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Original Article

Berberine Induced Apoptosis of Human Osteosarcoma Cells by Inhibiting Phosphoinositide 3 Kinase/Protein Kinase B (PI3K/Akt) Signal Pathway Activation

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Abstract

Background: Osteosarcoma is a malignant tumor with high mortality but effective therapy has not yet been developed. Berberine, an isoquinoline alkaloid component in several Chinese herbs including Huanglian, has been shown to induce growth inhibition and the apoptosis of certain cancer cells. The aim of this study was to determine the role of berberine on human osteosarcoma cell lines U2OS and its potential mechanism.

Methods: The proliferation effect of U20S was exanimed by 3- (4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) and the percentage of apoptotic cells were determined by flow cytometric analysis. The expression of PI3K, p-Akt, Bax, Bcl-2, cleavage-PARP and Caspase3 were detected by Western blott.

Results: Berberine treatment caused dose- dependent inhibiting proliferation and inducing apoptosis of U20S cell. Mechanistically, berberine inhibits PI3K/AKT activation that, in turn, results in up-regulating the expression of Bax, and PARP and down-regulating the expression of Bcl-2 and caspase3. In all, berberine can suppress the proliferation and induce the apoptosis of U2OS cell through inhibiting the PI3K/Akt signaling pathway activation.

Conclusion: Berberine can suppress the proliferation and induce the apoptosis of U2OS cell through inhibiting the PI3K/Akt signaling pathway activation.

Keywords: Berberine, Apoptosis, Osteosarcoma, PI3K/Akt

Introduction

Osteosarcoma is the most common primary malignant neoplasm of bone that progresses rapidly and has a poor prognosis (1-2). Standard treatment includes the use of "up-front" definitive surgery of the primary tumor, multiagent chemotherapy, and postoperative chemotherapy (3). Currently, chemotherapy treatment for osteosarcoma includes cisplatin, etoposide, epirubicin, cyclophosphamide and, methotrexate (4). These drugs are known to cause serious systemic toxicity. Moreover, osteosarcoma cells are not highly sensitive to most chemotherapeutic agents (4). Therefore, it is an urgent need to develop more available chemotherapy strategies or find safety and effective agents for the treatment of osteosarcoma.

Berberine (BBR, an isoquinoline alkaloid component in several Chinese herbs including Huanglian) have antimicrobial, anti-inflammatory, anti-diabetic and anti-angiogenesis and cholesterol-lowering effects (5). In China, berberine is commonly prescribed for the treatment of gastrointestinal complaints, diarrhea and other diseases (6). The therapeutic effects of berberine against dysentery and diarrhea probably lie in its inhibition of enterotoxin-induced secretion in the

intestines (7). Berberine possess anti-tumor activity, against cancer cells established from prostate cervical, esophageal, oral, colonic cancers leukemia melanoma and glioblastoma (8). Berberine inhibited tumor cell growth by inducing cell apoptosis, and the expression pattern of genes involved in the regulation of cell apoptosis and the inhibition of cellular proliferation (9). Berberine-induced growth inhibition of non-small cell lung cancer cells was mediated by PI3K/Akt signal pathway (10). However, it is largely unknown how berberine initiates the cascade that eventually leads to cell apoptosis.

In this study, we investigated the anti-tumor effects of the berberine on osteosarcoma cells and the involvement of PI3K/Akt signaling in this process. We demonstrate that inhibition of PI3K/Akt signaling by berberine may contribute to its anti-tumor activities in osteosarcoma cells.

Materials and Methods

Reagents

Berberine (purity, >98%, 2013) was purchased from Tianping Pharmaceutical Co. (Shanghai, China). The compound was dissolved in dimethyl sulfoxide (DMSO). The annexin V-FITC apoptosis detection kit was from Beckman Coulter (Fullerton, CA).

