

RESEARCH ARTICLE

Bufalin Induces Mitochondrial Pathway-Mediated Apoptosis in Lung Adenocarcinoma Cells

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Abstract

Background: To evaluate the effects of bufalin in A549 human lung adenocarcinoma epithelial cells in vitro and assess the underlying mechanisms. **Materials and Methods:** Human A549 non-small cell lung cancer (NSCLC) cells were treated with various concentrations of bufalin. Cell proliferation was measured by CCK-8 assay, apoptotic cell percentage was calculated by flow cytometry and morphological change was observed by inverted phase contrast microscopy/transmission electron microscopy. In addition, the membrane potential of mitochondria was detected by JC-1 fluorescence microscopy assay, and the related protein expression of cytochrome C and caspase-3 was analyzed by Western blotting. **Results:** Bufalin could inhibit the proliferation of A549 cells via induction of apoptosis, with the evidence of characteristic morphological changes in the nucleus and mitochondria. Furthermore, bufalin decreased the mitochondrial membrane potential with up-regulation of cytochrome C in the cytosol, and activation of caspase-3. **Conclusions:** Bufalin inhibits the proliferation of A549 cells and triggers mitochondria-dependent apoptosis, pointing to therapeutic application for NSCLC.

Keywords: Bufalin-lung cancer - A549 lung cancer cells - apoptosis - mitochondrial pathway

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Introduction

Lung cancer is one of the common diseases worldwide, also has the highest incidence and mortality rate of all malignant tumors (Jemal et al., 2011). On exploration of lung cancer, no matter base to clinic or diagnosis to treatment, researchers have paid more and more attentions. To improve the overall survival rate, treatments perform their own duties effectively such as surgery, radiotherapy, chemotherapy, targeted-therapy and so on. Currently, chemotherapy is still considered a mainstay of medical treatment. However, chemotherapy has reached a plateau of potency due to mutidrug resistance and complications related (Stinchcombe and Socinski, 2009). Therefore, the development of new chemotherapeutic agents with high effect and low toxicity against lung cancer is significant.

Bufalin is the major digoxin-like immunoreactive component of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of the toad (Krenn and Kopp, 1998), the molecular formula of which is C₂₄H₃₄O₄ with a relative molecular weight 386.5g/mol. The chemical structure of bufalin is shown in Figure 1A. Bufalin exhibits a variety of biological activities in pharmacological studies, such as cardiotoxic, anesthetic, blood pressure stimulation and analgesic (Takai et al., 2012). Recently bufalin has demonstrated its anti-tumor activity excellently in various cancer cells

(Yin et al., 2012), such as human gastric (Li et al., 2009), hepatocellular (Qi et al., 2011), endometrial and ovarian (Takai et al., 2008), leukemia (Chen et al., 2009) and other malignant cells (Xie et al., 2011). However, whether or not do bufalin inhibit the proliferation of human lung cancer cells and its molecular mechanisms are seldom reported. The aim of the present study was to provide insights into the antineoplastic activity and molecular mechanisms of bufalin against A549 cells, with emphasis on its ability to mediate intrinsic signaling pathway responsible for apoptosis, a relevant target in cancer prevention.

Materials and Methods

Materials and reagents

Human non-small cell lung cancer (NSCLC) cell line A549 was purchased from Shanghai cell bank of Chinese academy of sciences (Shanghai, China). Fetal bovine serum (FBS) and RPMI 1640 medium were obtained from Hyclone (Utah, USA). Bufalin and Cisplatin (DDP) as positive control drug were both provided by Dalian Meilun Biology Technology Co.,Ltd. (Liaoning, China), and dissolved in dimethylsulfoxide (DMSO), diluted with RPMI 1640, then filtered through a 0.22µm microfiltration membrane. Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Tokyo, Japan). Annexin V-FITC/PI apoptosis detection kit was obtained

