Berberine induces apoptosis by suppressing the arachidonic acid metabolic pathway in hepatocellular carcinoma

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Abstract. Berberine (BBR) has been suggested as a potential candidate anticancer agent due to its high anticancer activity and multiple mechanisms. In the present study, the inhibitory effect of BBR on hepatocellular carcinoma (HCC) via the suppression of the arachidonic acid (AA) metabolic pathway was investigated. BBR was demonstrated to reduce the viabilities of H22, HepG2 and Bel-7404 cells, in a dose- and time-dependent manner, and increase the number of apoptotic cells. BBR induced the translocation of apoptosis-inducing factor between the mitochondria and the nucleus, and had no effects on the protein expression levels of caspase-3 or -9. In addition, BBR significantly suppressed the protein expression levels of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase (COX)-2 and elevated the content ratio of AA to prostaglandin E2 (PGE2). Furthermore, BBR reduced the volume and weight of tumors in a H22 transplanted tumor model in mice. The results of the present study demonstrated that elevation in the ratio of AA to PGE2 via suppression of the protein expression of cPLA2 and COX-2 in the AA metabolic pathway is involved in the inhibitory effect of BBR in HCC.

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Abbreviations: BBR, berberine; HCC, hepatocellular carcinoma; AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; PGE2, prostaglandin E2; COX, cyclooxygenase; AIF, apoptosis-inducing factor

Key words: berberine, cytosolic phospholipase A2, cyclooxygenase 2, prostaglandin E2

Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent type of cancer, with high incidence and mortality rates worldwide (1). Although the current clinical management strategies, involving surgical resection, local ablation or liver transplantation, are able to cure a minority of cases in the early stages of HCC, the majority of patients are clinically diagnosed at advanced stages (2). HCC is not sensitive to conventional cytotoxic agents. Previously, the concept of targeted systemic therapies has been applied to the treatment of HCC. Sorafenib, a tyrosine protein kinase inhibitor, which selectively inhibits the mitogen-activated protein kinase/extracellular signal-related kinase signaling pathway, has been demonstrated to exhibit improved survival rates in patients with advanced HCC (3,4). This finding indicates that therapy, which targets metabolic pathways may enhance therapeutic efficacy and improve the prognosis for patients with HCC.

The arachidonic acid (AA) metabolic pathway has been recognized to be correlated with the occurrence and development of various types of cancer. Phospholipase A2 (PLA2) and cyclooxygenase (COX) are rate-limiting enzymes in the AA metabolic pathway, which are important in the development and progression of a number of types of cancer (5,6). PLA2 includes three subfamilies: Cytosolic (c)PLA2, secreted PLA2 and calcium-independent PLA2. Several studies have demonstrated that the overexpression of cPLA2α is correlated with angiogenesis and the expression of vascular endothelial growth factor in human colorectal cancer (7,8). By contrast, cancer cell proliferation and xenograft tumor growth are retarded following the suppression of cPLA2α (9,10). Similarly, COX-2 is regarded as a prognostic predictor for cancer in the liver, colon, pancreas, breast, prostate and lungs (11,12). A selective inhibitor of COX-2, celecoxib, is able to induce apoptosis by reducing the production of prostaglandin E2 (PGE2), which has been accepted as a potent promoter of cell proliferation, motility, invasion and angiogenesis (5). Accordingly, the development of drugs,

which act on the AA pathway may provide a novel avenue in identifying chemotherapeutic agents for the treatment of HCC.

Berberine (2,3-methylenedioxy-9,10-dimenthoxyprotoberberine chloride; BBR) is an isoquinoline alkaloid, purified from *Berberis* species, which has long been used as an anti-diarrhea drug in gastrointestinal disorders in traditional Chinese medicine (13,14). Previous studies have demonstrated that BBR has favorable anticancer actions against several types of tumor, including colon cancer, breast cancer, prostate cancer, melanoma and HCC (15-17). However, the molecular mechanisms underlying BBR-induced apoptosis mediated by metabolic pathways remain to be fully elucidated. In the present study, the inhibitory effect of BBR was investigated in HCC cell lines and in a transplanted tumor model in BALB/c mice, in order to elucidate whether BBR-induced apoptosis is correlated with the AA pathway.

