

The anti-migration and anti-invasion effects of Bruceine D in human triple-negative breast cancer MDA-MB-231 cells

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Abstract. Brucein D (BD) is a naturally occurring major active quassinoid extracted from the Chinese medicinal herb *Brucea javanica*, which has been previously demonstrated to exhibit anticancer activities. The present study aimed to investigate the anticancer effects of BD on MDA-MB-231 cells, a human triple-negative breast cancer (TNBC) cell line. An MTT assay was performed to assess cell viability, whilst wound healing and Transwell assay were applied to measure cell migration and invasion, respectively. Western blot analysis was performed to assess the expression of E-cadherin, vimentin and β -catenin, which are proteins associated with epithelial-mesenchymal transformation (EMT), and PI3K, AKT and p-AKT, which are key components of the PI3K/AKT signaling pathway. BD was indicated to reduce cell viability in a dose- and time-dependent manner, whilst cell invasion and migration were also significantly inhibited in a dose-dependent manner. Western blot analysis demonstrated that BD treatment significantly upregulated the expression of E-cadherin and downregulated the expression of vimentin and β -catenin. Additionally, BD downregulated the expression of PI3K and reduced AKT phosphorylation. In conclusion, BD can inhibit MDA-MB-231 cell viability, migration and invasion, suggesting the potential use of BD for the treatment of TNBC.

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that is characterized by the lack of estrogen receptor,

progesterone receptor and human epidermal growth factor receptor 2 expression (1). In total, TNBC accounts for ~10-25% of all breast cancer cases (1), and is associated with unique clinical and pathophysiological phenotypes, including strong invasiveness, low overall survival rate and poor prognosis (2). Compared with other types of breast cancer, TNBC carries an increased probability of metastasis and recurrence (3) due to metastasis to other organs, including the lungs, liver, and brain via the systemic circulation. Targeted therapy cannot be administered to patients with TNBC due to the lack of hormone receptor expression. Although previous studies have reported that TNBC is more sensitive to chemotherapy compared with other types of breast cancer, TNBC remains to exhibit the worst prognosis compared with other subtypes (4). Therefore, the development of novel anti-metastatic therapeutic strategies is required for the effective treatment of TNBC.

Brucea javanica is the fruit of the *Brucea javanica* (L.) Merr (Simaroubaceae). It has been used to treat a number of diseases, including cancer, dysentery, malaria and stomach ulcers (5). Bruceine D (BD; Fig. 1A) is a bioactive component that can be isolated from *Brucea javanica* (6), which has previously been reported to induce apoptosis in pancreatic cancer cell lines PANC1, SW1990 and Capan1 and inhibit hepatoma cell proliferation (7-10).

Metastasis is an important feature of malignant tumors that impede the clinical treatment of cancer (11). Epithelial-mesenchymal transition (EMT) is an important stage in the metastasis of cancer cells. During this process, epithelial cell polarity is lost, and is coupled with concomitant enhancements in migratory and invasive abilities (12). As a result, the epithelial phenotype disappears, whereas the mesenchymal phenotype gradually develops (12). The PI3K/AKT signaling pathway has been previously indicated to be associated with cancer cell proliferation, differentiation, migration and invasion (13). AKT activation in epithelial cells reduces cell polarity and intercellular adhesion and promotes EMT in cancer cells by altering the expression and distribution of epithelial and mesenchymal markers (14).

In the present study, the TNBC cell line MDA-MB-231 was used to investigate the potential inhibitory effects of BD on cell viability, migration and invasion. In addition, the effect of BD on the EMT process and the PI3K/AKT signaling pathway were evaluated in this cell type.

