Bufalin Induces Apoptosis of MDA-MB-231 Cell Through Activation of JNK/p53 Pathway

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Abstract: Cinobufacini has been widely used at oncology clinics in China to treat many kinds of cancers and bufalin is one active compound of it, but the anti-cancer effect and the underlying mechanisms of bufalin on breast cancer cells are still unclear. Our study demonstrated that bufalin could effectively decrease the viability of MDA-MB-231 cells with IC_{50} values of 152.2 ± 8.0 nM for 24 h and 22.4 ± 2.3 nM for 48 h, respectively. Further study showed that bufalin could trigger MDA-MB-231 cells to undergo apoptosis with up-regulating protein expression of p-JNK/JNK, p53, p-ERK/ERK, Puma and down-regulating protein expression of Bcl-2, supporting the possible application of bufalin to breast cancer treatment.

Keywords: Apoptosis, JNK, p53, Bcl-2, MDA-MB-231 cell.

INTRODUCTION

Approved by the Chinese State Food and Drug Administration (SFDA), cinobufacini has been widely used at oncology clinics in China to treat cancer [1], including hepatocellular carcinoma, non-small-cell lung cancer, pancreatic cancer and gallbladder carcinoma [2, 3]. Bufalin, one active compound of cinobufacini, also was reported to have the ability to induce cell cycle arrest and apoptosis of various cancer cells [4-10]. Some molecular mechanisms of bufalin-induced apoptosis had been illustrated, such as MAPK pathway, PI3K/Akt pathway [11]. Masuda *et al.* [12] reported that bufalin (> 0.1 µM) did not induce apoptosis in leukemia M1 cells, and Yongkui [13] reported that bufalin could trigger apoptosis in human leukemia HL-60 cells but not normal human leukocytes. These findings suggested that bufalin might have much less effect on human normal cells or murine cancer cells than human cancer cells, and bufalin might have the potential to become a drug candidate for cancer treatment. In addition to MAPK pathway and PI3K/Akt pathway, bufalin could up-regulate Fas expression to induce apoptosis of androgen-independent DU145 and PC3 cells [14]. Bufalin could significantly induce G_0/G_1 phase arrest in human endometrial and ovarian cancer cells [15], through decreasing cyclinA, cylinD3 expression and increasing $p21^{WAF1}$ expression. Bufalin was also reported to have the potential to induce differentiation of human leukemia cells HL60, U937 and ML1 toward macrophage/monocyte-like cells [16]. Recently, bufalin was found to have the ability to inhibit migration and invasion of bladder carcinoma cell, Osteosarcoma cell and hepatocellular carcinoma cell *in vitro*, through the inhibitions of matrix metalloproteinases and NF/kB signaling pathway [17-19]. But the anti-cancer effect and the underlying mechanisms of bufalin on breast cancer cells are still unclear.

Three most commonly diagnosed cancers among women in America in 2013 are breast, lung and bronchus, and colorectum, respectively [20]. Therefore, it's still urgent to find a way to effectively treat patients with breast cancer. In the present study, we found that bufalin could excellently induce apoptosis, but not cell cycle arrest in MDA-MB-231 cells through regulation of JNK, ERK, P53, Puma and Bcl-2. This suggests that bufalin, as an active compound of cinobufacini, has the potential to be used in breast cancer treatment.

MATERIALS AND METHODS

Cell Lines and Regents

MDA-MB-231 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were

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purchased from Gibco BRL (life technologies, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), ribonuclease A (RNase A) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), were supplied by Sigma (St. Louis, MO, USA). TACS^{TM} Annexin V-FITC/PI staining assay kit was obtained from Trevigen (Gaithersburg, MD, USA). Protease inhibitor cocktail tablets were purchased from Roche Applied Science (Mannheim, Ger). All antibodies were purchased from Cell Signaling Technology (Manchester, NH, US).

Cell Culture

MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (50 μg/ml of each), and grown in 5% CO_2 in air at 37^0C.

Cell Viability Assays

MDA-MB-231 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured overnight. Then cells were treated with various concentrations of bufalin. After 24 or 48 hours treatment, 30 μL of MTT (5 mg/mL, in PBS) was added to each well and incubated for another 4 hours. Then the medium was discarded and 150 μL of DMSO was added to each well in order to dissolve the purple formazan crystals. The absorbance in 570 nm was detected by a microplate reader (BioTek synergy HT). Cells treated with medium with 0.2% DMSO was regarded as negative control. IC_{50} value was determined by software GraphPad Prism 5.0.