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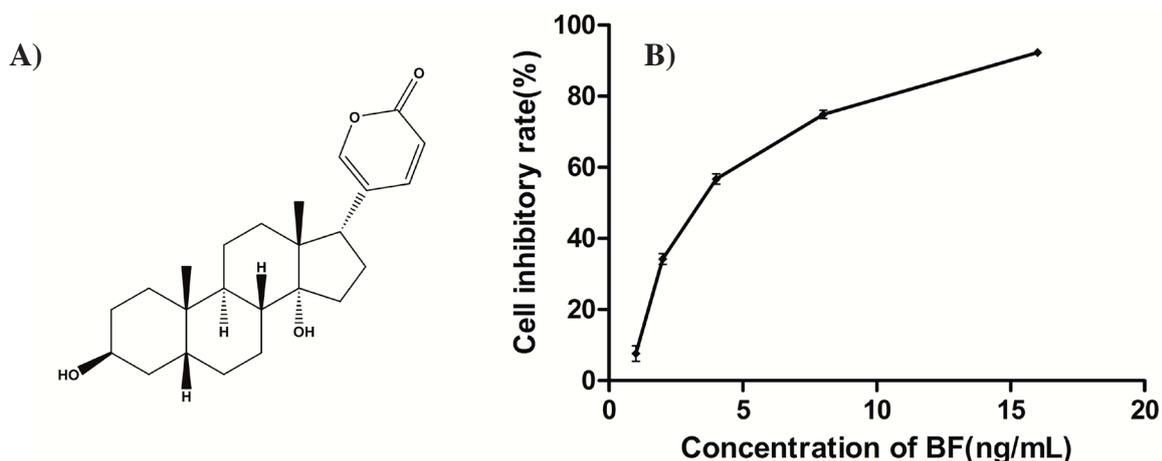


Figure 1. A) Chemical Structure of Bufalin (CAS No:465-21-4). B) The Inhibitory Effect of Bufalin on A549 Cells. Cells were exposed to 0, 1, 2, 4, 8 and 16 ng/mL of bufalin for 48h. The cell viability was measured by CCK-8 assay. Data were represented as mean \pm SD of three separate experiments

from GIBCO (California, USA) and mitochondrial membrane potential assay kit with JC-1 from KeyGEN Biotechnology Co., Ltd. (Nanjing, China). Antibodies against Cytochrome C (Cyt C), GAPDH and horseradish peroxidase conjugated secondary antibody were purchased from Bioworld (Minnesota, USA), and antibody against caspase-3 from CST (Danvers, USA).

Cell culture

Cells were cultured in RPMI 1640 medium containing 10% FBS, 100U/mL penicillin and 100mg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. The medium should be changed every 48h. Cells for further analysis were detached and harvested by a solution of 0.25% trypsin and 0.02% EDTA.

CCK-8 assay

The effect of bufalin on A549 cell proliferation was examined by measuring the CCK-8 dye absorbance of living cells. Briefly, 6×10^3 cells at logarithmic phase per well were seeded into a 96-well plate. After incubation for adherence, cells were exposed to different concentrations of bufalin (0, 1, 2, 4, 8 and 16 ng/ml) for 48h respectively, and DDP (4 μ g/ml) as positive control group. All exposures were performed in 5 wells. After treatment, 10 μ l of the CCK-8 solution was added into each well, followed by incubation for 1 h at 37°C. The value of optical density (OD) was measured at 450 nm with a 96-well multiscanner autoreader (Thermo Electron Corp, Waltham, MA, USA). The growth inhibitory rate was calculated according to the following formula: Inhibitory rate (%) = $(OD_{\text{control}} - OD_{\text{treated}}) / OD_{\text{control}} \times 100\%$. The procedure was repeated three times.

Flow cytometric analysis

The extent of apoptosis was analyzed by Annexin V-FITC/PI kit through flow cytometer (FCM). First, cells were seeded at 18×10^4 cells/well in a 6-well plate, and then exposed to varying concentration of bufalin (2, 4 and 8 ng/ml) for 48h. Subsequently, cells were harvested by trypsin without EDTA and washed twice with ice-cold PBS, pelleted and resuspended in 500 μ l binding buffer. After

the addition of 5 μ l Annexin V-FITC conjugate and 10 μ l PI buffer, cells were incubated at room temperature for 10 min in the dark. The samples were analyzed using BD Accuri[®] C6 flow cytometer (Becton, Dickinson and Company, New Jersey, USA). Positive cells were incubated with DDP (4 μ g/ml) and control cells with medium only. Each experiment was repeated three times.