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Abbreviations: BD, Bruceine D; EMT, epithelial-mesenchymal transformation; TNBC, triple-negative breast cancer

Key words: Bruceine D, triple-negative breast cancer, migration, invasion, epithelial-mesenchymal transition, PI3K/AKT

Materials and methods

Materials. The sample of Bruceine D ($\geq 98\%$ purity) used in the current study was provided by the Institute of Traditional Chinese Medicine and Natural Products, Jinan University (Guangzhou, China). RPMI-1640 medium and FBS were purchased from Gibco; Thermo Fisher Scientific, Inc. The MTT cell proliferation and cytotoxicity assay kits and penicillin/streptomycin solution (PS) were purchased from Nanjing KeyGen Biotech Co., Ltd. The antibody targeting PI3K (cat. no. AF5112) was obtained from Affinity Biosciences. Antibodies against AKT (cat. no. 60203-2-Ig), phosphorylated (p)-AKT (cat. no. 66444-1-Ig), E-cadherin (cat. no. 20874-1-AP) and β -catenin (cat. no. 51067-2-AP) were purchased from Proteintech Group, Inc. Vimentin antibody (cat. no. BS1491) was purchased from Bioworld Technology, Inc. Antibodies against GAPDH (cat. no. ab181602) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. ab6721) immunoglobulin (Ig) G were purchased from Abcam.

Cell culture. Human triple-negative breast cancer MDA-MB-231 cells were donated by Nanjing Pharmaceutical Co., Ltd. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% PS solution in a humidified atmosphere with 5% CO₂ at 37°C. BD was dissolved with DMSO and diluted in complete RPMI-1640 medium to required concentrations (1, 2 and 4 μ M). The final DMSO concentration in the culture medium was $\leq 0.1\%$ and control cells were treated with 0.1% DMSO at 37°C.

Cell viability assay. MTT assay was performed to measure cell viability. Cells were seeded into 96-well plates (5×10^3 cells/well), cultured overnight and subsequently treated at 37°C with ascending concentrations of BD (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 μ M) for 24, 48 and 72 h. Each well then received 20 μ l MTT (5 mg/ml) and the cells were cultured for an additional 4 h at 37°C. Subsequently, cells were rinsed using PBS and each well received 150 μ l DMSO. Optical density was then measured at the wavelength of 490 nm using a microplate reader (Mutiskan™ MK3; Thermo Fisher Scientific, Inc.). Data were presented as the percentage of survival rate relative to that of control.

Wound-healing assay. A wound-healing assay was performed to evaluate the migratory ability of MDA-MB-231 cells. Cells in the logarithmic growth phase were inoculated into six-well plates (5×10^3 cells/well). The following day, when $\sim 100\%$ of the surface was occupied a straight cell-free wound was introduced by scratching the bottom of the plate using a sterile pipette tip. Subsequently, the detached cells were washed twice with PBS and then re-incubated with BD (1, 2 and 4 μ M) or 0.1% DMSO dissolved in serum-free RPMI 1640 medium for 24 h at 37°C. The wound images were obtained using a fluorescence inverted microscope (magnification, $\times 100$) at 0 and 24 h, respectively, where the wound distance was measured using the following formula: Migration distance = scratch distance at 0 h - scratch distance at 24 h.

Transwell assay. The invasive capabilities of MDA-MB-231 cells were evaluated using a Transwell® assay (24 wells;

Matrigel gel; Corning, Inc.). The cells were first cultured in serum-free RPMI medium for 24 h at 37°C. Matrigel was incubated at 4°C overnight, and melted Matrigel was diluted twice with incomplete medium. A total of 30 μ l diluted Matrigel was added to the upper chamber of each Transwell insert, which was then incubated at 37°C for 120 min. The upper chamber of the Transwell contained 100 μ l cells (1×10^4 cells/chamber) suspended in serum-free RPMI medium containing different concentrations of BD (1, 2 and 4 μ M) or 0.1% DMSO, and the lower chamber was supplemented with 500 μ l RPMI-1640 medium containing 20% FBS. Following incubation for 24 h at 37°C, cells on the upper surface of the membrane were removed using a cotton swab, whereas cells on the bottom surface of insert membrane were fixed with 95% alcohol for 10 min at room temperature and subsequently stained with 0.1% crystal violet for 30 min at room temperature. Invasive cells were photographed and counted in five random fields of view under a fluorescence inverted microscope (magnification, $\times 200$).