DNA Content Analysis

DNA content analysis of MDA-MB-231 cells treated with or without bufalin was performed using a fluorescent probe PI. MDA-MB-231 cells (3×10^5 cells/well) were seeded in 6-well plates cultured overnight and treated with 100 nM bufalin for the indicated time. Then, cells collected and fixed with 75% ethanol overnight were incubated with 50 ng/mL PI staining solution (in PBS) and 0.1 mg/ml Rnase A for 15 min at room temperature in darkness. Using a Guava Easy Cytometer (Guava Technologies, Millipore, Hayward, CA, USA), DNA content of the cells were measured by detecting the red fluorescence intensity. Date was analyzed with ModFit LT software (Becton Dickinson, CA).

Apoptosis Assay with Annexin V-FITC/PI Staining

MDA-MB-231 cells (3×10^5 cells/well) were seeded in 6-well plates and cultured overnight. After that, cells were treated with 100 nM bufalin for the indicated time. TACS^{TM} Annexin V-FITC/PI staining assay kit was used to evaluate the apoptosis rate of MDA-MB-231 cells exposed to bufalin, according to the manufacturer's protocol. The detection of green fluorescence from AnnexinV-FITC and red fluorescence from PI was performed using a Guava Easy Cytometer.

Detection of Mitochondrial Transmembrane Potential (ΔΨm)

JC-1 can exhibit potential-dependent accumulation in mitochondria, showed clearly by a fluorescence emission shift from green (~525 nm) to red (~590 nm). A decrease in the ratio of red/green fluorescence intensity can indicate the depolarization of mitochondria. MDA-MB-231 cells (3×10^5 cells/well) were seeded in 6-well plates and cultured overnight before exposed to 100 nM bufalin for the indicated time. Then, cells were collected gently and incubated with 10 μg/ml JC-1 for 15 min at room temperature in the darkness. JC-1 fluorescence was detected using a Guava Easy Cytometer. Data was analyzed by FlowJo 7.6 software (Tree Star, Inc. USA).

Western Blotting

MDA-MB-231 cells (2×10^6 cells/dish) were seeded in 100 mm culture dishes and cultured overnight followed by bufalin treatment for the indicated time. After trypsinization, cells were transferred to centrifuge tubes and centrifugalized at 800 × g for 3 min. The supernatant was discarded and the cell pellets were washed with ice-cold PBS for two times. RIPA buffer containing 1 mM PMSF, protease inhibitor cocktail tablets and phosphatase inhibitor (Roche) was used to lysis the pellets for 30 min on ice. After the cell lysate was centrifugalized at 12,000 ×g at 4^0C for 15 min, supernatant was collected and stored at -80^0C. Using the BCA protein assay kit, protein concentration was determined. Electrophoresis and immunoblotting analysis was performed as descried previously [21].

Statistical Analysis

Each experiment was performed at least three times and analyzed by GraphPad Prism 5.0 software. The data are presented as means ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

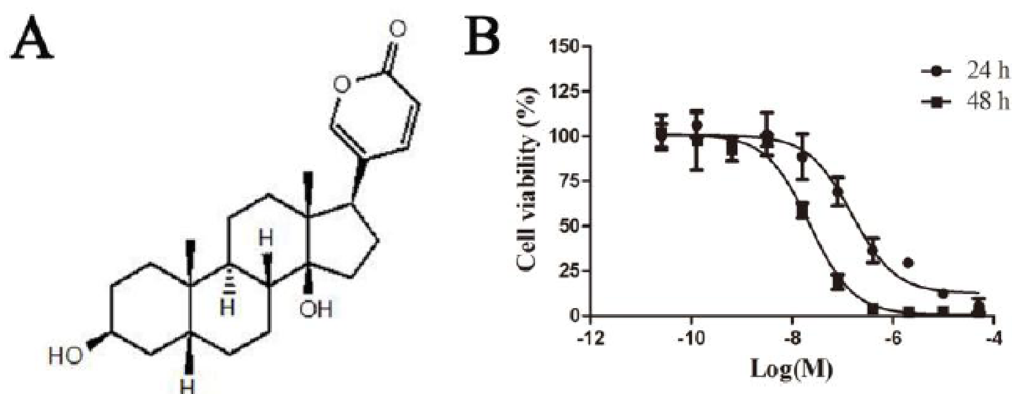

Figure 1: Inhibitory effect of bufalin on the viability of MAD-MB-231 cells.

