cells. As indicated in Fig. 3, loss of mitochondrial membrane potential was observed following treatment with Bigelovii A for 24 h (8.6, 13.9, 24.2 and 97.3% at 0, 5, 10 and 20 μM Bigelovii A, respectively). To further confirm that Bigelovii A induced mitochondrial damage, the levels of Bcl-2 family proteins (p-Bcl-2, Bcl-2 and Bcl-xl) were measured by western blot analysis. These anti-apoptotic Bcl-2 proteins (p-Bcl-2, Bcl-2 and Bcl-xl) were all markedly decreased following exposure to Bigelovii A for 24 h. In addition, as indicated in Fig. 2D, caspase-9 instead of caspase-8 was activated. Therefore, the apoptosis induced by Bigelovii A was mediated via the mitochondrial pathway.

Apoptosis induced by Bigelovii A involves inhibition of NF-κB. Bcl-2 and Bcl-xl are negative regulators of apoptosis and their overexpression can be induced by NF-κB (16,17). Significantly decreased expression levels of Bcl-2 and Bcl-xl in MCF7 cells were induced by Bigelovii A, suggesting that its pro-apoptotic effect may lead to NF-κB inactivation. In order to investigate the effect of Bigelovii A on the NF-κB pathway, the effect of Bigelovii A on the translocation and phosphorylation at serine residue 536 of p65 were analyzed, since phosphorylation is necessary for the transcriptional activity of p65. Cytoplasmic total p65 level was not altered, while cytoplasmic phosphorylated p65 was decreased. Meanwhile, nuclear total p65 expression and nuclear phosphorylated p65 expression were both reduced (Fig. 4A and B). These results indicated that Bigelovii A inhibited the translocation and phosphorylation of p65. Furthermore, immunocytochemical analysis demonstrated that Bigelovii A inhibited the translocation of p65 to the nucleus in MCF7 cells (Fig. 4C). For cells without any treatment, some p65 was translocated to the nucleus, whereas for cells treated with Bigelovii A, nearly all p65 was fixed in the cytoplasm. These results supported the notion that Bigelovii A inhibited the translocation of p65. Next, in order to investigate the role of Bigelovii A in regulating NF-κB DNA binding activity in MCF-7 breast cancer cells, cells were treated with 5, 10 or 20 μM Bigelovii A for 24 h and EMSA was performed. The results indicated that DNA binding activity of NF-κB decreased as the concentration of Bigelovii A increased (Fig. 4D). Phosphorylation of the inhibitor IκB by IKKs is a vital step for the nuclear translocation and activity of NF-κB (18). Fig. 4E and F indicated that Bigelovii A treatment for 4 h decreased phosphorylation levels of IKKα, IKKβ and IκBα, and increased

