

Proliferation, migration and invasion of triple negative breast cancer cells are suppressed by berbamine via the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signaling pathways

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Abstract. Breast cancer is the second most common cause of cancer-associated mortality among women worldwide, and triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer. Berbamine (BBM) is a traditional Chinese medicine used for the treatment of leukopenia without any obvious side effects. Recent reports found that BBM has anti-cancer effects. The present study aimed to investigate the effects of BBM on TNBC cell lines and the underlying molecular mechanism. MDA-MB-231 cells and MCF-7 cells, two TNBC cell lines, were treated with various concentrations of BBM. A series of bioassays including MTT, colony formation, EdU staining, apoptosis, trypan blue dye, wound healing, transwell, ELISA and western blotting assays were performed. The results showed that BBM significantly inhibited cell proliferation of MDA-MB-231 cells ($P<0.05$; $IC_{50}=22.72\ \mu\text{M}$) and MCF-7 cells ($P<0.05$; $IC_{50}=20.92\ \mu\text{M}$). BBM ($20\ \mu\text{M}$) decreased the apoptosis ratio (percentage of absorbance compared with the control group) by $28.4\pm 3.3\%$ ($P<0.05$) in MDA-MB-231 cells, and $62.4\pm 4.6\%$ ($P<0.05$) in MCF-7 cells. In addition, BBM inhibited cell migration and invasion of TNBC cells. Furthermore, the expression levels of PI3K, phosphorylated-Akt/Akt, COX-2, LOX, MDM2 and mTOR were downregulated by BBM, and the expression of p53 was upregulated by BBM. These results indicated that BBM may suppress the development of TNBC via regulation of

the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signal pathways. Therefore, BBM might be used as a drug candidate for the treatment of TNBC in the future.

Introduction

Breast cancer is the second most common cause of cancer-associated mortality in women globally, accounting for 16% of cancer-associated mortalities in adult women annually (1-3). Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and is characterized by a lack of hormone receptors, such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (4). Given that this subtype cannot be treated with molecular targeted therapies towards these receptors/hormones, the treatment of TNBC is challenging and these patients typically exhibit a poor prognosis (5,6). Currently, surgery, chemotherapy and radiation therapy are the standard methods of treatment for breast cancer; however, the 5-year survival rate for patients with breast cancer of advanced stages remains low from 2010-2015 (7). Additionally, treatments for patients with advanced breast cancer result in undesirable therapeutic effects, since relapse often occurs after treatment (1). Therefore, the development of effective anti-cancer drugs with minimum side effects is critical.

Traditional Chinese Medicine has provided multiple medicinal resources and materials for the treatment of a variety of cancers. Berbamine (BBM) is derived from *Berberis amunrensis*. As a small molecule of natural origin, BBM has been widely used to treat leukopenia caused by chemotherapy and/or radiotherapy, without any obvious side effects (8). Previous studies reported that BBM possesses biological activities including anti-oxidation, anti-inflammation and protective effects against ischemia/reperfusion injury (9-11). In addition, BBM exhibits anti-tumor activity and inhibits the proliferation of breast cancer cell lines (8,12). Although previous studies have demonstrated that BBM downregulates the protein levels of Bax and Bcl-2 (12), the molecular mechanism underlying its anti-tumor function is still unclear. In addition, few studies have explored the effects and mechanism of BBM in breast cancer.

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Hyperactivation of the phosphatidylinositol 3 kinase/protein kinase B (PI3K/Akt) signaling pathway is associated with tumor growth, maintenance and chemotherapy resistance in several types of cancer, such as breast cancer, endometrial cancer and liver cancer (13-21). Dysregulation of signaling via the PI3K/Akt signaling pathway is one of the most frequent oncogenic aberrations associated with TNBC (22). Pierobon *et al* (21) reported that breast cancer with liver metastasis may be associated with increased incidence of PIK3CA mutations and activation of the PI3K/Akt/mTOR signaling pathway. These studies suggest that the mechanism underlying the effect of BBM treatment on tumors may involve the PI3K/Akt pathway. Therefore, it was hypothesized that BBM may inhibit the proliferation, invasion and metastasis of TNBC cells via the PI3K/Akt signaling pathway. Murine double minute 2 (MDM2) and mTOR were the downstream targets of Akt (4,23), MDM2 is a master regulator of the p53 tumor suppressor (24). Li *et al* (10) reported that HBXIP promotes human breast cancer growth by activating phosphorylated (p)-Akt, which in turn phosphorylates MDM2, thus enhancing the interaction between MDM2 and p53 and resulting in p53 degradation. Rinaldi *et al* (25) found that the mTOR pathway is related to tumor growth. Therefore, MDM2 and mTOR may serve an important role in the anti-breast cancer effects of BBM. Lysyl oxidase (LOX) and cyclooxygenase (COX)-2 are also downstream targets of the PI3K/Akt pathway (26,27). LOX is overexpressed in patients with TNBC and is closely associated with tumor metastasis (28). Moreover, the expression of LOX is enhanced by the activation of PI3K (26). COX-2 is also overexpressed in patients with TNBC (29) and COX-2 promotes migration in osteosarcoma MG-63 cells via the PI3K/Akt signal pathway (27). Previous studies have demonstrated that BBM may inhibit the proliferation and metastasis of tumor cells by regulating the expression levels of several proteins, including p-Akt (30-33). However, to the best of our knowledge, there have not been previous studies which indicate that BBM inhibits breast cancer by inhibiting the expression of PI3K, and there are no studies investigating the association between the downstream targets of Akt mentioned above (including MDM2, mTOR, LOX, and COX-2) and BBM. The present study will investigate the effects of BBM on TNBC cell proliferation, invasion and migration and will explore its underlying mechanisms. The current study will examine whether BBM inhibits the proliferation, migration and invasion of TNBC cells via regulating the PI3K/Akt pathway and downstream targets such as MDM2, p53, mTOR, COX-2 and LOX.

