A novel chalcone-based molecule, BDP inhibits MDA-MB-231 triple-negative breast cancer cell growth by suppressing Hsp90 function

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Abstract. Triple-negative breast cancer (TNBC) is a molecularly diverse and heterogeneous disease and the molecular heterogeneity of TNBC increases the difficulty in improving survival rates. To date, therapeutic approaches for the treatment of TNBC such as hormonal chemotherapy and trastuzumab-based therapy have been limited by the lack of target receptors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2), emphasizing the urgent need for identifying new therapeutic options. In this regard, heat shock protein 90 (Hsp90) has emerged as an attractive therapeutic target for TNBC. Hsp90 plays a central role in regulating correct folding, stability, and function of numerous oncogenic proteins. In the present study, we evaluated the *in vitro* effect of a small molecule Hsp90 inhibitor, (*E*)-3-(2-bromo-3,4,5-trimethoxyphenyl)-1-(2,4-dihydroxyphenyl)prop-2-en-1-one (BDP) on TNBC cell line, MDA-MB-231. This study indicated that BDP efficiently inhibited the growth of MDA-MB-231 cells in a dose- and time-dependent manner. BDP induced overall degradation of multiple oncogenic proteins including EGFR, Her2, Met, Akt, c-Raf, and Cdk4, consequently leading to apoptotic cell death. The flow cytometric analysis revealed that BDP promoted cell cycle arrest at G_2/M phases. Moreover, BDP treatment attenuated the migration of MDA-MB-231 cells and impaired MMP9 activity, which are essential processes for tumor metastasis. Collectively, BDP represents a new class of Hsp90 inhibitor and shows therapeutic potential for TNBC treatment.

Introduction

Triple-negative breast cancers (TNBCs), which are characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (Her2), account for 15-20% of all breast cancers (1). These cancers exhibit a more aggressive phenotype and a poorer clinical outcome compared to other breast cancer subtypes, and that is because of the high propensity for metastatic progression and the absence of specific targeted treatment options (2,3). Besides, the molecular heterogeneity of TNBC increases the difficulty in improving survival rates and developing specific targeted therapy.

Due to the lack of ER, PR, and Her2 receptors, patients with TNBC could not benefit from hormonal and trastuzumab-based targeted therapies and thus conventional chemotherapy such as taxanes and anthracyclines remains the mainstay for the treatment of TNBC (4). Although a significant number of TNBC patients respond well to the conventional chemotherapy, the prognosis of TNBC patients remains poor and alternative therapeutic approaches are therefore highly needed.

Heat shock proteins 90 (Hsp90) is an adenosine triphosphate (ATP) dependent molecular chaperone protein, which is widely expressed in breast cancers (5-7). Hsp90 plays a critical role in the correct folding, stability, and function of its substrate proteins, referred to as 'client proteins' (8-10). These client proteins include epidermal growth factor receptor (EGFR/ErbB1), human epidermal growth factor receptor 2 (Her2/ErbB2), mesenchymal-epithelial transition factor (Met), anaplastic lymphoma kinase (Alk), protein kinase B (Akt/PKB), cellular rapidly accelerated fibrosarcoma (c-Raf), cyclin-dependent kinase 4 (Cdk4), hypoxia-inducible factor 1 (Hif-1α), matrix metalloproteinase 2 (MMP2) (11-14). Hsp90 has received significant attention and emerged as an attractive target for cancer therapy, in that the inhibition of single Hsp90 protein induces client protein degradation via the ubiquitin-proteasome pathway, and subsequently results in simultaneous blockage of multiple signaling pathways in the heterogeneous cancers.

Chalcones represent an important group of naturally occurring molecules, which are especially abundant in edible plants such as green tea, licorice, and bean sprouts. Chemically, chalcones are aromatic ketone with two phenyl linked by three-carbon enone moiety (Fig. 1). Chalcones exhibit a wide spectrum of biological activities including anti-oxidative (15), anti-inflammatory (16), and anticancer activities (17-21). More importantly, chalcones have been shown to interfere with each step of carcinogenesis including initiation, promotion and progression, suggesting that chalcones and their derivatives could serve as promising candidates for anticancer drugs.

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In our ongoing effort to develop a new Hsp90 inhibitor (22-26), we have recently found that a chalcone-based small molecule (*E*)-3-(2-bromo-3,4,5-trimethoxyphenyl)-1-(2,4-dihydroxy-phenyl)prop-2-en-1-one (BDP) impairs the growth of cancer cells and this observation prompted us to direct our efforts toward investigating its biological activities and the underlying mechanisms of action.

Materials and methods

Cell culture and material. Triple-negative breast cancer cells MDA-MB-231 were grown in DMEM high glucose, supplemented with streptomycin (500 mg/ml), penicillin (100 U/ml), and 10% fetal bovine serum (FBS). Cells were grown to confluence at 37°C in humidified atmosphere with 5% CO_2. BDP was prepared following the previously reported procedure (26). For *in vitro* studies, BDP and geldanamycin (Alexis Biochemical, Farmingdale, NY, USA) were dissolved in DMSO. Antibodies for EGFR, Her2, Met, Akt, c-Raf, Cdk4, Hsp90, Hsp70, PARP, caspase 3, cleaved caspase 8, Bax and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-2, anti-mouse, and anti-rabbit antibodies were purchased from Santa Cruz Biothechnolgy (Dallas, TX, USA).

