Bisdemethoxycurcumin suppresses human osteosarcoma U-2 OS cell migration and invasion via affecting the PI3K/Akt/NF-κB, PI3K/Akt/GSK3β and MAPK signaling pathways *in vitro*

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Abstract. The metastasis of human osteosarcoma (OS) shows a difficult-to-treat clinical scenario and results in decreased quality of life and diminished survival rates. Finding or developing novel treatments to improve the life quality of patients is urgent. Bisdemethoxycurcumin (BDMC), a natural product, was obtained from the rhizome of turmeric (*Curcuma longa*) and exerts antitumor activities in numerous human cancer cell lines. At present, there is no study showing BDMC effects on OS cell migration and invasion. In the present study, the effects of BDMC on cell migration and invasion of OS U-2 OS cells were investigated *in vitro*. Cell viability and proliferation were measured by flow cytometric and MTT assays, respectively. Cell motility, MMP-2 and -9 activity, and cell migration and invasion were assayed by scratch wound healing, gelatin zymography, and Transwell chamber assays, respectively. The protein expression levels were measured by western blotting. BDMC at 20 and 40 μM significantly reduced total cell viability, and BDMC at 5 and 10 μM significantly inhibited cell motility in U-2 OS cells. BDMC significantly suppressed the activities of MMP-2 and MMP-9 in U-2 OS cells. BDMC suppressed cell invasion and migration after 24 h treatment in U-2 OS cells, and these effects were in a dose-dependently manner. Results from western blotting indicated that BDMC significantly decreased the protein expression levels of PI3K/Akt/NF-κB, PI3K/Akt/GSK3β, and MAPK pathway in U-2 OS cells. Furthermore, BDMC inhibited uPA, MMP-2, MMP-9, MMP-13, N-cadherin, VE-cadherin, and vimentin but increased E-cadherin in U-2 OS cells. Based on these observations, it was suggested that BDMC may be a potential candidate against migration and invasion of human OS cells in the future.

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Introduction

Osteosarcoma (OS), the primary malignant bone tumor, is found in children, adolescents and young adults (1). OS exhibits high destructive and metastatic potential in patients (2,3). A total of ~15-20% of OS patients have clinically detectable metastases, more than 85% of the metastatic site occurs in the lung (4), and the other is the distant bone (5). Therefore, OS metastasis is an obstacle to disease treatment. After clinical treatment, ~80% of OS patients with metastatic disease have suffered relapse (6). The current treatment for OS patients, including radiotherapy, surgery, chemotherapy, bisphosphonates, calcitonin and analgesics (7,8), is accompanyed with adverse side effects (9,10). Thus, it has been with the addition of adjuvant chemotherapy after surgery. Although both

surgical techniques with adjuvant chemotherapy have been improved for patient survival, OS remains a primary cause of mortality for patients (11). Therefore, new drugs from natural products are attractive for patients with OS.

Tumor metastasis, the primary problem for tumor therapy (5), is a complicated, multi-step process and accounts for the vast majority of cancer-related deaths (12). Metastasis is a series of sequential and interrelated multi-step processes where tumor cells disseminate from the primary tumor to colonize distant organs (13). These steps include cancer cells must detach and move from the primary tumor and survive. Then these cells intravasate into the circulatory and lymphatic systems and evade immune attacks at distant capillary beds. Subsequently, cancer cells exit the bloodstream to colonize a distant target organ and finally proliferate and grow at different organ sites resulting in malignant secondary tumors (14-19). Urokinase and matrix metalloproteinases (MMPs) that degrade the extracellular matrix and basement membrane are involved in metastasis (20) for cell movement (18) and metastasize (21). The epithelial-mesenchymal transition (EMT), which involves cell polarity and cell-cell junction, plays a vital role in the process of metastasis (22). Both MMP-2 and MMP-9 are overexpressed and associated with enhanced metastatic ability in human OS cell lines (23). Inhibiting MMPs and their related pathways may be the potential strategies for inhibiting OS metastasis.

Numerous pharmaceutical drugs are obtained from natural products, and numerous studies have focused on screening phytochemicals for treating human diseases. Curcuminoids, yellow, lipid-soluble polyphenols, are extracted from the rhizome of turmeric (*Curcuma longa*). Three major components of curcuminoids are curcumin (Cur), demethoxycurcumin and bisdemethoxycurcumin (BDMC) (24). Curcuminoids exist in various biological activities, including anti-oxidant, anti-inflammatory and cytotoxic in numerous human cancer cell types (25). The lack of methoxyl groups at the *ortho* position on the BDMC aromatic ring renders it more stable in physiological media than Cur (26). BDMC presents anticancer effects on human breast cancer MCF-7 cells (27) and gastric adenocarcinoma cells (28). In addition, BDMC prevents kidney fibrosis by activating fibroblast apoptosis (29). Previously, BDMC was revealed to suppress migration and invasion in human cervical cancer HeLa cells (30) and highly metastatic lung cancer 95D cells (31).

BDMC has been revealed to inhibit the proliferation and increase the apoptotic rate of cancer cells. U-2 OS cells with functional *p53* and *pRb* genes result in the lowest level of chromosomal numerical variations compared with other OS (32). In addition, abundant osteoid production and infiltrating immune cells were detected in U-2 OS-derived tumors. Therefore, the U-2 OS cells are widely used for studying the cancer treatment, bone formation arthritis, and the interaction between immune system and tumor (33,34). However, there is no available information to show the effect and molecular mechanism of BDMC on cell migration and invasion in human OS cells. Therefore, the present study investigated the possible effects and molecular mechanisms of BDMC on cell migration and invasion of U-2 OS cells *in vitro*. The results indicated that BDMC inhibited cell migration and invasion by suppressing MMP-2 and MMP-9 signaling pathways in U-2 OS cells. This information may provide the clinical trial treatment of human OS, which is similar to U-2 OS cell line, metastasis in the future.

Materials and methods

Test chemicals, reagents, antibodies and culture medium. BDMC, dimethyl sulfoxide (DMSO), EDTA, gelatin, Tris-HCl, trypan blue, trypsin, propidium iodide (PI) and Coomassie blue R-250 with purity higher than 98% were purchased from Sigma-Aldrich; Merck KGaA. All chemicals were used as received without any further purification. McCoy's 5A medium, penicillin-streptomycin and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. The antibodies against Akt (1:1,000; cat. no. 4691), E-cadherin (1:1,000; cat. no. 14472), EGFR (1:1,000; cat. no. 4267), ERK1/2 (1:1,000; cat. no. 4695), JNK (1:1,000; cat. no. 9252), MMP-2 (1:1,000; cat. no. 87809), N-cadherin (1:1,000; cat. no. 14215), NF-κB (1:1,000; cat. no. 8242), P38 (1:1,000; cat. no. 8690), phosphorylated (p)-Akt^{Thr308} (1:1,000; cat. no. 4056), p-EGFR^{Tyr1068} (1:1,000; cat. no. 2234), vimentin (1:1,000; cat. no. 3932), goat anti-rabbit IgG, horseradish peroxidase (HRP)-linked antibody (1:5,000; cat. no. 7074) and horse anti-mouse IgG, HRP-linked antibody (1:5,000; cat. no. 7076) were purchased from Cell Signaling Technology, Inc. The antibodies against c-Jun (1:4,000; cat. no. 558036), GRB2 (1:5,000; cat. no. 610112), PI3K (1:2,500; cat. no. 610046), protein kinase C (PKC; 1:250; cat. no. 554207), Ras (1:500; cat. no. 610002) and SOS1 (1:250; cat. no. 610095) were obtained from BD Pharmingen; BD Biosciences. The antibodies against p-ERK1/2 (1:1,000; cat. no. sc-16982-R), p-JNK (1:1,000; cat. no. sc-6254), p-P38 (1:1,000; cat. no. sc-17852-R), Rho A (1:1,000; cat. no. sc-418) and uPA (1:1,000; cat. no. sc-14019) were purchased from Santa-Cruz Biotechnology, Inc. The antibodies against GSK3β (1:500; cat. no. 05-412) and p-Akt^{Ser473} (1:500; cat. no. 05-669), were obtained from Millipore. The antibodies against β-catenin (1:2,000; cat. no. GTX101435), MMP-9 (1:1,000; cat. no. GTX62122) and MMP-13 (1:500; cat. no. GTX55707) were purchased from GeneTex, Inc. The antibodies against β-actin (1:5,000; cat. no. A5316), p-c-Jun (1:1,000; cat. no. J2253) and VE-cadherin (1:500; cat. no. V1514) were obtained from Sigma-Aldrich; Merck KGaA. BDMC was dissolved in DMSO (carrier solvent), and 1% DMSO was used in control groups (as 0 concentration).