(**A**) Chemical structure of bufalin. (**B**) MDA-MB-231 cells seeded in 96-well plates were exposed to bufalin at the concentration of 50 μM, 10 μM, 2 μM, 0.4 μM, 80 nM, 16 nM, 3.2 nM and so on for 24 h and 48 h. Cell viability was determined using the MTT assay. Data represents three independent experiments.

RESULTS

Anti-Proliferation Effect of Bufalin on MDA-MB-231 Cells *In Vitro*

Bufalin (Figure **1A**) showed an excellent anti-proliferation effect on MDA-MB-231 cells which were determined by MTT assay (Figure **1B**). Bufalin significantly decreased cell viability of MDA-MB-231 cells in both dose- and time-dependent manner. The IC_{50} values were 152.2 ± 8.0 nM for 24 h and 22.4 ± 2.3 nM for 48 h, respectively.

Apoptosis Induction of MDA-MB-231 Cells by Bufalin

In order to detect weather the anti-proliferation effect of bufalin on MDA-MB-231 cells was caused by its cell cycle arrest effect or not, DNA content assay was performed to detect the change of DNA content of MDA-MB-231 cells. Data showed that bufalin did not significantly induce cell cycle arrest on MDA-MB-231 cells. But MDA-MB-231 cells treated with 100 nM bufalin for 24 h, 36 h strongly showed Sub-G_1 phase (Figure **2A**) indicating that occurrence of DNA fragmentation [22]. It's known that in early phase of apoptosis, phosphatidylserine (PS) translocates from the inner to the outer surface of cellular membrane. To confirm the occurrence of apoptosis, annexin V-FITC/PI double staining assay was performed. Data showed that treated with 100 nM bufalin for 12 h, cell population in early apoptotic phase increased from 4.56% to 22.8% compared with vehicle-treated control (Figure **2B**). Another universal phenomenon occurring in apoptosis is that PARP is cleaved by caspases thereby abolishing PARP's catalytic activity [23]. Confirmed by western blotting, cleaved PARP was strongly showed in MDA-MB-231 cells treated with 100 nM bufalin for 24 h (Figure **2E**).

Decrease of Mitochondrial Transmembrane Potential and Caspases Activation Induced by Bufalin

It's a universal recognition that there're two major pathways which can lead to cell apoptosis, one is called mitochondrial pathway, another is called TNF receptor pathway [24]. JC-1 [25] was used to monitor the change of mitochondrial transmembrane potential of MAD-MB-231 cells treated with bufalin. As showed in Figure **3A**, cells treated with 100 nM bufalin for 6 h and 12 h respectively showed 16.2% and 46.0% cell population with low mitochondrial transmembrane potential, which was much larger than 6.76% in vehicle-treated control. A great number of reports indicate that loss of mitochondrial membrane potential can lead to caspases cascade. Western blotting showed that caspase 9 and caspase 3 were obviously activated in MDA-MB-231 cells treated with bufalin for 12 h, 24 h and 36 h (Figure **3C**).

JNK/p53 Pathway Involved in Bufalin-Induced Apoptosis of MDA-MB-231 Cells

According to the above findings, bufalin could induce the decrease of mitochondrial transmembrane potential. Expression level of proteins related to the regulation of mitochondrial membrane potential in MDA-MB-231 cells treated with bufalin were detected by western blotting. As Bcl-2 family protein are known as mitochondrial functions mediators [26], expression of pro-apoptotic proteins Bax, Puma and anti-apoptotic protein Bcl-2 were determined. The expression level of Puma, but not Bax, was up-regulated and the