Western blot analysis. Cells were first treated with BD (1–4 μ M) or 0.1% DMSO for 24 h at 37°C before being washed with cold PBS, lysed using RIPA buffer (Gibco; Thermo Fisher Scientific, Inc.) and centrifuged at 13,000 \times g for 15 min at 4°C. The supernatant was collected and stored at -20°C for further use. A BCA protein assay kit (Nanjing KeyGen Biotech Co., Ltd.) was used to quantify the total protein concentration for each sample. Protein samples (30 μ g) were subsequently separated by 12% SDS-PAGE (90 min, 100 V) and transferred onto nitrocellulose membranes (90 min; 300 mA). The membranes were then blocked with 5% non-fat dry milk for 1 h at room temperature and washed three times with TBS supplemented with Tween-20 (TBS-T) following which the membranes were incubated with primary antibodies against vimentin (1:1,000), E-cadherin (1:5,000), β -catenin (1:5,000), PI3K (1:1,000), AKT (1:5,000), p-AKT (1:5,000) or GAPDH (1:10,000) overnight at 4°C. The membranes with the primary antibodies were then incubated on a shaker at room temperature for 30 min. After further rinsing with TBS-T three times, the membranes were incubated with HRP-conjugated goat anti-rabbit (1:2,000) IgG secondary antibodies at room temperature for 1 h. The protein bands were then visualized with enhanced chemiluminescent substrates (cat. no. 32106; Thermo Fisher Scientific, Inc.) using a Syngene G:BOX Chemi XR5 imaging system (Syngene International). Gel-Pro Analyzer software (version 4.0; Media Cybernetics, Inc.) was used to perform densitometric analysis on each membrane. GAPDH was used as a loading control.

Statistical analysis. All experimental data were presented as the mean \pm standard deviation. All experiments were repeated in triplicate. The data were analyzed using a one-way ANOVA, followed by Tukey's test using GraphPad 7 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BD reduces MDA-MB-231 cell viability. MTT assay was performed to evaluate the effect of BD on the viability of MDA-MB-231 cells. The cells were treated with varying