Morphological changes in cell and mitochondria

A549 cells incubated adherently were treated with 0, 1, 2, 4, 8 and 16 ng/ml of bufalin for 48h, and then examined and photographed by inverted phase contrast microscope at 200 \times . Apoptotic morphology of cells can be observed directly using transmission electron microscopy (TEM) (PHILIPS, Amsterdam, Holland). Briefly, cells treated with 4 ng/ml bufalin for 48h were harvested and fixed in 2.5% glutaraldehyde overnight at 4°C. After removal of the fixative, each cell mass was washed twice with cold PBS, then dehydrated in graded ethanol, immersed in propylene oxide and embedded in epoxy resin. Ultra thin sections were double-stained with lead citrate/uranyl acetate previously and examined by TEM.

Measurement of the mitochondrial membrane potential ($\Delta\psi_m$)

JC-1 mitochondrial membrane potential detection assay kit is for detection of mitochondrial depolarization during the early stages of apoptosis. A549 cells were treated with 4 ng/ml bufalin for 48h and control cells were treated with medium only. Consequently, cells were harvested and washed twice with cold PBS. Followed by addition of 100 μ l JC-1 dye, cells were incubated at room temperature for 15 min and resuspended again. $\Delta\psi_m$ can be measured by JC-1 dye, in which mitochondria depolarization was indicated by a switch from red to green fluorescence intensity. Pictures were taken under a fluorescent microscope (Olympus, Tokyo, Japan).

Western blot analysis

A549 cells were treated with DMSO and different concentrations (2, 4, 8 ng/ml and 4 ng/ml + Z-VAD-FMK) of bufalin. After 48h of incubation, cells were

collected and lysates were analyzed by western blot. In brief, the harvested cells were lysed with cell lysis buffer in an ice-cold tissue grinder. The products were transferred and centrifuged at 12,000 rpm for 10 min at 4°C to gather supernatant which used as the total cell protein. For Cyt C detection, the collected cells acted with mitochondria isolation kit to obtain the cytosolic component. Concentration of the resultant protein can be detected by BCA protein assay kit. These samples were added into 5×SDS sample buffer and heated to 100°C for 5 min. Equal amounts of sample-protein were electrophoresed on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were then blocked for 1 h at room temperature with 5% nonfat dry milk/TBST, followed by incubation with primary antibodies for Cyt C, caspase-3 (at 1:1000 dilution) overnight at 4°C. Consequently, membranes were washed three times for 10 min in TBST at room temperature, and incubated with horseradish peroxidase-conjugated secondary antibodies (at 1:5000 dilution) for 1 h at room temperature. The immunoreactive bands were visualized by incubation with ECL, followed by exposure to radiograph film. Each experiment was repeated twice and antibody for GAPDH acted as an internal loading.

Statistical analysis

Statistical analyses were performed using the SPSS19.0 software package. The results were expressed as mean±standard deviation (SD). Statistical differences between groups were performed with one-way analysis of variance (ANOVA) and the Student's t-test. A P-value of 0.05 or less was considered statistically significant.

Results

Bufalin inhibited the proliferation of A549 cells in vitro

CCK-8 was used to evaluate the anti-proliferation effect of bufalin on A549 cells *in vitro*. The results showed that the effect of growth inhibition was enhanced along with the increasing concentration from 1 to 16 ng/ml of bufalin ($p < 0.05$). The exhibition of dose-response relationship could be observed in Figure 1B.