Materials and methods

Drugs and reagents. BBM (purity $\geq 98\%$) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. and was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM as a stock solution and diluted into indicated concentrations using DMEM medium (Gibco; Thermo Fisher Scientific, Inc.), as previously described (30,31). The final DMSO concentration was $<0.1\%$ in all experiments. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's Medium (DMEM) were purchased from Gibco; Thermo Fisher Scientific, Inc. Matrigel was purchased from BD Biosciences.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Cell Death Detection ELISA kits were purchased from Sigma-Aldrich; Merck KGaA. Primary antibodies against PI3K, MDM2, Akt, phosphorylated (p)-Akt, p53 and GAPDH, and the ELISA kit for mTOR, were purchased from Abcam. LOX, COX-2 and β -actin antibodies were purchased from Santa Cruz Biotechnology, Inc. Trypan Blue assay kits and all other reagents for western blotting were purchased from Beyotime Institute of Biotechnology.

Cell culture. The human TNBC cell lines MDA-MB-231 and MCF-7 were purchased from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin and stored in a humidified incubator at 37°C with $5\% \text{CO}_2$.

MTT assay. MDA-MB-231 cells and MCF-7 cells were seeded into a 96-well plate (5×10^4 cells/well) and treated with vehicle (DMEM medium with 0.08% DMSO) or different concentrations of BBM (1.25, 2.5, 5, 10, 20, 40, and $80 \mu\text{M}$) for 24 h at 37°C . MTT solution (5 mg/ml) was added to each well and the plate was incubated for another 4 h at 37°C . Next, the supernatant was removed and DMSO solution was added. The absorbance was read by a Multi-Well Micro-Plate Reader (Bio-Rad Laboratories, Inc.) at 560 nm. Cell viability was expressed as a percentage of the vehicle (Ctrl) group.

Colony formation assay. The colony formation assay was performed as previously described (29). MDA-MB-231 cells and MCF-7 cells were cultured in a 6-well plate (1×10^3 cells/well) and treated with vehicle or different concentrations of BBM (10, 20 and $40 \mu\text{M}$) for 24 h at 37°C . The medium was replaced by a drug-free, complete DMEM (with 10% FBS). The medium was changed every 3 days for MDA-MB-231 cells and every 2 days for MCF-7 cells. After 7 days, the cells were fixed with 4% paraformaldehyde for 30 min at 25°C and stained with crystal violet solution (0.05% w/v) for 20 min at 25°C . The number of clones >10 cells was counted under a light microscope (Leica DMI 4000; magnification, $\times 100$).

5-Ethynyl-2'-deoxyuridine (EdU) staining assay. EdU staining assay was performed using the EdU Apollo-567 *in vitro* kit (Guangzhou RiboBio Co., Ltd; cat. no. C10310-1). Briefly, MDA-MB-231 cells and MCF-7 cells were seeded into 96-well plates (1×10^4 cells/well) and cultured overnight at 37°C . EdU ($10 \mu\text{M}$) was added into each well and incubated for 2 h at 37°C . After the cells were fixed with 4% polyoxymethylene at 25°C for 30 min and decolorized with glycine (2 mg/ml) at 25°C for 5 min, the cell nuclei were stained with Hoechst 33342 at 25°C for 30 min. The fluorescence of cells was observed using an inverted fluorescence microscope (magnification, $\times 100$; Leica Microsystems GmbH). The EdU ratio was calculated as follows: Number of EdU-positive cells/number of Hoechst 33342-positive cells $\times 100\%$.

Trypan Blue Dye assay. MDA-MB-231 cells (1×10^5 cells/well) were seeded into 6-well plates in the presence or absence of different concentrations of BBM (10, 20 and $40 \mu\text{M}$) and cultured at 37°C for 48 h. The cells were collected and re-suspended using 0.4% Trypan blue at 25°C . Then the cells