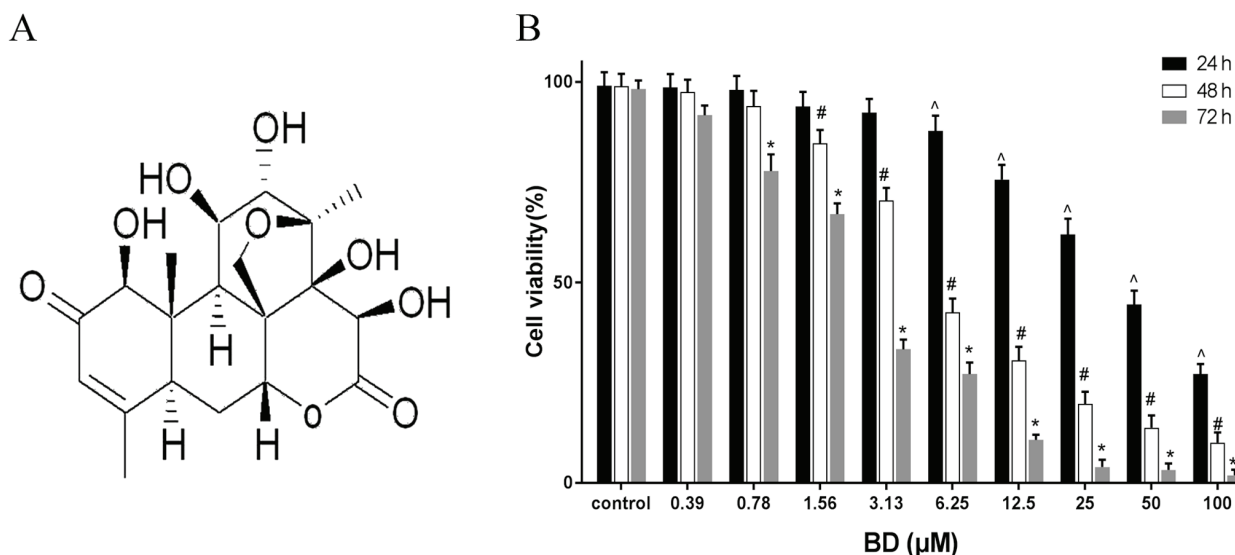


Figure 1. The molecular structure of BD and the inhibitory effect of BD on the growth of MDA-MB-231 cells. (A) Molecular structure of BD. (B) BD reduced the viability of MDA-MB-231 cells in a time- and dose-dependent manner. ^{*}P<0.05 vs. control at 24 h; [#]P<0.05 vs. control at 48 h and [^]P<0.05 vs. control at 72 h. BD, bruceine D.

concentrations of BD (0-100 μ M) for 24, 48 and 72 h at 37°C. BD treatment markedly reduced cell viability in a time- and dose-dependent manner (Fig. 1B). The half maximal inhibitory concentrations (IC_{50}) of BD were calculated to be 40.805, 5.84 and 2.364 μ M at 24, 48 and 72 h, respectively.

BD suppresses the migratory and invasive capabilities of MDA-MB-231 cells. Wound healing and Transwell assays were performed to explore the effects of BD on cell migration and invasion, respectively. In this assay, a low dose of BD (1, 2 and 4 μ M), which exerted little to no effects on cell viability, was used. BD reduced the wound healing ability of MDA-MB-231 cells in a dose-dependent manner (Fig. 2A). The width of the wounds observed following treatment with BD at 1, 2 and 4 μ M were 715.79 ± 0.8 , 714.62 ± 1.01 and 718.71 ± 1.01 μ m at 0 h, respectively, which were not significantly different compared with the control group (719.01 ± 1.34 μ m; Fig. 2A and B). Following treatment with different concentrations of BD for 24 h, the width of the wounds were 425.44 ± 13.82 , 460.53 ± 12.16 and 549.12 ± 14.15 μ m in the 1, 2 and 4 μ M treatment groups, respectively, all of which were significantly higher compared with that in the control group (318.13 ± 11.68 μ m; Fig. 2A and B). In addition, BD reduced the invasive capabilities of MDA-MB-231 cells in a dose-dependent manner. The numbers of invasive cells were 148.33 ± 5.53 , 110 ± 9.21 and 82.33 ± 5.55 in the 1, 2 and 4 μ M treatment groups, respectively (Fig. 2C and D), all of which were significantly lower compared with the control group (192.33 ± 6.08). Altogether, these results indicated that BD suppressed the migratory and invasive capabilities of MDA-MB-231 cells in a dose-dependent manner.

BD reverses the EMT process of MDA-MB-231 cells. EMT is an important physiological process in the migration and invasion of malignant tumor cells (12). Following treatment with 1-4 μ M BD or 0.1% DMSO for 24 h, western blot analysis was performed to evaluate the expression of E-cadherin,

vimentin and β -catenin, which are proteins associated with EMT. E-cadherin expression in cells treated with BD were found to be significantly higher compared with the control group (Fig. 3A and B). In contrast, the expression of vimentin and β -catenin were significantly lower compared with the control group (Fig. 3A and B). Additionally, the upregulation of E-cadherin expression and the downregulation of vimentin and β -catenin expression appeared to be dependent on the dose of BD applied. These observations suggest that BD reversed the EMT process in MDA-MB-231 cells in a dose-dependent manner.

BD inhibits the activation of the PI3K/AKT pathway in MDA-MB-231 cells. The PI3K/AKT signaling pathway has been previously reported to serve a role in cancer cell migration and invasion (13). Therefore, western blot analysis was performed to determine the expression of PI3K, AKT, and p-AKT, which are key components in the PI3K/AKT signaling pathway. BD significantly reduced PI3K expression and AKT phosphorylation in a dose-dependent manner after 24 h, whilst no significant changes were observed in total AKT levels (Fig. 4). These results indicated that BD inhibited the PI3K/AKT signaling pathway in MDA-MB-231 cells in a dose-dependent manner.