Cell proliferation assay. MDA-MB-231 cells ($2x10^3$ cells/well) were seeded in 96-well plate, the medium volume was brought to 100 μl, and the cells were allowed to attach for 24 h. The cells were then incubated with BDP (10, 30, 50, 70 and 100 μM) or GA (1 μM) at 37°C with 5% CO_2 for 1, 2 and 3 days. CellTiter 96 Aqueous One Solution reagent (Promega, Madison, WI, USA) was added into each well following the manufacturer's instructions. The absorbance of each sample was determined by Tecan Infinite F200 Pro plate reader at 490 and 690 nm as the reference wavelength.

Assessment of cell morphology. MDA-MB-231 cells ($1x10^5$ cells/well) were seeded in 12-well plate, and the cells were allowed to attach for 24 h. Culture medium was then changed to fresh complete medium containing BDP (10, 30 and 50 μM). After being incubated for 24 h, the cell morphology was observed with inverted phase contrast microscope (Olympus, Japan) at 20x objective.

Western blotting. After being treated with BDP (10, 30, 50 and 70 μM) or GA (1 μM) for 24 h, MDA-MB-231 cells were harvested and lysed in ice-cold lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). The 30 μg of lysate per lane was separated by SDS-PAGE and followed by transferring to a PVDF membrane (Bio-Rad, Hercules, CA, USA). After being blocked with 5% skim milk in TBS with 0.1% Tween-20 (TBS-T), the membrane was incubated with the corresponding antibodies in TBS-T at 4°C overnight. Proteins were visualized by using enhanced chemiluminescence (ECL) reagent according to the manufacturer's instructions (GE Healthcare, Pittsburgh, PA, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was performed using the RT-PCR kit (Bio-Rad) following the manufacturer's protocol. Briefly, total RNA was extracted from cultured cells using TRIzol reagent (Fisher Scientific, Hampton, NH, USA), reverse transcribed, and then subjected to PCR. The following primers were used for the amplification of human Met, Akt and β-actin: Met, 5'-AAG AGGGCATTTTGGTTGTG-3' (forward) and 3'-GATGAT TCCCTCGGTCAGAA-5'(reverse); Akt, 5'-TTTTATTTCTCG GGTGCAT-3' (forward) and 3'-CATTTCCCTACGTGAA TCGG-5' (reverse); β-actin, 5'-AGAAAATCTGGCACCAC ACC-3' (forward) and 3'-CCATCTCTTGCTCGAAGTCC-5' (reverse).

Apoptosis assay. After being treated with BDP (10, 30, 50 and 70 μM) or GA (1 μM) for 24 h, MDA-MB-231 cells were resuspended in 1X Annexin V binding buffer, and stained with Annexin V for 15 min. The cells were treated with FACS buffer and propidium iodide prior to FACS analysis. Apoptotic cells were analyzed by fluorescent-activated cell sorting (FACS) flow cytometer (BD Bioscience, San Jose, CA, USA) and BD CellQuest™ Pro software.

Cell cycle arrest. For cell cycle assay, MDA-MB-231 cells were treated with BDP (10, 30, 50 and 70 μM) or GA (1 μM) for 24 h. The cells were resuspended in 300 μl of PBS, treated with 700 μl of 95% ethanol, and gently vortexed. The cells were then incubated at 4°C for 2 h, washed with PBS, resuspended in 500 μl of PBS containing 50 μg/ml of propidium iodide and 1 μg/ml of RNAse A. After being incubated for an additional 30 min at room temperature in the dark, the cells were analyzed by FACS flow cytometer and BD CellQuest Pro software.

Wound healing assay. MDA-MB-231 cells ($3x10^5$ cells/well) were seeded in 6-well plate, and the cells were allowed to attach for 24 h. The 80% confluent cells were wounded with a linear scratch by using disposable 200 μl micropipette tip. The cells were washed with medium to remove cell debris and covered with serum-free medium containing BDP (10 μM). After being incubated for 24 h, migrated cells were determined under inverted phase contrast microscope (Olympus) at 10x objective.