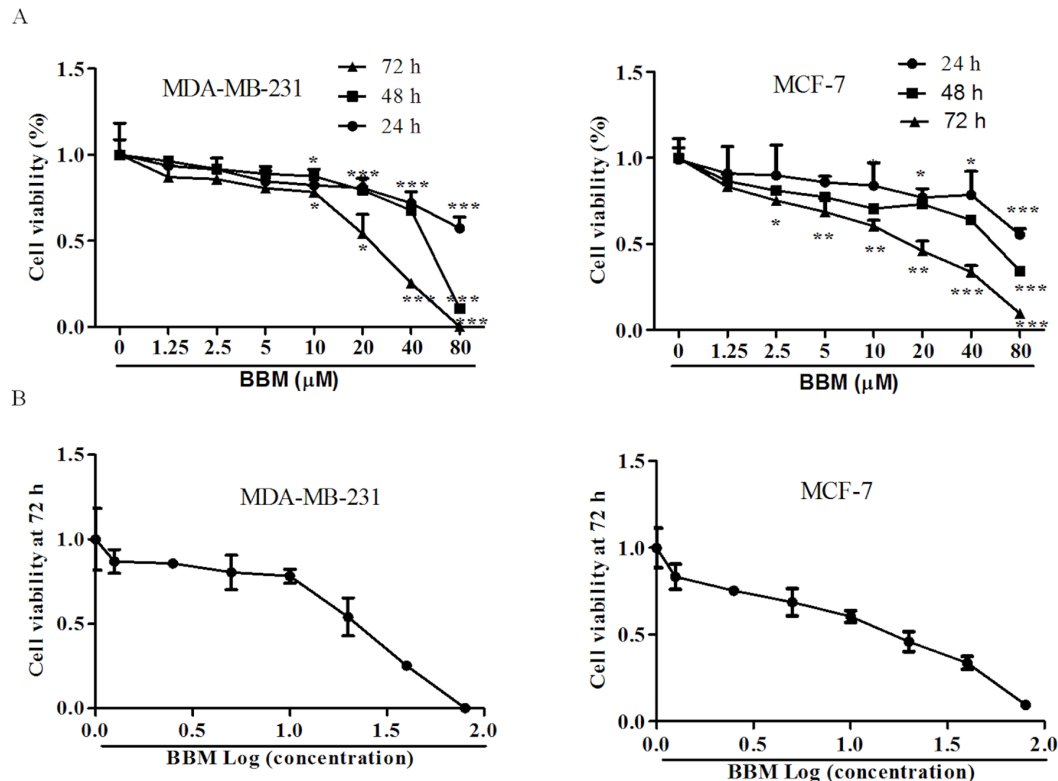


Figure 1. BBM is cytotoxic to triple negative breast cancer cells. The cells were treated with applied concentrations (1.25, 2.5, 5, 10, 20, 40 or 80 μ M) of BBM, cell viability was tested using MTT assay. (A) Effects of different concentrations of BBM on cell proliferation at different times. (B) The effect of BBM on cell proliferation after treatment for 72 h. The Ctrl group was the cells treated with solvent (medium containing DMSO, DMSO <0.8%). Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl group. BBM, Berbamine; Ctrl, control.

visualized under a light microscope (Leica DMI 4000; magnification, x100).

Apoptosis assay. Cell apoptosis was detected using a photometric enzyme immunoassay (Cell Death Detection ELISA kit, cat. no. 11544675001; Sigma-Aldrich Merck KGaA), as previously described (30). This is based on the quantitative sandwich immunoassay employing antibodies against histone and DNA. MDA-MB-231 cells and MCF-7 cells (1×10^5 cells/well) were seeded into a 6-well plate in the presence or absence of different concentrations of BBM (10, 20 and 40 μ M) and cultured for 24 h at 37°C. The cells were washed with PBS and then lysed in RIPA lysis buffer (containing 1 mM PMSF). The supernatant was collected via centrifugation at 12,000 \times g for 15 min at 4°C. and used for testing. The mono- and oligonucleosomal fragmented DNA was measured according to the instructions of the manufacturer.

Wound healing assay. MDA-MB-231 cells and MCF-7 cells were seeded (2×10^5 cells/well) into 6-well plates and cultured for 48 h at 37°C. Once the cells reached a confluence of 90%, a 200 μ l plastic tip was used to make a straight line in the monolayer, and the plate was washed with PBS. Fresh serum-free medium with vehicle or different concentrations of BBM (10, 20 and 40 μ M) and mitomycin (2 μ g/ml) was added into each well. The cells were observed under an inverted microscope coupled to a camera (100x, Leica DMI 4000) at 25°C, and this time was designated at 0 h, then the cells were placed in an incubator with 5% CO₂ for 24 h (MDA-MB-231 cells) or

72 h (MCF-7 cells) at 37°C. The cells were observed under an inverted microscope coupled to a camera (100x, Leica DMI 4000) at 25°C. The migration distance was measured using Image J (National Institutes of Health, USA, v 1.5.1), and the migration percentage was calculated, the percentage of wound closure=(the scratch area of 0 h-the scratch area of 24 h)/the scratch area of 0 h.

Transwell assay. MDA-MB-231 cells and MCF-7 cells (8×10^4 cells) in the presence or absence of different concentrations of BBM (10, 20 and 40 μ M) were suspended in serum-free medium and then seeded into the upper chamber of a transwell chamber with an 8- μ m pore (Corning, Inc.). The upper chambers were pre-coated at 37°C with (Transwell invasion assay) or without (Transwell migration assay) Matrigel (1 mg/ml), and the lower chambers contained medium with 20% FBS. The chambers were incubated for 36 h (MDA-MB-231) or 48 h (MCF-7) at 37°C. The cells on the surface of the membrane were fixed with 4% polyoxymethylene at 25°C for 30 min and stained with crystal violet staining solution (1%) at 25°C for 5 min. Mitomycin (2 μ g/ml) was added when the cells were seeded into the upper chambers to exclude the proliferation of the cells. Cells visualized under a light microscope (Leica DM 4000; magnification, x100).

Western blotting assay. Cells were lysed using RIPA lysis buffer. Protein concentration was determined using a BCA assay kit (Beyotime Institute of Biotechnology; cat. no. P0012). Lysate proteins (20 μ g) were then separated