Cell culture

Human osteosarcoma cell lines U2OS were purchased from the American Type Culture Collection (Manassas, VA). U2OS was cultured in McCoy's

5A modified medium (Gibco, Invitrogen). All media contained 10% FBS (Gibco, Invitrogen), 100 μg/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37 ˚C. Cells were passaged twice weekly and routinely examined for mycoplasma contamination. For assessing morphological changes, 50–60% confluent cells were treated with different concentrations of BBR, whereas DMSO treated cells served as control. After 48 h of treatment, photographs were taken using a phase-contrast microscope at 200·magnification (Olympus, Japan).

Cell growth/cell viability assay

Proliferation of cells was determined by the MTT assay. Approximately 3×10^3 U20S cells were plated in each well of 96-well plates. After overnight incubation, the cells were treated with BBR (0–50μg/mL) for 48 h. At 48h following BBR treatment, the medium was removed and MTT (20 μl of 5 mg/mL) was added to each well and incubated at 37 ˚C for 4 h. The plates were spun, and the purple colored precipitates of formazan were dissolved in 150 μL of dimethyl sulfoxide. Absorbance was measured at 490 nm using an ELISA plate reader. The reduction in viability of in BBR-treated U20S cells was expressed as a percentage compared to non-BBR treated control cells. Control cells were considered 100% viable.

Colony formation assay

The anti-proliferative effect of BBR on U20S cells was assessed by colony formation assay, as described previously (11).

Briefly, 1000 cells were plated into each well of 6-well plates in triplicate for 48 h. Thereafter, cells were treated with BBR (0–50 μg/mL). The cells were kept in an incubator at 37 ˚C for 7 days. On day 8, the colonies were washed with PBS, fixed with formalin (10%), and stained with Giemsa (11). The colonies that had P50 cells per colony were counted. The number of colonies formed in the presence of varying concentrations of BBR was expressed as a percentage of untreated controls.

Detection of apoptotic cells by fluorescence staining and flow cytometry

Apoptosis was assessed by adding an acridine orange (100 mg/mL)/ethidium bromide (100 mg/mL) (1/1 v/v) mixture to the cell suspension and the percentage of cells undergoing apoptosis was determined with a Leica TCSSP Confocal Microscope (Wetzlar, Germany) as described (12-13). Live cells have green fluorescence (with acridine orange) while dead cells fluoresce orange

(with ethidium bromide). A quantitative assessment of apoptosis was made by determining the percentage of cells with nuclei that were highly condensed or fragmented. Annexin V and PI double stainingwas performed using the Annexin V-FITC Apoptosis Detection Kit as described by the manufacturer.

Western blot analysis

Total proteins from U20S in different groups were separated by 12% SDS-PAGE, and the separated proteins were next electro-transferred onto nitrocellulose membranes using a transblot system (Bio-Rad, Hercules, CA, USA). The membranes were first blocked with 5% non-fat milk for 2 h at room temperature, followed by incubating with indicated primary Polyclonal rabbit antibody to PI3K (CST, USA, 1:500), p-Akt (CST, USA, 1:500), Akt (CST, USA, 1:1000), PARP (CST,, USA, 1:500), Caspase3 (CST, USA, 1:500), Bax (CST, USA, 1:400), Bcl-2 (CST, USA, 1:1000), β-actin (Abmart, China,1:300) were used as a primary antibody. Anti-rabbit antibody conjugated to HRP (Jackson ImmunoResearch Laboratories, West Grove,PA) was used as a secondary antibody. β-Actin was used as an intrinsic quality control. The bands were incubated in ECL-Plus reagent (Amersham, Piscataway, NJ) and chemiluminescence was detected on BioMax MR Film (Kodak, Rochester, NY). The density of the bands was quantified using a Labworks image acquisition and analysis software (UVP, USA).

Statistical analysis

All experiments were performed in triplicates. The results are expressed as mean ± SD. For statistical analysis, Student's *t*-tests were performed using SPSS software (Chicago, IL, USA). Statistical significance was accepted at the level of $P<0.05$.