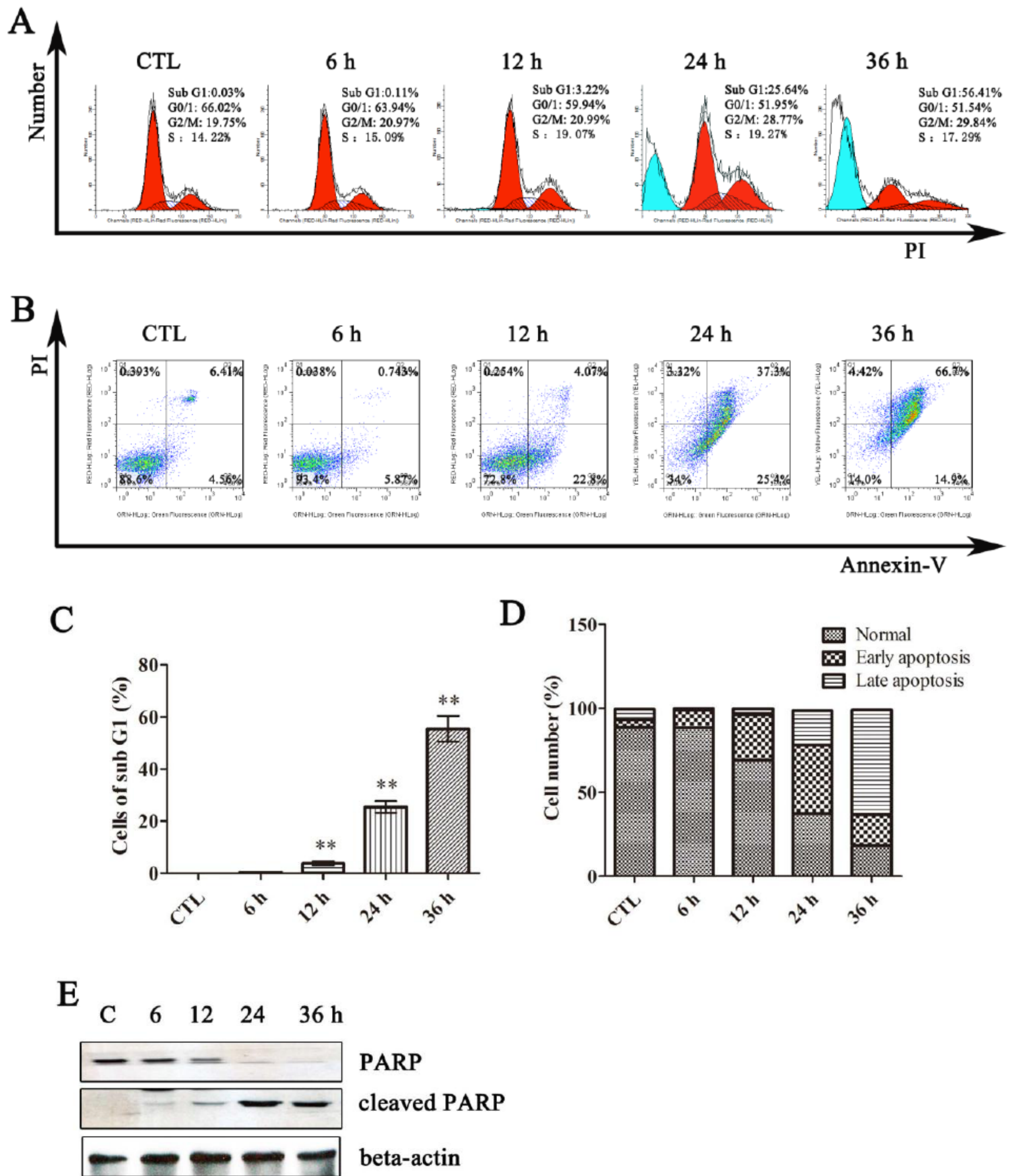

Figure 2: Induction of apoptosis in MDA-MB-231 cells by bufalin.

(**A**) DNA content analysis of MDA-MB-231 cells. Cells treated with 100 nM of bufalin for the indicated time were collected, fixed with 70% ethanol overnight at 4^0C, and then stained with PI for 15 min. Flow cytometric analysis was conducted to determine the DNA content. (**B**) Phosphatidylserine translocation of MDA-MB-231 cells. Cells treated with 100 nM bufalin were collected and stained with PI and Annexin V-FITC in darkness for 15 min and analyzed by flow cytometry. The population in the region of PI $^-$/Annexin V $^+$ was considered to be normal cells, the population in the region of PI $^-$/Annexin V $^+$ was considered to be early apoptotic cells, while that of PI $^+$/Annexin V $^+$ was considered to be late apoptotic cells. (**C**) Quantifications of cells population in Sub-G1 phase, (**D**) Statistical analysis of early/late apoptotic cells induced by bufalin. Each column represents the means ± SD of three independent experiments. **, P < 0.001 versus the control. (**E**) PARP cleavage in MDA-MB-231 cells after 100 nM bufalin treatment.