Cell culture. The human OS cell line (U-2 OS) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.). U-2 OS cells were cultured in McCoy's 5A medium containing 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin at 37˚C with 5% CO_2 in a humidified atmosphere as previously described (35).

Cell viability assays. U-2 OS cells ($8x10^4$ cells/well) were placed in each well of 12-well plates with McCoy's 5A medium for 24 h. Cells were treated with 0, 2.5, 5, 7.5, 10, 15, 20 and 40 μM BDMC in triplicate for 24 and 48 h. Cells were harvested, washed, and stained with PI (5 μg/ml) on the ice and then immediately to determine cell viability by using flow cytometry (Becton Dickinson and Company) as previously described (35).

Cell proliferation assays. U-2 OS cells ($5x10^3$ cells/well) were placed in each well of 96-well plates with McCoy's 5A medium for 24 h. Cells were treated with 0, 2.5, 5, 7.5, 10, 15, 20 and 40 μM BDMC in triplicate for 24 and 48 h. After treatment, 10 μl MTT reagent (5 mg/ml) was added for another 4 h, and then 10% SDS solution (in 0.4 N HCl) was used to dissolve the formazan crystals overnight. The absorbance was measured at $OD_{595\ nm}$ for analyzing cell proliferation by Bio-Rad Model 680 microplate reader as previously described (36).

Wound healing motility assay. U-2 OS cells were placed in a 12-well plate at $8x10^4$ cells/well and cultured in McCoy's 5A medium containing 10% FBS to almost 100% confluence of the cell monolayer. After 12 h of starvation (McCoy's 5A medium containing 2% FBS), the cell monolayers were carefully wounded using a 200-μl pipette tip, and cell debris was removed and then treated with 0, 5 and 10 μM of BDMC in serum-free medium for 12 and 24 h. Cell healing images were captured under a phase-contrast microscope in the denuded zone at different periods (0, 12 and 24 h) as previously described (35).

Gelatin zymography assay. U-2 OS cells ($8x10^4$ cells/well) were plated in 12-well plates in McCoy's 5A culture medium overnight and replaced with serum-free medium containing BDMC (0, 5 and 10 μM) for 48 h. The conditioned medium from each treatment was harvested for gelatinase activity assay on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel containing 0.2% gelatin and run in the SDS running buffer. At the end of the process, all gels were washed twice with the renaturing solution containing 2.5% Triton X-100 for 45 min and then incubation with zymogen developing buffer [550 mM Tris (pH 7.5), 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% Brij-35] followed for 24 h at 37˚C. The bands corresponding to MMP-2 and MMP-9 activities in gels were stained using 0.2% Coomassie Brilliant Blue. Then the gels were destained using 50% methanol and 10% acetic acid and images were captured. All bands (gelatinolytic activity) were measured using NIH ImageJ software (version 1.47; National Institutes of Health) (35,37).

Transwell assay. A commercial Transwell chamber insert (8-μm pore size; Millipore) was used to measure cell migration and invasion ability. For measuring cell migration and invasion ability, chambers were precoated with collagens (Sigma-Aldrich; Merck KGaA) and with Matrigel, reapectively, then put it in the incubator at 37˚C overnight. U-2 OS cells ($2x10^4$ cells/well) were suspended in serum-free McCoy's 5A medium containing 0, 5 and 10 μM of BDMC and seeded in the upper chamber. The lower chambers were filled with 800 μl of McCoy's 5A medium supplemented with 10% FBS for 24 h. After treatment, cells adhere to the upper surface of the cham swab. Cells on the underside of the membrane (migratory cells) were fixed with 100% methanol at room temperature for 10 min, stained with 0.1% crystal violet solution at room temperature for 10 min, examined and images were captured under a light microscope. In the cell invasion experiment, all subsequent steps were performed as the cell migration assay, except for the fact that chamber membranes were coated with Matrigel as previously described (35,37).

Western blot analysis. The U-2 OS cells ($1x10^6$ cells/dish) were placed in 10-cm culture dishes for 24 h and incubated with 0, 5 and 10 μM of BDMC for 24 and 48 h. Cells were collected, washed with cold PBS and lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT) containing protease inhibitor cocktail (Roche Diagnostics) for 30 min on ice. Then total protein concentration was quantitated following the guideline of the protein assay kit (cat. no. 5000006; Bio-Rad Laboratories, Inc.). A defined amount (40 μg) of proteins were electrophoresed on 8-12% SDS-PAGE gels and transferred to PVDF membranes. After blocked in blocking buffer (PBS with 2% FBS and 0.1% Tween 20) at room temperature for 1 h, the membranes were probed with specific primary antibodies against Akt, β-actin, β-catenin, c-Jun, E-cadherin, EGRF, ERK1/2, GRB2, GSK3β, JNK, MMP-2, MMP-9, MMP-13, N-cadherin, NF-κB, P38, p-Akt^{Thr308}, p-Akt^{Ser473}, p-c-Jun, p-$EGFR^{Tyr1068}$, p-c-Jun, p-ERK1/2, PI3K, PKC, p-JNK, p-P38, Ras, Rho A, SOS1, uPA, VE-cadherin and vimentin at 4˚C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and visualized by the ECL detection system (cat. no. WBKLS0500; MilliporeSigma). Finally, the ImageJ software was used for densitometry of the respective protein band intensity in all blots (35,37).

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. The statistical analysis was performed by one-way ANOVA analysis of variance, and then the Dunnett's post-hoc test was used to compare all groups against control, or the Tukey's post hoc test was used for multiple group comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

BDMC decreases the total cell viability and cell proliferation of U-2 OS cells. After U-2 OS cells were treated with BDMC (0, 2.5, 5, 7.5, 10, 15, 20, and 40 μM) for 24 and 48 h, the total viable cell number was counted (Figs. S1, 1A and B). Tretament with 20 and 40 μM BDMC at 24 and 48 h significantly decreased the percentage of cell viability. However, 2.5-15 μM treatment of BDMC did not exhibit a significant decrease in total cell viability of U-2 OS cells.

For the cell proliferation assay, U-2 OS cells were treated with BDMC (0, 2.5, 5, 7.5, 10, 15, 20 and 40 μM) for 24 and 48 h and subsequently the cell proliferation was determined. As revealed in Fig. 1C, BDMC at 10-40 μM significantly reduced the proliferation (16-84%, respectively). According to the results of cell viability and cell proliferation assays, 5 and 10 μM of BDMC were selected for further experiments, as these concentrations did not influence the cell viability but slightly inhibited the cell proliferation.

