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Original Article

Bufalin attenuates the proliferation of breast cancer MCF-7 cells *in vitro* and *in vivo* by inhibiting the PI3K/Akt pathway

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Abstract: The traditional Chinese medicine bufalin, extracted from toad's skin, has been demonstrated to exert anticancer activities in various kinds of human cancers. In the present study, we used MCF-7 human breast cancer cells as the experimental model to evaluate the potential of bufalin in breast cancer chemotherapy. MCF-7 cells were treated with bufalin, then the proliferation was detected by CCK8 assay and apoptosis was detected by flow cytometry analysis. In addition, MCF-7 cells were treated by Akt inhibitor LY294002 in combination with bufalin and the activation of Akt as well as the expression levels of Bax, Bcl-2 were examined by Western blot analysis. The results showed that bufalin inhibits the activation of PI3K/Akt pathway to regulate the expression of pro-apoptotic and anti-apoptotic proteins, leading to the execution of apoptosis. We confirmed that bufalin suppresses breast cancer growth in tumor xenograft mice. These results suggest that bufalin may have potential applications in the treatment of Breast cancer.

Keywords: Breast cancer, proliferation, bufalin, PI3K/Akt, MCF-7 cells

Introduction

Breast cancer is the second leading cause of cancer-related deaths among women in American and northwestern European women [1]. Its rate in China and other Asian countries is also increasing rapidly [2]. It is important to find novel natural compounds with high selectivity and low toxicity. To date, chemotherapy has been the most frequently used treatment for breast cancer and other cancers. However, some normal cells are destroyed by this method of treatment as well [3]. Due to their low toxicity and wide range of biological activities, some natural products have been used as alternative treatments for cancers including breast cancer.

Bufalin is a class of toxic steroids purified from Chinese traditional medicine chan'su [4, 5]. Several studies have shown that Bufalin has been used to treat various cancers including breast cancer. However, the mechanisms of action of bufalin against cancers such as human breast cancer are largely unknown.

Resistance to apoptosis is an important hallmark of tumor cells [6]. Apoptosis of tumor cells is known to be regulated by several apoptotic pathways such as PI3K/Akt pathway. Akt has been shown to regulate apoptosis related proteins such as Bcl-2, Bax and is crucially involved in anticancer drug induced apoptosis of cancer cells [7].

In this study, we demonstrate that the PI3K/Akt pathway modulates bufalin-induced apoptosis in MCF-7 breast cancer cells and affects Bcl-2, Bax expression, suggesting its potential application in breast cancer chemotherapy. This study further elaborates the mechanisms of bufalin-induced apoptosis in breast cancer cells and explored potential biological targets and signaling pathways.

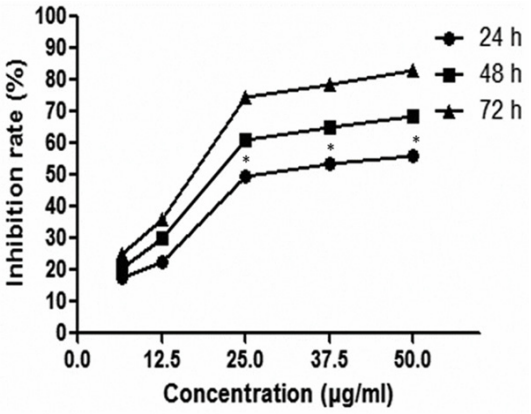

Figure 1. CCK8 assay of bufalin on MCF-7 cells proliferation.

Materials and methods

Reagents and antibodies

Bufalin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in ethylalcohol to make 0.01 mol/L stock solution, which was kept at -20°C and diluted in phosphate buffer saline (PBS) when used. LY294002 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in DMSO to make 0.01 mol/L stock solution, which was kept at 4°C. Mouse antibodies specific for Caspase-3, Bcl-2, p-Akt, Akt, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology. TACS™ Annexin V-FITC/PI staining assay kit was obtained from Trevigen Inc (Gaithersburg, MD. USA).