Results

BBR inhibits U20S cell growth and clonogenic survival

The cytotoxic effect of BBR on osteosarcoma U20S cells was determined with varying concentrations of BBR by MTT assay. As shown in Fig. 1A, the lowest concentration of BBR that exhibited an effect on cell viability was 12.5 μg/mL at 48 h. Inhibition of cell viability by BBR was dose dependent for 48 h. Reduction in cell viability with BBR treatment at concentrations from 12.5 μg/mL to 50 μg/mL after 48 h ranged from 20% to 76%. Based on these observations, we selected a dose range of 0–50 μg/mL and a time of 48h post-BBR treatment for further mechanistic studies.

We also examined the effect of BBR on the clonogenic survival of U20S cells. There was a drastic decrease in the ability of the U20S cells to form colonies with increasing doses of BBR (0–50 μg/mL) (Fig. 1B). BBR at dosages of 50 μg/ml completely inhibited the proliferation of cells with no colonies formed by 48 h. These observations indicated that BBR has anti-proliferative and anti-carcinogenic effects on U20S cells.

BBR induces apoptosis in U20S cells

We next determined whether BBR-mediated loss of U20S cell viability was the result of the induction of apoptosis. As shown in Fig. 2A, BBR treatment resulted in induction of apoptosis in a dose-dependent manner (Fig. 2B). These data also show that BBR treatment resulted in cell necrosis, which may be a secondary event in the apoptotic process. The pro-apoptotic effect of BBR was confirmed by PI staining and the annexin V method (Fig. 2A). Phasecontrast photomicrographs taken 48 h after BBR treatment revealed a dose dependent decrease in cell density.

Changes in cell morphology and cell membrane blebbing, which are characteristics of apoptosis, were also detected. We next quantified the extent of apoptosis by flow-cytometric analysis of BBR-treated cells labeled with PI and annexin V. As shown by PI staining and the annexin V method, BBR caused a dosage dependent increase in U20S cell apoptosis (Fig. 2A). Treatment of U251 cells with 12.5 and 50 μg/mL of BBR for 48 h increased the number of early apoptotic cells (LR) from 0.7% to 13.1%, respectively, in a dose-dependent manner.

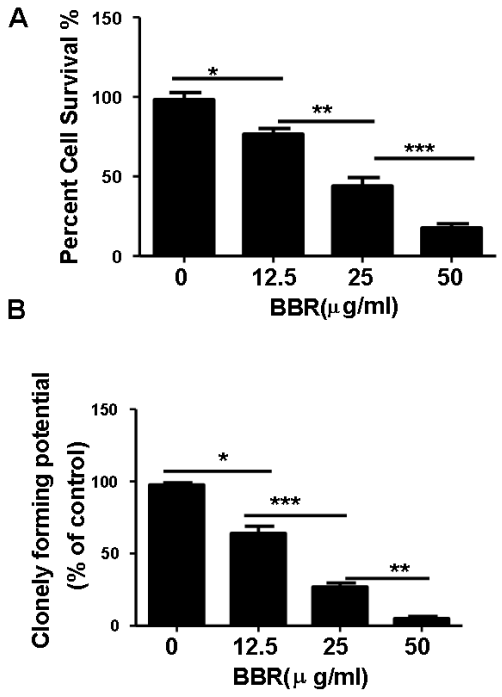

Fig. 1: BBR inhibits cell viability and proliferation of human Osteosarcoma cells in a dose-dependent manner. (A) Dose -dependent effect of BBR on U20S cell viability. Viability of cells was determined by the MTT assay as described in Materials and methods. Reduced cell viability was observed with BBR treatment (12.5–50 μg/mL) concentrations at 48 h. The data are presented as means ± SD (n = 8). (B)
Cellular proliferation assayed by the clonogenic assay as described in Materials and methods. A value of 100 corresponds the number of colonies obtained with control (non-BBR treated) cells. Treatment of U20S cells with 50μg/mL of BBR almost completely inhibited colony formation. The experiment was done in triplicate, and each time point indicates the effect of BBR on colony formation compared to controls (means ± SD). ND, not detectable