Materials and methods

Chemicals. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS), SDS, dimethyl sulfoxide (DMSO), Tris and Tween-20 were purchased from Beijing Dingguo Changsheng Biological Technology Co., Ltd. (Beijing, China). The Bradford Protein Assay kit, radioimmunoprecipitation assay (RIPA) lysis buffer, Annexin V-fluorescein isothiocyanate (FITC) Apoptotic Detection kit and Caspase Activity Assay kit were purchased from Beyotime Institute of Biotechnology (Haimen, China). The primary antibodies against apoptosis-inducing factor (AIF; mouse monoclonal; cat. no. sc-55519), COX-2 (rabbit polyclonal; cat. no. sc-7951), cPLA2 (rabbit polyclonal; cat. no. sc-438) and secondary antibodies (rabbit anti-mouse IgG-HRP, cat. no. sc-358914; goat anti-rabbit IgG-HRP, cat. no. sc-2004) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-caspase-3 (rabbit polyclonal; cat. no. 9662) and anti-caspase-9 (mouse monoclonal; cat. no. 9508) antibodies were from Cell Signaling Technology, Inc. (Shanghai, China). β-actin primary antibody (rabbit monoclonal, cat. no. 1854-1) and GAPDH primary antibody (rabbit monoclonal, cat. no. 2251-1) were obtained from Epitomics, Inc. (Burlingame, CA, USA). BBR (Purity >99%) was donated by the Northeast Pharmaceutical Group Co., Ltd. (Shenyang, China) and was dissolved with 0.2% DMSO culture medium to a final concentration of 200 μM as a stock solution. All other chemicals and reagents were of analytical grade.

MTT assay and determination of the half maximal inhibitory concentration (IC_{50}). H22, HepG2 and Bel-7404 hepatoma cell lines and normal hepatic embryo HL-7702 cells, were maintained in DMEM high glucose medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, in an atmosphere of 95% air and 5% CO_2 at 37˚C. An MTT assay was performed to assess cell viability, which was performed in 96-well plates in octuplicate. The cells were seeded at a density of $5x10^3$ cells/well overnight, and treated with BBR at final concentrations of 0, 12.5, 25, 50 and 100 μM for 24, 48 or 72 h. Subsequently 20 μl MTT (5 mg/ml) was added to each well for the final 3 h of the 24, 48 or 72 h BBR treatment periods. Following this, the cell supernatants were discarded, the MTT crystals were dissolved with DMSO and the optical density (OD) was measured at 490 nm wavelength (3360063; Tecan Austria GmbH, Grödig, Austria). The ratio of cell proliferation to control group was calculated from the MTT data. IC_{50} values of BBR were calculated from the percentages of cell viability obtained from the MTT assay.

Apoptosis detection using flow cytometry. Cell death and apoptosis were detected using an Annexin V-FITC Apoptotic Detection kit by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). In brief, following treatment with 0, 50 and 100 μM BBR at 37˚C for 24 h, the HepG2 cells plated in six-well plates at a density of $5x10^6$ cells/well were harvested and washed twice with cold phosphate-buffered saline. Following this, the cell pellets were suspended with binding buffer (Beyotime Institute of Biotechnology) at a density of $1x10^6$ cells/ml. Following the addition of 5 μl FITC-conjugated annexin V to the suspension, the suspension was incubated for 15 min at 4˚C in the dark, followed by the addition of 5 μl propidium iodide (PI; Beyotime Institute of Biotechnology) for 5 min. The samples were subsequently analyzed using flow cytometry (BD FACSCalibur; BD Biosciences).

Western blot analysis. The cells were collected by centrifugation at 12,000 x g for 15 min at 4˚C and were then washed twice with cold PBS. The cell pellets were suspended in 200 μl RIPA lysis buffer for 30 min at 4˚C, vortexed every 10 min, then centrifuged at 12,000 x g for 15 min at 4˚C. The supernatant was collected as total protein extract. In order to investigate AIF translocalization into the nucleus, the nuclear protein extract was collected using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. The protein concentration was measured using a Bradford Protein Assay kit. The protein extract (50 μg) was aliquoted by 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The blots were blocked with Tris-buffered saline containing 0.05% Tween-20 and 5% non-fat dried milk for 1 h at room temperature, then they were incubated with rabbit anti-mouse primary anti-COX-2, anti-cPLA2, anti-caspase-3, anti-caspase-9 and anti-AIF antibodies (all 1:1,000). The reaction was incubated overnight at 4˚C with gentle agitation, following which the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,000) for 2 h at room temperature with gentle agitation. The membranes were then washed and the bands were visualized using an Enhanced Chemiluminescence Western Blotting Detection system (GE Healthcare Life Sciences, Chalfont, UK).