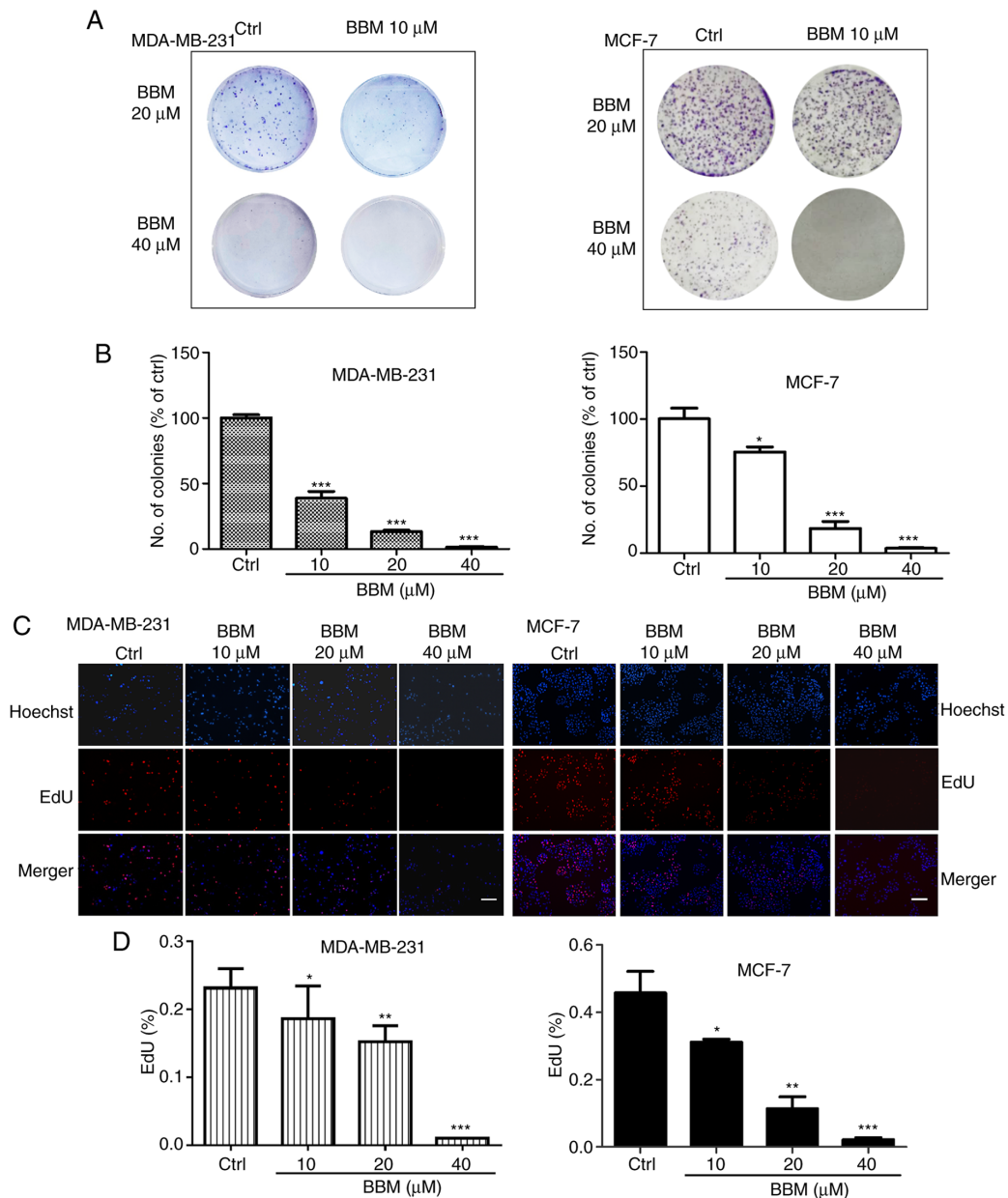


Figure 2. BBM inhibits the proliferation of triple negative breast cancer cells. Cells were treated with applied concentrations (10, 20 and 40 μ M) of BBM. (A) The cell proliferation was tested using a colony formation assay and medium was renewed every 3 days. (B) Quantification of colony forming assay results. (C) EdU assay images and (D) quantification (scale bar, 100 μ m). The Ctrl group was untreated cells. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl group. BBM, Berberine; Ctrl, control.

by 10% SDS-PAGE and transferred to a PVDF membrane (EMD Millipore). The membranes were blocked using TBST (containing tween 20, 0.05%) containing 5% non-fat milk at room temperature for 2 h and then incubated with primary antibodies against PI3K (cat. no. ab70912; 1:1,000), MDM2 (cat. no. ab16895; 1:500), Akt (cat. no. ab8805; 1:1,000), p-Akt (cat. no. ab38449; 1:1,000) COX-2 (cat. no. sc-19999; 1:200), LOX (cat. no. sc-373995; 1:500), GAPDH (Abcam; cat. no. ab9845; 1:1,000) and β -actin (cat. no. sc-8432; 1:1,000) at 4°C overnight. After washing with TBST, the membranes were incubated with secondary antibodies (GenScript; cat. no. A00098; 1:10,000) conjugated to horseradish peroxidase at room temperature for 2 h. Bands were detected using THE Hypersensitive ECL chemiluminescence kit (Beyotime Institute of Biotechnology;

cat. no. P10018FS). Optical density of bands (relative to GAPDH or β -actin) were quantified using Image J (National Institutes of Health; v1.5.1).

ELISA. mTOR (pSer2448) level was assayed using the mTOR ELISA kit (Abcam, cat. no. ab176657). Cells were lysed in RIPA buffer as described above and mTOR was detected according to the manufacturer's instructions.

Statistical analysis. All statistical analyses were performed using SPSS 21.0 (IBM Corp.). Data are presented as mean \pm standard deviation (SD). Significance differences were determined using one-way ANOVA, followed by Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