BDMC inhibits cell motility in U-2 OS cells. After U-2 OS cells were treated with BDMC (0, 5 and 10 μM) for 12 and 24 h, cell motility was observed and images were captured (Fig. 2A). The percentage of inhibition of cell motility was calculated (Fig. 2B). After 12 and 24 h of treatment, both cell

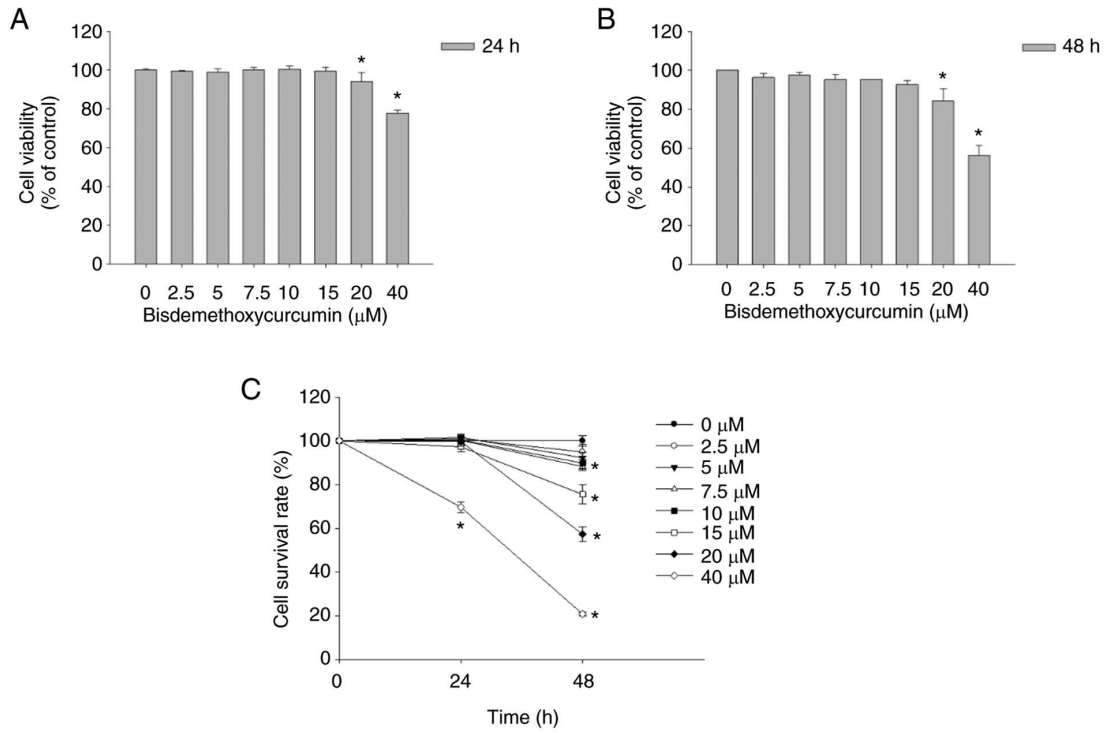

Figure 1. BDMC decreases cell viability and cell proliferation of U-2 OS cells. (A and B) Cells ($8x10^4$ cells/well) were incubated with BDMC (0, 2.5, 5, 7.5, 10, 15, 20 and 40 μM) for (A) 24 and (B) 48 h and harvested to measure the total cell viability. (C) Cells ($5x10^3$ cells/well) were incubated with BDMC (0, 2.5, 5, 7.5, 10, 15, 20 and 40 μM) for 48 h. After treatment, cell solutions were added MTT reagent and the cell proliferation was determined. *P<0.05 (one-way ANOVA followed by Dunnett's post hoc test). BDMC, bisdemethoxycurcumin.

migration movement and the scratch in the control group were basically covered; however, the scratch areas of the higher dose (10 μM) of BDMC treatment were more evident than that of lower dose (5 μM). Moreover, the wound areas of the BDMC-treated groups were higher than that of the control group (Fig. 2A and B). The results revealed that BDMC significantly inhibited the motility of U-2 OS cells.

BDMC affects matrix metalloproteinase activity in U-2 OS cells. After U-2 OS cells were treated with 0, 5 and 10 μM of BDMC for 48 h, conditioned medium in the well was harvested for examining the gelatinase activities of MMP-2 and MMP-9. Both activities were measured using gelatin zymography assay (Fig. 3). BDMC at 5 and 10 μM significantly inhibited MMP-2 (active form; 64 kDa) and MMP-9 (pro-form; 92 kDa) activity at 48 h of treatment (Fig. 3A and B). Moreover, the higher dose (10 μM) of BDMC demonstrated a higher inhibition of MMP-2 (active form) and MMP-9 (pro-form) activities than the lower dose (5 μM) of BDMC at 48 h of treatment in U-2 OS cells.

BDMC affects cell migration and invasion in U-2 OS cells. After being exposed to BDMC at the final concentrations of 0, 5 and 10 μM for 24 h, cells were assayed for cell migration and invasion by using the Transwell chambers. As revealed in Fig. 4A, BDMC at 5 and 10 μM significantly inhibited cell migration of U-2 OS cells ~56-66% compared with untreated cells. The results indicated that BDMC at 5 and 10 μM significantly inhibited cell invasion of U-2 OS cells ~16-34% compared with untreated groups (Fig. 4B). Both results indicated that BDMC reduced cell migration and invasion in a dose-dependent manner.

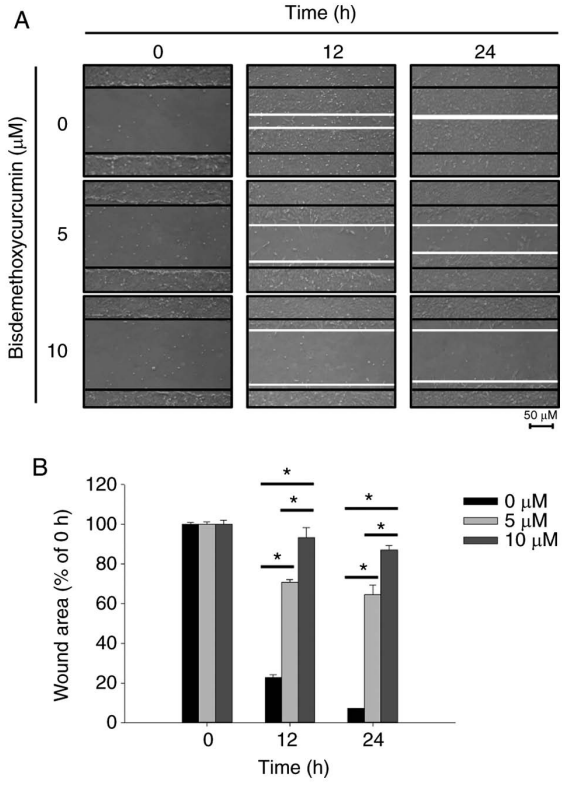

Figure 2. BDMC inhibits *in vitro* wound closure of U-2 OS cells. (A and B) Cells ($8x10^4$ cells/well) were kept in 12-well plate, and the cells were scratched (wounded). Then cells were incubated with BDMC (0, 5 and 10 μM) for 12 and 24 h. (A) Images of the relative wound closures were captured using phase-contrast microscopy. (B) The percentage of wound area was calculated. *P<0.05 (one-way ANOVA followed by Tukey's post hoc test). BDMC, bisdemethoxycurcumin.

