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Original Article

Bruceine D suppresses viability, metastasis and EMT of human breast cancer MDA-MB-231 cells

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Abstract: Bruceine D (BD), a major quassinoid extracted from the Chinese medicinal herb *Brucea javanica*, has been demonstrated to exert anticancer effects on various tumor cells. However, the anti-metastatic effect of BD has not been reported. The present study was designed to investigate the anti-metastatic effect of BD on MDA-MB-231 cells. The effects of BD on cell viability, migration and invasion were examined by MTT assay, wound healing assay and transwell assay, respectively. The effect of BD on epithelial-mesenchymal transition (EMT)-associated proteins, including E-cadherin and vimentin was tested by Western blot. At the same time, we detected the expression of β-catenin, which is a key molecular target for promoting cell metastasis. The results showed that BD inhibited the viability of MDA-MB-231 cells in a dose and time-dependent manner, and it also reduced cell migration and invasion in a dose-dependent manner. Western blot showed that BD reversed EMT through suppressing vimentin but enhancing E-cadherin expression. Furthermore, BD obviously decreased the expression of β-catenin in a dose-dependent manner. Taken together, all these results suggest that BD suppresses viability, metastasis and EMT of human breast cancer MDA-MB-231 cells.

Keywords: Bruceine D, β-catenin, breast cancer, EMT, metastasis

Introduction

Breast cancer is one of the three most common cancers in women, accounting for 30% of all new cancer diagnoses in women [1]. It is also the second leading cause of cancer death among women after lung cancer [2]. Breast cancer starts as a local disease, but it can metastasize to various distant organs through blood vessels or lymph nodes. Compared with local breast cancer, metastatic breast cancer greatly reduces the patient's long-term survival rate from 90% to 5% [3]. After metastasis, breast cancer cells are found to be less responsive to chemotherapy, which contributes to the failure of breast cancer treatment [4]. Therefore, the inhibition of breast cancer metastasis is a huge challenge in clinical treatment and anti-metastasis drugs are also urgently needed.

The medicinal value of plants has been widely recognized since immemorial time. At present, natural products have become a vital source of new drug development, and more and more natural products are used to improve human health [5]. In fact, more than 50% of chemotherapy drugs, such as Taxol and Vinblastine, are derived from natural products [6]. The Chinese medicinal plant *Brucea javanica (L.)* is widely distributed in southern regions of China and its seeds have been used for treatment of dysentery, malaria and cancer in traditional medicine [7]. BD (**Figure 1A**), a quassinoid extracted from *Brucea javanica*, was reported to possess antiphytoviral and anti-diabetic activities [8, 9]. Furthermore, recent studies have shown that BD has obvious anti-tumor effects on various cancer cells, including pancreatic cancer cells (PANC1, SW1990 and CAPAN1) [10-12], hepatocellular carcinoma cells (Bel7404, HepG2, Hep3B, Huh7 and PLC) [13] and chronic myeloid leukemia cells (K562) [7]. These studies have mainly explored the role of