Determination of the levels of AA and PGE2. The cells were plated in 96-well plates in RPMI-1640 medium containing 10% (v/v) FBS. When ~70% confluence was reached, the cells were treated with BBR at concentrations of 0, 12.5, 25, 50 or 100 μM for 24 h. At the end of treatment, the culture supernatant was collected and centrifuged (10,000 x g, 3 min

at 25˚C) to remove cells or cell debris. The concentrations of PGE2 and AA in the supernatant were measured using an enzyme immunoassay (PGE2 EIA and AA monoclonal EIA kits; Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions. The production of PGE2 (pg/ml) was normalized to the cell counts/well.

H22 transplanted tumor model in mice. A total of 50 male BALB/c mice (6-8 weeks old, weighing 18-22 g) were purchased from the Experimental Animal Center of Norman Bethune College of Medicine, Jilin University (Changchun, China). The mice were housed 5/cage under a 12/12 h light/dark cycle with *ad libitum* access to food and water. The mouse production permit number was SCXK (JI) 2010-0001 and the usage license was SYXK (JI) 2010-0001. The animal experiments in the present study were performed in accordance with the Good Laboratory Practice Guidelines (18) and the experimental protocol was approved by the Ethics Committee of the Norman Bethune Health Science Center of Jilin University (Changchun, China). In brief, H22 cells ($2x10^6$) suspended in 0.1 ml PBS were injected subcutaneously into the right side of the dorsal flank of each mouse. The mice were randomly divided into the following five groups, each containing 10 mice: Control group, positive control cyclophosphamide (CTX) group, 12.5 mg/kg BBR group, 25 mg/kg BBR group and 50 mg/kg BBR group. The mice in the control group were treated with vehicle (water; 2 ml/kg body weight). Mice in the BBR groups were administrated with a daily gavage of BBR (dissolved in water), beginning the day following transplantation of H22 mice hepatoma tumor cells. The tumor volumes were measured every day, calculated according to the following formula: Length x $width^2$ x 0.52. Body weights were also measured every day. The mice were sacrificed ~2 weeks subsequent to treatment.

Statistical analysis. All data are expressed as the mean ± standard error of the mean. The statistical significance of the data was compared using Student's t-test with SPSS software, version 14.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

BBR inhibits viability of HCC cell lines in a time- and dose-dependent manner. In order to evaluate the anticancer characteristics of BBR, the viability of the cells was measured using an MTT assay following exposure to BBR for different time-periods (24, 48 or 72 h). It was found that BBR significantly reduced cell viability in mouse model and human hepatoma cell lines in a time- and dose-dependent manner. BBR also affected the viability of normal cells folloing longer durations of exposure (48 and 72 h), however, the IC_{50} values in the HL-7702 hepatic embryonic cell line (122.4 μM) at 72 h were markedly higher than the Bel-7404, H22 and HepG2 HCC cell lines (9.21, 43.2 and 82.8 μM, respectively). The observed IC_{50} values indicated that hepatoma cells are more susceptible to BBR, compared with normal cells (Fig. 1).

BBR increases apoptosis in HepG2 cells. The HepG2 cells were treated with 50 and 100 μM of BBR for 24 h. Apoptosis was detected using flow cytometric analysis with annexin V-PI staining. As shown in Fig. 2, HepG2 cells without BBR treatment were observed in the quadrant (Q)3, however, BBR treatment significantly induced early apoptosis, indicated by the distribution of the cells in Q4, and late apoptotic cells, indicated by distribution of the cells in Q2, in a dose-dependent manner. The total apoptotic rates were 24.13±2.14 and 55.55±2.86% in the 50 and 100 μM BBR treatment groups, respectively. These results suggested that the BBR-induced apoptosis in the tumor cells contributed to its anti-proliferative and cytotoxic efficacies.