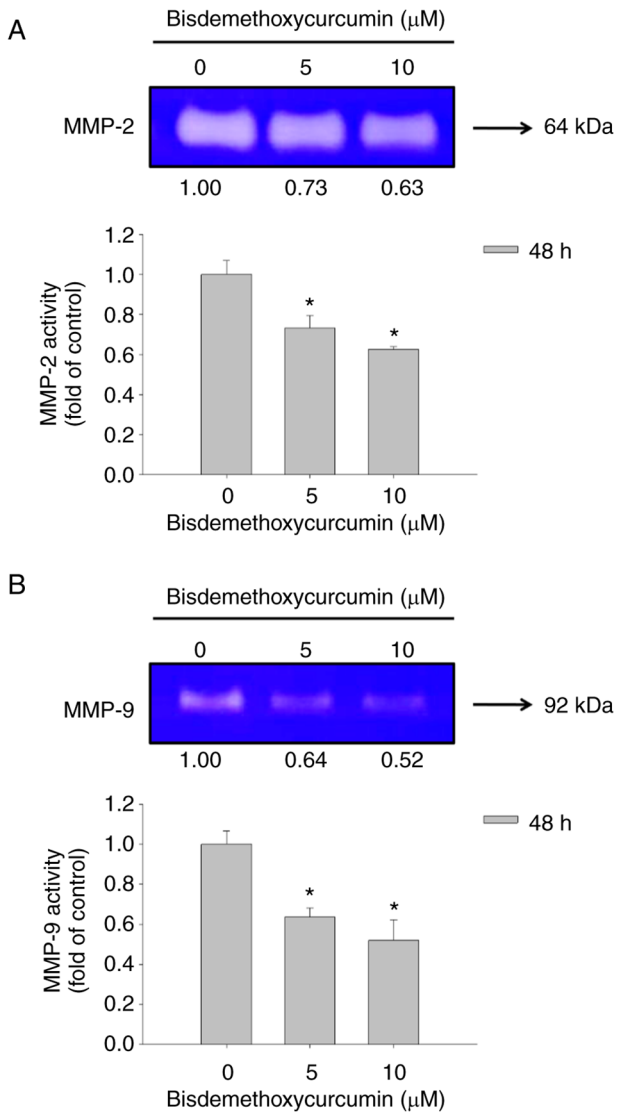

Figure 3. BDMC inhibits MMP-2 and MMP-9 activities of U-2 OS cells. (A and B) Cells ($1x10^5$ cells/well) were placed at 12-well plate and treated with BDMC (0, 5 and 10 μM) for 48 h. Then the conditioned medium was harvested for gelatin zymography assay. Representative gelatin gel pictures and the quantification of the clear zone of (A) MMP-2 and (B) MMP-9. *P<0.05 (one-way ANOVA followed by Dunnett's post hoc test). BDMC, bisdemethoxycurcumin; MMP, matrix metalloproteinase.

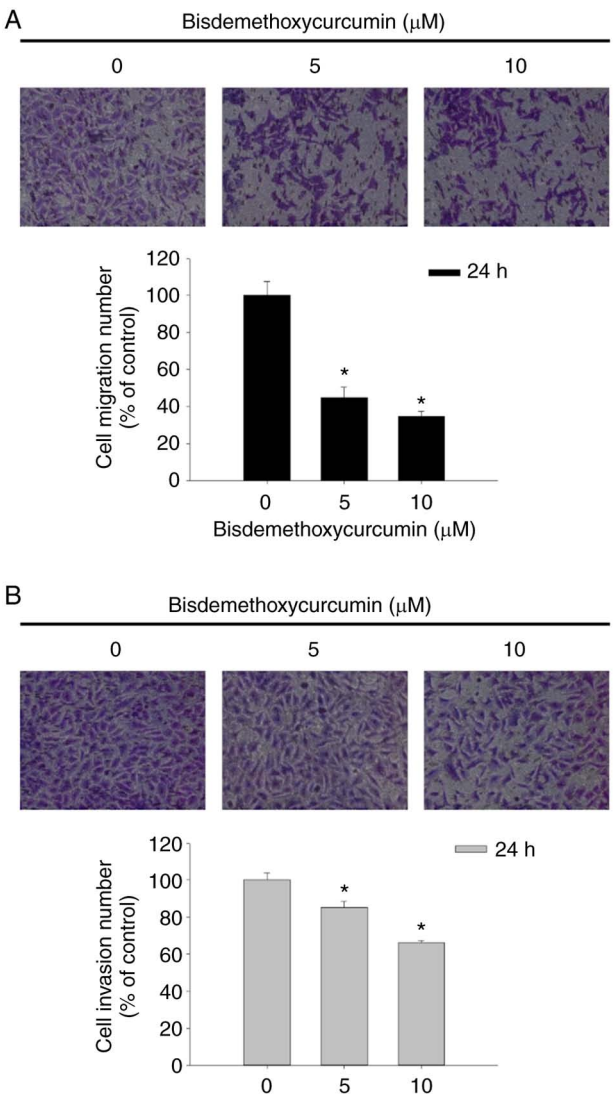

Figure 4. BDMC suppresses cell migration and invasion of U-2 OS cells. (A and B) Cells ($2x10^4$ cells/well) were placed on Transwell insert coated with collagen for migration or Matrigel for invasion and were treated with BDMC (0, 5 and 10 μM) for 24 h. U-2 OS cells penetrated to the lower surface of the transwell membrane for (A) migration and (B) invasion were stained with crystal violet. Images of the cells were captured under a light microscope (magnification, x200) and penetrated cells were counted. *P<0.05 (one-way ANOVA followed by Dunnett's post hoc test). BDMC, bisdemethoxycurcumin.

BDMC affects key metastasis-related proteins in U-2 OS cells. In order to understand the effects and mechanism of BDMC on inhibiting cell migration and invasion of U-2 OS cells, cells were treated with 0, 5 and 10 μM of BDMC for 24 and 48 h and harvested for western blotting. The results indicated that BDMC (5 and 10 μM) at 24 h of treatment increased p-EGFR^{Tyr1068}, but 48 h treatment led to a decrease in the expression of p-EGFR^{Tyr1068} (Fig. 5A). The ratio of p-EGFR^{Tyr1068}/EGFR only decreased at 24 h. BDMC at 5 and 10 μM decreased the expression of SOS1, GRB2, Ras, PKC, Rho A and uPA at 24 and 48 h treatment. BDMC (5 and 10 μM) inhibited the expression of p-ERK1/2, p-JNK, p-P38, p-c-Jun and c-Jun (Fig. 5B). These results indicated that BDMC affected the protein expression levels of the MAPK signaling pathway. In addition, BDMC (5 and 10 μM) reduced the expression levels of PI3K, p-Akt^{Thr308}, p-Akt^{Ser473}, NF-κB, GSK3β and β-catenin at both periods, indicating the effects of BDMC on the PI3K/Akt/NF-κB and PI3K/Akt/GSK3β signaling pathways in U-2 OS cells (Fig. 5C). BDMC (5 and 10 μM) significantly decreased the expression levels of MMP-2, MMP-9 and MMP-13 at both periods (Fig. 5D). Furthermore, BDMC (5 and 10 μM) increased E-cadherin and decreased N-cadherin, VE-cadherin and vimentin at both periods in U-2 OS cells (Fig. 5E).

Discussion

Cell metastasis involves multi-step processes in which tumor cells disseminate from the primary tumor and colonize distant organs (13). Cancer cell metastasis has been recognized to account for more than 90% of all cancer-related

