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Original Article

Inhibitory effect of bufalin on retinoblastoma cells (HXO-RB44) via the independent mitochondrial and death receptor pathway

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Abstract: Cinobufacini (Huachansu) is a Chinese medicine prepared from the skin of *Bufo bufo gargarizans* Cantor (Bufonidae), and has long been used in traditional Chinese medicine. In the present study, the anti-retinoblastoma constituent bufalin obtained from Cinobufacini was investigated. Treatment of human retinoblastoma (HXO-RB44) cells with bufalin induced apoptosis which was accompanied by a decrease in mitochondrial membrane potential, activation of caspase-9, caspase-8 and caspase-3, as well as changes in the expression of cytochrome C. Bufalin induced the cleavage of caspase-3 and apoptosis, and it was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Taken together, these results demonstrate that bufalin-induced apoptosis in human retinoblastoma (HXO-RB44) cells involved both intrinsic and extrinsic pathways.

Keywords: Apoptosis, bufalin, mitochondrial transmembrane potential, retinoblastoma

Introduction

Retinoblastoma is the most common primary intracellular malignancy in childhood with an incidence of 1/15,000 to 1/20,000 births [1]. Untreated retinoblastoma is always fatal and patients die of intracranial extension and disseminated disease within two years [1, 2]. In the heritable form, the patient usually inherits one defective gene from the parents and a subsequent "hit" of the uninvolved gene results in tumor formation. The heritable form is more often bilateral than the non-heritable form of the disease. Despite progress in the treatment of retinoblastoma [2], significant problems remain unsolved. Metastatic disease is often fatal [3]. Although several treatments are available for retinoblastoma, including enucleation and/or the combination of chemotherapy, laser and cryotherapy, each has major drawbacks in pediatric patients. There is a need for alternative new treatment modalities for retinoblastoma with better safety and efficacy profiles.

Preliminary studies have shown that bufalin has anti-tumor effects by inducing apoptosis and inhibiting the proliferation of many different cancer cells including cervical, stomach, breast, and lung cancers, as well as hepatocellular carcinoma, leukemia, and multiple myeloma [4-6]. The ability of bufalin to inhibit tumor growth has been proposed to be via the modulation of apoptosis- and/or proliferation-related genes and proteins [7-12]. Moreover, a recent study reported that bufalin inhibited pancreatic cancer growth through inhibition of the PI3K/Akt pathway [13]. Unfortunately, very few studies have been carried out on the inhibitory effect of bufalin on retinoblastoma and the mechanisms of the anticancer capacity remain poorly understood. In this study, we investigated bufalin-mediated toxicity and apoptosis in human retinoblastoma HXO-RB44 cells.

Materials and methods

Chemicals and reagents

Bufalin (**Figure 1**) was purchased from Sigma (St. Louis, MO, USA). The compound was prepared in dimethylsulfoxide (DMSO) as a 1000

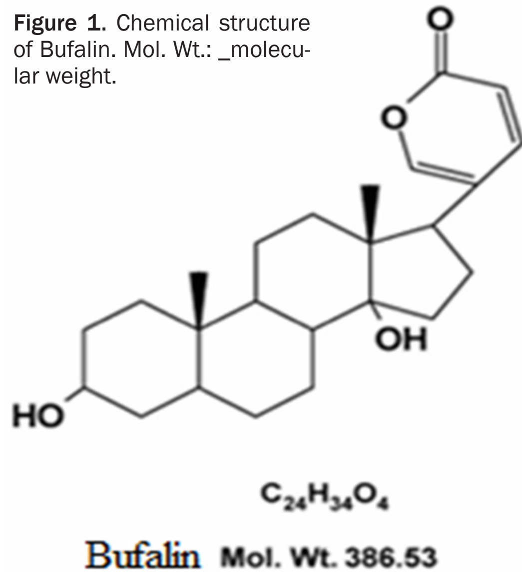

Figure 1. Chemical structure of Bufalin. Mol. Wt.: _molecular weight.