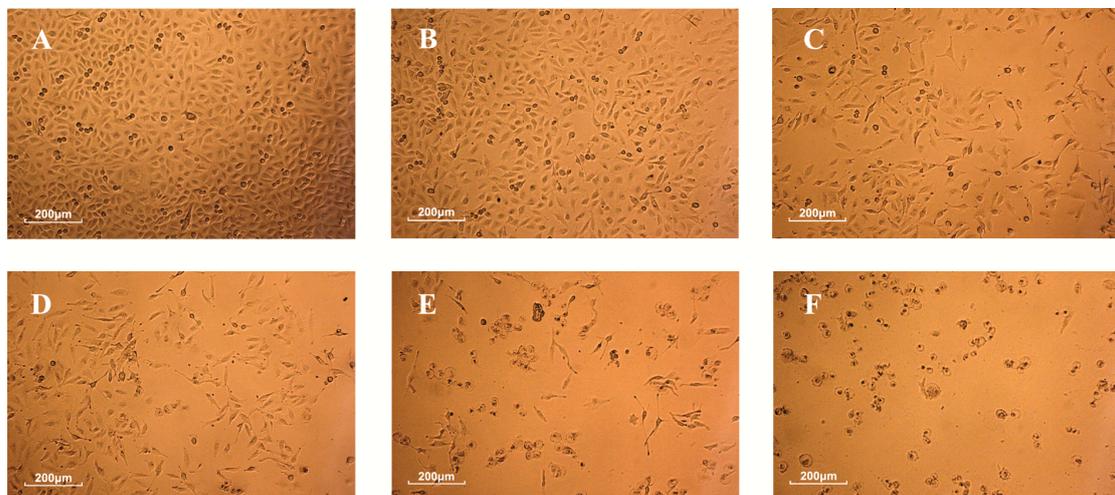


Figure 3. Micrographs of A549 Cells Treated With Various Concentrations of Bufalin. The morphology of cells were dramatically altered with increasing doses (0, 1, 2, 4, 8, 16 ng/mL for A, B, C, D, E, F respectively) of bufalin. Cells were observed using inverted microscopy at 200×

Bufalin induced cellular apoptosis in A549 cells

Apoptosis induced by bufalin was further evaluated using Annexin V-FITC/PI staining assay. Results assessed by FCM showed bufalin a clear potency of promoting apoptosis on A549 cells. As displayed in Figure 2, the proportion of Annexin V-positive/PI-negative cells increased from 21.8% to 37.4% after incubation with increasing dose of bufalin for 48h, which were significantly different from 4.1% in the control group ($p < 0.05$). DDP (4 µg/ml) was used as a positive control.

Bufalin influenced the morphological character of A549

Compared to the control group, A549 cells exposed to various concentration of bufalin for 48h presented typical apoptotic changes in morphology by inverted phase contrast microscope, such as cell shrinkage, nuclear condensation, reduction of cell density, increase in cytoplasmic granules (Figure 3).

TEM also revealed various morphological alterations in A549 cells after treated with bufalin (4 ng/ml) for 48 h. As shown in Figure 4, chromatin condensation and marginalization could be observed in treated cells, as well as mitochondrial reduction in the amount and damage in the cristae structure.

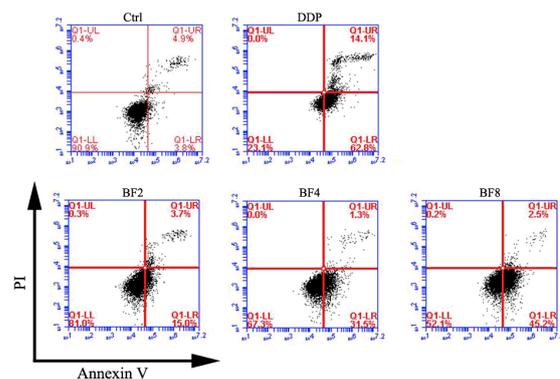


Figure 2. Flow Cytometry Analysis of Bufalin-induced Apoptosis in A549 Cells. Cells were treated with 0, DDP, 2, 4 and 8 ng/ml bufalin for 48h. FCM analysis showed the right lower region as the early-stage apoptosis (Annexin V+/PI-). Data represented one of three experiments yielding similar results

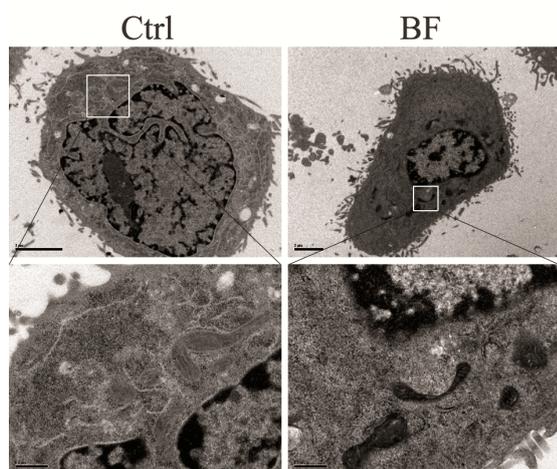


Figure 4. Electron Micrographs of A549 Cells Treated with Bufalin. Cells treated with bufalin(4ng/ml) for 48h showed ultrastructural changes in the nucleus and mitochondria at 3700x and 17500x respectively