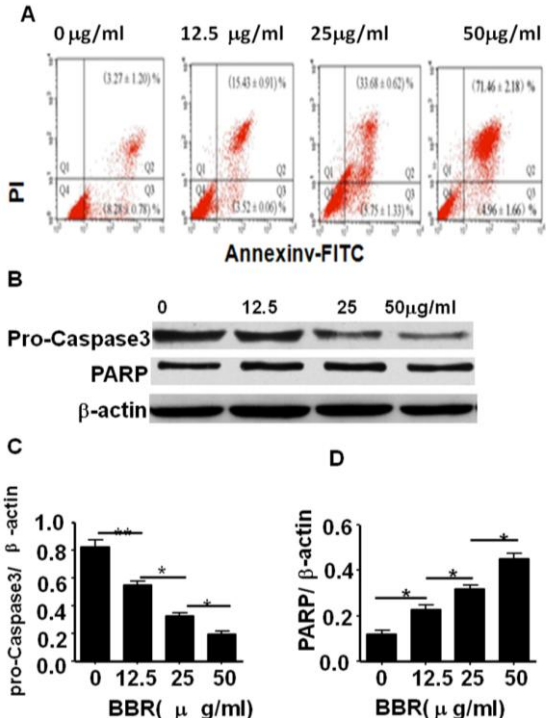

Fig. 2: BBR treatment induces dose-dependent apoptosis in U20S cells
(A) Flow cytometry of BBR treated U20S cells using a double-staining method with FITC-conjugated annexin-V and PI. The LR quadrant indicates the percentage of early apoptotic cells (Annexin V-stained cells) and the UR quadrant the percentage of late apoptotic cells (Annexin V + propidium iodide-stained cells). (B) A representative result for Western blot analysis the expression of Pro-caspase3 and PARP. C. Semi-quantitative analysis of U20S studied in each group. The relative amount of Pro-caspase3 in each group was normalized by β-actin. *P<0.05;C. Semi-quantitative analysis of U20S cell studied in each group. The relative amount of PARP in each group was normalized by β-actin. *P<0.05

Compared to 0.3% in untreated control cells, the number of late apoptotic cells (UR) increased from 0.9% to 26.0% compared with 0.5% in non-BBR treated cells. The total percent of apoptotic cells (UR + LR) increased from 0.8% in untreated U20S cells to 39.1% with 50 μg/mL of BBR treatment for 48 h.

Consistent with the phase-contrast andconfocal microscopy data, flow cytometry using PI staining and annexin V revealed that treatment of U20S cells with BBR resulted in a dose-dependent induction of apoptosis.
For these reasons, we determined the effect of BBR on the expression of these proteins. As shown in Fig. 2B, the cleaved PARP fragment was detected in BBR-treated samples. Fig. 2B

also shows that the level of procaspase-3 was diminished at low doses (12.5μg/mL) of BBR, indicating activation of this caspase. Taken together, Western blot analysis indicated that treatment of U20S cells with BBR resulted in a dose-dependent activation of caspase-3 and PARP (Fig. 2B) proteins 24 h after BBR treatment. Thus, the significant induction of apoptosis caused by BBR confirms the observed reduction in cell viability and the anticarcinogenic effect on U20S human osteosarcoma cells.

BBR induces apoptosis in U20S cells by suppressing PI3K/AKT signaling

We next investigated whether or not PI3K/Akt was involved in BBR-mediated apoptosis of U20S cells. As shown in Fig. 3A and B, treatment resulted in a significant down-regulation of protein expression of PI3K, p-Akt, and without an effect on total Akt expression in U20S cells. BBR induced the degradation of PI3K, which give a signal to phosphorylates Akt. Inhibition of p-AKT by BBR may be attributed to inactivation of cell survival pathways resulting in apoptosis. These observations suggest that the PI3K/Akt pathway could be involved in the anti-proliferative/pro-apoptotic response of Osteosarcoma cells to BBR.