mM stock solution and kept at 4°C. Dilutions of the drug were performed on the day of medium change. The final concentration of DMSO in the samples was less than 0.01% (v/v). Materials used included the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Becton Dickinson, Franklin Lakes, NJ, USA), Hoechst-propidium iodide (PI) staining assay kit (Beyotime Institute of Biotechnology, Shanghai, China); anti-caspase-9, anti-caspase-3, anti-caspase-8, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell line and culture conditions

The retinoblastoma HXO-RB44 cells were maintained in Dulbecco Minimum Essential Medium DMEM supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C with 5% CO_2 [14]. The cells were kept in an exponential growth phase during the experiments.

Cell proliferation assays

Cell growth inhibition by bufalin was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HXO-RB44 cells were seeded in 96-well plates at a density of 6 × 10^3 cells per well. After treatment with various concentrations of bufalin (0-10^{-1} μM) for 48 h and 72 h, 20 μl MTT (5 mg/ml) was added. Four hours later, 100 μl DMSO was added to each well to dissolve the resulting formazan crystals. Absorbance was read at 490 nm using an enzyme-linked immunosorbent assay reader (SpectraMax; Molecular Devices, Sunnyvale, CA, USA). Data were collected from three separate experiments and the percentage of bufalin-induced cell growth inhibition was determined by comparing with control cells. All experiments were performed at least three times.

Cell apoptosis analyses

Annexin V-FITC/PI double staining was employed to quantify the apoptosis of retinoblastoma cells treated with bufalin. Briefly, cells were seeded in 6-well plates (2 × 10^5 cells/ml) and exposed to bufalin (0-10^{-1} μM) for 24 h. The cells were then stained using the Annexin V-FITC/PI double fluorescence apoptosis detection kit (Biouniquer Technology, USA) following the manufacturer's instructions. Samples were analyzed using a FACS Calibur Flow cytometer within 1 h after staining. Cells were grown in six-well plates for 12 h and treated with Z-LETD-FMK (a selective caspase-9 inhibitor) and (or) Z-IETD-FMK (a selective caspase-8 inhibitor) for 1 h before treatment with bufalin. After 36 h or 60 h, the cells were washed twice with phosphate buffer solution (PBS), adjusted to 100 μl of the solution and transferred to a 1-ml centrifuge tube (1 × 10^5 cells). 10 μl of Annexin V-FITC and 10 μl of PI were added and the cells were incubated for 15 min at room temperature (25°C) in the dark before analysis as described previously.

Measurement of mitochondrial transmembrane potential using the fluorescent dye JC-1

Cells cultured as described previously were resuspended in culture medium to a concentration of 1 × 10^5 cells/ml and incubated with the fluorescent dye JC-1 (20 nM) at 37°C for 30 min in the dark. JC-1 fluorescence was then immediately analyzed with a flow cytometer (Becton Dickinson).

Western blot analyses

Cells were washed separately, collected and homogenized in lysis buffer (10 mM Tris-HCl, pH 8, 0.32 mM sucrose, 5 mM Ethylene Diamine Tetraacetic Acid (EDTA), 2 mM dithioth-