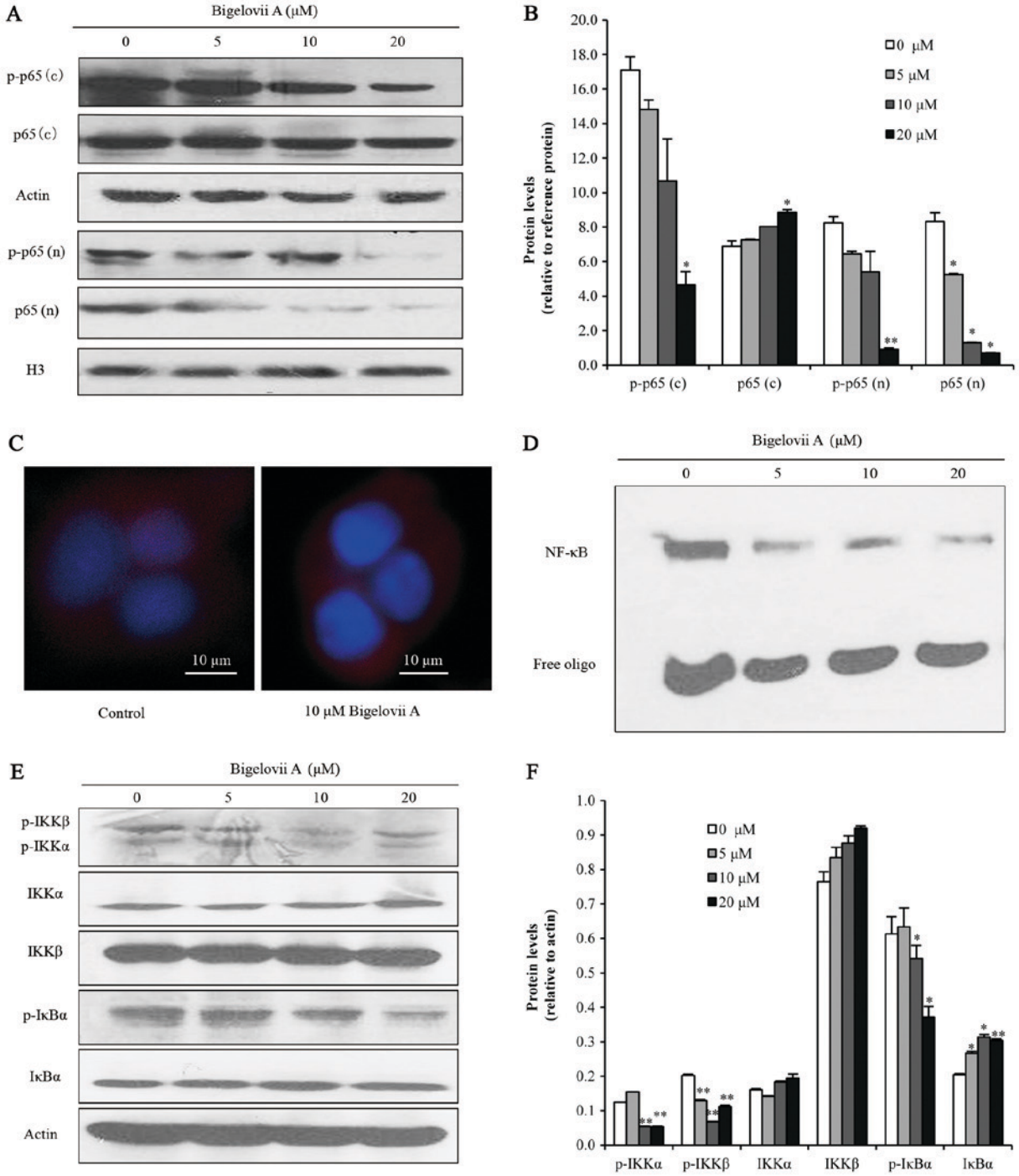

Figure 4. Bigelovii A blocked the IKK/NF-κB pathway. (A and B) Protein levels of p65 and phosphorylated p65 in the cytoplasm (C) and nucleus (N) were analyzed by western blotting and quantified by Image J. For loading control of cytoplasmic and nuclear proteins, the membranes were reblotted with β-actin and H3 antibody, respectively. (C) Nuclear translocation of p65 was analyzed by immunocytochemistry. Blue indicates nuclei and red indicates p65. (D) NF-κB DNA binding activity was assayed by EMSA with nuclear extracts. (E and F) Following treatment with Bigelovii A for 4 h, total protein extracts were analyzed and quantified for the expression levels of p-IKK, IKKα, IKKβ, p-IκBα and IκBα. *P<0.05, **P<0.01 vs. 0 μM. EMSA, electrophoretic mobility shift assay; NF, nuclear factor; IKK, IκB kinase.

the expression levels of IκBα, while there was no effect on the expression levels of IKKα and IKKβ. In addition to Bcl-2 and Bcl-xl, COX-2 and Cyclin D1 are also NF-κB-regulated genes (19,20). It was identified that in MCF7 cells, Bigelovii A decreased protein expression of COX-2 and Cyclin D1 (Fig. 5A). RT-qPCR analysis was performed to further confirm Bcl-2, Bcl-xl, COX-2 and Cyclin D1 gene alterations. As indicated in Fig. 5B, Bigelovii A suppressed Bcl-2, Bcl-xl, COX-2 and Cyclin D1 mRNA levels. Fig. 6 demonstrated the role of Bigelovii A in blocking NF-κB activation and mediating cell apoptosis.