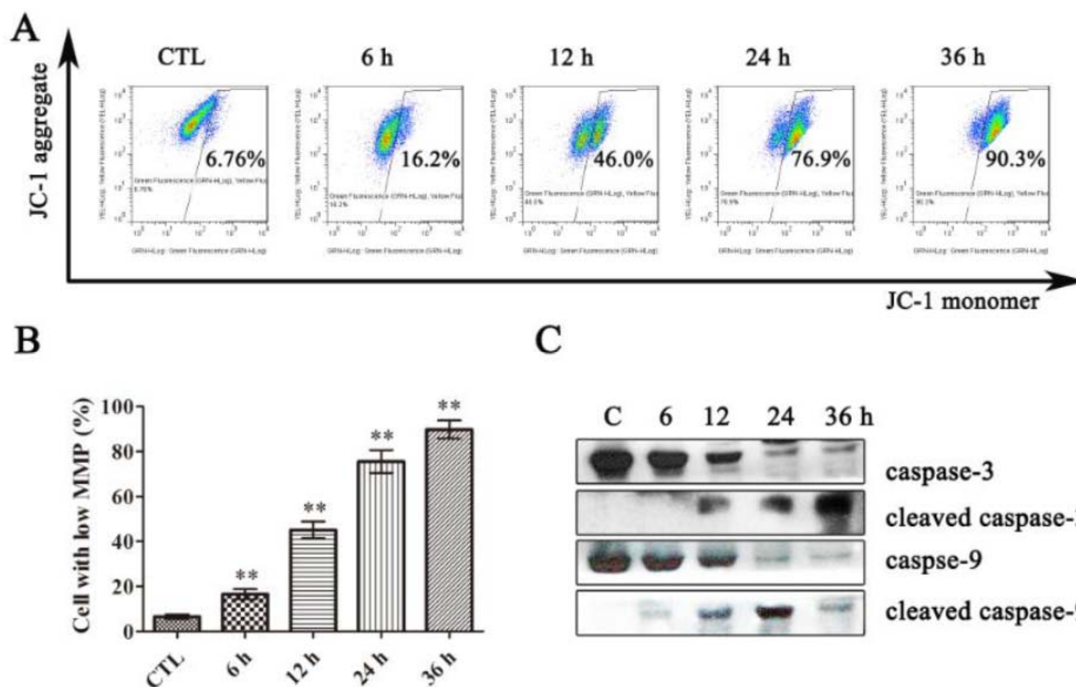

Figure 3: Bufalin induced apoptosis *via* mitochondrial pathway in MDA-MB-231 cells.

(**A**) Decrease of mitochondrial membrane potential in MDA-MB-231cells induced by bufalin. Cells treated with 100 nM bufalin for 6 h, 12 h, 24 h, and 36 h were stained with JC-1 for 15 min and analyzed by flow cytometry. (**B**) Quantifications of cells population with low mitochondrial membrane potential. Each column represents the means ± SD of three independent experiments. **, $P < 0.001$ versus the control. (**C**) Caspase 3, cleaved caspase 3, caspase 9, cleaved caspase 9, Bax and Bcl-2 protein expression level in MDA-MB-231 treated with 100 nM bufalin for the indicated time were detected by western blotting.

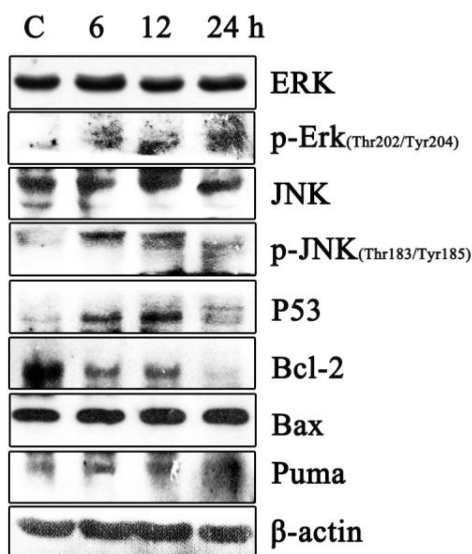

Figure 4: JNK/p53 pathway involved in bufalin-induced apoptosis of MDA-MB-231 cells.

MDA-MB-231 cells treated with 100 nM bufalin for the indicated time were collected and protein extracts were used for western blotting analysis. Protein expression of p-JNK/JNK, p-ERK/ERK, p53 and Puma were up-regulated. Protein expression of Bcl-2 was decreased. Slight change in Bax protein level was shown.

expression level of Bcl-2 was down-regulated (Figure **4**). The expression of p53, which can up-regulate the expression of Puma through transcription regulation [27], and p-JNK, which was reported to have the ability to stabilize p53 [28], were up-regulated after 6 h treatment of 100 nM bufalin (Figure **4**). Additionally, bufalin also increased the ratio of p-ERK/ERK in MDA-MB-231 cells.