BBR-induced apoptosis is regulated by PI3K/AKT signaling in U20S cells

To further investigate whether PI3K activity directly influences the biological effects of BBR in U20S cells, an expression vector encoding a constitutively-PDK1or an empty control vector were transfected into U20S cells. Transfected cells were confirmed by Western blot analysis (Fig. 4A Left). Expression of constitutively PI3K in U2OS cell promoted resistance to the anti-proliferative and pro-apoptotic effects of BBR (Fig. 4A Right). BBR treatment inhibited PI3K-regulated signaling for tumor cell survival and proliferation (Fig. 4B).

BBR treatment results in modulation of the levels of Bcl-2 family proteins in a manner that promotes apoptosis in U20S cells

Therefore, we examined the effect of BBR treatment on Bcl-2 in U20S cells. As shown in Fig. 4A, BBR treatment of U20S cell lines resulted in a decrease in anti-apoptotic Bcl-2 and a concomitant increase in pro-apoptotic Bax proteins.

The ratio of Bax/Bcl-2 was significantly increased in a dose-dependent manner with BBR treatment (Fig. 4B), suggesting that U20S cell apoptosis occurs with the involvement of Bcl-2 family proteins. Thus, there was an overall shift in the ratio of anti-apoptotic and pro-apoptotic proteins following BBR treatment.

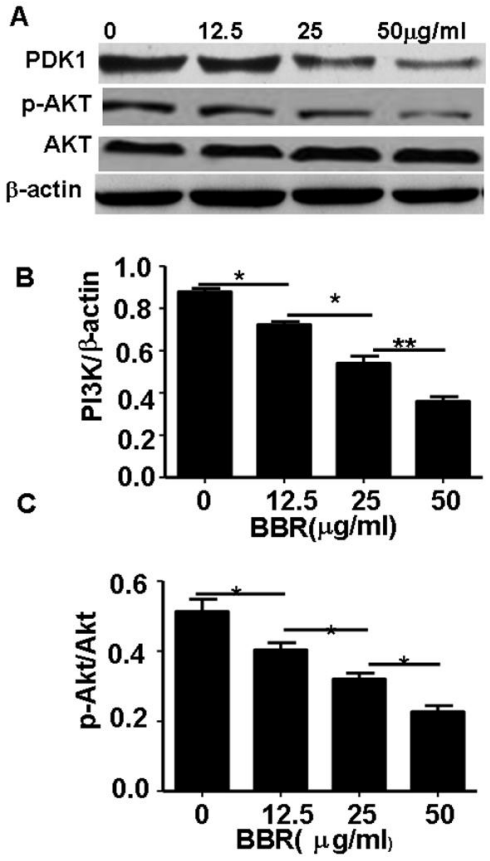

Fig. 3: Effect of BBR on the expression of PI3K and Akt

A: A representative result for Western blot analysis the expression of PI3K and Akt; B: Semi-quantitative analysis of U20S cell studied in each group. The relative amount of PI3K in each group was normalized

by β-actin, *P<0.05; C: Semi-quantitative analysis of U20S cell studied in each group. The relative amount of Akt and p-Akt in each group was normalized by β-actin and presented as a ratio between p-Akt and Akt.