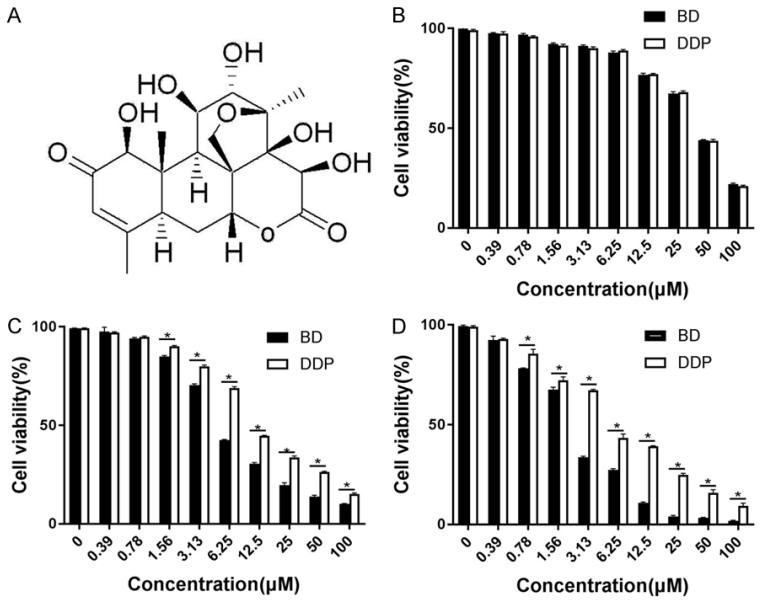

Figure 1. Structure of Bruceine D (BD), and anti-viability effects of BD and DDP on MDA-MB-231 cells. A. Chemical structure of BD. B. Cell viability after treatment with BD or DDP for 24 h. C. Cell viability after treatment with BD or DDP for 48 h. D. Cell viability after treatment with BD or DDP for 72 h. DDP represents DDP-treated group, BD represents BD-treated group. *P<0.05 vs. DDP-treated group.

BD in inducing apoptosis; however, the anti-metastatic effect of BD has not been reported.

Epithelial-mesenchymal transition (EMT) is a key step in the migration and invasion of tumor cells [14]. In this process, the epithelial cells lose their cell polarity; the junctions between cells become loose; and the cytoskeleton is remodeled. All of these changes allow cells to acquire migratory and invasive traits [15]. The molecular characteristics of EMT are the down-regulation of epithelial marker E-cadherin and up-regulation of mesenchymal marker vimentin.

In this study, we used human breast cancer MDA-MB-231 cells, an aggressive cell line generally thought to be a well-established cell line to examine cancer metastasis [16, 17], to explore the anti-metastasis effect of BD. Meanwhile, the present study provides the first evidence for BD as an anti-metastatic drug for the treatment of breast cancer.

Materials and methods

Reagents and antibodies

The sample of Bruceine D (purity ≥98) used in this experiment was obtained from the Institute of Traditional Chinese Medicine and Natural Products, Jinan University (Guangzhou, China). Cisplatin was obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). RPMI-1640 medium and Fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Vimentin antibody was purchased from Nanjing Enogene Biotech. Co., Ltd (Nanjing, China). E-cadherin and β-catenin antibodies were purchased from Proteintech Group, Inc. (Wuhan, China). GAPDH was obtained from Nanjing KeyGen Biotech Co., Ltd (Nanjing, China). Anti-rabbit immunoglobulin (Ig) G was obtained from Nanjing Key Gen Biotech Co., Ltd (Nanjing, China).

Cell line and cell culture

MDA-MB-231 cells were donated by Nanjing Ogpharmaceutical Co., Ltd (Nanjing, China). Cells were cultured in PRMI-1640 medium supplement with 10% FBS and 1% PS solution in a humidifying thermostat with 5% CO_2 at 37°C. BD was dissolved with dimethyl sulphoxide (DMSO) and diluted to the required concentration with RPMI-1640 complete medium. The final DMSO concentration was ≤0.1%. The cells were divided into the following four groups: control group (control), 1 μM BD-treated group (1 μM), 2 μM BD-treated group (2 μM) and 4 μM BD-treated group (4 μM). BD-treated groups were treated with 1, 2 and 4 μM BD. Control group was treated with the same volume of 0.1% DMSO.

Cell viability assay

The MTT assay was used to measure cell viability. Cisplatin (DDP) was used in the positive control group. Cells were seeded into 96-well plates (5×10^3 cells/well) and cultured for 24 h, then different concentrations of DDP (0-100 μM) and BD (0-100 μM) were added for further incubation for 24, 48 and 72 h. Subsequently, 20 μl MTT solution was added to each well. After 4 h, the medium was discarded and 150 μl DMSO was added. Finally, the optical density

(OD) value of each well at 490 nm was measured to evaluate the viability of the cells. Percentage cell viability = (OD of the experiment group/OD of the control group) × 100.