Cell culture and treatments

MCF-7 was originated from the American Type Culture Collection. Cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FCS) and 1% penicillin/streptomycin. Cells were incubated in the humidified incubator at 37°C with 5% CO_2. MCF-7 cells at exponential growth stage were employed in all of the experiments.

Flow cytometry analysis

Apoptosis was quantified with annexin V-FITC/PI double staining kit (Beyotime, China). After treatment, 1×10^6 cells were harvested and washed in PBS, then responded in 500 μl annexin binding buffer. Annexin V-FITC and propidium iodide (PI) were then added to the cells. The cell mixture was then incubated in the dark at room temperature for 15 min according to the manufacturer's instructions. Apoptosis was measured using a flow cytometer (Becton Dickinson, USA). Annexin V single positive cells were identified as early apoptotic cells. PI single positive cells were identified as necrotic cells. Annexin V/PI double positive cells were identified as late apoptotic cells.

Cell proliferation and viability assay

The Cell Counting Kit 8 (CCK-8; Dojindo, Kumamoto, Japan) is a sensitive nonradioactive colorimetric assay for determining cell growth [8]. Briefly, control and treated MCF-7 cells were seeded at 5×10^3 cells/ml in 96-well microplates and allowed to attach for 24 h, 48 h, 72 h. CCK8 (10 μl) was added to each well and incubated for 3 h at 37°C, then cell proliferation and cytotoxicity were assessed by measuring the absorbance at 450 nm using microplate reader (BioTek, USA).

Western blot analysis

Western blot analysis was performed using standard techniques as described previously. Briefly, the cells were lysed in RIPA buffer for 40 min on the ice. Lysates were collected after centrifuging at 12,000 rpm for 20 min at 4°C. Protein concentrations were quantified using the bicinchoninic acid (BCA) method with the protein assay kit (Thermo Scientific, Rockford, IL). Equivalent amounts of protein (50 ug/lane) were separated by 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and eletrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in PBS containing 5% nonfat dry milk (w/v) for 1 h, and then incubated with primary antibodies overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. After incubation with antibodies, specific bands of interest were detected by chemiluminescence (Amersham ECL Plus; GE Healthcare, Uppsala, Sweden) and exposed to Hyperfilm (Amersham, Arlington Heights, IL, USA).