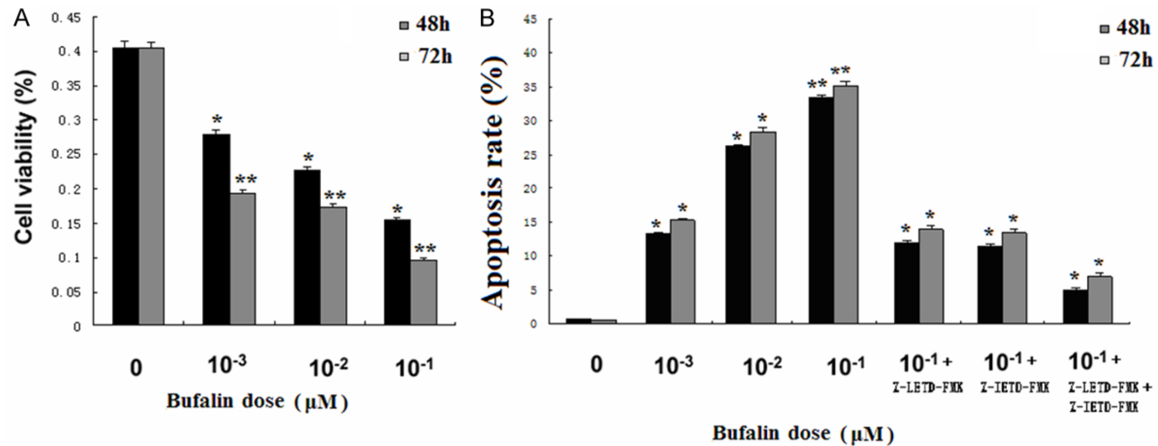

Figure 2. The effects of bufalin on HXO-RB44 cells. A. HXO-RB44 cells were treated with or without different concentrations of bufalin for 48 or 72 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out as described. Data are presented as means ± standard deviation from three independent experiments. B. Flow cytometric analysis of HXO-RB44 apoptotic cells stained with Annexin V + propidium iodide (PI) after treatment with 0-10^{-1} μM bufalin, and Z-IETD-FMK or Z-LETD-FMK. All bufalin treated groups showed significant increases in apoptosis compared with the control groups. When the caspase inhibitor Z-LETD-FMK (5 mM) and (or) Z-LETD-FMK (5 mM) were added before exposure to 10^{-1} μM bufalin for 48 h, the apoptotic rates decreased ($^{*}P < 0.05$; $^{**}P < 0.01$).

reitol, 1 mM phenylmethyl sulfonylfluoride, and 1% Triton X-100), and centrifuged (13,000 *g*, 10 min, 4°C). To ensure that an equal amount of protein was loaded in each case, western blots were also carried out, using the Bradford protein assay. Equal amounts of proteins (50 μg) were subjected to electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel (10%). The gel-separated proteins were transferred to Nitropure nitrocellulose membranes (Santa Cruz Biotechnology) and the membranes were blocked with 10% bovine serum albumin in TBST [10 mM Tris-HCl (pH 8.0), 137 mM NaCl, and 0.05% Tween-20 by vol.] overnight at 4°C and probed with primary antibodies at 37°C for 2 h. Each of the targeted proteins was immunostained by specific antibodies. The membranes were washed three times with TBST and then incubated for 1 h at room temperature with alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology) before being visualized using a chemiluminescence detection kit (Beyotime Institute of Biotechnology).

Western blot analysis of cytosolic cytochrome C

Cells were harvested 48 h after incubation with bufalin at the indicated concentration. The pellets were suspended in a 5-fold volume of cytosol extraction buffer containing 1,4-dithiothreitol (DTT) and protease inhibitors (Abcam plc. USA). After incubation on ice for 10 min, the pellets were homogenized in an ice-cold Dounce tissue grinder. The cells were then centrifuged at 700 *g* for 10 min at 4°C. The supernatant was collected into a fresh 1.5 ml tube, and centrifuged at 10,000 *g* for 30 min at 4°C. The final supernatant was used as the cytosolic fraction. Finally, cytosolic cytochrome C was identified by western blot analysis as described before.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). The independent t-test was used to compare between two groups, and comparisons of multiple groups were performed with one-way analysis of variance (ANOVA) followed by the LSD-t (for equal variances assumed) or Dunnett's (for equal variances not assumed) test. $P < 0.05$ was considered statistically significant.