Wound-healing assay

MDA-MB-231 cells were seeded into 6-well plates and cultured at 37°C with 5% CO_2. The cells were slowly and gently scratched with a 200-uL pipette tip when the cells reached 80% confluence. Subsequently, the detached cells were washed twice with phosphate-buffered saline (PBS) and BD was added for 24 h. For each scratch, images were captured at 0 and 24 h by using a microscope and the cell migration distance was measured using the following formula: Migration distance = 0 h scratch distance - 24 h scratch distance.

Transwell assay

Cell invasions were analyzed in 24-well transwell unit. Matrigel was double diluted with incomplete medium and covered in the Transwell upper chamber. The cell concentration was adjusted to 1 × 10^5/ml using incomplete medium and cells were pretreated with BD (1-4 μM) for 72 h, 100 μl of the cell suspension was added to the Transwell upper chamber, and 500 μl of medium containing 20% FBS was added to the lower chamber. After incubating for 24 h at 37°C in a 5% CO_2 incubator, the matrigel and the cells in the upper chamber were wiped off with a cotton swab. Subsequently, the cells were fixed with methanol and stained with 0.1% crystal violet. Finally, it was washed with PBS and the invading cells were counted on 5 random fields using an inverted microscope.

Western blot analysis

The total protein of MDA-MB-231 cells was extracted using a total protein extraction kit (Sigma, USA). The bicinchoninic acid protein assay kit (Nanjing Key Gen Biotech Co., Ltd.) was used to quantify the total protein concentration of each sample. Protein samples from each group were separated by 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk powder for 1 h at room temperature, and then washed three times with TBST. Subsequently, the membranes were incubated with primary antibodies against vimentin (1:2000), E-cadherin (1:2000), β-catenin (1:2000) and GAPDH (1:400) overnight at 4°C. Membranes were then incubated with the appropriate HRP-conjugated goat anti-rabbit (1:2000) IgG secondary antibody for 1 h, stained with enhanced chemiluminescence, imaged using G:BOX chemiXR5 and analyzed for gray using Gel-Pro32 software. GAPDH was used as an internal control to guarantee the uniformity of equally loaded proteins among all groups.

Statistics

All experiments were carried out in triplicate and expressed as mean ± SD, and statistically significant differences between groups were determined using Dunnet t test and one-way ANOVA in GraphPad 7 software. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BD inhibits the viability of MDA-MB-231 cells

To detect the effect of BD on cell viability, the cells were subjected to MTT assay after being exposed to different concentrations of BD. Cisplatin (DDP) is a commonly used chemotherapy drug and we used it as the positive control medicine in our cell viability analysis. **Figure 1B** showed that BD and DDP inhibited the viability of MDA-MB-231 cells in a dose-dependent manner after being treated with BD or DDP for 24 h, but there was no significant difference in cell viability between the two groups. However, the viability of BD-treated cells was greatly lower than that of DDP-treated cells after cells were treated with BD or DDP for 48 h (**Figure 1C**) and 72 h (**Figure 1D**). All these results indicated that BD inhibited the viability of MDA-MB-231 cells.

BD inhibits the migration and invasion of MDA-MB-231 cells

Migration and invasion are the important features of cancer cells. In this study, the effects of BD on migration and invasion in MDA-MB-231 cells were assessed using wound healing assay and transwell assay, respectively. We used a low concentration of BD that had little effect on cell viability to investigate the anti-metastasis effect of BD. As shown in **Figure 2A** and **2B**, the wound healing ability of BD-treated