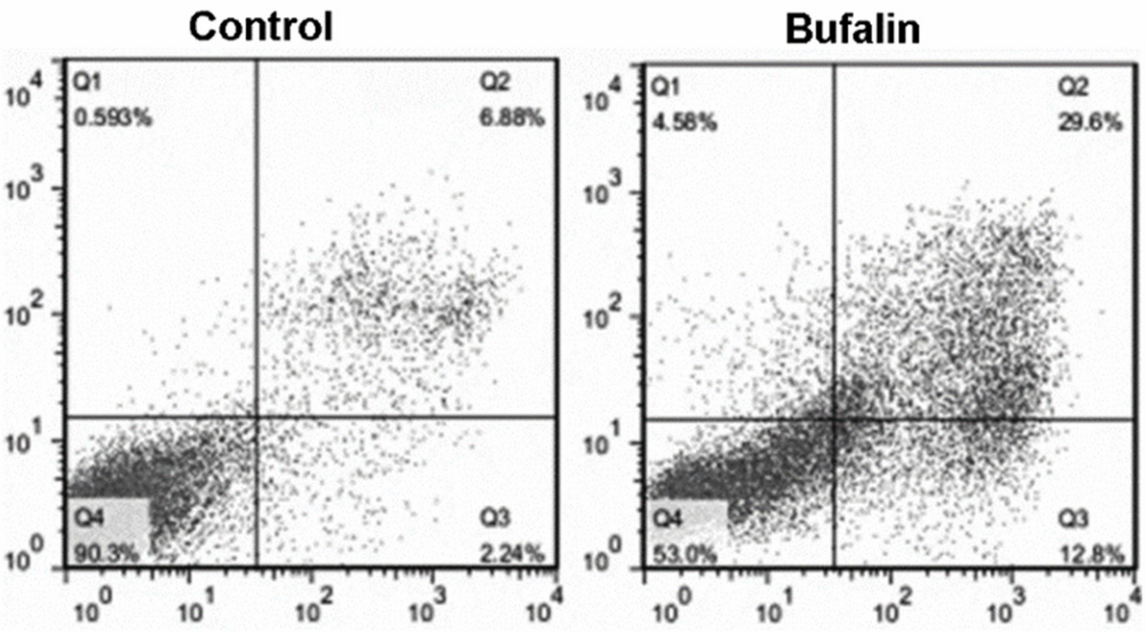

Figure 2. Flow cytometry analysis of bufaln-induced MCF-7 cell apoptosis.

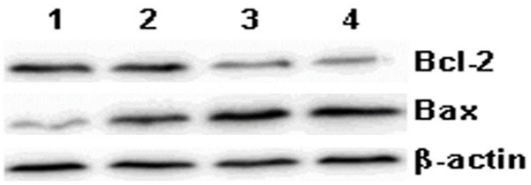

Figure 3. Bufalin regulates the expression of apoptosis related proteins in MCF-7 cells. Western blot analysis Bcl-2 and Bax protein level in MCF-7 cells treated with 12.5 ug/ml bufalin for different time: 1. 0 h; 2. 12 h; 3. 24 h; 4. 48 h. B-actin served as loading control.

In vivo experiments

Pathogen-free male BALB/C nude mice (aged 4-5 wk, SPF grade and weighing 18-20 g) were purchased from the center of experimental animal, the Academy of Military Medical Science (Beijing, China). All animal experiments and protocols were performed strictly in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals were kept and the experiments were performed in accordance with committee's criteria for the care and use of laboratory animals. Approval for animal experiments was obtained from the institutional animal welfare committee. MCF-7 cells (1×10^7) in 0.2 ml of serum-free medium were injected subcutaneously into the right flanks of the mice, Ten days after the injection of the cells, mice were randomly divided into treatment and control groups. The animals were intraperitoneally treated with bufalin (1 mg/kg, intraperitoneally daily for 20 days), whereas control animals were treated with an equal volume of PBS. The body weight and tumor size were measured every 4 days, and the tumor volume was determined by caliper measurements and calculated using the following formula: length × width^2/2. After 20 days of the treatment, the mice were sacrificed and the tumors were removed.

Statistical analysis

All values are reported as mean ± SE. Where appropriate, differences were compared using Student's t-tests or analyses of variance. A value < 0.05 was considered statistical significance. All statistical analyses were carried out with the GraphPad Prism software version 5.01 (GraphPad, San Diego, CA).

Results

Bufalin inhibits the proliferation of MCF-7 cells

To investigate the effects of bufalin on MCF-7 cells, we tested the effect of various doses of bufalin on the viability of MCF-7 cells using CCK8 assay. The cells were treated with 0, 6.5, 12.5, 25, 37, 50 μg/ml bufalin, after incubation