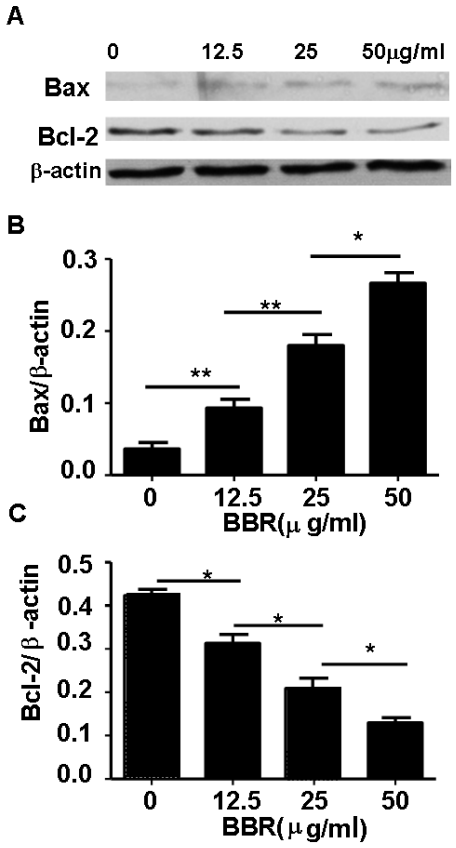

Fig.4: BBR regulates the expression of apoptotic genes in U20S cells

A. A representative result for Western blot analysis the expression of Bax and Bcl-2. B. Semi-quantitative analysis of U20S studied in each group. The relative amount of Bax in each group was normalized by β-actin. *P<0.05; C. Semi-quantitative analysis of U20S cell studied in each group. The relative amount of Bcl-2 in each group was normalized by β-actin. *P<0.05

Discussion

In this study, we evaluated the chemopreventive/therapeutic potential of BBR against osteosarcoma and its mechanism of action. Consistent with earlier observations we found that BBR suppress the proliferation and viability of osteosarcoma cells by inducing apoptosis. It is well know that hyperactivation of AKT kinases is a common event in many human cancers including osteosarcoma, and this activation results in tumor cell survival and enhanced resistance to apoptosis through multiple mechanisms (14). Our data show that BBR treatment resulted in a significant dose-dependent inhibition of constitutively degradation of PI3K and p-AKT in U20S cells. Since Akt is a downstream target of PI3K, the observed inhibition of p-AKT suggests that BBR also inhibits PI3K. This argument shows that BBR is an inhibitor of PI3K (15). Accumulating evidence indicates an important role for the PI3K signaling pathway in osteosarcoma. The PI3k/Akt pathway is being explored as a target the development of osteosarcoma therapies. BBR suppressed the activation of Akt and, therefore, could be useful in the prevention and treatment of osteosarcoma.

Activation of the PI3K/Akt pathway leads to increase the expression of Bcl-2 with the decrease the expression of Bax (16, 17); therefore we assessed the effect of BBR on Bcl-2 and Bax proteins. Our data show treatment of U20S cells with BBR could increase the level of pro-apoptotic Bax and decrease the expression of Bcl-2. Overall, there is a shift in the ratio between the anti-apoptotic and pro-apoptotic proteins following BBR treatment. The ratio of Bax/Bcl-2 proteins expression plays an important role in apoptotic signal (18). As shown in Fig. 4B, the ratio of Bax/Bcl-2 was significantly increased dose dependently after BBR treatment. BBR treatment significantly reduced the levels of p-Akt in U20S cells.

Alteration in the ratio of Bax/Bcl-2 stimulates the release of cytochrome c from mitochondria into cytosol. Cytosolic cytochrome c then binds to Apaf-1 and leads to the activation of caspase-3 and PARP (19). Activated caspase 3 is the key mediator of cell apoptosis cleaving intracellular proteins vital for cell survival and growth, such as PARP. In our in vitro system, BBR activated caspase-3 (Fig. 2B), leading to PARP cleavage (Fig. 2B) and the induction of apoptosis in BBR treated U20S cells.

The data from our study indicate that BBR induced apoptosis in human osteosarcoma cancer U20S cells. This is mediated through the PI3K/AKT pathway.
That is associated with Bcl-2, Bax and activation of Caspase-3 and PARP. BBR may be an novel agent for osteosarcoma. However, further studies are needed to ascertain the relationship between the PI3K/Akt pathway and BBR action.

Conclusions

We demonstrated convincing evidence that berberine had the effects of inducing growth inhibition and the apoptosis of in vitro through inhibiting PI3K/Akt pathway signaling activation. Altogether, our data supports that berberine could be a good alternative therapy for treatment of Osteosarcoma in the clinical practice.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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