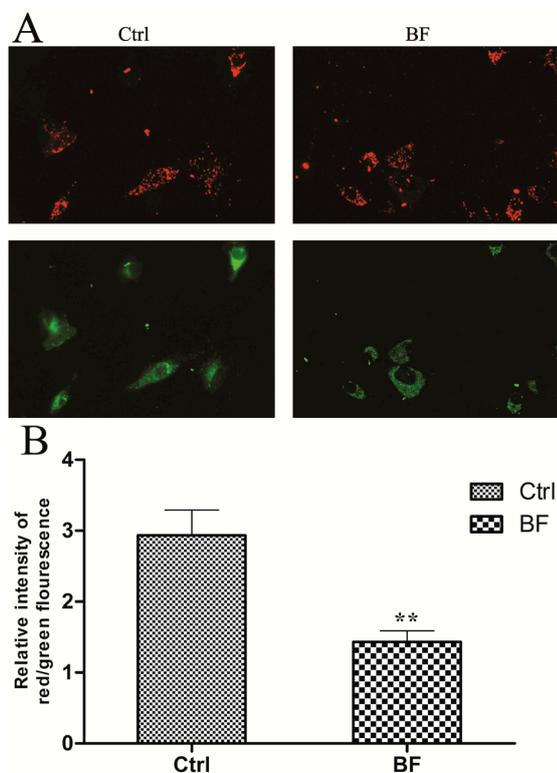


Figure 5. A)Disruption of $\Delta\psi_m$ Induced by Bufalin. B)The Relative Ratio of Red/Green Fluorescence Intensity. A549 cells were treated with 0,4ng/ml of bufalin for 48h. The alteration in $\Delta\psi_m$ were detected by fluorescence microscopy. The value was shown as mean \pm SeD(n=3), **P<0.01 versus untreated controls

Bufalin led to alterations in $\Delta\psi_m$

For the fluorimetric analysis of $\Delta\psi_m$ in the intact cells the lipophilic cationic probe JC-1 was used. Depending on the membrane potential, the JC-1 monomer could form J-aggregates in the mitochondrial matrix, which brought a shift from green to red fluorescence. As shown in Figure 5A and B, compared to the control group, a $\Delta\psi_m$ loss was observed as a significant decrease in the red/green fluorescence intensity ratio when cells treated with bufalin (4ng/ml) for 48h. The data indicated that apoptosis triggered by bufalin could be related to disruption of $\Delta\psi_m$.

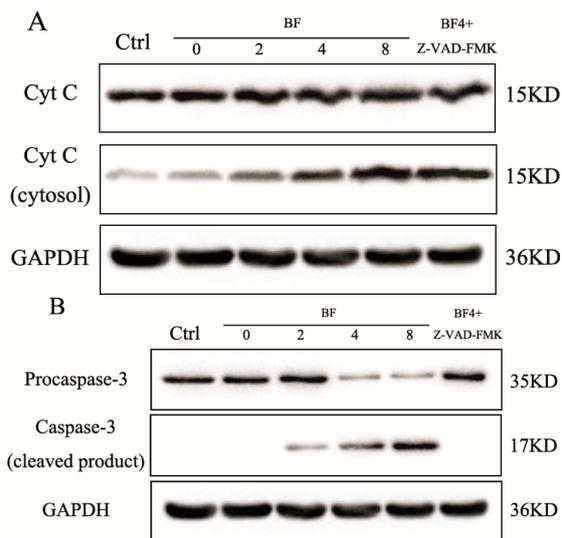


Figure 6. Effects of Bufalin on Protein Expressions of Cytosolic and Mitochondrial Cytochrome C A), Caspase-3/procaspase-3 B) in A549 Cells. After incubation with bufalin in various groups for 48h, the changes on expression of proteins associated with intrinsic signaling pathway were detected. The images were representative of three experiments. GAPDH was used as an internal loading

Bufalin regulated the expression of mitochondrial apoptosis related proteins in A549 cells

In order to realize the molecular basis of mitochondrial apoptosis induced by bufalin deeply, we investigated the expression levels of Cyt C in the cytosolic and total protein by Western blot analysis. The results revealed an up-regulation in cytosolic Cyt C after bufalin treatment in A549 cells, meanwhile, the relative density of Cyt C in the cytosol fraction increased compared to the control (Figure 6A). Accordingly we considered that a release of Cyt C was involved in bufalin-mediated apoptosis.

Furthermore, we examined the activation of apoptotic effector caspase-3 via detecting the cleaved form of caspase-3. With the increasing dose of bufalin treatment, the density of cleaved caspase-3 enhanced (Figure 6B). To clear that bufalin activated caspase-3, we employed Z-VAD-FMK, a specific inhibitor of caspases, and found that it could inhibit expression of cleaved caspase-3 induced by bufalin in A549 cells. Taken together, these results suggested that bufalin caused the release of Cyt C from the mitochondria and subsequently increased the activity of caspase-3, which finally induced apoptosis in A549 cells.