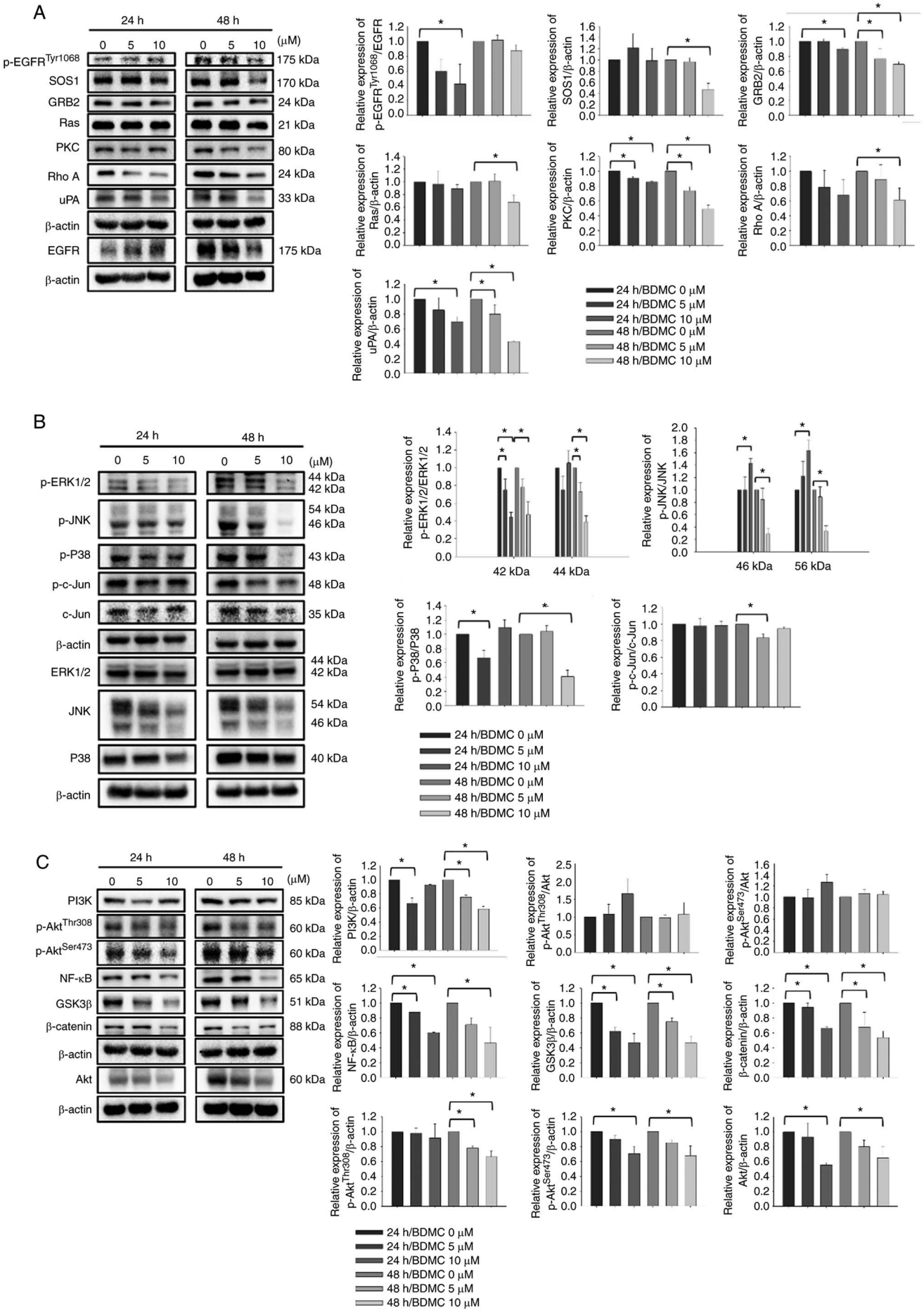

Figure 5. Continued.

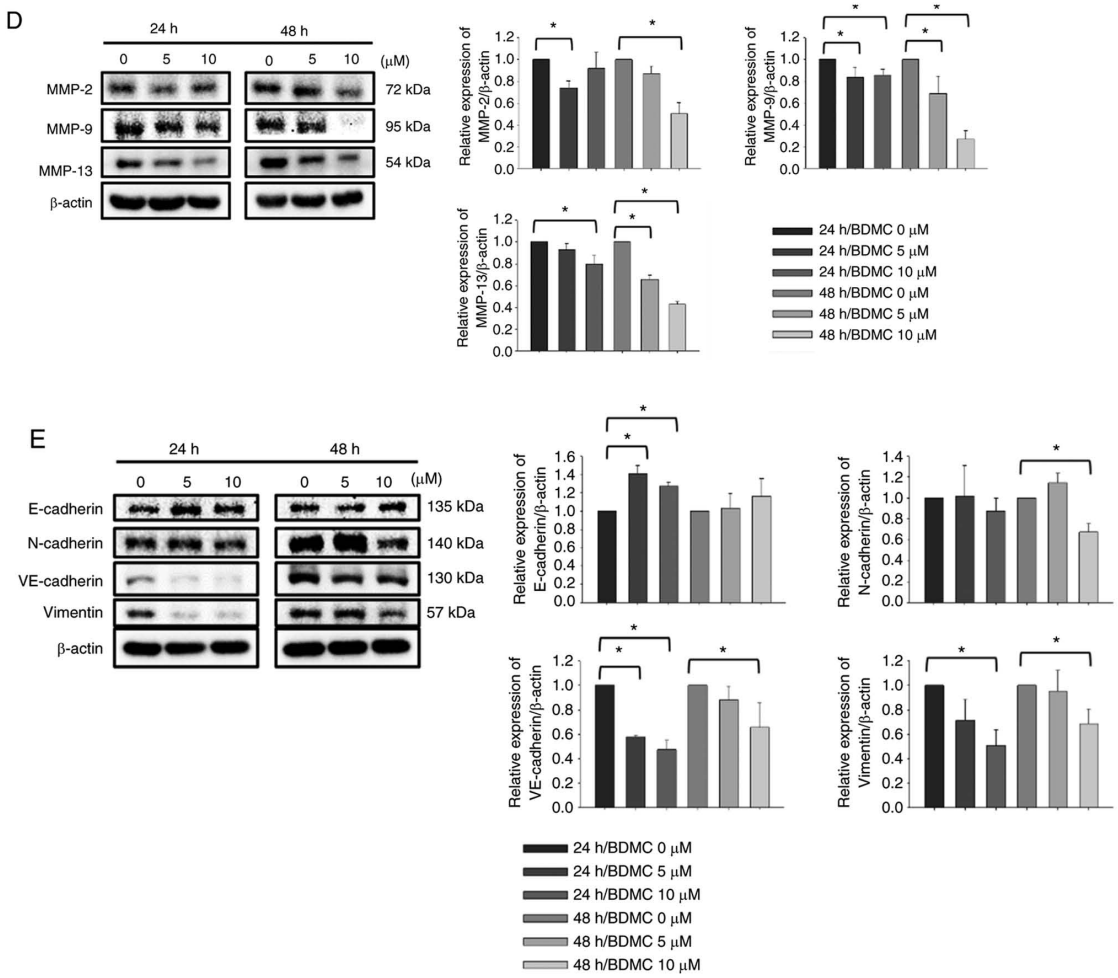

Figure 5. BDMC affects the levels of metastasis-associated proteins in U-2 OS cells. (A-E) Cells ($1x10^6$ cells/dish) were treated with BDMC (0, 5 and 10 μM) for 24 and 48 h. Cells were harvested for total protein evaluation using western blotting and the band intensity was quantitated by ImageJ software. The protein levels of (A) p-EGFR^{Tyr1068}, SOS1, GRB2, Ras, PKC, Rho A, uPA and EGFR; (B) p-ERK1/2, p-JNK, p-p38, p-c-Jun, c-Jun, ERK1/2, JNK and p38; (C) PI3K, p-Akt^{Thr308}, p-Akt^{Ser473}, NF-κB, GSK3β, β-catenin and Akt; (D) MMP-2, MMP-9 and MMP-13; and (E) E-cadherin, N-cadherin, VE-cadherin and Vimentin were analyzed. *P<0.05 (one-way ANOVA followed by Dunnett's post hoc test). BDMC, bisdemethoxycurcumin; p-, phosphorylated; PKC, protein kinase C.

deaths (38,39). Thus, investigating the mechanisms driving cancer cell motility and invasion is crucial to understanding metastasis and inhibiting cancer cell growth in other organs. To prevent bone metastasis, the molecular pathways involved in bone metastasis need to be comprehended (40,41). Thus, agents that block cancer cell migration and invasion or inhibit metastasis-associated molecular pathways may be potential strategies to inhibit cancer metastasis. BDMC, one of the natural plants, has been identified to induce cancer cell apoptosis and inhibit cell migration and invasion in numerous human cancer cell lines. At present, there are no studies revealing that BDMC suppresses cell migration and invasion in human OS cells. Herein, the present studies were focused on BDMC and whether or not it could inhibit U-2 OS cell migration and invasion *in vitro*.