BBR induces AIF-dependent apoptosis in HepG2 cells. In order to elucidate how BBR exerts its effects on apoptosis

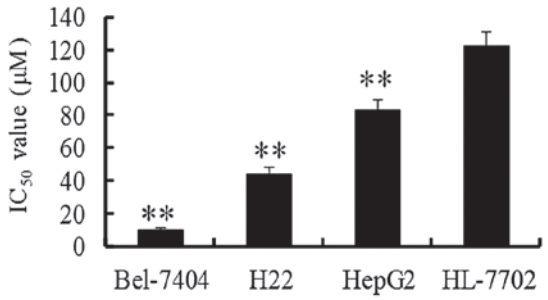

Figure 1. IC_{50} values in hepatoma and normal cell lines following 72 h treatment with BBR. An MTT assay was performed to determine the cell viability following 72 h-BBR treatment in three independent experiments, and the IC_{50} values were calculated from the MTT results. The data are presented as the mean ± standard error of the mean; $^{**}P<0.01$ vs. HL-7702 group. IC_{50}, half maximal inhibitory concentration; BBR, berberine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

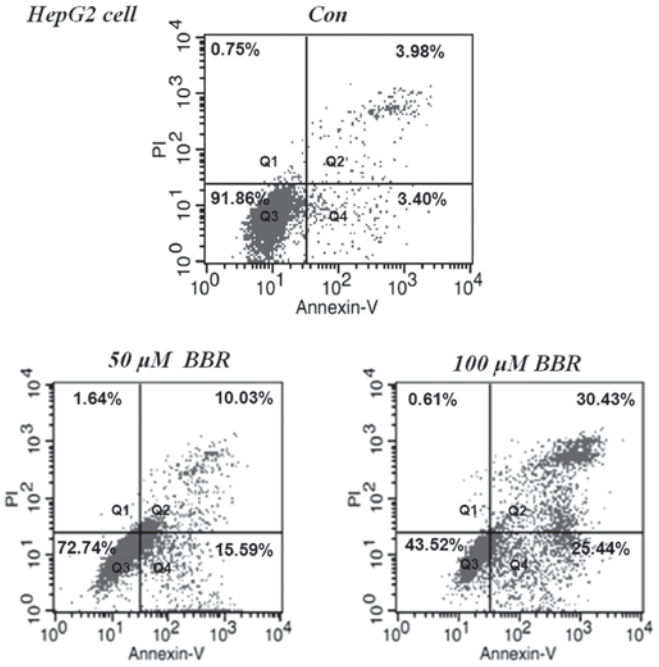

Figure 2. BBR induces apoptosis in HepG2 cells. Apoptosis was detected using flow cytometric analysis with annexin V-fluorescein isothiocyanate/PI double staining. The cells were incubated either in the absence or the presence of 50 or 100 μM BBR for 24 h. Q4 and Q2 quadrants represent early and late apoptosis following treatment with BBR. BBR, berberine; con, control; PI, propidium iodide.

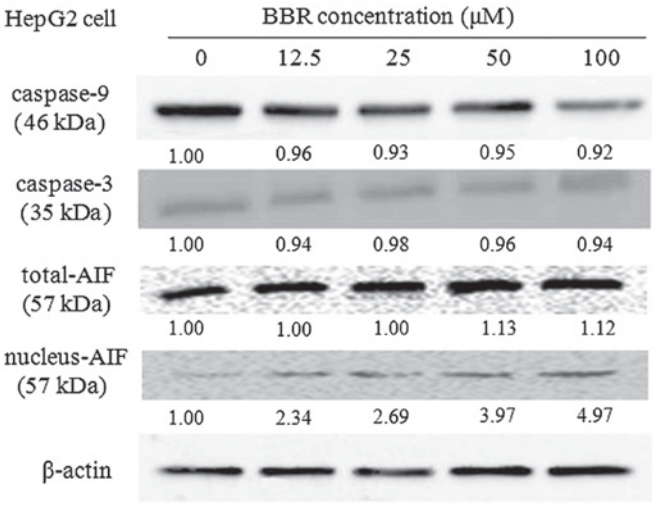

Figure 3. Protein expression levels of caspase-3, caspase-9, total AIF and nucleic AIF following treatment for 24 h with BBR, determined using western blot analysis. The HepG2 cells were treated with 0, 12.5, 25, 50 and 100 μM BBR for 24 h. The numbers below the band indicate the relative density ratio of each protein normalized to the internal control (β-actin). The experiments were performed three times. AIF, apoptosis-inducing factor; BBR, berberine.