Discussion

BD is a potent quassinoid extracted from the *Brucea javanica* plant, which has been demonstrated to exhibit anticancer effects in a number of previous studies (15). Xiao *et al* (10) reported that BD induced apoptosis of hepatoma cells by regulating microRNA-95 expression without affecting the growth of normal hepatocytes. Cheng *et al* (16) demonstrated that BD inhibited liver cancer proliferation by synergizing with the protein kinase inhibitor sorafenib to induce tumor necrosis and apoptosis. However, since the anticancer effects of BD on TNBC cells remain unclear, the present study examined the

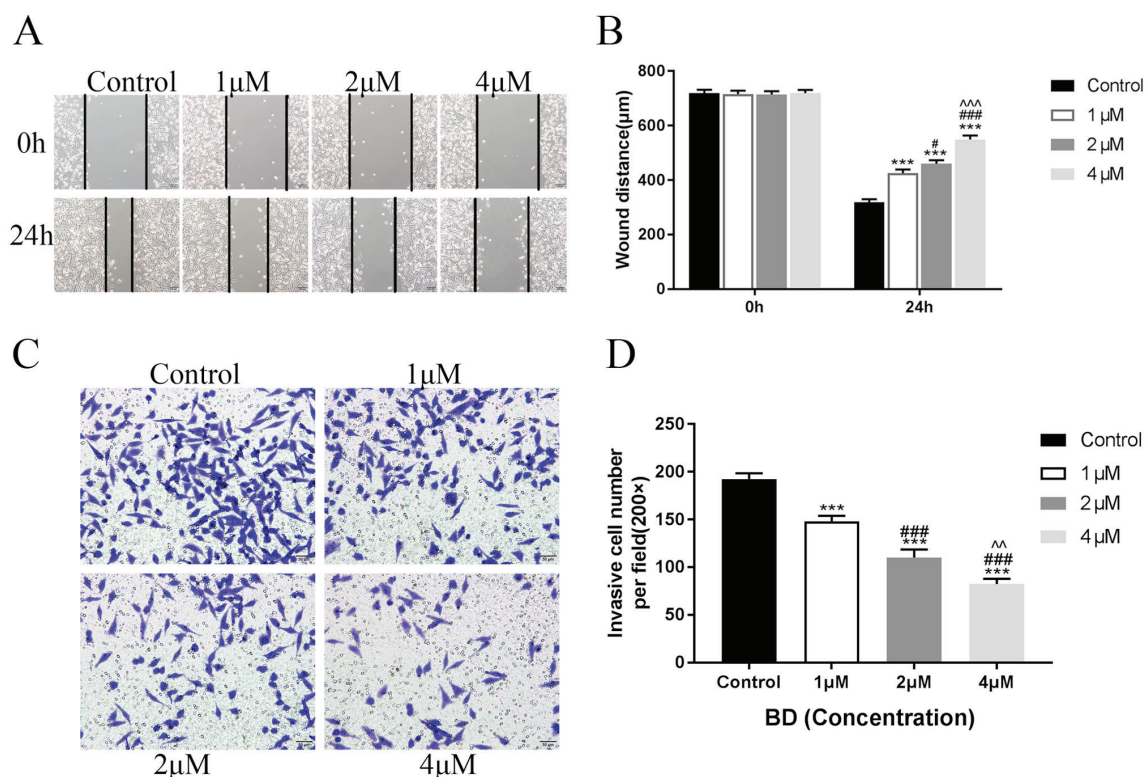


Figure 2. BD inhibits invasive and migratory abilities of MDA-MB-231 cells. Cells were treated with 0.1% DMSO or BD (1-4 μ M) for 24 h at 37°C. (A and B) A wound healing assay was performed to determine the effects of BD on cell migration. (A) Representative images of the wounds 0 and 24 h after treatment with 1, 2 or 4 μ M BD. (B) Quantified data of wound distances in the respective treatment groups. (C and D) Transwell assay was performed to determine the effects of BD on cell invasion. (C) Images captured of invasive cells for each treatment condition with (D) subsequent quantification. *** P <0.001 vs. control; # P <0.05 and ### P <0.001 vs. 1 μ M; ^ P <0.01 and ^^^ P <0.001 vs. 2 μ M. BD, bruceine D.