Gelatin zymography assay. MDA-MB-231 cells ($5x10^5$ cells/dish) were seeded in 60 mm dish and the cells were allowed to attach for 24 h. The cells were then washed with PBS and incubated with serum-free medium containing BDP (10 and 30 μM) or GA (1 μM) for 24 h. Conditioned media from cell cultures treated with BDP or GA were collected, centrifuged, and mixed with sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol) without reducing agents. The corresponding samples were loaded on 10% polyacrylamide gels with gelatin (1 mg/ml) and separated by gel electrophoresis. Gels were then washed with 2.5% Triton X-100 for 40 min and incubated in incubation buffer (50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% sodium azide) at 37°C for 72 h. Gels were stained with 1% coomassie staining solution containing 10% acetic acid and 20% methanol, and incubated at room temperature for 1 h. Proteolytic activity was detected as clear band against the background stain of undigested substrate.
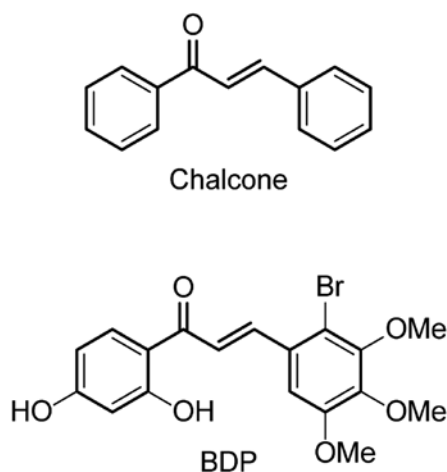
Figure 1. Structure of a chalcone-based small molecule, BDP.

Statistical analysis. Quantitative data are presented as mean value ± SD. The statistical significance of compared values was determined by using Student's t-test, and p<0.001, p<0.01, and p<0.05 were considered to indicate statistically significant results.

Results

BDP has anti-proliferative effect on MDA-MB-231 cells. We first evaluated the dose- and time-dependent effect of BDP on the growth of MDA-MB-231 cells. MDA-MB-231 cells were treated with various concentrations (0, 10, 30, 70 and 100 μM) of BDP for 1, 2 and 3 days and cell viability was determined using MTS colorimetric assay (Fig. 2A). The assay indicated that BDP afforded a potent growth-inhibitory effect on MDA-MB-231 cells in a dose- and time-dependent manner.

The treatment of cells with BDP (30 μM) effectively impaired nearly 50% of MDA-MB-231 cell growth.

We also observed the morphology changes of MDA-MB-231 cells (Fig. 2B). MDA-MB-231 cells became round and floating upon the treatment of cells with 30 or 50 μM of BDP for 24 h, which indicated typical characteristic of apoptosis.

BDP inhibits the chaperone function of Hsp90 and down-regulates the Hsp90 client proteins through the proteasomal pathway. To determine whether the observed anti-proliferative effect of BDP was associated with Hsp90 inhibition, we next evaluated the dose-dependent effect of BDP on the cellular biomarkers of Hsp90 inhibition. Since Hsp90 is responsible for maintaining the stability of EGFR, Her2, Met, Akt, c-Raf, and Cdk4, the inhibition of Hsp90 will induce the degradation of Hsp90 client proteins through the ubiquitin-proteasome pathway. As shown in Fig. 3A, the treatment of MDA-MB-231 cells with the indicated concentration of BDP for 24 h caused a dose-dependent decrease of EGFR, Her2, Met, Akt, c-Raf, and Cdk4. Interestingly, Her2 and Cdk4 more sensitively responded to the exposure of cells with BDP than other client proteins. The expression levels of Her2 and Cdk4 were almost completely depleted with 30 μM of BDP. On the contrary, BDP upregulated the cellular protein level of Hsp70, the upregulation of which is considered a cellular hallmark of Hsp90 inhibition.

To further determine whether the observed downregulation of Hsp90 client proteins was a consequence of Hsp90-mediated proteasomal degradation, we performed a recovery experiment using a proteasomal inhibitor, MG-132 (Fig. 3B). BDP (30 μM) significantly decreased the protein level of Met and Akt, whereas the pretreatment of MDA-MB-231 cells with 1 μM of MG-132 recovered the protein abundance of these proteins.

We also investigated the dose-dependent effect of BDP on the transcriptional level of Met and Akt (Fig. 3C). As expected,
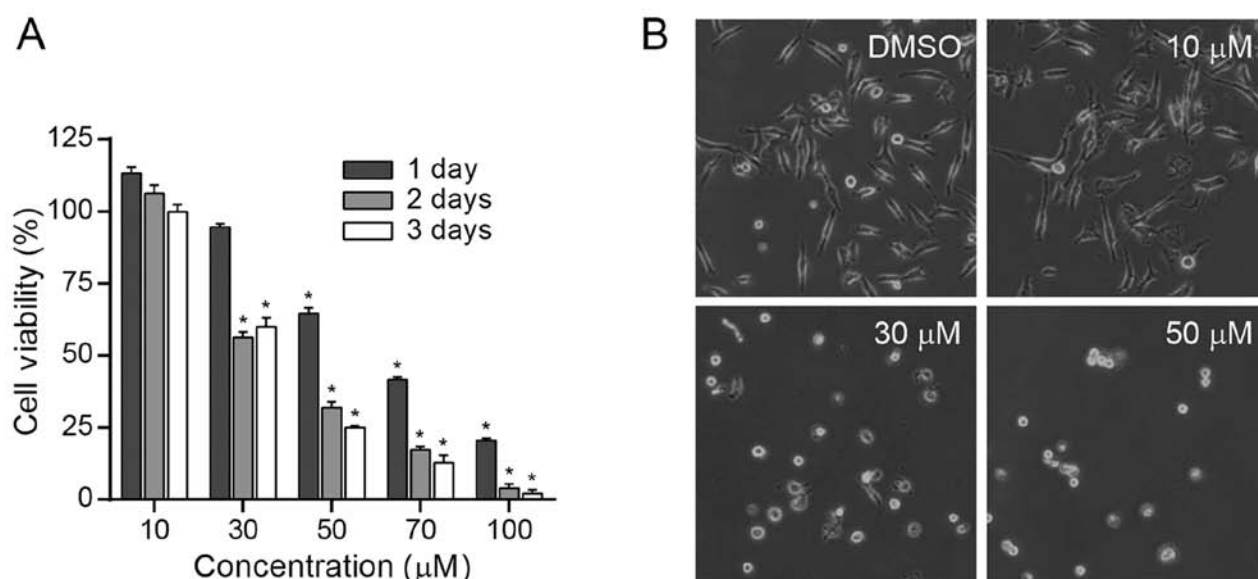