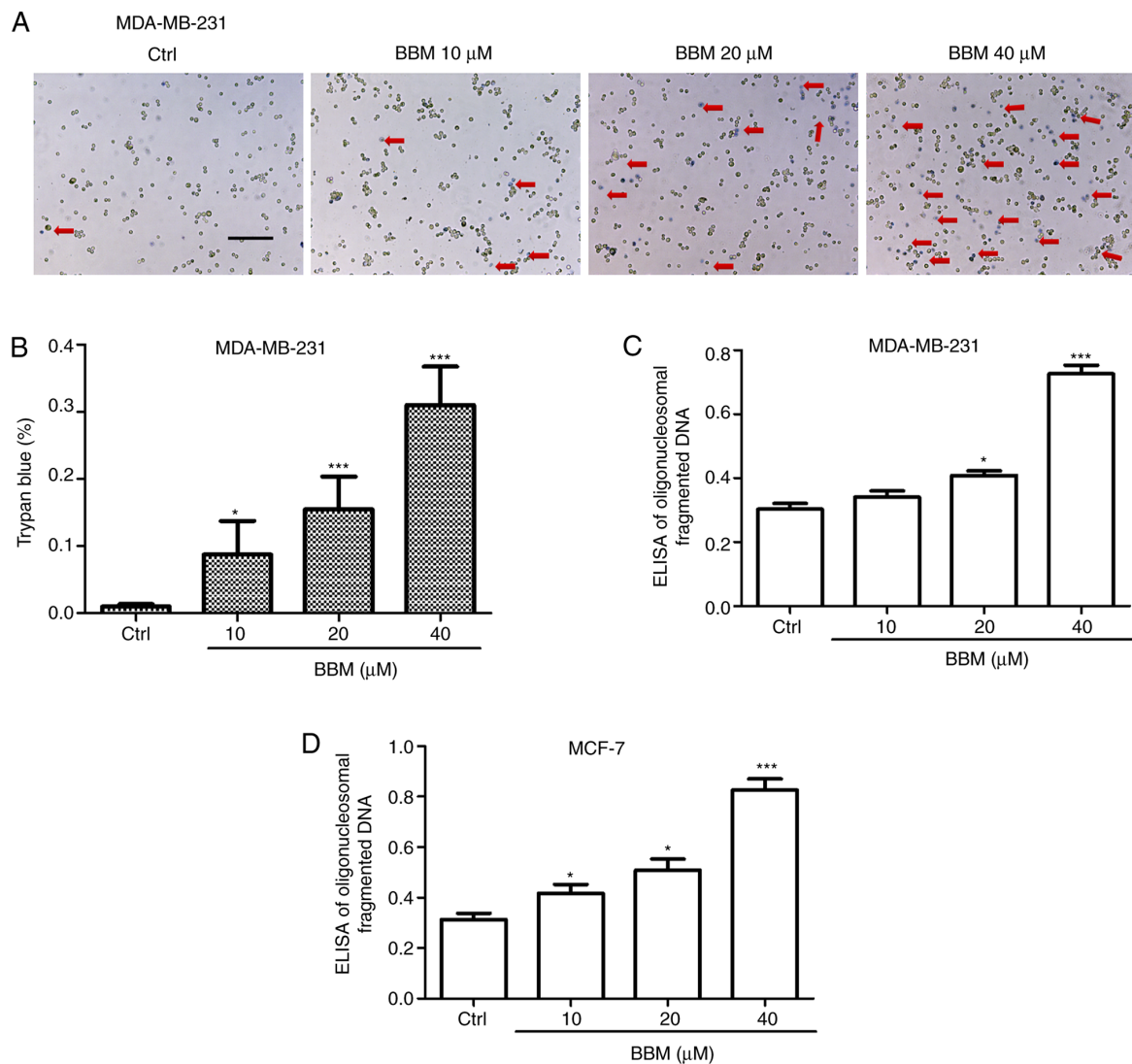


Figure 3. BBM induces triple negative breast cancer cell death. Cells were untreated (Ctrl) or treated with BBM (10, 20 and 40 μ M). Cell death and apoptosis were (A) detected using a Trypan blue dye assay (scale bar, 100 μ m, the arrows indicated that the cells were stained with Trypan blue) and (B) quantified. Cell Death Detection ELISA assays in (C) MCF-7 and (D) MDA-MB-231 cells. The Ctrl group was untreated cells. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, ***P<0.001 vs. Ctrl group. BBM, Berbamine; Ctrl, control.

Results

BBM inhibits the cell viabilities of MDA-MB-231 and MCF-7 cells. MTT assay was used to detect the effects of BBM on the proliferation of human TNBC cell lines. The results demonstrated that BBM significantly inhibited the cell viabilities of MDA-MB-231 cells and MCF-7 cells in a time- and dose-dependent manner; (Fig. 1A). In MDA-MB-231 cells, after incubation for 24, 48, and 72 h, the minimum concentration of BBM inhibited cell proliferation was 10 μ M. Moreover, with the extension of incubation time, the proliferation rate of cells inhibited by BBM was significantly decreased at the same concentration. In MCF-7 cells, the minimum concentration of BBM inhibited cell proliferation was 20 μ M for 24 h treated cells, 10 μ M for 48 h, and 2.5 μ M for 72 h. In addition, with the extension of incubation time, the proliferation rate of cells inhibited by BBM was significantly decreased as well. The IC₅₀ values of BBM for 72 h on MDA-MB-231 cells and MCF-7 cells were 22.7 \pm 3.3, and 20.9 \pm 1.5 μ M, respectively (Fig. 1B).

IC₅₀ value of 72 h was selected as the basis to exclude the effect of cell proliferation. Thus, BBM of 10, 20 and 40 μ M were selected for the subsequent assays.

BBM inhibits the proliferation of MDA-MB-231 and MCF-7 cells. The effects of BBM on cell proliferation were evaluated using colony formation and EdU staining assays. In MDA-MB-231 cells, compared with the Ctrl group, BBM at 10, 20 and 40 μ M decreased the number of the colonies in a dose-dependent manner (P<0.001; Fig. 2A and B). In MCF-7 cells, BBM at 10, 20 and 40 μ M decreased the number of the colonies in a dose-dependent manner as well (P<0.05, P<0.001 and P<0.001, respectively (Fig. 2A and B). To further elucidate the effect of BBM on the cell proliferation of TNBC cells, EdU staining assay was performed. The results indicated that BBM inhibited cell proliferation in a concentration-dependent manner (Fig. 2C and D). In MDA-MB-231 cells, BBM at 10, 20 and 40 μ M significantly inhibited cell proliferation compared with the Ctrl group (P<0.05, P<0.01 and P<0.001,