Discussion

Breast cancer is the leading cause of cancer-associated cases of mortality in women worldwide (21). Plant-derived triterpenoids are considered to be promising agents in treating

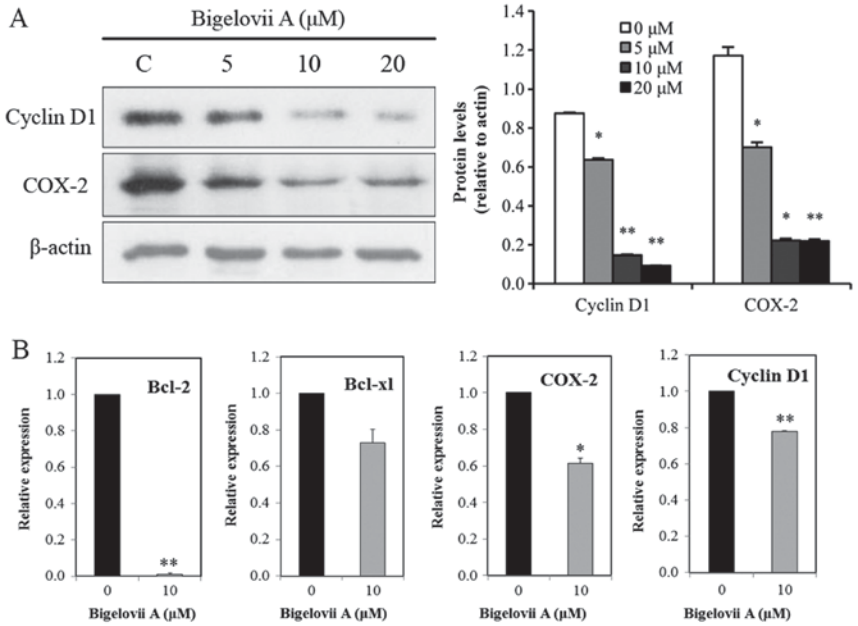

Figure 5. Effects on NF-κB-dependent gene expression of Bigelovii A. (A) Western blotting analysis. Following treatment with Bigelovii A for 24 h, total protein extracts were isolated and subjected to western blotting for Cyclin D1 and COX-2. (B) Effects of Bigelovii A on levels of Bcl-2, Bcl-xl, COX-2 and Cyclin D1 mRNA. Cells were treated with 10 μM Bigelovii A. Total RNA was extracted at 24 h, and was examined by RT-qPCR for Bcl-2, Bcl-xl, COX-2 and Cyclin D1. β-actin was used as an internal control. *P<0.05, **P<0.01 vs. 0 μM. NF, nuclear factor; Bcl-xl, B-cell lymphoma-extra large; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

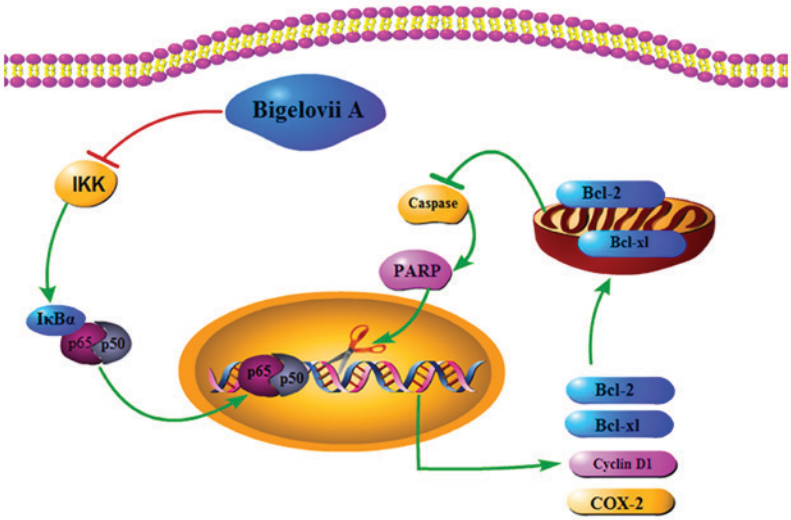

Figure 6. Proposed mechanism of the anti-tumor effects of Bigelovii A. Bigelovii A downregulates the constitutive activation of IκBα kinase and nuclear factor-κB in human breast cancer cells, leading to inhibition of proliferation and induction of apoptosis. Bcl-xl, B-cell lymphoma-extra large; IKK, IκB kinase.

breast cancer (22). In the current study, Bigelovii A, a new triterpenoid isolated from *Salicornia bigelovii* Torr., was shown to inhibit the growth of MCF7 cells in dose-dependent and time-dependent manners. MCF-7 cells were arrested in the G1 phase of the cell cycle and underwent apoptosis in a dose-dependent manner following treatment with Bigelovii A, as indicated by chromatin condensation, externalization of phosphatidylserine on the plasma membrane, hypodiploid DNA, caspase activation and PARP cleavage.