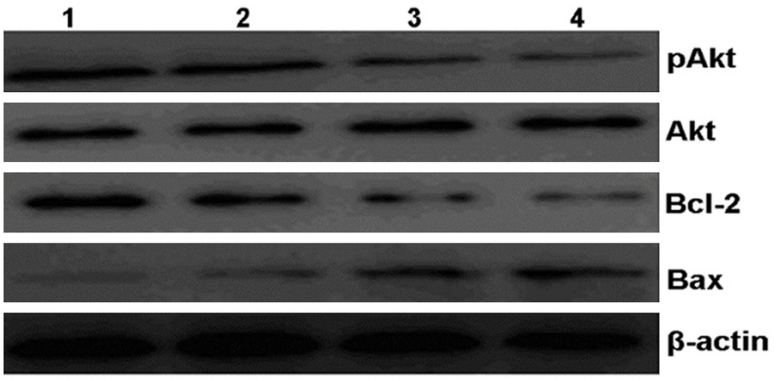

Figure 4. Bufalin modulates the activation of PI3K/Akt apthway in MCF-7 cells. Western blot analysis of the levels of Bcl-2 and Bax in differently treated MCF-7 cells: 1.0 h; 2. 25 umol/L LY294002 for 2 h; 3. 12.5 ug/ml bufalin for 24 h; 4. 2.25 umol/L LY294002 for 2 h plus 12.5 ug/ml bufalin for 24 h.

for 24, 48, 72 h, as shown in **Figure 1**, cell viability was inhibited by bufalin in a dose and time dependent manner. Bufalin at concentrations below 12.5 μg/ml had minor toxicity for MCF-7 cells while bufalin between 12.5-25 μg/ml significantly inhibited MCF-7 cells proliferation. Therefore, we used 12.5 μg/ml bufalin in the following experiments.

Bufalin induces apoptosis in MCF-7 cells

To evaluate the effect of bufalin on MCF-7 cell apoptosis, we used Annexin V-FITC and PI fluorescence staining. Annexin V-FITC and PI signals were visible in response to 12.5 μg/ml for 48 h, while could barely be detected in vehicle control cells. These findings suggested that bufalin is capable of inducing cancer cell apoptosis, in particular for Breast cancer (**Figure 2**).

Bufalin regulates the expression of apoptosis related proteins in MCF-7 cells

To explore the molecular mechanism by which bufalin induces the apoptosis of MCF-7 cells. MCF-7 cells were treated with 12.5 μg/ml bufalin for 0 h, 12 h, 24 h, 48 h and subjected to Western blot analysis. These results suggested that bufalin decreased Bcl-2 expression while increased Bax expression at protein level in a time dependent manner (**Figure 3**).

Bufalin modulates the activation of PI3K/Akt pathway in MCF-7 cells

We detected the activation of Akt in MCF-7 cells treated with 12.5 μg/ml bufalin. Western blot analysis showed that, compared to control cells, the level of p-Akt was reduced in bufalin treated cells, although the level of Akt remained the same, and also demonstrated that specific Akt inhibitor LY294002 pretreatment (25 μmol/L for 2 h) could synergize with bufalin treatment to up-regulated Bax expression, down-regulate Bcl-2 expression, and promote the activation of Caspase-3 (**Figure 4**). In addition, we found that pretreatment with LY294002 (25 μmol/L) could synergize bufalin treatment (12.5 μg/ml) to induce the apoptosis of MCF-7 cells as assessed by flow cytometry analysis (**Figure 5**). These results suggest that bufalin could inhibit the activation of PI3K/Akt pathway to induce the apoptosis of MCF-7 cells.

Effect of bufalin on tumor growth in vivo

The antitumor effect of bufalin was tested in nude mice bearing tumor. Compared with the control group, bufalin significantly inhibited tumor growth in a dose-dependent manner. At the end of treatment, the tumor volume of nude mice in the bufalin group was significantly less than that in the control group ($P < 0.05$). Additional, the average weight of the tumor was also significantly lower in the treated mice compared to the control mice (not shown). However, bufalin administration did not have an effect on the overall body weight during the experimental period, suggesting no apparent toxicity (**Figure 6**). These results indicated that bufalin administration inhibited growth of the MCF-7 xenograft tumors.