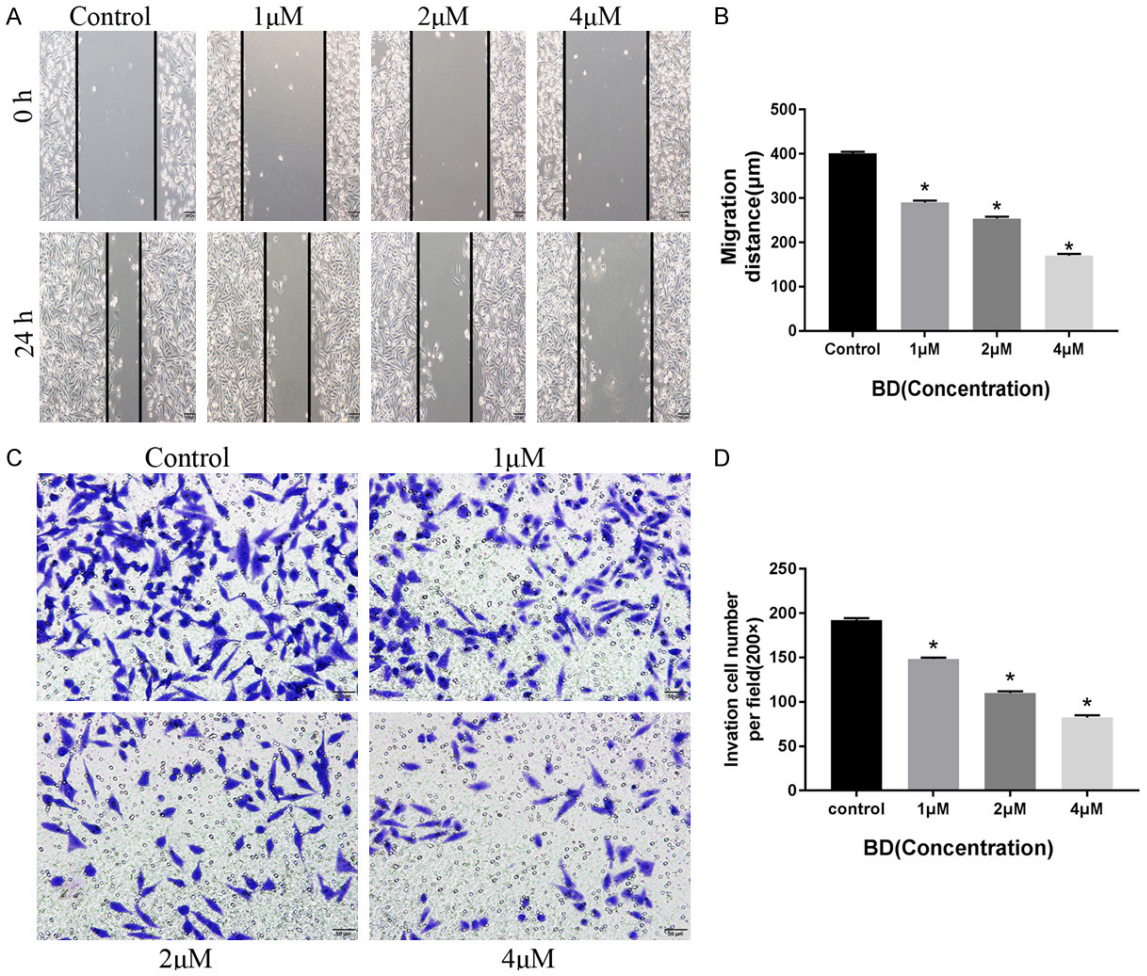

Figure 2. BD inhibits the migration and invasion of MDA-MB-231 cells. A. Wound healing assay was used to examine the effect of BD on cell migration. The cells were scratched with a pipette tip and exposed to 1-4 μM for 24 h. Photos were taken at 0 and 24 h respectively, and migration distance was calculated. B. Migration distance after 24 h. C. Transwell assay was used to examine the effect of BD on cell invasion. After treatment with 1-4 μM BD for 24 h, the invading cells were photographed. D. The number of invading cells per field was counted. 1 μM represents 1 μM BD-treated group, 2 μM represents 2 μM BD-treated group, 4 μM represents 4 μM BD-treated group, control represents control group. *P<0.05 vs. the control group.

cells was weakened in a concentration-dependent manner compared with control cells. Transwell assay showed that the number of invading cells in BD treatment group was significantly reduced in a dose-dependent manner when compared with the control group (**Figure 2C** and **2D**). Taken together, these results indicated that BD inhibited the invasion and migration of human breast cancer MDA-MB-231 cells in a dose-dependent manner.