in HepG2 cells, the effects of BBR on the intrinsic apoptotic pathways of caspase-3 and caspase-9 were investigated. The results demonstrated that the protein levels of total caspase-3 and total caspase-9 were not significantly altered following BBR treatment. In addition, the no protein expression of cleaved caspase-3 or cleaved caspase-9 proteins were detected using western blotting (Fig. 3). Similar results were observed in the caspase-3 activity assay, which demonstrated that BBR had no significant effect on the activity of caspase-3 (data not shown). To further examine the apoptotic mechanisms of BBR, total AIF and nucleic AIF were assessed using western blot analysis. The results demonstrated that a wide range of BBR concentrations between 12.5 and 100 μM, induced significant increases in the protein expression of nucleic AIF, however, the protein levels of total AIF were not affected. These results indicated that there is a potential translocation of AIF between the mitochondria and the nucleus, which is likely to be a pivotal factor of apoptosis in the BBR-treated HCCs.

BBR affects the AA pathway and increases the content ratio of AA to PGE2. To investigate how BBR affects the AA metabolic pathway, two key enzymes (cPLA2 and COX-2) in the pathway were assessed using western blot analysis. As shown in Fig. 4A, the protein expression levels of cPLA2 and COX-2 were suppressed significantly in a dose-dependent manner between concentrations of 12.5-100 μM BBR in the H22 and HepG2 cells. The contents of AA and PGE2 in the culture medium of the H22 and HepG2 cells treated with BBR for 24 h were then measured. As shown in Fig. 4B, the content of AA was significantly increased, and increased as the dose of BBR increased. The level of PGE2 was significantly reduced, even with treated with a low dose of BBR (12.5 μM). The concentration ratio of AA to PGE2 was increased following BBR treatment in the H22 and HepG2 cells, suggesting that the ratio may be important in BBR-induced apoptosis in the tumor cells.

BBR inhibits H22 transplanted tumor growth. To evaluate the efficacy of BBR on tumor growth *in vivo*, a H22 transplanted tumor model was established in BALB/c mice. As shown in Fig. 5A, the tumor volume in the control group reached the logarithmic growth phase 12 days following inoculation, and the same inhibitory effects were observed in the 50 mg/kg BBR and the CTX positive control group. Treatment with BBR reduced tumor volume in a dose-dependent matter (Fig. 5A), and this was confirmed by the ratios of tumor weight relative to mouse body weight (Fig. 5B).

Discussion

HCC has higher prevalence and mortality rates, compared with other types of cancer, including breast, prostate and stomach cancer (1). Therefore, prolonging life expectancy and improving quality of life have become key objectives in the treatment and management of patients with advanced HCC. The anticancer properties of BBR have been previously investigated (15,16,19-21). A key factor for the use of natural BBR as an alternative to the chemotherapeutic approach is its low cytotoxicity (22). In the present study the effects of BBR on cell viability and apoptosis were investigated in normal and cancer cell lines. BBR was found to suppress cell growth in dose- and time-dependent manner in mouse H22 and in human HepG2 and Bel-7404 HCC cell lines. This indicated that the inhibitory effect of BBR on liver tumor was not species-specific, and was associated with a specific type of HCC cells, as previously reported (22). In the control, the normal HL-7702 hepatic embryo cells exhibited depressed cell viability only in the higher dose (50-100 μM) BBR and longer (48-72 h) duration treatment groups, and exhibited higher IC_{50} values, compared with all the HCC cell lines. Comparable results of a previous study indicated that BBR does not significantly affect cell viability in an immortalized non-tumor cell line, which is consistent to Chang liver cells, following 48 h treatment (22). The higher IC_{50} observed in the normal cells in the present study indicated that BBR selectively reduces tumor cell viability with lower toxicity in normal cells. The divergent actions of BBR in the normal and hepatic cancer cells suggested that BBR may be a practical alternative therapeutic agent for HCC.

Apoptosis may proceed predominantly through a death receptor-dependent pathway (extrinsic pathway) or a mitochondria-dependent pathway (intrinsic pathway) (23). The permeabilization of the outer mitochondrial membrane and the subsequent release of pro-apoptotic proteins from the intermembrane space of mitochondria are key events in caspase-dependent and caspase-independent pathways. AIF is one of the mitochondrial proteins, which is released into the cytosol and translocated to the nucleus, where it binds to DNA and provokes caspase-independent chromatin condensation resulting in apoptosis (24-26). Therefore, AIF is a reliable indicator of caspase-independent apoptosis in cells. The results of the present study demonstrated that the intrinsic mitochondrial pathway, triggered by the activation of caspase-9 and -3 activation appear to be independent of