Figure 2. BDP inhibits the cell proliferation of triple-negative breast carcinoma MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with the indicated concentration of BDP for 1, 2 and 3 days and cell viability was measured using MTS assay. Data are presented as mean ± SD; *p<0.05, significant difference compared with DMSO-treated control (n=4). (B) MDA-MB-231 cells were treated with indicated concentration of BDP for 24 h and cell morphology was analyzed by a microscope.
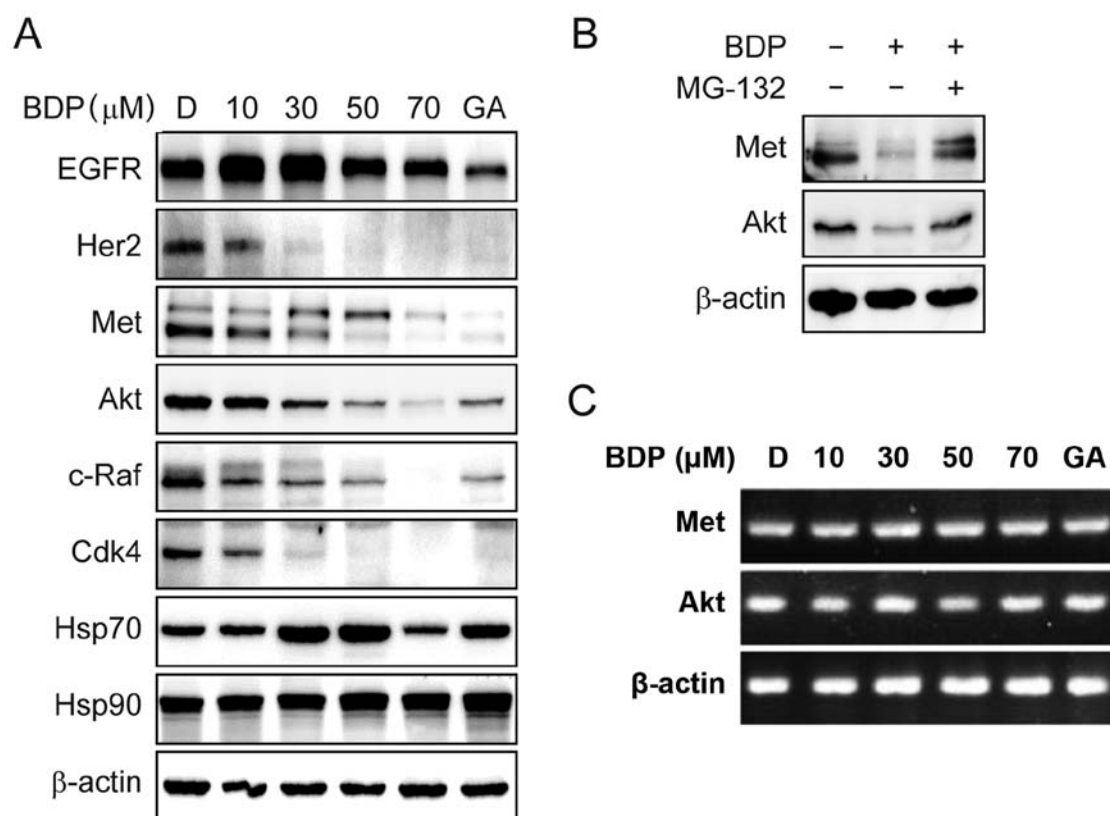
Figure 3. BDP induces the degradation of Hsp90 client proteins and the upregulation of Hsp70. (A) MDA-MB-231 cells were treated with the indicated concentration of BDP for 24 h and the expression of EGFR, Her2, Met, Akt, c-Raf, Cdk4, Hsp70, Hsp90, and β-actin was analyzed using western blotting. Geldanamycin (GA, 1 μM) and DMSO (D, 0.5%) were employed as a positive and a negative control, respectively. (B) MDA-MB-231 cells were pretreated with 1 μM of MG-132 for 3 h and then incubated with 30 μM of BDP for additional 24 h. The expression of Met, Akt, and β-actin were analyzed using western blotting. (C) Effect of BDP on the transcriptional levels of Met and Akt were measured by semi-quantitative RT-PCR. MDA-MB-231 cells were treated with indicated concentration of BDP or GA (1 μM) for 24 h. Total RNA was isolated from the cells, reverse transcribed to cDNA, and then assessed by semi-quantitative RT-PCR.