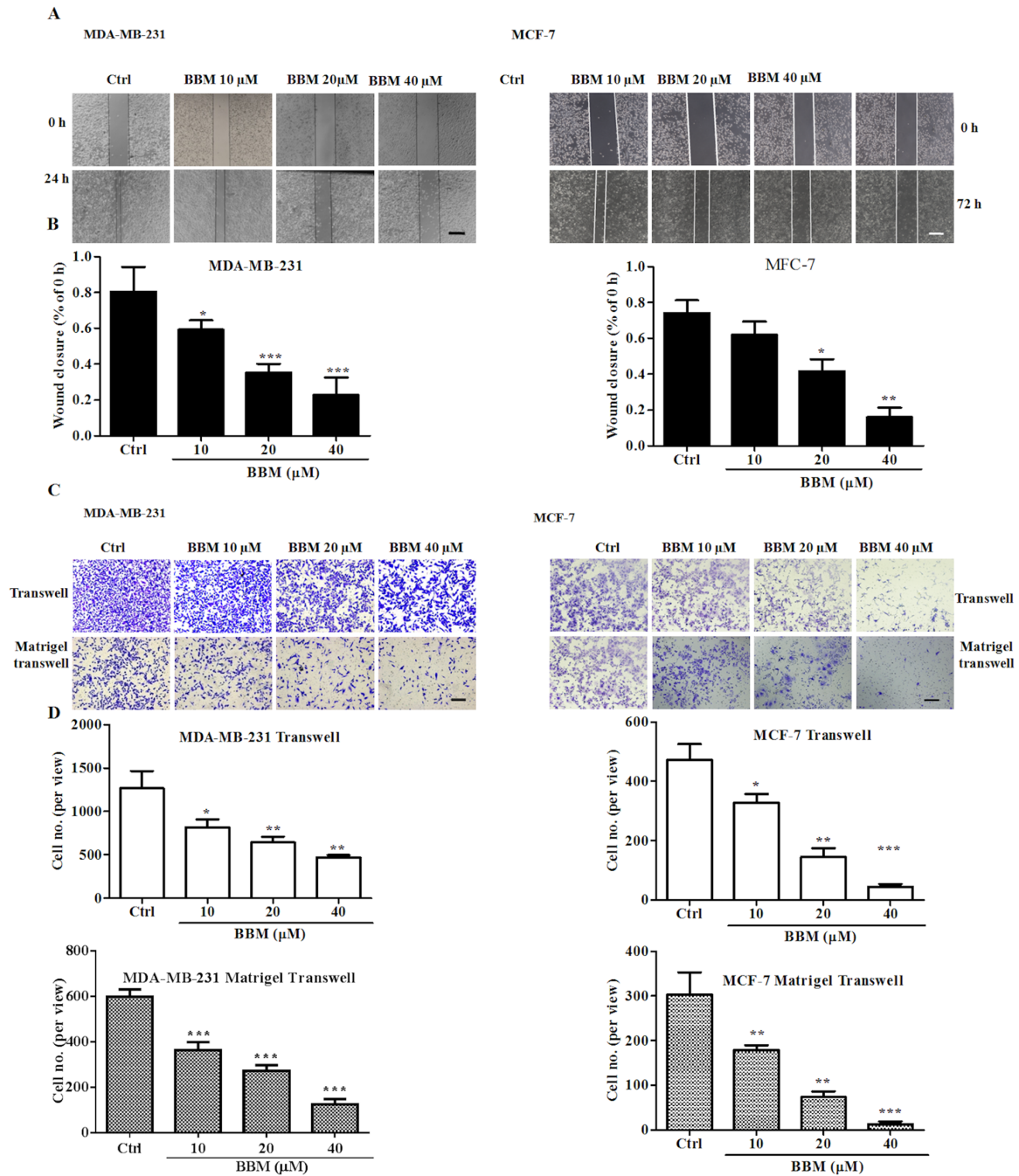


Figure 4. BBM inhibits the migration and invasion of triple negative breast cancer cells. Cells were treated with BBM (10, 20 and 40 μ M), migration and invasion were tested using (A) wound closure assays (scale bar, 100 μ m) and (B) quantified. (C) Transwell and Matrigel transwell assay cell images (scale bar, 100 μ m) and (D) quantification of the results. The Ctrl group was untreated cells. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl group. BBM, Berbamine; Ctrl, control.

respectively). In MCF-7 cells, BBM at 10, 20, and 40 μ M significantly inhibited cell proliferation compared with the Ctrl group (P<0.05, P<0.01 and P<0.001, respectively).

BBM induces apoptosis of MDA-MB-231 and MCF-7 cells. To investigate the effects of BBM on TNBC cell apoptosis, trypan blue and Cell Death Detection ELISA assays were performed. The results showed that BBM induced cell death in a concentration-dependent manner. BBM at 10, 20, and 40 μ M significantly induced cell death compared to the Ctrl group (P<0.05, P<0.001 and P<0.001, respectively; Fig. 3A and B). In

addition, the ELISA assay revealed that BBM at 20 and 40 μ M significantly induced death of MDA-MB-231 cells compared with the Ctrl group (P<0.05 and P<0.001, respectively; Fig. 3C). Moreover, BBM at 10, 20, and 40 μ M induced the cell death of MCF-7 cells compared with the Ctrl group (Fig. 3D) (P<0.05, P<0.05 and P<0.001, respectively).

BBM inhibits cell migration and invasion in MDA-MB-231 and MCF-7 cells. Wound healing and transwell assays were performed to evaluate the effects of BBM on cell migration and invasion. The results showed that BBM inhibited wound