Results

Bufalin inhibited the proliferation of retinoblastoma cells in a dose- and time-dependent manner

To investigate the effects of bufalin on the proliferation of retinoblastoma cells, we measured the growth of the retinoblastoma cell line using

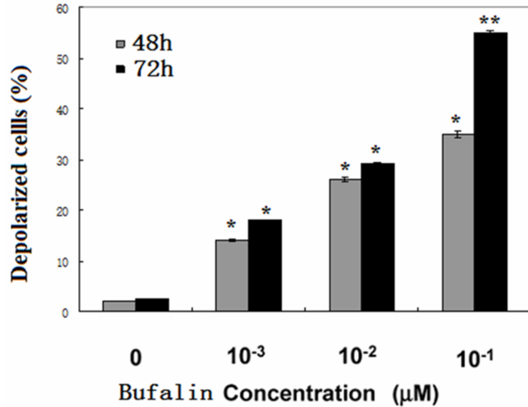

Figure 3. The effects of bufalin on the depolarized cell ratio in HXO-RB44 cells. Mitochondrial membrane hyperpolarization was induced by bufalin in HXO-RB44 cells. Cells were treated with various concentrations of bufalin for 48 h and 72 h, and then stained with fluorescent JC-1 and incubated at 37°C for 30 min in the dark. The mean fluorescence intensity was detected using a flow cytometer. Data are presented as means ± standard deviation of three independent experiments, and each experiment was carried out in triplicate (*$P < 0.01$).

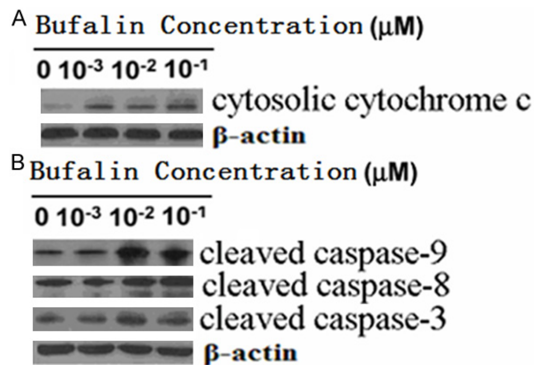

Figure 4. Bufalin induced apoptosis through intrinsic and extrinsic signaling pathways in HXO-RB44 cells. A. Bufalin induced the release of cytochrome C from the mitochondrial membrane to the cytosol. Cytosolic cytochrome C was identified by western blot analysis. β-actin protein levels were used as a cytosolic control. All experiments were performed at least thrice, and a representative experiment is shown in each panel. B. Bufalin induced activation of caspase-9 and caspase-8 and the cleavage of caspase-3. The whole-cell lysate was assayed by western blot and corresponding antibodies. β-actin protein levels were used as a loading control. All experiments were performed at least thrice, and a representative experiment is presented in each panel.

the MTT incorporation assay. The treatment of HXO-RB44 cells with 0-10^{-1} μM bufalin resulted in a dose- and time-dependent inhibition of cell growth (**Figure 2A**).

Bufalin induced apoptosis of retinoblastoma cells

Flow cytometry assays showed marked changes in cell profiles after treatment with 0-10^{-1} μM bufalin, which strongly indicated that bufalin induced apoptosis. All groups treated with bufalin showed significant increases in apoptosis compared with the control groups ($P < 0.01$). The 10^{-1} μM bufalin group showed the highest apoptosis in all experiments (compared with the control group, $P < 0.01$). In addition, when Z-LETD-FMK (a selective caspase-9 inhibitor) and (or) Z-IETD-FMK (a selective caspase-8 inhibitor) were added before exposure to 10^{-1} μM bufalin for 48 h, the apoptotic rates decreased ($P < 0.01$) (**Figure 2B**).

Measurement of mitochondrial transmembrane potential using the fluorescent dye JC-1

Mitochondrial transmembrane potential (MMP) decreased in a dose-dependent manner in response to bufalin (**Figure 3**). Cells treated with 10^{-3} μM bufalin showed a 13.3 ± 2.48% decrease in MMP ($P < 0.01$), cells treated with 10^{-2} μM bufalin showed a 21.7 ± 1.55% decrease in MMP ($P < 0.01$) and cells treated with 10^{-1} μM bufalin showed a 45.3 ± 2.29% decrease in MMP ($P < 0.01$).