The U-2 OS cells were treated with various concentrations of BDMC for 24 and 48 h and the results indicated that BDMC significantly decreased total viable cell number (cell viability) and the cell proliferation of U-2 OS cells. Therefore, for further experiments, the concentrations of 5 and 10 μM of BDMC were selected, which did not influence cell survival and slightly inhibited cell proliferation. Cancer cell motility is involved in tumor cell metastasis and wound healing cell motility assay is used to measure cell motility (35,42). The results indicated that BDMC inhibited cell motility of U-2 OS cells in a dose-dependent manner. The present study, to the best of our knowledge, is the first to identify that BDMC suppresses the cell motility of U-2 OS cells *in vitro*.

MMPs play a critical cascade in cancer cell migration and invasion. BDMC shows excellent effects on degradation-associated proteins in several cells, including uPA, MMP-2, MMP-9, membrane Type 1 MMP (MT1-MMP) and tissue inhibitors of MMPs (TIMP-2) (30,43,44). Whether or not reduced motility regarding the MMP-2 and MMP-9 activities was affected by BDMC in U-2 OS cells, the gelatin zymography method was assayed in U-2 OS cells after exposure to BDMC. Gelatin zymography detects proteolytic enzymes, including MMP-2 (gelatinase A) and MMP-9 (gelatinase B), based on both enzymes having potent gelatin-degrading activity (45). Both MMP-2 and MMP-9 were found to be overexpressed in OS cells (35). Moreover, the increased expression of MMP-2 in the tumor tissue has been shown to involve clinical stages, including cancer cell metastases, recurrence and survival (46). The present results indicated that BDMC significantly reduced

MMP-2 (active form) and MMP-9 (pro-form) activities. Furthermore, the Transwell system for examining cell migration and invasion across endothelial monolayer *in vitro* was used to evaluate cancer cell metastasis ability (46-48). BDMC significantly inhibited cell migration and invasion in U-2 OS cells *in vitro*. These effects are in a dose-dependent manner.

For further investigating the protein expression levels regarding BDMC suppressing cell migration and invasion of U-2 OS cells *in vitro*, western blot analysis was used. Previous studies reported that numerous signaling pathways, including PI3K/Akt/mTOR, ERK/MAPK and Slit-Robo pathways, were involved in tumor metastasis (49,50). The results indicated that BDMC decreased p-EGFR^{Tyr1068}, SOS1, GRB2, Ras, PKC and Rho A after 48 h treatment. The Rho GTPases and downstream effector proteins have been shown to mediate tumor cell migration, invasion and metastasis via the cytoskeleton (51). Moreover, BDMC significantly inhibited the expression levels of p-ERK1/2, p-JNK, p-p38, p-c-Jun and c-Jun in U-2 OS cells. These were consistent with previous studies that indicated that the MAPK pathway (including ERK, Jun and p38) (52) and the Ras/Raf/MEK/ERK pathways are associated with OS-lung metastasis (53). The present results may suggest that these pathways were associated with the U-2 OS cell migration and invasion. In addition, the p38/MAPK signaling pathways are involved in cell metastasis (54). Therefore, a feasible and promising approach for OS treatment is to block the Ras/MAPK kinase cascade (55).

BDMC reduced the expression of PI3K, p-Akt^{Ser473}, NF-κB, GSK3β and β-catenin in U-2 OS cells. In cancer cells, overexpressed PI3K/Akt/GSK3β signaling pathways will promote cancer cell invasion and metastasis (56,57). Furthermore, NF-κB induces the expression of diverse target genes to stimulate cancer cell invasion and metastasis (58). Notably, Aurora-B has also been revealed to activate the PTK2/PI3K/Akt/NF-κB pathway to promote the malignant phenotype of OS cells (59). Thus, if agents could inhibit the PI3K/Akt/GSK3β and PI3K/Akt/NF-κB signaling pathways, that may benefit treating cancer patients with advanced metastasis. BDMC inhibited the expression of β-catenin in U-2 OS cells. The activation of Wnt/β-catenin has been revealed to induce actin to alter the cytoskeleton to acquire a migratory phenotype (60). Thus, BDMC inhibiting U-2 OS cell migration and invasion may also mediate the inhibition of β-catenin.

BDMC significantly inhibited MMP-2, MMP-9 and MMP-13 in U-2 OS cells. It was also confirmed that MMP-2 and MMP-9 activities were suppressed by BDMC, and MMP-2 and MMP-9 were involved in cancer invasion and metastasis (61,62). The regulation of EMT, a decrease or the loss of E-cadherin expression, or the induction of N-cadherin or vimentin in cancer cells associated with cell migration and invasion is an evaluated strategy for agents to affect cancer cell metastasis. In the present study, E-cadherin, N-cadherin and VE-cadherin were analyzed in U-2 OS cells after exposure to BDMC. The results indicated that BDMC increased E-cadherin but decreased N-cadherin, VE-cadherin and vimentin in U-2 OS cells. In prostate cancer cells, cells migrated to bone metastases often switched the cadherin type from E-cadherin to Cad11 by EMT (63). The EMT process was associated with decreased

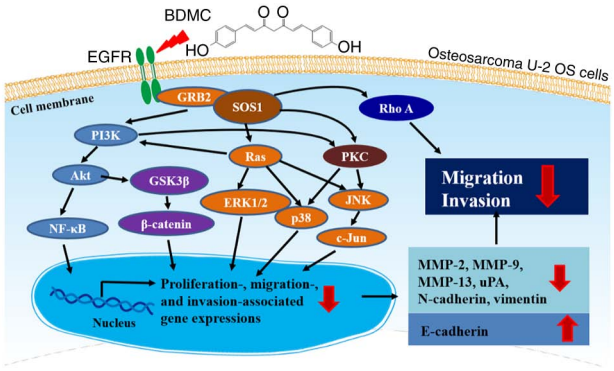

Figure 6. The possible signaling pathways for BDMC suppress cell migration and invasion in U-2 OS cells *in vitro*. BDMC, bisdemethoxycurcumin.

E-cadherin (cell adhesion molecule) and increased vimentin and N-cadherin expression (64,65). Furthermore, inhibition of EMT activation, including the downregulation of N-cadherin, vimentin, MMP-2 and MMP-9, or the upregulation of E-cadherin and tissue inhibitor of MMPs (TIMP-2), could lead to suppressing cancer cell migration and invasion in cervical cancer (66).

BDMC affects the PI3K/Akt/GSK3β, PI3K/Akt/NF-κB and Ras/MAPK signaling pathways in U-2 OS cells and these pathways are cross-talked in the present study. PI3K is one of the main Ras effectors and regulates important cellular functions, including cell viability or angiogenesis upon oncogenic Ras activation (67). PKC is a family of serine/threonine kinases and stimulates survival- or proliferation- or metastasis-associated signaling pathways, including the Ras/Raf/MEK/ERK or PI3K/Akt/mTOR pathways (68). Therefore, PI3K, Ras and PKC play a cross-talked role in connecting each other (Fig. 6). BDMC, targeting PI3K, Ras and PKC, may indicate potential therapies in the metastasis inhibition of OS U-2 OS cells in the future.

In the present study, BDMC inhibited the migration and invasion of U-2 OS cells by affecting the PI3K/Akt/NF-κB, PI3K/Akt/GSK3β and MAPK signaling pathways *in vitro*. However, there are certain limitations to the present study. The associated signaling pathway of BDMC on U-2 OS cells was not confirmed by related inhibitors. In addition, further research will be needed to investigate the effects of cell migration and invasion of BDMC in other OS with different genetic backgrounds.