DISCUSSION

Bufalin is one representative constituent of bufadienolides which are the major active compounds from the skin of the toad *Bufo bufo gargarizans Cantor*. It's been reported that bufalin can induce apoptosis of various tumor cells, such as gallbladder carcinoma cells [8], osteosarcoma cells [10], lung cancer cells [9], prostate cancer cell PC3 [7] and so on. It has also been found that bufalin can induce cell cycle arrest of hepatocellular carcinoma cells [6], human bladder cancer cells [4] and esophageal squamous carcinoma cells [5]. But there is rare report about bufalin-induced apoptosis or cell cycle arrest of breast carcinoma cells. MTT assay confirmed that MDA-MB-231 cells treated with 152.2 nM bufalin for 24 h, 22.43 nM for 48 h showed 50% cell viability inhibition.

To determine whether bufalin induced apoptosis or cell cycle arrest of MDA-MB-231 cells, DNA content

assay and Annexin V-FITC/PI double staining assay were performed. DNA content assay showed that there was little cells involved in cell cycle arrestwhen MDA-MB-231 cells were treated with bufalin for 6, 12, 24 and 36 h, but 25.64% of bufalin-treated cells at 24 h exhibited DNA fragment indicated by the subG1 phase. Annexin V-FITC/PI double staining assay indicated that 22.8% of MDA-MB-231 cells underwent early apoptosis when treated with 100 nM bufalin for 12 h and cleaved PARP was formed. Hepatocellular carcinoma cells, human bladder cancer cells and esophageal squamous carcinoma cells were reported to show cell cycle arrest induced by bufalin [4-6], but not MDA-MB-231 cells, according to our data. It might be due to that different cells could exhibit different reactions when exposed to the same compound.

Besides extrinsic pathway of apoptosis, mitochondria is one of the most important organelles to regulate cell apoptosis [24]. Mitochondrial transmembrane potential of 16.2% MDA-MB-231 cells exposed to 100 nM bufalin for 6 h was decreased, and the ration was increased up to 46.0% after 12 h treatment. Activated caspase 3 and caspase 9 were obviously detected by western blotting in MDA-MB-231 cells with 12 h bufalin treatment. It's reasonable to speculate that bufalin treatment resulted in decrease of the mitochondrial transmembrane potential and subsequent apoptosis of MDA-MB-231 cells.

Our data proved that bufalin could elevate the expression level of p53, Puma and p-JNK, which indicated activation of JNK and strongly down-regulate the expression of Bcl-2. It's reported that MEKK1/JNK signaling can increase p53 stability and transcriptional activation, thus potentiating the ability of p53 to initiate programmed cell death [28]. The increased expression of p53 may be an outcome of up-regulation of p-JNK by bufalin. Meanwhile, JNK can promote the translocation of endogenous Bax, a pro-apoptosis protein, to mitochondria [29], and accumulation of integral mitochondrial membrane Bax can enhance cytochrome *c* release to cytoplasm [30], causing the activation of caspases. Furthermore, direct activation of Bax by p53 can also enhance mitochondrial membrane permeabilization and apoptosis [31]. Thus, the up-regulating of p-JNK and p53 by bufalin might contribute to the apoptosis of MDA-MB-231 cells through enhancing mitochondria translocation of Bax. Down-regulated Bcl-2 and up-regulated Puma, a transcription target of p53, might also participate in the apoptosis of MDA-MB-231 treated with bufalin since Puma was reported to has the ability to bind to Bcl-2, and then locate to mitochondria to induce cytochrome *c* release [27]. Although activation of ERK is generally considered to promote cell proliferation and inhibit apoptosis, our data shows persistent activation of ERK1/2 in bufalin-treated MDA-MB-231 cells. Watabe *et al.* [32] also reported that abnormal and continuous activation of ERK was involved in the apoptosis of U937 cells induced by bufalin. So, whether the activation of ERK participated in the apoptosis of MDA-MB-231 cells treated by bufalin or not is still unclear and further investigation is still needed.

In conclusion, our study demonstrated that bufalin (100 nM) could effectively trigger MDA-MB-231 cells to undergo apoptosis with up-regulating protein expression of p-JNK/JNK, p53, p-ERK/ERK, Puma and down-regulating protein expression of Bcl-2, supporting the possible application of bufalin to breast cancer treatment.

CONFLICTS OF INTEREST STATEMENT

There are no conflicts of interest.

ACKNOWLEDGEMENTS

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