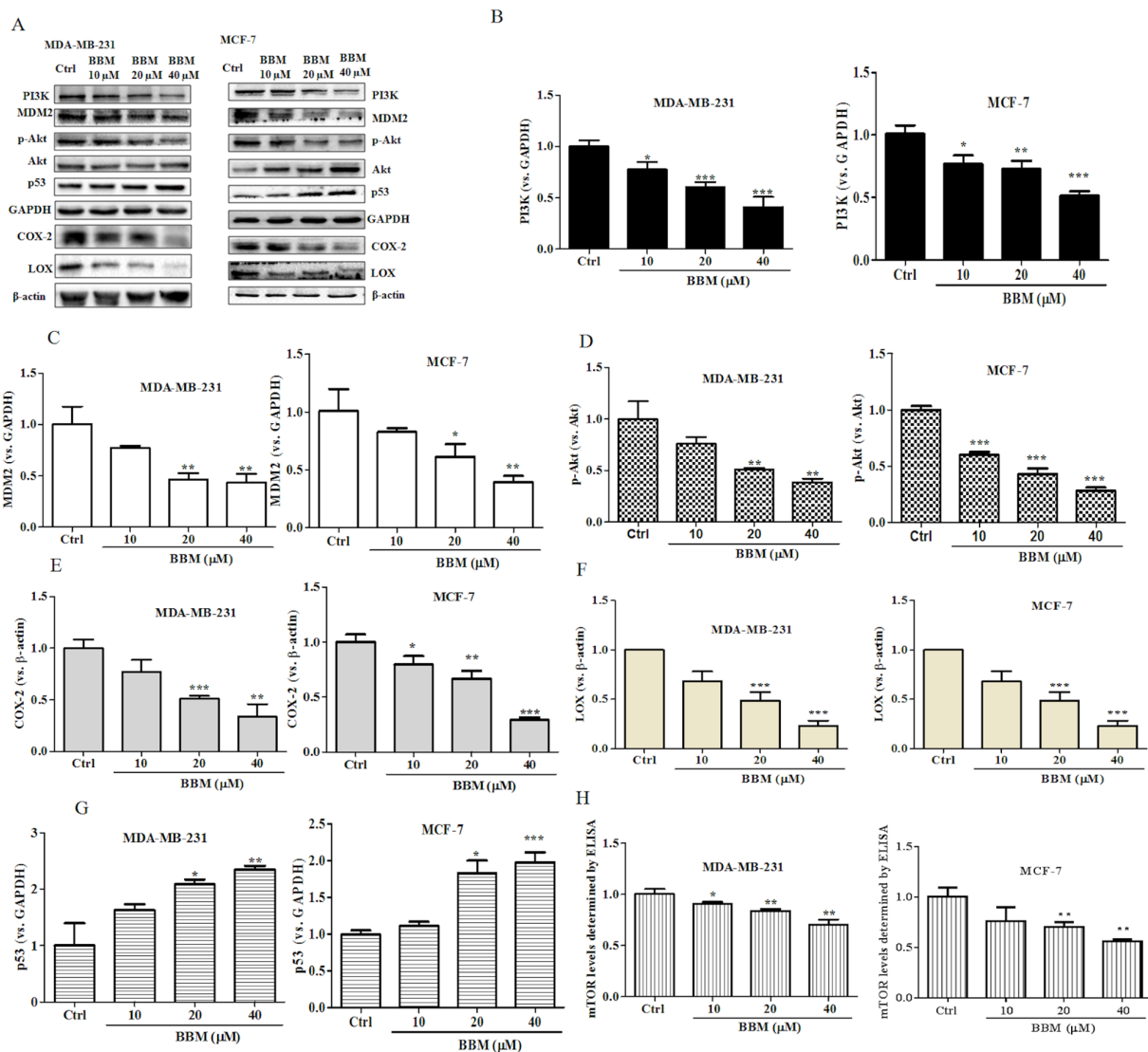


Figure 5. BBM disrupts the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signaling pathways in triple negative breast cancer cells. Cells were untreated (Ctrl) or treated with BBM (10, 20 and 40 μ M). (A) Western blots were performed on both cell lines. The expression levels of (B) PI3K, (C) MDM2, (D) p-Akt/Akt, (E) COX-2, (F) LOX and (G) p53 were quantified. (H) The expression of p-mTOR was tested using an ELISA. The Ctrl group was untreated cells. Data are presented as the mean \pm standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl group. BBM, Berberine; Ctrl, control; p-, phosphorylated.

closure in a concentration-dependent manner (Fig. 4A and B). BBM at 10, 20, and 40 μ M significantly inhibited the migration of MDA-MB-231 cells compared to the Ctrl group (P<0.05, P<0.001 and P<0.001, respectively). BBM at 20, and 40 μ M significantly inhibited the migration of MCF-7 cells compared to the Ctrl group (P<0.05 and P<0.01, respectively).

It was also revealed that BBM significantly inhibited cell migration and invasion of TNBC cells in a concentration-dependent manner (Fig. 4C and D). In MDA-MB-231 cells, BBM at 20, and 40 μ M significantly reduced the number of the migrated cells compared to the Ctrl group (P<0.05, P<0.01 and P<0.01, respectively). Furthermore, BBM at 20, and 40 μ M significantly reduced the number of invaded cells compared to the Ctrl group (all P<0.001). In MCF-7 cells, BBM at 20, and 40 μ M significantly reduced the number of migrated cells compared to the Ctrl group (P<0.05, P<0.01 and P<0.001, respectively). BBM at 10, 20, and 40 μ M significantly reduced the number of invaded cells compared to the Ctrl group (P<0.01, P<0.01 and P<0.001, respectively). These

results indicated that BBM inhibited the cell migration and invasion in TNBC cells.

BBM regulates the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signaling pathways in MDA-MB-231 and MCF-7 cells. The present results revealed that BBM exhibited strong inhibitory effects on cell growth, proliferation, metastasis and invasion in TNBC cells. It was speculated that BBM may serve an anti-cancer role in breast cells via regulation of the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signaling pathways. To verify this hypothesis, the expression levels of PI3K, p-Akt/Akt, MDM2 and p53 were evaluated using western blotting. BBM at 10, 20 and 40 μ M significantly inhibited the expression of PI3K (Fig. 5A and B) both in MDA-MB-231 cells (P<0.05) and in MCF-7 cells (P<0.05). BBM at 20 and 40 μ M significantly inhibited the expression of MDM2 (Fig. 5A and C) both in MDA-MB-231 cells (P<0.01) and in MCF-7 cells (P<0.05). BBM significantly inhibited the expression levels of p-Akt/Akt in MDA-MB-231 cells at

the concentrations of 20 and 40 μM ($P < 0.01$) and in MCF-7 cells at the concentration of 10, 20 and 40 μM ($P < 0.001$) (Fig. 5A and D). BBM significantly inhibited the expression of COX-2 in MDA-MB-231 cells at the concentrations of 20 and 40 μM ($P < 0.001$) and in MCF-7 cells at the concentration of 10, 20 and 40 μM ($P < 0.001$) (Fig. 5A and E). BBM at 20 and 40 μM significantly inhibited the expression of LOX both in MDA-MB-231 cells ($P < 0.001$) and MCF-7 cells ($P < 0.001$) (Fig. 5A and F). Notably, BBM at 20 and 40 μM increased the expression p53 in MDA-MB-231 ($P < 0.05$) cells in MCF-7 cells ($P < 0.05$) (Fig. 5A and G). The expression level of mTOR was tested by ELISA. BBM significantly inhibited the expression of mTOR in MDA-MB-231 cells at of the doses of 10 μM ($P < 0.05$), 20 μM ($P < 0.01$) and 40 μM ($P < 0.01$) and in MCF-7 cells at the dose of 20 μM ($P < 0.01$) and 40 μM ($P < 0.01$) (Fig. 5H). In summary, the results of the western blot assay indicated that the expression levels of PI3K, MDM2, p-Akt/Akt, COX-2, LOX and mTOR were significantly downregulated by BBM, while the expression of p53 was upregulated by BBM.