BDP did not affect the transcriptional levels of Met and Akt, suggesting the downregulation of the Hsp90 client proteins, Met and Akt was not associated with the transcriptional regulation, but the proteasomal degradation.

Collectively, this result clearly suggested that BDP inhibited the chaperone function of Hsp90 and downregulated the Hsp90 client proteins via Hsp90-mediated proteasomal degradation pathway.

BDP induces apoptotic cell death in MDA-MB-231 breast cancer cells. To address the question of whether the anti-proliferative effect of BDP was associated with the induction of apoptotic cell death, MDA-MB-231 cells were treated with the indicated concentration of BDP for 24 h, stained with Annexin V and propidium iodide, and then analyzed by fluorescence-activated cell sorting (FACS). As shown in Fig. 4A and B, the exposure of MDA-MB-231 cells with BDP induced the early and late apoptosis in a dose-dependent manner.

The activation of caspases is an important indicator of apoptosis, which is stimulated by various apoptotic stimuli. To elucidate the mechanism of BDP-induced apoptotic cell death in MDA-MB-231 cells, we next examined the cellular levels of apoptotic biomarker proteins including poly(ADP-ribose) polymerase (PARP), cleaved PARP, caspase 3, cleaved caspase 3, cleaved caspase 8, B-cell

lymphoma 2 (Bcl-2), and Bcl-2 associated X protein (Bax), shown in Fig. 3C. Upon the exposure to BDP, the cleavage of PARP, caspase 3, and caspase 8 were remarkably induced and the anti-apoptotic protein, Bcl-2 was significantly decreased in a dose-dependent manner, while the pro-apoptotic protein, Bax was unchanged. The result clearly suggested that BDP efficiently inhibited the growth of MDA-MB-231 cells through inducing apoptosis.

BDP induces cell cycle arrest at the G_2/M phases in MDA-MB-231 cells. To determine whether BDP affected the cell cycle, we analyzed the cell cycle distribution using flow cytometry. After exposure to the indicated concentration of BDP for 24 h, the cell cycle distribution of MDA-MB-231 cells was analyzed. As shown in Fig. 5, the treatment of cells with BDP remarkably induced the cell cycle arrest at the G_2/M phases compared to the DMSO control. Twenty-four-hour exposure to 10 μM BDP increased the G_2/M fraction from 21.3 to 30.1% and this increase was more marked when exposed to 30 μM BDP (G_2/M fraction, 47.9%). Similar results were also observed when exposed to 50 μM BDP (G_2/M fraction, 43.1%) or 70 μM BDP (G_2/M fraction, 46.2%). Consequently, the treatment of cells with BDP results in a reduction of the cell population at the G_0/G_1 phases, while the cell population at the S phase was not significantly
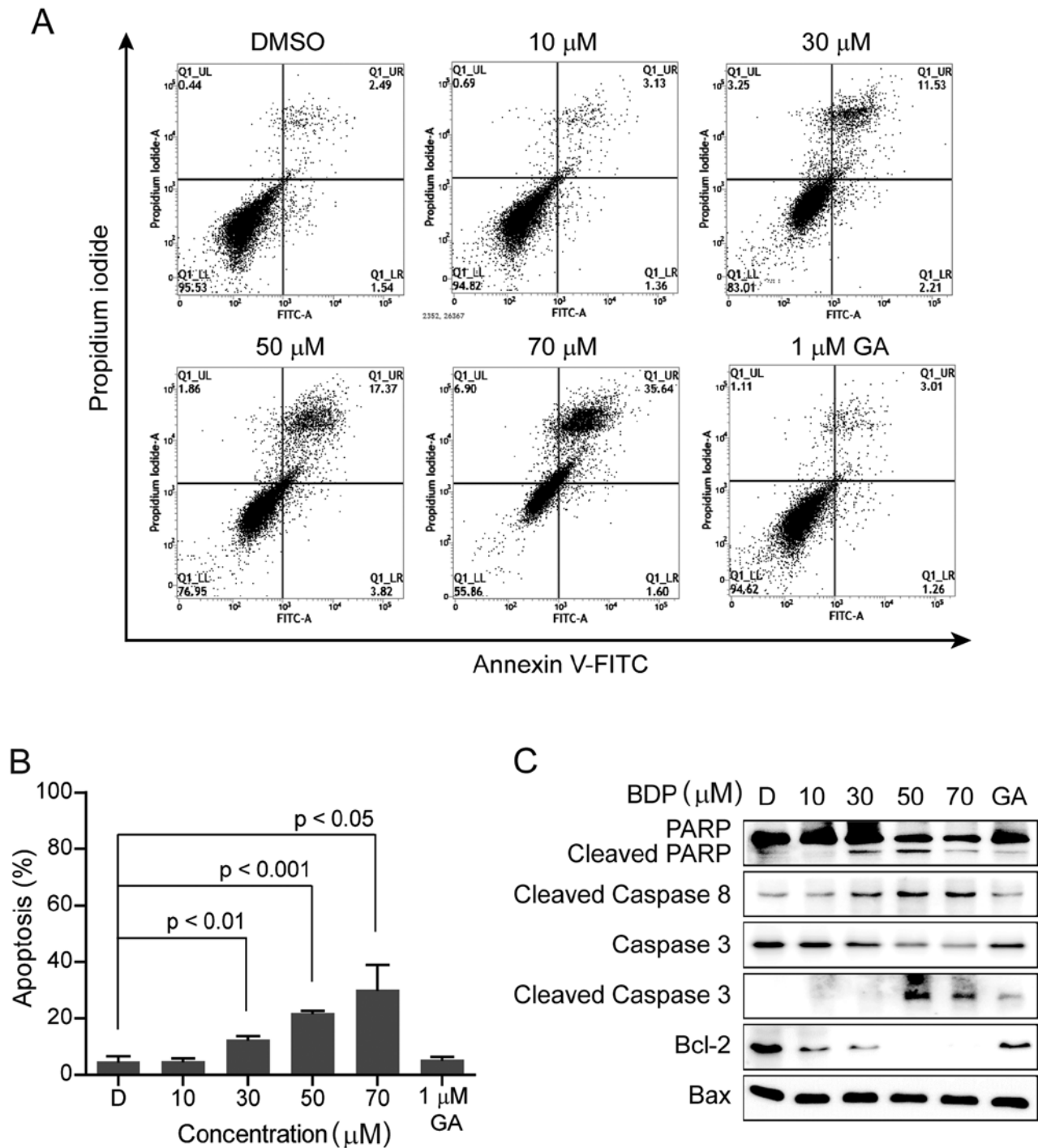
Figure 4. BDP induces apoptosis in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with the indicated concentration of BDP for 24 h, and cells were then analyzed by flow cytometric analysis with Annexin V and propidium iodide staining. (B) Bar graphs represent the percentage of apoptotic MDA-MB-231 cells from three independent experiments by FACS analysis. Values are presented as mean ± SD (n=3). (C) MDA-MB-231 cells were incubated with the indicated concentration of BDP for 24 h and the expression of the apoptotic marker proteins (PARP, caspase 8, caspase 3, Bcl-2, and Bax) was analyzed using western blotting. Geldanamycin (GA, 1 μM) and DMSO (D, 0.5%) were employed as a positive and a negative control, respectively.