In conclusion, in the present study, BDMC significantly inhibited cell motility, migration and invasion of U-2 OS cells *in vitro* involved in the inhibitions of the PI3K/Akt/GSK3β, PI3K/Akt/NF-κB and Ras/MAPK signaling pathways. Furthermore, it also reduced the levels of MMP-2, MMP-9, MMP-13, N-cadherin, vimentin and uPA but increased E-cadherin. Therefore, BDMC or BDMC nanocarrier which improve its water solubility may become a potential drug or adjuvant for treating other OS with wild-type *p53* and *pRb* genes in the future.

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Availability of data and materials

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

Authors' contributions

YSM, CLL, and TCH conceived and designed the study. SFP, RSCW, FSC and WWH acquired the data. PYC, CLK and ACH analyzed and interpretated the data. CLL and TCH wrote the draft of the manuscript. CLL and TCH critically revised the manuscript. CLL and TCH confirm the authenticity of all the raw data. All Authors discussed the results and commented on the article. All authors read and approved the final version of the 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.

References

1. Taran SJ, Taran R and Malipatil NB: Pediatric osteosarcoma: An updated review. Indian J Med Paediatr Oncol 38: 33-43, 2017.
2. Deng Z, Liu X, Jin J, Xu H, Gao Q, Wang Y and Zhao J: Histone deacetylase inhibitor trichostatin a promotes the apoptosis of osteosarcoma cells through p53 signaling pathway activation. Int J Biol Sci 12: 1298-1308, 2016.
3. Moirangthem A, Bondhopadhyay B, Mukherjee M, Bandyopadhyay A, Mukherjee N, Konar K, Bhattacharya S and Basu A: Simultaneous knockdown of uPA and MMP9 can reduce breast cancer progression by increasing cell-cell adhesion and modulating EMT genes. Sci Rep 6: 21903, 2016.
4. Lu J, Song G, Tang Q, Zou C, Han F, Zhao Z, Yong B, Yin J, Xu H, Xie X, *et al*: IRX1 hypomethylation promotes osteosarcoma metastasis via induction of CXCL14/NF-κB signaling. J Clin Invest 125: 1839-1856, 2015.
5. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, *et al*: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: An analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20: 776-790, 2002.
6. Wang JY, Wu PK, Chen PC, Yen CC, Hung GY, Chen CF, Hung SC, Tsai SF, Liu CL, Chen TH and Chen WM: Manipulation therapy prior to diagnosis induced primary osteosarcoma metastasis-from clinical to basic research. PLoS One 9: e96571, 2014.
7. Mercadante S: Malignant bone pain: Pathophysiology and treatment. Pain 69: 1-18, 1997.
8. Mercadante S and Fulfaro F: Management of painful bone metastases. Curr Opin Oncol 19: 308-314, 2007.
9. Harris JD: Management of expected and unexpected opioid-related side effects. Clin J Pain 24 (Suppl 10): S8-S13, 2008.
10. Weber M and Huber C: Documentation of severe pain, opioid doses, and opioid-related side effects in outpatients with cancer: A retrospective study. J Pain Symptom Manage 17: 49-54, 1999.
11. Yi XJ, Zhao YH, Qiao LX, Jin CL, Tian J and Li QS: Aberrant Wnt/β-catenin signaling and elevated expression of stem cell proteins are associated with osteosarcoma side population cells of high tumorigenicity. Mol Med Rep 12: 5042-5048, 2015.
12. Gupta GP and Massagué J: Cancer metastasis: Building a framework. Cell 127: 679-695, 2006.
13. Talmadge JE and Fidler IJ: AACR centennial series: The biology of cancer metastasis: Historical perspective. Cancer Res 70: 5649-5669, 2010.
14. Bacac M and Stamenkovic I: Metastatic cancer cell. Annu Rev Pathol 3: 221-247, 2008.
15. Chambers AF, Groom AC and MacDonald IC: Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer 2: 563-572, 2002.
16. Duffy MJ, McGowan PM and Gallagher WM: Cancer invasion and metastasis: Changing views. J Pathol 214: 283-293, 2008.
17. Fidler IJ: The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. Nat Rev Cancer 3: 453-458, 2003.
18. Steeg PS: Tumor metastasis: Mechanistic insights and clinical challenges. Nat Med 12: 895-904, 2006.
19. Tarin D: Cell and tissue interactions in carcinogenesis and metastasis and their clinical significance. Semin Cancer Biol 21: 72-82, 2011.
20. Yadav L, Puri N, Rastogi V, Satpute P, Ahmad R and Kaur G: Matrix metalloproteinases and cancer-roles in threat and therapy. Asian Pac J Cancer Prev 15: 1085-1091, 2014.
21. Yilmaz M, Christofori G and Lehembre F: Distinct mechanisms of tumor invasion and metastasis. Trends Mol Med 13: 535-541, 2007.
22. Nieto MA, Huang RY, Jackson RA and Thiery JP: EMT: 2016. Cell 166: 21-45, 2016.
23. Kansara M and Thomas DM: Molecular pathogenesis of osteosarcoma. DNA Cell Biol 26: 1-18, 2007.
24. Jayaprakasha GK, Jaganmohan Rao L and Sakariah KK: Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Food Chem 98: 720-724, 2006.
25. Shishodia S: Molecular mechanisms of curcumin action: Gene expression. Biofactors 39: 37-55, 2013.
26. Masuda T, Hidaka K, Shinohara A, Maekawa T, Takeda Y and Yamaguchi H: Chemical studies on antioxidant mechanism of curcuminoid: Analysis of radical reaction products from curcumin. J Agric Food Chem 47: 71-77, 1999.
27. Li YB, Gao JL, Zhong ZF, Hoi PM, Lee SM and Wang YT: Bisdemethoxycurcumin suppresses MCF-7 cells proliferation by inducing ROS accumulation and modulating senescence-related pathways. Pharmacol Rep 65: 700-709, 2013.
28. Luo C, Du Z, Wei X, Chen G and Fu Z: Bisdemethoxycurcumin attenuates gastric adenocarcinoma growth by inducing mitochondrial dysfunction. Oncol Lett 9: 270-274, 2015.
29. Hongtao C, Youling F, Fang H, Huihua P, Jiying Z and Jun Z: Curcumin alleviates ischemia reperfusion-induced late kidney fibrosis through the APPL1/Akt signaling pathway. J Cell Physiol 233: 8588-8596, 2018.
30. Liao CL, Chu YL, Lin HY, Chen CY, Hsu MJ, Liu KC, Lai KC, Huang AC and Chung JG: Bisdemethoxycurcumin suppresses migration and invasion of human cervical cancer HeLa cells via inhibition of NF-κB, MMP-2 and -9 pathways. Anticancer Res 38: 3989-3997, 2018.
31. Xu J, Yang H, Zhou X, Wang H, Gong L and Tang C: Bisdemethoxycurcumin suppresses migration and invasion of highly metastatic 95D lung cancer cells by regulating E-cadherin and vimentin expression, and inducing autophagy. Mol Med Rep 12: 7603-7608, 2015.
32. Isfort RJ, Cody DB, Lovell G and Doersen CJ: Analysis of oncogenes, tumor suppressor genes, autocrine growth-factor production, and differentiation state of human osteosarcoma cell lines. Mol Carcinog 14: 170-178, 1995.
33. Gorgoulis VG, Vassiliou LV, Karakaidos P, Zacharatos P, Kotsinas A, Liloglou T, Venere M, Ditullio RA Jr, Kastrinakis NG, Levy B, *et al*: Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 434: 907-913, 2005.