Discussion

Apoptosis is a cell defense mechanism to eliminate malignant cells and plays an important role in preventing tumor development [9]. In fact, many anti-cancer drugs function primarily to induce apoptosis through regulating apoptosis-associated signaling [10]. Bcl-2 family members are known to regulate the mitochondrial apoptosis pathway [11]. The balance between pro- and anti-apoptotic proteins is thought to be critical to cell survival and apoptosis [12]. Bcl-2 exerts anti-apoptotic activity by inhibiting the pro-apoptotic molecule Bax to maintain the

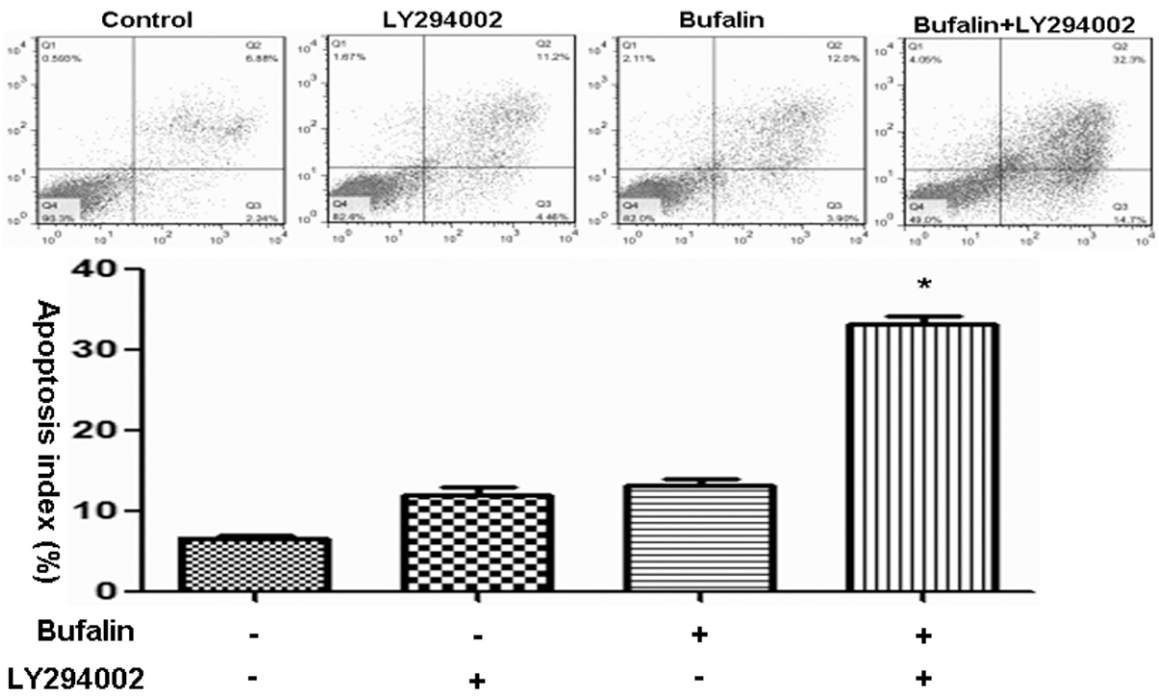

Figure 5. Flow cytometry analysis of bufaln-induced MCF-7 cell apoptosis. MCF-7 cells were pretreated with 25 umol/L LY294002 for 2 h followed by treatment with bufalin as indicated and the apoptosis was examined by flow cytometry. *P < 0.01. vs left three groups of cells.