altered. Taken together, these findings revealed that BDP significantly induced cell cycle arrest at the G_2/M phases in MDA-MB-231 cells.

BDP impairs the migration of MDA-MB-231 cells. An increase of mobility has been associated with the metastatic potential of cancer cells. We thus evaluated the impact of BDP on the mobility of MDA-MB-231 cells. Wounds were

formed by scratching the cell monolayer with a pipette tip and wound closure of MDA-MB-231 monolayer in the presence or absence of 10 μM BDP was measured by counting the number of cells that had infiltrated the wounded area at 24 h. As shown in Fig. 6A and B, the treatment of cells with 10 μM BDP for 24 h suppressed the migration of MDA-MB-231 cells, in that the number of migrated cells was significantly decreased (p<0.01) in the BDP-treated group,
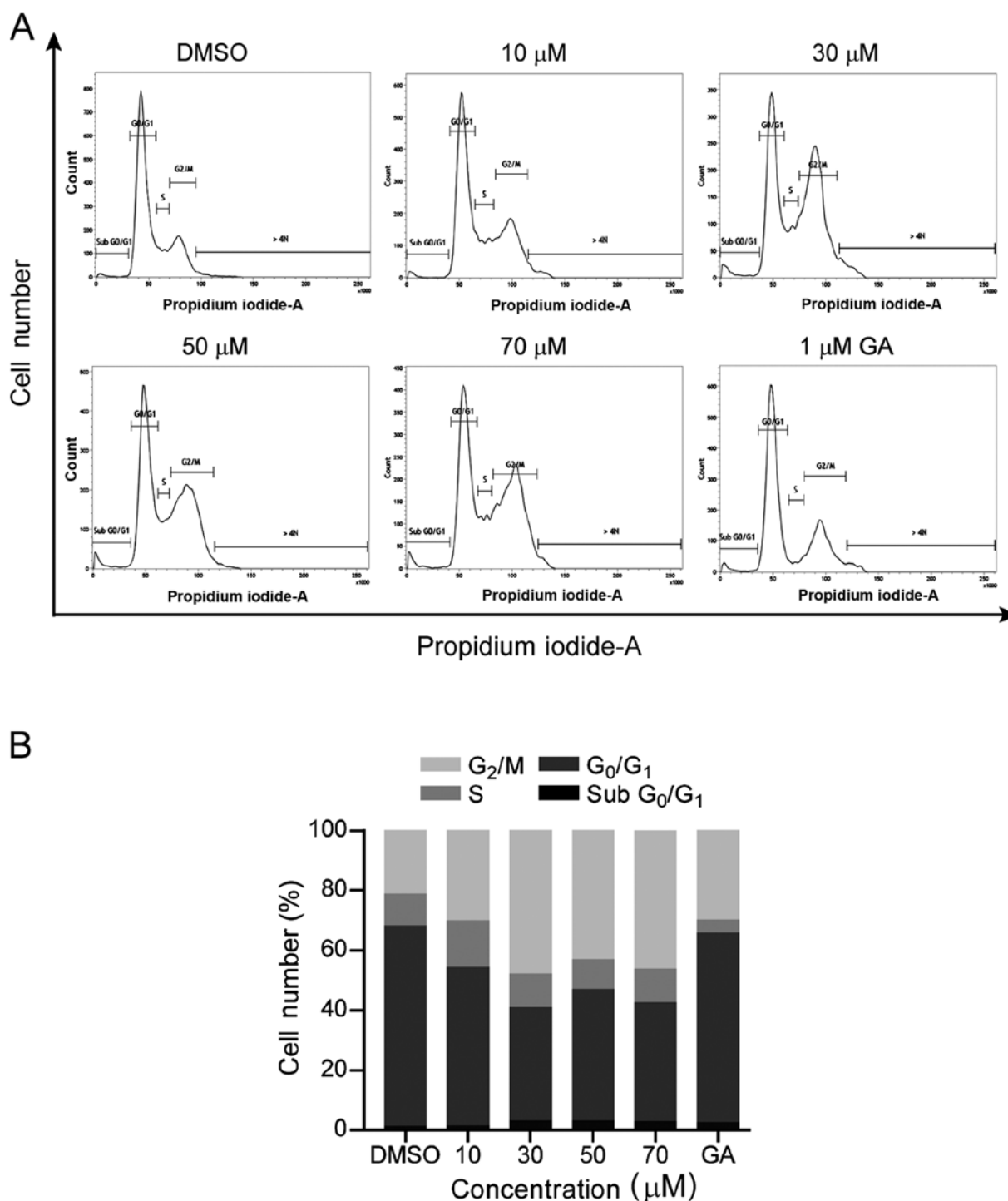
Figure 5. Effect of BDP on the cell cycle distribution in MDA-MB-231 cells. (A) After being treated with the indicated concentration of BDP or GA (1 μM) for 24 h, MDA-MB-231 cells were collected, fixed, stained with propidium iodide, and analyzed by flow cytometry. The values represent the numbers of cells in the indicated phases of the cell cycle. (B) Stacked bar graphs represent percentage of cells in each cell cycle phase.

compared to the non-treated group without impacting cell viability (Fig. 6C). The assay indicated that BDP inhibited the migration of MDA-MB-231 cells.