Discussion

As a physiological process of programmed cell death, apoptosis is always disrupted in various cancers. It has been considered as an anti-cancer strategy effectively by inducing apoptosis of malignant cells, which is one of the eternal themes in cancer research (Khan et al., 2014). A precise and complex network system regulates apoptosis of cells, involving multi-factors and multi-steps. There are two known signaling pathways: the extrinsic pathway mediated by cell surface death receptors and the intrinsic one initiated in the mitochondria (Igney and

Krammer, 2002). Much more than the “powerhouse” of the cell, mitochondria also lies in the center of cell apoptotic pathways (Fogg et al., 2011). Often before the characteristic changes in nucleus, mitochondria has contributed to apoptosis through the remarkable transformation of morphology and function. Once depolarization of mitochondrial membrane occurred, apoptosis will start irreversibly. Therefore, when activated by a variety of apoptotic signals, mitochondrial permeability transition pore (MPTP) opens and $\Delta\psi_m$ declines, results in the release of Cyt C and other apoptogenic proteins to the cytosol, commits cells to apoptosis in turn (Ulivieri, 2010).

In our study, the results of CCK-8 assay indicated that bufalin can inhibit the proliferation of A549 cells in a dose-dependent relation, and defined its optimal concentration. Furthermore, we confirmed that bufalin induce apoptosis in A549 cells by FCM, in which the early-stage accounted for a large proportion. Meanwhile, significant morphological changes were clearly observed when cells were treated with bufalin for 48h, such as cellular shrinkage, mitochondrial reduction in the amount along with damage in the cristae structure, even chromatin condensation and marginalization, but no classical apoptotic bodies. Based on these findings, we concluded that the mechanism of bufalin in triggering apoptosis may be related to mitochondria and the signal pathway it referred.

To further investigate the mitochondrial pathway and molecular mechanism performed by bufalin, we then used JC-1 probe to confirm the changes in $\Delta\psi_m$ before and after, accordingly thought mitochondria to be a target spot in mediating apoptosis. Apoptosis can be triggered by various stimuli, including death receptor-mediated signaling and intracellular stress (Banjerdpongchai and Kongtawelert, 2011). It is well known that Cyt C triggers the activation of caspase cascade in the intrinsic apoptotic pathway if released from mitochondrial membrane gap into cytoplasm (Garrido et al., 2006; Xue et al., 2014). Cyt C is so called the Achilles’ heel in apoptosis, for its promotion in the process of apoptosis irreversibly (Kulikov et al., 2012). Dysfunctional mitochondria releases Cyt C, which in turn activates procaspase-9 and forms a complex with the help of the adapter protein Apaf-1. Finally, as an executive effector on apoptosis, caspase-3 is activated by the steady complex to cleave many substrates and structural proteins to engender apoptosis (Kothakota et al., 1997). In this study we found that bufalin-induced apoptosis was associated with the transformation of $\Delta\psi_m$, mechanistically the up-regulation of Cyt C in the cytosol and activation of caspase-3, consistent with previous report that mitochondrial pathway could notably mediated apoptosis in cells (Jiang et al., 2010). The application of caspase inhibitor Z-VAD-FMK could significantly indicated that bufalin-caused apoptosis involved the activation of caspase-3. Nevertheless, mitochondria and its mediated pathway may be influenced by many factors in cells due to its irreplaceable value on energy metabolism and apoptosis (Zou et al., 2012). So whether do bufalin play a direct role on mitochondria need further research. Also as a promising agent with anti-cancer properties,

bufalin remains to be proved its virtue *in vivo*, and the relationship with other cellular signaling pathways for apoptosis.

In summary, our present results have demonstrated that bufalin inhibited the proliferation of A549 cells in a dose-dependent manner. The cytotoxic effect of bufalin-induced cell death was due to the induction of apoptosis, as indicated by the current data. Bufalin exposure led to decrease of membrane potential of mitochondria, activation of intrinsic signaling pathway, which caused the up-regulation of Cyt C in the cytosol, as well as the activation of caspase-3. All these findings suggest that bufalin deserves further study and may show the widely therapeutic and adjuvant application in the treatment of human NSCLC.

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