34. Mancini L, Paul-Clark MJ, Rosignoli G, Hannon R, Martin JE, Macintyre I and Perretti M: Calcitonin and prednisolone display antagonistic actions on bone and have synergistic effects in experimental arthritis. Am J Pathol 170: 1018-1027, 2007.
35. Shih YL, Au MK, Liu KL, Yeh MY, Lee CH, Lee MH, Lu HF, Yang JL, Wu RS and Chung JG: Ouabain impairs cell migration, and invasion and alters gene expression of human osteosarcoma U-2 OS cells. Environ Toxicol 32: 2400-2413, 2017.
36. Chen HY, Yang MD, Chou YC, Ma YS, Peng SF, Liao CL, Chen PY, Hsia TC, Lien JC and Chen CH: Ouabain suppresses cell migration and invasion in human gastric cancer AGS cells through the inhibition of MMP signaling pathways. Anticancer Res 41: 4365-4375, 2021.
37. Ma YS, Hsiao YT, Lin JJ, Liao CL, Lin CC and Chung JG: Phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) inhibit human melanoma A375.S2 cell migration and invasion by affecting MAPK signaling pathway in vitro. Anticancer Res 37: 6223-6234, 2017.
38. Siegel R, Ward E, Brawley O and Jemal A: Cancer statistics, 2011: The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 61: 212-236, 2011.
39. Sporn MB: The war on cancer. Lancet 347: 1377-1381, 1996.
40. Roato I, Caldo D, Godio L, D'Amico L, Giannoni P, Morello E, Quarto R, Molfetta L, Buracco P, Mussa A and Ferracini R: Bone invading NSCLC cells produce IL-7: Mice model and human histologic data. BMC Cancer 10: 12, 2010.
41. Santini D, Galluzzo S, Zoccoli A, Pantano F, Fratto ME, Vincenzi B, Lombardi L, Gucciardino C, Silvestris N, Riva E, *et al*: New molecular targets in bone metastases. Cancer Treat Rev 36 (Suppl 3): S6-S10, 2010.
42. Liu Y, Bi T, Shen G, Li Z, Wu G, Wang Z, Qian L and Gao Q: Lupeol induces apoptosis and inhibits invasion in gallbladder carcinoma GBC-SD cells by suppression of EGFR/MMP-9 signaling pathway. Cytotechnology 68: 123-133, 2016.
43. Pei H, Yang Y, Cui L, Yang J, Li X, Yang Y and Duan H: Bisdemethoxycurcumin inhibits ovarian cancer via reducing oxidative stress mediated MMPs expressions. Sci Rep 6: 28773, 2016.
44. Yodkeeree S, Chaiwangyen W, Garbisa S and Limtrakul P: Curcumin, demethoxycurcumin and bisdemethoxycurcumin differentially inhibit cancer cell invasion through the down-regulation of MMPs and uPA. J Nutr Biochem 20: 87-95, 2009.
45. Toth M, Sohail A and Fridman R: Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods Mol Biol 878: 121-135, 2012.
46. Grzelczyk WL, Szemraj J and Józefowicz-Korczyńska M: The matrix metalloproteinase in larynx cancer. Postepy Hig Med Dosw (Online) 70: 1190-1197, 2016.
47. Hendrix MJ, Seftor EA, Seftor RE and Fidler IJ: A simple quantitative assay for studying the invasive potential of high and low human metastatic variants. Cancer Lett 38: 137-147, 1987.
48. Li YH and Zhu C: A modified Boyden chamber assay for tumor cell transendothelial migration in vitro. Clin Exp Metastasis 17: 423-429, 1999.
49. Huang T, Kang W, Cheng AS, Yu J and To KF: The emerging role of Slit-Robo pathway in gastric and other gastro intestinal cancers. BMC Cancer 15: 950, 2015.
50. Yang M and Huang CZ: Mitogen-activated protein kinase signaling pathway and invasion and metastasis of gastric cancer. World J Gastroenterol 21: 11673-11679, 2015.
51. Fife CM, McCarroll JA and Kavallaris M: Movers and shakers: Cell cytoskeleton in cancer metastasis. Br J Pharmacol 171: 5507-5523, 2014.
52. Koul HK, Pal M and Koul S: Role of p38 MAP kinase signal transduction in solid tumors. Genes Cancer 4: 342-359, 2013.
53. Yu Y, Luk F, Yang JL and Walsh WR: Ras/Raf/MEK/ERK pathway is associated with lung metastasis of osteosarcoma in an orthotopic mouse model. Anticancer Res 31: 1147-1152, 2011.
54. Wada T and Penninger JM: Mitogen-activated protein kinases in apoptosis regulation. Oncogene 23: 2838-2849, 2004.
55. Wang C, Zhou X, Li W, Li M, Tu T, Ba X, Wu Y, Huang Z, Fan G, Zhou G, *et al*: Macrophage migration inhibitory factor promotes osteosarcoma growth and lung metastasis through activating the RAS/MAPK pathway. Cancer Lett 403: 271-279, 2017.
56. Liu Y, Yuan X, Li W, Cao Q and Shu Y: Aspirin-triggered resolvin D1 inhibits TGF-β1-induced EMT through the inhibition of the mTOR pathway by reducing the expression of PKM2 and is closely linked to oxidative stress. Int J Mol Med 38: 1235-1242, 2016.
57. Wang H, Zhang C, Xu L, Zang K, Ning Z, Jiang F, Chi H, Zhu X and Meng Z: Bufalin suppresses hepatocellular carcinoma invasion and metastasis by targeting HIF-1α via the PI3K/AKT/mTOR pathway. Oncotarget 7: 20193-20208, 2016.
58. Mirzaei S, Saghari S, Bassiri F, Raesi R, Zarrabi A, Hushmandi K, Sethi G and Tergaonkar V: NF-κB as a regulator of cancer metastasis and therapy response: A focus on epithelial-mesenchymal transition. J Cell Physiol 237: 2770-2795, 2022.
59. Pi WS, Cao ZY, Liu JM, Peng AF, Chen WZ, Chen JW, Huang SH and Liu ZL: Potential molecular mechanisms of AURKB in the oncogenesis and progression of osteosarcoma cells: A label-free quantitative proteomics analysis. Technol Cancer Res Treat 18: 1533033819853262, 2018.
60. Akiyama T and Kawasaki Y: Wnt signalling and the actin cytoskeleton. Oncogene 25: 7538-7544, 2006.
61. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A and Engler JA: Matrix metalloproteinases: A review. Crit Rev Oral Biol Med 4: 197-250, 1993.
62. Dutta A, Li J, Lu H, Akech J, Pratap J, Wang T, Zerlanko BJ, FitzGerald TJ, Jiang Z, Birbe R, *et al*: Integrin αvβ6 promotes an osteolytic program in cancer cells by upregulating MMP2. Cancer Res 74: 1598-1608, 2014.
63. Chu K, Cheng CJ, Ye X, Lee YC, Zurita AJ, Chen DT, Yu-Lee LY, Zhang S, Yeh ET, Hu MC, *et al*: Cadherin-11 promotes the metastasis of prostate cancer cells to bone. Mol Cancer Res 6: 1259-1267, 2008.
64. Chang JW, Kang SU, Shin YS, Seo SJ, Kim YS, Yang SS, Lee JS, Moon E, Lee K and Kim CH: Combination of NTP with cetuximab inhibited invasion/migration of cetuximab-resistant OSCC cells: Involvement of NF-κB signaling. Sci Rep 5: 18208, 2015.
65. Guarino M, Rubino B and Ballabio G: The role of epithelial-mesenchymal transition in cancer pathology. Pathology 39: 305-318, 2007.
66. Yuan Y, Ye HQ and Ren QC: Upregulation of the BDNF/TrKB pathway promotes epithelial-mesenchymal transition, as well as the migration and invasion of cervical cancer. Int J Oncol 52: 461-472, 2018.
67. Cuesta C, Arévalo-Alameda C and Castellano E: The importance of being PI3K in the RAS signaling network. Genes (Basel) 12: 1094, 2021.
68. Matsuoka H, Tsubaki M, Yamazoe Y, Ogaki M, Satou T, Itoh T, Kusunoki T and Nishida S: Tamoxifen inhibits tumor cell invasion and metastasis in mouse melanoma through suppression of PKC/MEK/ERK and PKC/PI3K/Akt pathways. Exp Cell Res 315: 2022-2032, 2009.