BD regulates the expression of E-cadherin and vimentin in MDA-MB-231 cells

We assessed the effects of BD on the expression of E-cadherin and vimentin by western blot. In addition, E-cadherin and vimentin are the hallmark proteins of EMT and play an important role in cell metastasis. Following treatment with BD (1-4 μM) for 24 h, the expression of E-cadherin was increased (**Figure 3A** and **3B**), whereas the expression of vimentin was decreased (**Figure 3A** and **3C**). The enhanced expression of E-cadherin and the down-regulation of vimentin were relative to BD concentration. These findings showed that BD suppressed EMT in a dose-dependent manner.

BD decreases the expression of β-catenin in MDA-MB-231 cells

β-catenin is a key molecular target for promoting cell metastasis [18]. After treatment with BD (1-4 μM) for 24 h, we used western blot to

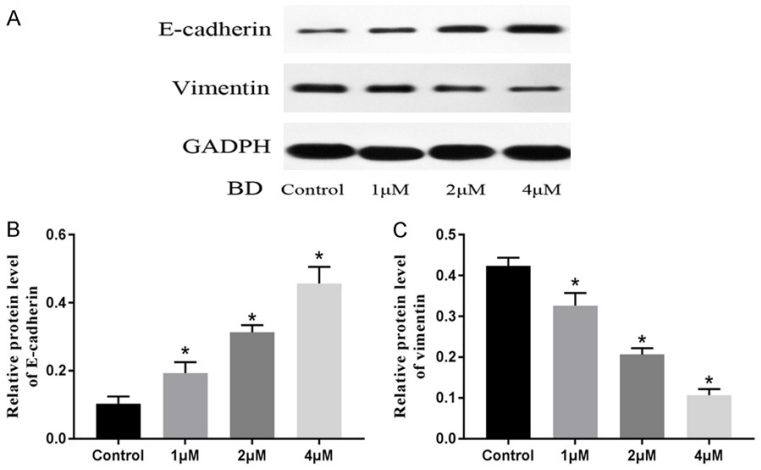

Figure 3. BD regulates the expression of E-cadherin and vimentin in MDA-MB-231 cells. A. Representative Western blot results. B. E-cadherin expression increased in a dose-dependent manner in BD-treated group. C. Vimentin expression decreased in a dose-dependent manner in BD-treated group. 1 μM represents 1 μM BD-treated group, 2 μM represents 2 μM BD-treated group, 4 μM represents 4 μM BD-treated group, control represents control group. *P<0.05 vs. the control group.

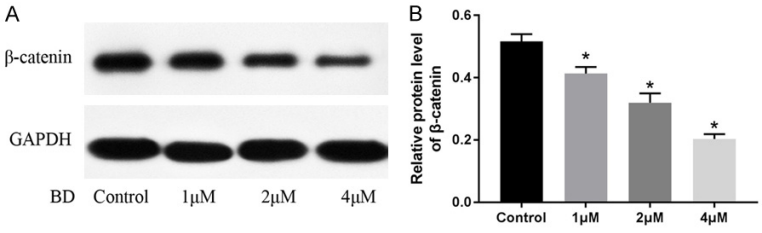

Figure 4. BD decreases β-catenin expression in MDA-MB-231 cells. A. Representative Western blot results. B. β-catenin expression decreased in a dose-dependent manner in BD-treated group. 1 μM represents 1 μM BD-treated group, 2 μM represents 2 μM BD-treated group, 4 μM represents 4 μM BD-treated group, control represents control group. *P<0.05 vs. the control group.

examine the expression of β-catenin. As shown in **Figure 4**, BD reduced the expression of β-catenin in a dose-department manner compared with the control group.