BDP inhibits the activity of MMP-9 in MDA-MB-231 cells. Gelatinases, such as MMP-2 and MMP-9, play a critical role in regulating extracellular matrix degradation. One of the gelatinases, MMP-9 is secreted by various types of malignant cells and contributes to tumor metastasis by breaking

down various extracellular matrix molecules, which allows metastatic cells to be more invasive. In order to investigate the effect of BDP on MMP-9 activity, we performed gelatin zymography. As shown in Fig. 7A and B, BDP efficiently inhibited MMP-9 activity in MDA-MB-231 cells, compared to the DMSO control. BDP (30 μM) significantly decreased the activity of MMP-9 to 21.3% (p<0.01). The result clearly indicated that BDP inhibited the activity of MMP-9 in MDA-MB-231 cells.
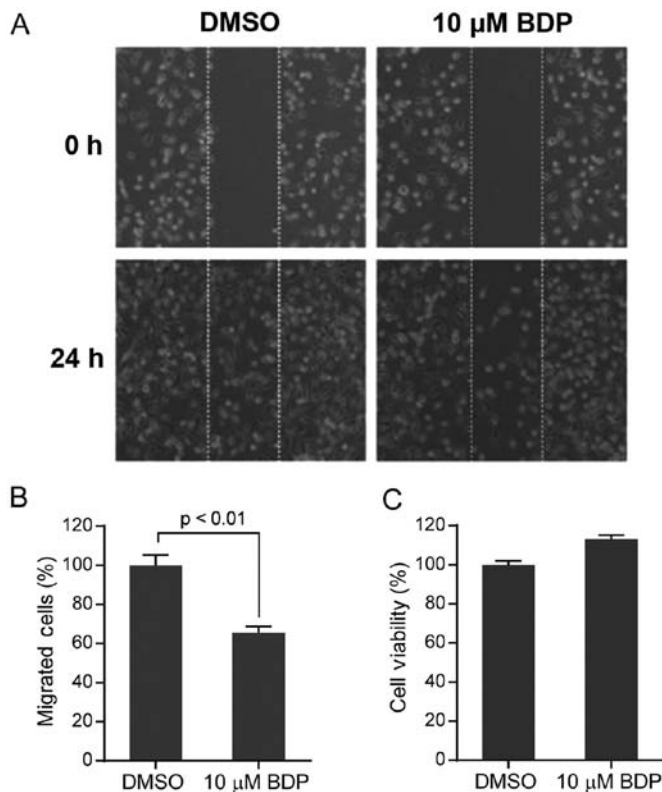
Figure 6. Effect of BDP on the migration of MDA-MB-231 cells. (A) Scratch wound healing assay was performed to examine the effect of BDP on the migration of MDA-MB-231 cells, 80-90% confluent monolayers of MDA-MB-231 cells were scratched, and then incubated with DMSO or 10 μM of BDP for 24 h. Images of wounded monolayers were taken at 0 h and 24 h. (B) Migration was measured by counting cell numbers within the wounded region. (C) MDA-MB-231 cells were incubated with the indicated concentration of BDP for 24 h and cell viability was measured using MTS assay. Data are presented as mean ± SD (n=4).
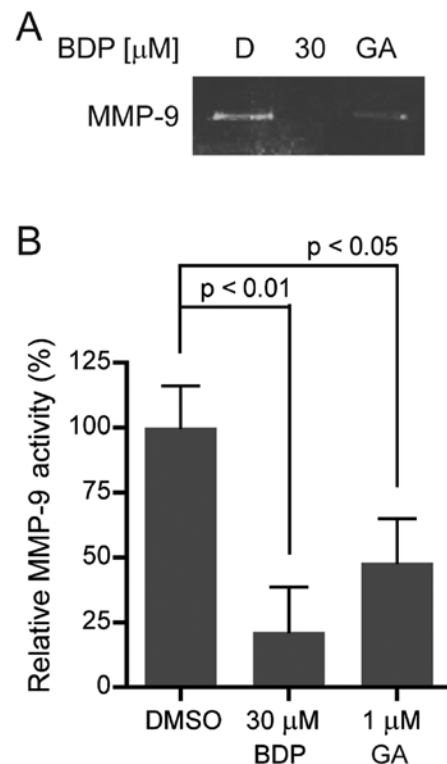