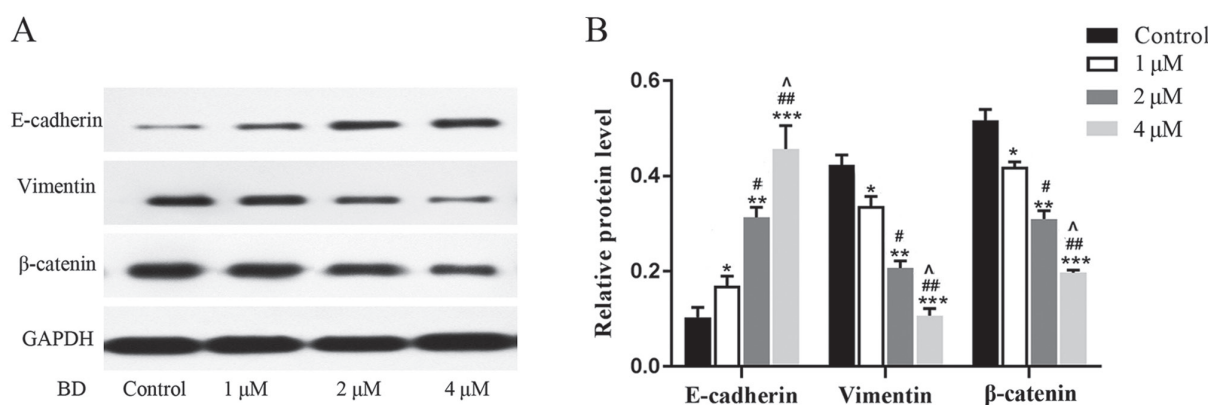


Figure 3. BD reverses the EMT in MDA-MB-231 cells. (A) Representative blots showing the expression of E-cadherin, vimentin and β -catenin, proteins associated with EMT, following treatment with 1, 2 or 4 μ M BD. (B) BD treatment upregulated the expression of E-cadherin and downregulated the expression of vimentin and β -catenin in a dose-dependent manner. GAPDH was used as the loading control. * P <0.05, ** P <0.01 and *** P <0.001 vs. control; # P <0.01 and ## P <0.001 vs. 1 μ M; ^ P <0.01 vs. 2 μ M. BD, bruceine D; EMT, epithelial-mesenchymal transition.

potential effects of BD on the MDA-MB-231 cell line. Data from the present study demonstrated that BD reduced the viability of MDA-MB-231 cells in a time- and dose-dependent manner. Additionally, low concentrations of BD (1-4 μ M) were used for wound healing and Transwell assays, which indicated that BD significantly inhibited MDA-MB-231 cell migration and invasion in a dose-dependent manner, suggesting that BD exerts a potent anti-migratory effect on MDA-MB-231 cells.

Tumor metastasis is a process in which malignant tumor cells migrate from the primary site to other organs via lymphatic

channels, blood vessels and body cavities (11). Previous studies have revealed that the EMT serves a pivotal role in the primary invasion and secondary metastasis of breast, colon and liver cancer (17), where the reduction or loss of E-cadherin expression is a key landmark change during the EMT process (18). E-cadherin is an important protein that is associated with cell-cell adhesion and attachment, and promotes adhesion between cells to maintain structural integrity (19). A number of studies have previously reported that E-cadherin is involved in the metastasis of malignancies, including colorectal,