Discussion

BD is a natural compound extracted from Chinese medicine *Brucea javanica* and has been proven to have anticancer activity [19]. Numerous studies have indicated that BD induces apoptosis of human pancreatic cancer cells through mitochondrial pathway [12] and NF-kB signaling pathway [10], inhibiting the growth of hepatocellular carcinoma by regulating microRNA 95 [13] and targeting β-catenin/jagged1 pathway [19]. Breast cancer is a heterogeneous disease, and metastasis is the primary factor contributing to breast cancer-related death [20]. The development of anti-metastatic drugs is critical to the treatment of breast cancer. In order to detect the effect of BD on cell viability, we used DDP as a positive control. DDP is an effective chemotherapeutic agent commonly used in clinical practice. The results revealed that BD had stronger anti-viability effect than DDP. Next, we detected the anti-metastasis effect of BD. It showed that low concentrations of BD that had little effect on cell viability, inhibited the migration and invasion of MDA-MB-231 cells in a dose-dependent manner, implying that BD may be a potential anti-metastasis drug for breast cancer.

To further investigate the anti-metastasis effect of BD in MDA-MB-231 cells, we examined the expression of EMT-related proteins, including E-cadherin and vimentin. E-cadherin is a kind of cell adhesion molecule that exists on the surface of epidermal cells, playing a crucial role in cell adhesion. The loss or reduction of E-cadherin can induce EMT and promote metastasis of tumor cells [21, 22]. In addition, increased expression of E-cadherin is associated with good survival in serous ovarian cancer [23] and endometrial cancer patients [24]. Vimentin is a representative molecule of mesenchymal cells and a necessary regulator of mesenchymal cell migration. It also can be expressed in epithelial cells and promote tumor cell invasiveness [25]. Tanaka et al. demonstrated that vimentin expression was a poor prognostic factor for breast cancer [26]. Our results indicated that BD up-regulated the expression of E-cadherin and down-regulated the expression of vimentin in MDA-MB-231 cells, and this regulation was related to the con-

centration of BD, suggesting that BD effectively suppressed EMT.

β-catenin is a key molecular target to inhibit tumor metastasis and promote the development of EMT in tumor cells [27]. It binds the cytoplasmic domain of E-cadherin and plays an important role in E-cadherin-mediated adhesion [28]. A study indicated that inhibition of β-catenin in breast cancer cells positively inhibited vimentin expression and stimulated E-cadherin expression, thereby reversing EMT [29]. Furthermore, it was reported that vimentin promoter is a target of β-catenin/TCF pathway in human breast cancer cells, suggesting that β-catenin is involved in EMT regulation [30]. In addition, β-catenin occupies a central position in the Wnt/β-catenin signal pathway [31], which is associated with tumor migration and invasion [32, 33]. Furthermore, β-catenin is also a significant protein in other EMT-related signaling pathways, including NF-kB and TGF-β pathways [34]. Cheng et al. [19] found that BD inhibited the expression of β-catenin in hepatocellular carcinoma cells. Similarly, our results showed that BD inhibited the expression of β-catenin in MDA-MB-231 cells. However, whether β-catenin is the key molecular target for BD to inhibit tumor cell metastasis has not been explored in the current study, and we will study it further in the future.

In conclusion, the present study is the first to demonstrate the anti-metastasis effect of BD against breast cancer MDA-MB-231cells *in vitro*. Our data showed that BD suppressed viability, metastasis and EMT of human breast cancer MDA-MB-231 cells. However, we have only studied it *in vitro*, and in terms of whether BD can suppress the migration and invasion of cancer cells *in vivo*, more research needs to be done.

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Disclosure of conflict of interest

None.

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