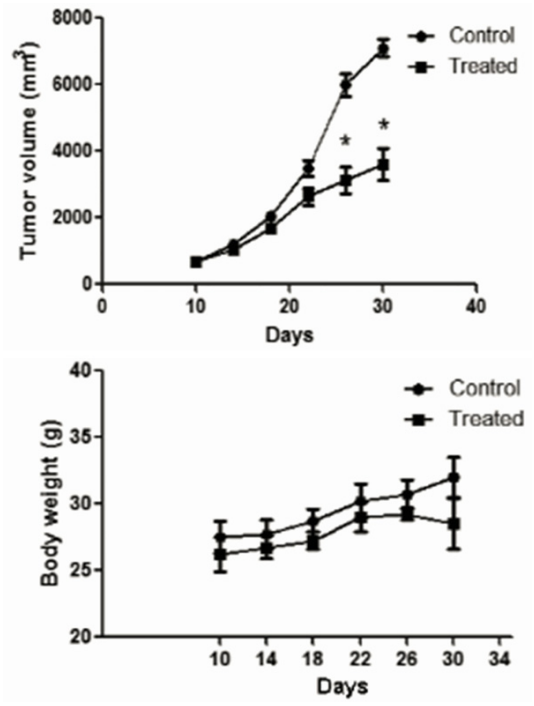

Figure 6. Effect of bufalin on tumor growth in xenografted nude mice. The mice were subcutaneously injected with MCF-7 cells and intraperitoneally administered with bufalin. Tumor volumes were measured with a caliper every fourth days, and the body weight changes were monitored during the test period.

integrity of the mitochondrial outer membrane and prevent the release of Cytochrome c and other apoptotic factors, while Bax exerts pro-apoptotic activity by translocation from the sytosol to the mitochondria, where it induces Cyt-c release [13].

In this study we demonstrate that bufalin treated MCF-7 breast cancer cells exhibited significantly higher rates of apoptosis than control treated cells. These results were consistent with the CCK8 assay showing that bufalin inhibited the proliferation of MCF-7 cells in a dose and time dependent manner. Therefore, we concluded that the loss of viability of MCF-7 cells treated by bufalin is partly due to the induction of apoptotic cell death. Notably, we found that bufalin induced apoptosis is associated with the down-regulation of Bcl-2 expression and up-regulation of Bax expression in MCF-7 cells. In vivo experiments, we also found that the bufalin significantly inhibited tumor growth in a dose-dependent manner.

PI3K/Akt pathway plays an essential role in mammalian cell survival and resistance to apoptosis [14]. PI3K/Akt signaling has been shown to be activated in a variety of cancers and activated Akt acts to phosphorylate Bad

and Caspase-9 to promote the resistance of cancer cells to apoptosis [15, 16]. However, the functional role of PI3K/Akt pathway in bufalin induced cancer cell apoptosis remains largely unclear. In the present study we found that bufalin treatment reduced the level of phosphorylated Akt, indicating the inhibition of Akt activation. Furthermore, by employing Akt specific inhibitor LY294002 we could demonstrate the synergistic effects of bufalin and LY294002 on the induction of apoptosis and regulation of apoptosis related proteins in MCF-7 cells. The expression of anti-apoptotic molecules such as Bcl-2 was substantially down-regulated by pretreatment with LY294002 followed by bufalin treatment of MCF-7 cells, while the expression of pro-apoptotic protein Bax was substantially up-regulated. Collectively, these results suggest that bufalin inhibits the activation of PI3K/Akt pathway to promote the apoptosis of cancer cells. However, further studies are needed to elucidate the mechanism by which bufalin modulates the activation of PI3K/Akt and other signaling pathways that are crucial for cancer cell survival and chemoresistance.

In conclusion, the results of this study indicate that bufalin can inhibit the proliferation of MCF-7 cells by inducing the apoptosis in a dose and time dependent manner. Mechanistically, we found that bufalin inhibits the activation of PI3K/Akt pathway to regulate the expression of pro-apoptotic and anti-apoptotic proteins, leading to the execution of apoptosis. Interestingly, the marked ability of bufalin to synergize with Akt inhibitor to induce cancer cell apoptosis suggests that bufalin is a potential regimen for combined chemotherapy to overcome the resistance of cancer cells to chemotherapeutics induced apoptosis. In vivo experiments, we also found that the bufalin significantly inhibited tumor growth in a dose-dependent manner. These results suggest that bufalin may have potential applications in the treatment of Breast cancer.

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Disclosure of conflict of interest

None.

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