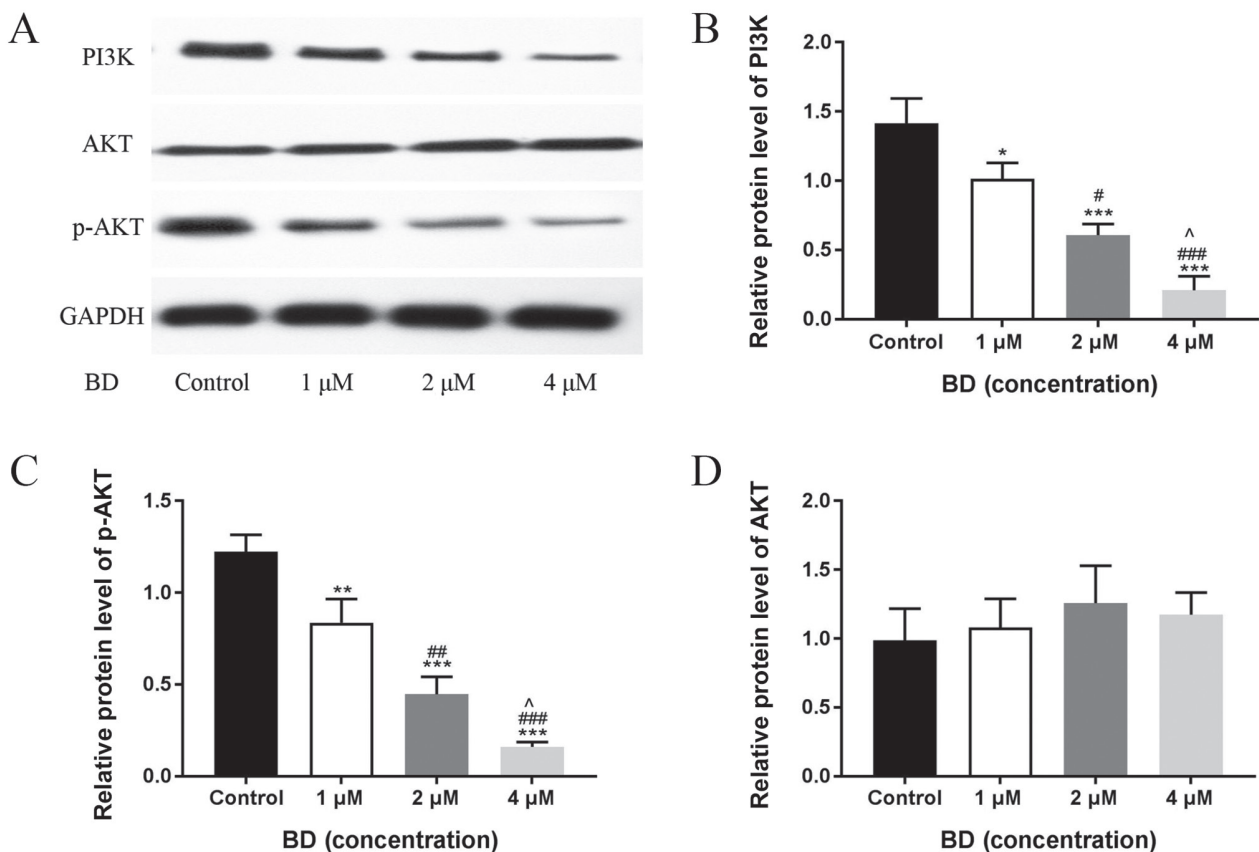


Figure 4. BD inhibits the activation of the PI3K/AKT pathway in MDA-MB-231 cells. (A) The protein levels of PI3K, AKT and p-AKT were evaluated using western blotting. BD increased the expression of (B) PI3K and (C) p-AKT, while (D) AKT expression was not affected by BD. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. #P<0.05, ##P<0.01 and ###P<0.001 vs. the 1 μ M group. ^P<0.05 vs. the 2 μ M group. BD, bruceine D.