Discussion

TNBC is the most aggressive subtype of breast cancer and accounts for 15~20% of all breast cancers worldwide (34). Metastasis is the primary cause of mortality in patients with breast cancer (35). BBM is a traditional medicine derived from *Berberis amurensis*. Previous studies have demonstrated the anti-tumor effects of BBM in a variety of cancers (36,37). Jin and Wu (38) demonstrated that BBM significantly downregulates the expressions of apoptosis-related proteins including Bcl-2 and Bcl-xL and inhibits cell proliferation in pancreatic carcinoma. Du *et al* (30) found that BBM induces cell apoptosis and inhibits cell proliferation via the PI3K/Akt pathway in lymphoma. In addition, BBM inhibits cell proliferation and destroys mitochondria in prostate cancer cells (39). These studies support the potential of BBM as a drug for cancer treatment.

In the present study, the effect of BBM on the proliferation, apoptosis, invasion and migration of TNBC cells was evaluated. The present study indicated that the cell proliferation of MAD-MB-231 cells and MCF cells was inhibited by BBM. Moreover, BBM induces the apoptosis of TNBC cells. Subsequent experiments revealed that BBM inhibits the invasion and metastasis of TNBC cells. These results are consistent with previous studies (8,12,17) showing that BBM has inhibitory effects on tumors. Western blot was used to detect the effect of BBM on protein expression in TNBC cells to elucidate the possible mechanism of BBM inhibition of proliferation, invasion and metastasis of TNBC cells.

The PI3K/Akt pathway is involved in cell proliferation, apoptosis invasion, and migration, and aberrant activation of this pathway is associated with the development of many types of cancers, breast cancer and lung cancer (40). The present results revealed that cell proliferation, invasion and migration of TNBC cells were inhibited by BBM, and apoptosis was induced by BBM. In addition, the expressions of PI3K and p-Akt/Akt were downregulated following BBM treatment. These results, combined with previous studies, indicate that BBM may serve an anti-tumor role in TNBC by inhibiting the

PI3K/Akt signaling pathway. Several studies have reported that proteins downstream of Akt regulate cancer cell apoptosis and other cellular events (41-43). For instance, MDM2 is downstream of Akt and inhibits the expression of p53. Overexpression of MDM2 may induce breast cancer via the activation of other oncogenes, such as by promoting ubiquitination/degradation of E-cadherin, resulting in increased invasion of cancer cells (44).

COX-2 is overexpressed in TNBC (28) and may increase the phosphorylation of MDM2, resulting in impaired p53 function (45). Zhang *et al* (27) reported that COX-2 promotes angiogenesis and tissue invasion in osteosarcoma. The current results revealed that the invasion of TNBC cells was inhibited by BBM. And the expressions of MDM2 and COX-2 were downregulated by BBM treatment, while the expression of p53 was upregulated. Combined with the aforementioned studies, these findings suggest that BBM inhibits the invasion of TNBC cells via regulating the MDM2-p53 pathway, and the pathway may also be regulated by COX-2. In addition, overexpression of Akt2 induces cell metastasis and invasion via upregulation of integrin $\beta 1$ in breast and ovarian cancers (3,4). mTOR is an important effector of the PI3K/Akt signaling pathway and is expressed in most mammalian cells, resulting in a rise of cellular protein mass and inhibition of autophagy (8). Gao *et al* (46) found that cepharanthine can induce apoptosis and autophagy by inhibiting the Akt/mTOR signaling pathway (46). Cepharanthine is a natural product and used for >70 years in Japan to treat a variety of diseases, including leukopenia (42). These characteristics of cepharanthine are very similar to BBM (47). Moreover, cepharanthine was reported to inhibit the metastasis and invasion, and induce apoptosis of breast cancer cells (46,48). Therefore, we hypothesized that BBM also served an anti-tumor role via the Akt/ mTOR signaling pathway. Furthermore, LOX is high expressed in patients with TNBC, a previous study revealed that LOX promotes cell proliferation and inhibits apoptotic cell death, and this effect may be achieved by activation of the Akt/mTOR signaling pathway (49). The present results indicate that BBM induced the apoptosis of TNBC cells, and the expressions of mTOR and LOX were downregulated following treatment with BBM. Collectively, the current results suggest that BBM may induce the apoptosis of TNBC cells by inhibiting the expressions of mTOR and LOX. MDM2 and mTOR are downstream targets of the PI3K/Akt signaling pathway (4,21,50,51). Thus, it was hypothesized that BBM may induce the apoptosis of TNBC cells via the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signal pathways.

In conclusion, the proliferation, migration and invasion of TNBC cells were suppressed following treatment with BBM, and this effect may be achieved by modulating the PI3K/Akt/MDM2/p53 and PI3K/Akt/mTOR signaling pathways. However, this study is only a preliminary *in vitro* study, future studies such as *in vivo* experiments may need to be performed to verify that BBM has low toxicity, and either alone or in combination with other chemotherapy may represent a novel treatment strategy for breast cancer.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JY and LL designed the present study, analyzed the data, drafted the initial manuscript and revised it for important intellectual content. YC and YY performed the experiments. BY, LT, XS and LL were responsible for acquiring, analyzing and interpreting the data. SW designed the present study and revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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