Bufalin induced apoptosis through intrinsic pathways in human retinoblastoma cells

MMP is critical for correct cellular functions. Disruption of MMP altered the membrane dynamics of the mitochondria leading to the release of cytochrome C, formation of apoptosome complexes, and activation of caspase-9. These changes initiated a cascade of caspase activation leading to apoptosis. To observe the release of cytochrome C and activation of caspase-9, HXO-RB44 cells were treated with 0-10^{-1} μM bufalin for 48 h. Cytosolic cytochrome C was identified by western blot analysis. As shown in (**Figure 4A**), cytosolic cytochrome C was increased after bufalin treatment. The release of cytochrome C triggered caspase-9 and caspase-3 activation (**Figure 4B**).

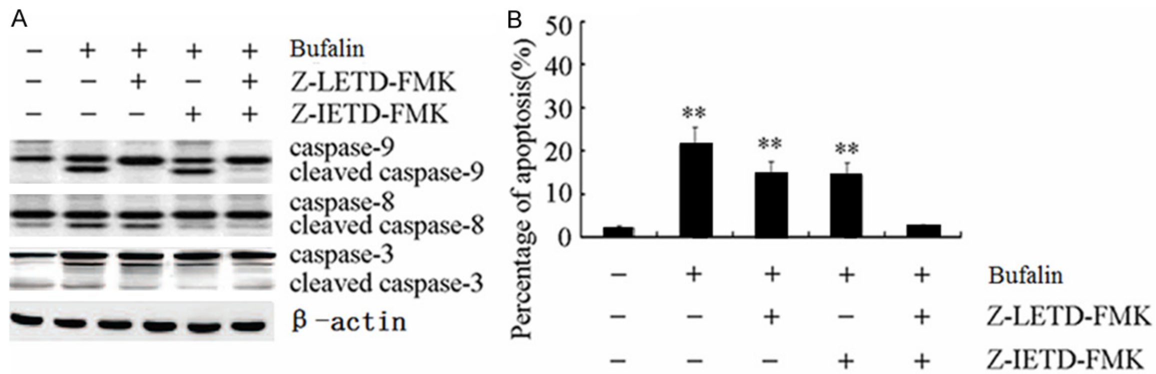

Figure 5. Bufalin-induced apoptosis involved both intrinsic and extrinsic signaling in cells. A. Bufalin-induced apoptosis was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Cells were plated in six-well culture plates. After pre-incubation with 0.1 μmol/l Z-IETDFMK or Z-LETD-FMK for 12 h, the cells were treated with bufalin for 48 h, respectively. Cells were harvested and stained with Annexin V-FITC and PI. Stained apoptotic cells were classified by flow cytometry. B. Cleavage of caspase-3 was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment. Cells were plated in six-well culture plates. After pre-incubation with 20 μmol/l Z-IETD-FMK or Z-LETD-FMK for 12 h, the cells were treated with 0.1 μmol/l bufalin for 48 h, respectively. Cells were harvested and total protein was extracted. The whole-cell lysates were assayed by western blot and corresponding antibodies. β-actin protein levels were used as a loading control. All experiments were performed at least thrice, and a representative experiment is presented in each panel. **P < 0.01 as compared with respective controls.

Bufalin induced apoptosis via extrinsic signaling pathways in human retinoblastoma cells

In order to investigate whether bufalin induced apoptosis through extrinsic pathways, we assessed the activation of caspase-8. As shown in **Figure 4B**, cleaved caspase-8 was detected after bufalin treatment. These results suggest that extrinsic pathways are involved in bufalin-mediated apoptosis in human retinoblastoma HXO-RB44 cells.