Figure 7. BDP inhibits MMP-9 activity in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated with the indicated concentration of BDP for 24 h and proteolytic activity of MMP-9 was measured by gelatin zymography assay. (B) Bar graphs represent relative MMP-9 activity. Data are presented as mean ± SD (n=3).

Discussion

TNBC exhibits an aggressive subtype and a poor prognosis because these cancers lack ER, PR and Her2 receptors (27-29). Therefore, TNBC does not respond to hormone therapies or Her2 targeted therapies, which makes it difficult to treat TNBC (30). The molecular chaperone protein Hsp90 is widely expressed in breast cancers and plays a key role in regulating the stability and functions of many oncogenic proteins (31). Therefore, Hsp90 inhibition represents a promising anticancer strategy to treat TNBCs.

In this study, we investigated the biological activity of a chalcone-based small molecule, BDP and found that BDP efficiently impaired the growth of MDA-MB-231 breast cancer cells. Our data indicated that BDP treatment of MDA-MB-231 cells significantly led to the degradation of Hsp90 client proteins such as EGFR, Her2, Met, Akt, c-Raf, and Cdk4, while BDP upregulated Hsp70, which is a cellular hallmark of Hsp90 inhibition (32,33). The downregulation of Hsp90 client proteins, Met and Akt could be reversed by adding a proteasome inhibitor, MG-132 indicating that BDP caused the degradation of Hsp90 client proteins by ubiquitin-proteasome pathway. The investigation of the transcription level demonstrated that BDP did not alter mRNA level of Met and Akt, further suggesting that the downregulation of the Hsp90 client proteins, Met and Akt was not associated with the transcriptional regulation, but the proteasomal degradation.

Apoptosis is a tightly regulated cell suicide program that plays an essential role in maintaining the physiological balance between life and death of cells (34,35). Accordingly, the proliferation of cancer can be suppressed by triggering apoptotic signaling pathways. As expected, BDP treatment of MDA-MB-231 cells induced the cleavage of PARP, caspase 3, and caspase 8, while the anti-apoptotic protein, Bcl-2 was significantly downregulated in a dose-dependent manner. The result clearly suggested that BDP efficiently induced the apoptosis of MDA-MB-231 cells. BDP also caused cell cycle arrest in G_2/M phase in MDA-MB-231 cells. As the percentage of cells in G_2/M phase increased, the percentage of cells in G_0/G_1 phase decreased.

An increase of mobility has been associated with the metastatic potential of cancer cells and patients with TNBC have a tendency to metastasize to bone, lung, liver, and brain, which contributes to the poor prognosis with short overall survival (36,37). In particular, MMPs are associated with cancer invasion and metastasis. Scratch wound healing assay and gelatin zymography assay demonstrated that BDP significantly abrogated the migratory and invasive capacity of MDA-MB-231 cells.

In conclusion, BDP suppressed the proliferation of triple-negative MDA-MB-231 breast cancer cells by inducing apoptosis, coupled with augmented G_2/M phase arrest. Moreover, BDP displayed significant degradation of Hsp90 client proteins, including EGFR, Her2, Met, Akt, c-Raf, and

Cdk4, and the upregulation of Hsp70. Our data also indicated the treatment with BDP attenuated the migratory and invasive capacity of MDA-MB-231 cells. Overall, these findings strongly supported that BDP could serve as a potential drug candidate to treat TNBC.

Acknowledgements

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