Two apoptotic pathways have been identified in cells, regulated by either the death receptor or mitochondria (23). The mitochondrion transduction pathway is regulated by the Bcl-2 protein family (15). Pro-apoptotic protein Bax is translocated to the mitochondrial outer membrane, after which cytochrome c is released and MMP is disrupted. By contrast, mitochondrial integrity is preserved by anti-apoptotic Bcl-2 in order to inhibit the process. Caspase-9 is recruited and cleaved by mitochondria-dependent death signal, while activation of caspase-2, -8, or -10 is induced by death receptors. These caspases then activate caspase-3, -6 and -7 (24), and PARP is cleaved into p24 and p89, leading to DNA fragmentation and inducing apoptosis (25,26). The current results suggested that Bigelovii A disrupted the mitochondrial membrane potential. Western blotting indicated that Bcl-2 anti-apoptotic protein was

decreased, and caspase-7 and caspase-9, instead of caspase-8, were activated following Bigelovii A treatment. Therefore, Bigelovii A induced apoptosis via the mitochondrion-mediated pathway.

Aberrant NF-κB activation is responsible for the development of various cancer types (5). Inflammatory cytokines, including tumor necrosis factor-α and interleukin-1β, are abundant in breast cancer and activate NF-κB pathway in cancer cells (5,27). The current results indicated that MCF7 cells had the ability to constitutively express active NF-κB, which was consistent with two recent reports by Liu *et al* (28) and Wang *et al* (29), which indicated constitutive NF-κB activation in MCF7 cells on western blot analysis. In the current study, Bigelovii A was demonstrated to inhibit constitutive NF-κB activation in MCF7 cells. These results were in accordance with previous reports from our laboratory (9) and other groups, that triterpenoids are potent inhibitors of NF-κB activation (30). By using antibodies that specifically detect the phosphorylated form of IκBα, we showed that Bigelovii A blocks consitutive phosphorylation of IκBα. The phosphorylation of IκBα is regulated by a large number of kinases including IKK-α, IKK-β, IKK-γ, NIK, TAK1, Akt, and mitogen-activated protein kinase kinase kinase 1 (31). Akt and NIK are primarily known to activate IKK-α, whereas mitogen-activated protein kinase kinase kinase 1 and atypical protein kinase C activate IKK-β. Bigelovii A inhibited IKK activity without directly interfering with the IKK protein. Thus, more detailed studies are warranted to identify one or more of the upstream kinases responsible for IKK activation. Inhibition of NF-κB by Bigelovii A significantly decreased expression of several gene products regulated by NF-κB. The expression of Bcl-2, Bcl-xl, cyclin D1 and COX-2, are known to be regulated by NF-κB, whose synthesis process was inhibited by Bigelovii A. Therefore, it was not surprising to identify that Bigelovii A induced G1 arrest and apoptosis.

In conclusion, Bigelovii A decreased IKK kinase activity, suppressed constitutive IκBα phosphorylation and nuclear p65 expression, and reduced expression of the NF-κB-regulated gene products Bcl-2, Bcl-xl, cyclin D1 and COX-2. These effects inhibited the proliferation of MCF7 cells, arrested cells at the G1 phase boundary of the cell cycle and induced apoptosis.

Acknowledgements

The authors would like to thank Dr Sheng Xu, Dr Lu Yan and Dr Xiaoqin Ding at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences for performing RT-qPCR and statistical analysis.

Funding

The present study was funded by the National Natural Science Foundation of China (grant nos. 81402829, 31470425 and 31570359) and the Jiangsu Provincial Natural Science Foundation of China (grant no. BK20131338).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FQG, YS and XF designed the study. FQG, MY, FL, YYZ and JHZ performed the experiments. FQG, QZW, MW and YC analyzed the data. FQG, MY and FL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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