breast and cervical squamous cell carcinoma (20-23). Sloan and Anderson (24) confirmed that breast cancer patients with lower E-cadherin expression were associated with higher rates of bone and lung metastasis. Indeed, the intracellular cytoskeletal structure is altered during the EMT, which is mainly characterized by upregulated vimentin expression (25). During the EMT process in breast cancer, the cytoskeleton profile changes from cytokeratin to vimentin, significantly increasing cell viability (26). In contrast, silencing vimentin expression reduces the invasiveness of breast cancer (27,28). β -catenin is a cytoskeletal protein that binds to E-cadherin and α -catenin to form the E-cadherin/catenin complex, which serves an important role in cell adhesion and maintaining the structural integrity of epithelial cells (29,30). However, the downregulation of E-cadherin expression results in the dissociation of this complex, releasing β -catenin into the nucleus to activate the TCF/Lef transcription factor, inducing the transcription of genes that regulate invasion and migration (31). Cheng *et al* (16) indicated that BD inhibited the expression of β -catenin in hepatoma cells. The results of the present study demonstrated that BD upregulates E-cadherin expression whilst downregulating β -catenin and vimentin expression in a concentration-dependent manner, suggesting that BD effectively reversed EMT in MDA-MB-231 cells.

The PI3K/AKT signaling pathway has been found to be aberrantly activated in a large number of tumor cells (32). PI3K is a specific class of kinases that catalyze the synthesis of phosphatidylinositol lipids (33). Activated PI3K activates

its downstream target AKT via a second messenger, phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P₃] (34). Previous studies have demonstrated that inhibition of PI3K inhibits AKT activation, subsequently downregulating the expression of key regulatory factors of the cytoskeleton by suppressing adhesion, thereby inhibiting EMT (13,35,36). Wang *et al* (37) reported that inhibition of AKT activation inhibited ovarian cancer cell proliferation and invasion. In another study, Nakanishi *et al* (38) detected AKT phosphorylation in serum-free cultured Li7 cells, which is a liver cancer cell line, suggesting that AKT activation is associated with intrahepatic hematogenous metastasis. Treatment with a PI3K/AKT blocker LY294002 in the implanted model inhibited intrahepatic metastasis, demonstrating that PI3K/AKT serves an important role in metastasis (38). Lai *et al* (39) previously confirmed that BD inhibited the PI3K/AKT signaling pathway in pancreatic cancer cells. Similarly, the present study revealed that BD treatment reduced PI3K expression and AKT activation in a concentration-dependent manner, suggesting that BD inhibits the PI3K/AKT signaling pathway in MDA-MB-231 cells, which can serve as the underlying mechanism behind the anti-invasion and anti-migratory effects of BD.

However, it should be noted that the present study only explored the inhibitory effects of BD on the PI3K/AKT signaling pathway. Whether this inhibition was mediated by BD directly and if this leads to the suppression of EMT remains unclear, since multiple signaling pathways are likely to be involved in the regulation of EMT. In addition, whether

BD directly regulates the expression of genes downstream of AKT was not been investigated in the present study. Since only one cell line was used, whether BD exerts similar anti-tumor effects on other TNBC cell lines remains to be determined. The present study was conducted *in vitro*, *in vivo* studies are required to investigate whether BD exerts similar anti-migratory and anti-invasive effects on TNBC xenografts or human samples. Treatment with 1-4 μ M BD exerted anti-cancer effects *in vitro*, but whether it can safely reach the amount used *in vivo* will also need to be studied in the future. Therefore, addressing these questions will be the subject for further research.

In conclusion, the present study demonstrated that BD inhibited MDA-MB-231 cell viability migration and invasion whilst suppressing EMT and PI3K/AKT signaling activation. These results highlighted the use of BD as a therapeutic agent for the treatment of TNBC.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZJ and CL designed the study. CL was a major contributor in writing the manuscript. CL and YW performed cell culture and MTT assay experiments. CL and CW performed the wound healing and Transwell assay. CL and YC performed western blotting. All authors 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 interests.

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