Bufalin induced apoptosis through both intrinsic and extrinsic signaling pathways in human retinoblastoma HXO-RB44 cells

Cross-talk has been found between intrinsic and extrinsic apoptosis signaling pathways. In order to investigate which apoptotic signaling pathways bufalin is involved in, Z-LETD-FMK (a selective caspase-9 inhibitor) and Z-IETD-FMK (a selective caspase-8 inhibitor) were used to block the intrinsic or extrinsic apoptosis signaling pathway. As shown in **Figure 4A**, Z-LETD-FMK only inhibited bufalin-mediated activation of caspase-9, and Z-IETD-FMK only inhibited bufalin-mediated activation of caspase-8. When both Z-LETD-FMK and Z-IE-TD-FMK were added, bufalin-mediated activation of caspase-9 and caspase-8 was inhibited, following inhibition of caspase-3 cleavage. The analysis of apoptosis showed that Z-LETD-FMK or Z-IETD-FMK partially inhibited bufalin-mediated apoptosis. Bufalin-mediated apoptosis was inhibited by both Z-LETD-FMK and Z-IETD-FMK treatment (**Figure 5B**).

Discussion

Retinoblastoma is a malignant tumor of the retina and generally affects children under the age of six years. Retinoblastoma affects approximately 1 in 15,000 live births. Worldwide, approximately 5000 new cases occur each year [14-16]. It is the most common eye cancer in children and is caused by a mutation on chromosome 13, called the *RB1 gene*. In some children, the defective RB1 gene can be inherited from either parent; however, the mutation occurs in the early stages of fetal development. Characterized by the typical cat's eye or the white pupil reflex (leukocoria) noted by parents, approximately 63% of all retinoblastomas arise in the first two years of life. In some cases, retinoblastoma metastasizes to extraocular organs including bone, lung and brain. Although non-metastatic tumors can be treated by enucleation (removal of the eye), there is currently no treatment for metastatic retinoblastoma. The standard treatment for retinoblastoma is neoadjuvant chemotherapy. However, the effectiveness of cytotoxic drugs often declines due to acquired chemoresistance. Therefore, the identification of new therapeutic agents to

target the malignant behavior of retinoblastoma cells is important for improving prognosis.

The results of the present study show that bufalin at concentrations, ranging from 0 to 10^{-1} μM for 48 and 72 h inhibited the growth of HXO-RB44 cells and significantly decreased the proliferation of HXO-RB44 cells in a concentration- and time-dependent manner (**Figure 3A**). Apoptosis induced by bufalin also occurred in a time- and concentration-dependent manner (**Figure 3B**).

Apoptosis can be triggered by internal and external signals. The loss of MMP has been suggested to cause cytochrome C release [17]. Released cytochrome C is essential for activating caspase-9. This results in internal damage to cells [18]. Death activators transmit a signal to the cytoplasm that leads to activation of caspase-8. This initiates the extrinsic pathway leading to phagocytosis of cells [19]. In the present study, we observed cleaved caspase-8 and caspase-9, followed by a cascade of caspase activation (**Figure 4B**). It is increasingly thought that crosstalk exists between intrinsic and extrinsic apoptosis signaling pathways [20]. For example, the cleavage of Bid caused by caspase-8 can lead to the release of cytochrome C from mitochondria, which subsequently activates the caspase-9-mediated intrinsic apoptosis pathway [21]. We also investigated the pathways involved. The results showed that Z-LETD-FMK or Z-IETD-FMK only inhibited bufalin-mediated caspase-9 or caspase-8 activation and partial apoptosis (**Figure 4**). However, the combination of Z-LETD-FMK and Z-IETD-FMK inhibited the bufalin-mediated cascade of caspase activation and apoptosis (**Figure 5A**, **5B**). These results suggested that bufalin can trigger both intrinsic and extrinsic apoptosis pathways in human retinoblastoma HXO-RB44 cells.

In conclusion, bufalin induced apoptosis of HXO-RB44 cells by perturbing mitochondrial permeability transition pores and caspase activation. Moreover, the release of cytochrome C into the cytosol, and the activation of caspase-9 triggered the mitochondrial pathway. Taken together, these findings provide a basis for further analysis of bufalin as a promising candidate for the treatment of retinoblastoma.

Acknowledgements

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Disclosure of conflict of interest

None.

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