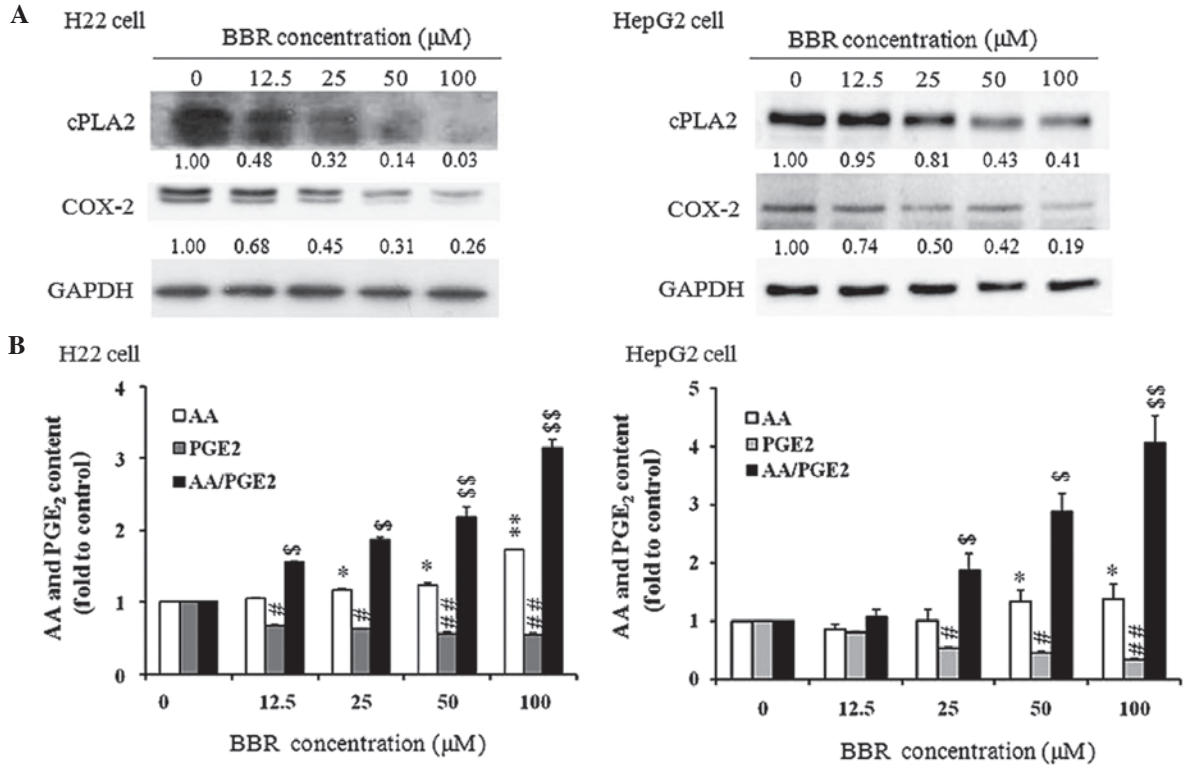

Figure 4. Effect of BBR on the AA metabolic pathway. (A) Protein expression levels of cPLA2 and COX-2 in the H22 and HepG2 cells. Cells were treated with 0, 12.5, 25, 50 and 100 μM BBR for 24 h. The numbers below the bands indicate the relative density ratio of each protein, normalized by the internal control (GAPDH). The experiments were performed three times and produced similar results. (B) Ratio of AA to PGE2 in the culture medium of H22 and HepG2 cells. The cells were treated with BBR at concentrations of 0, 12.5, 25, 50 or 100 μM for 24 h. At the end of treatment, the culture supernatant was collected and used to measure the content of PGE2 and AA using an enzyme immunoassay, according to the manufacturer's instructions. The values of PGE2, AA and the ratio of AA/PGE2 in the control group were set as 1 as normalization. Data are expressed as the mean ± standard error of the mean. *P<0.05 and **P<0.01, vs. control group for PGE2; $^{\$}$P<0.05 and $^{\$\$}$P<0.01, vs. control group for AA; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. control group for AA/PGE2. BBR, berberine; AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; COX-2, cyclooxygenase 2; PGE2, prostaglandin E2.

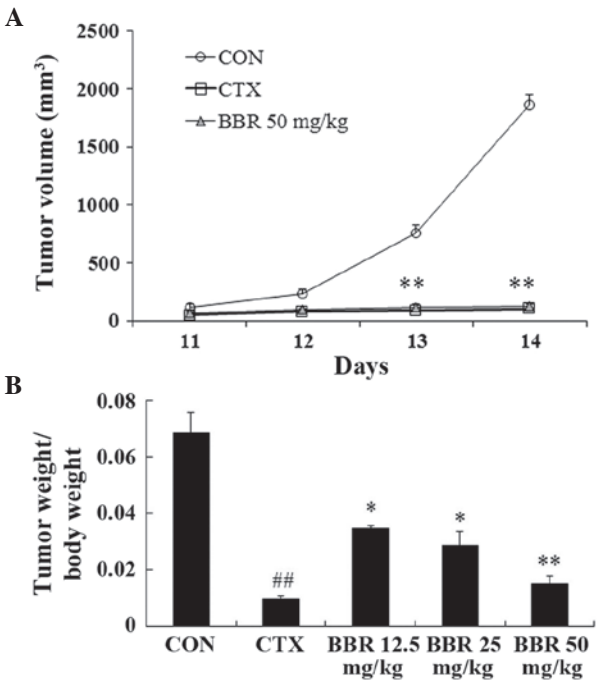

Figure 5. BBR inhibits hepatoma H22 transplanted tumor growth in mice. (A) Tumor volume curve following treatment with 50 μM BBR. (B) Ratio of tumor weight to relative mouse body weight at 14 days. All data are presented as the mean ± standard error of the mean. *P<0.05 and **P<0.01 vs. control group; $^{\#\#}$P<0.01, CTX group, vs. control group. BBR, berberine; CTX, cyclophosphamide.

BBR-induced apoptosis due to the fact that the protein expression levels of cleaved caspase-9 and -3 were undetectable, and that the activity of caspase-3 remained unchanged following treatment with BBR. This was consistent with reports from previous studies, which indicated that the caspase pathway was not significantly activated in HepG2 cells and other cancer cells following treatment with BBR (19,27). However, a number of previous studies have presented contradictory results. Hwang *et al* (22) demonstrated increases in the expression levels of cleaved caspase-3 and -8 in HepG2 cells, and proposed that the potential of anti-hepatoma activity of BBR may be mediated through a caspase-dependent pathway. Wang *et al* (19) reported that the increase of activity observed in caspase-3, -8 and -9 is involved in HepG2 cell apoptosis induced by BBR. There is no clear explanation for the contradictory results at present, which require further investigation. The results of the present study also demonstrated that BBR induced the translocation of AIF from the mitochondria to the nucleus in a dose-dependent manner in the HepG2 cells.

PLA2 and COX-2 in the AA metabolic pathway have been demonstrated to be important in the development and progression of various types of cancer (7,9,28,29). Tumor tissues surgically obtained from patients with HCC have been previously observed to exhibit significantly higher enzymatic activities, and protein and mRNA levels of PLA2, compared with those in surrounding liver tissues or control liver

tissues (30). The activation of cPLA2 may lead to the accumulation of intracellular AA and/or lysophospholipid, inducing the alteration of mitochondrial function (31). However, the released AA is degraded by COX-2, which is also overexpressed in the development of cancer (12,32). Consequently, PLA2 and COX-2 affect the production of AA and PGE2, with the final biological effects depending on the coupling of the PLA2 and COX-2 pathways and the combined actions of AA and PGE2. The present study demonstrated that overexpression of cPLA2 and COX2 in the H22 and HepG2 cells was significantly depressed by treatment with BBR, and the production of PGE2 was also reduced in a dose-dependent manner. In addition, the ratio of AA to PGE2 was consistently elevated in the two cell lines, indicating that the ratio of pro-apoptotic AA and anti-apoptotic PGE2 determined the survival of the cancer cells.

In conclusion, the present study demonstrated that BBR selectively exerted cytotoxicity towards the HCC cell lines and suppressed H22 transplanted tumor growth in BALB/c mice. In addition, BBR induced AIF-mediated apoptosis and increased the ratio of AA to PGE2, through suppressing the protein expression of cPLA2 and COX-2, which was involved in the inhibitory effect of BBR on HCC. These results suggested that BBR may be used as a potent and alternative chemotherapeutic agent for HCC treatment.

Acknowledgements

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