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*Bis*demethoxycurcumin Suppresses Migration and Invasion of Human Cervical Cancer HeLa Cells *via* Inhibition of NF-κB, MMP-2 and -9 Pathways

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Abstract. *Background/Aim: Bisdemethoxycurcumin (BDMC) exhibits biological activities including anticancer and anti-metastasis in human cancer cell lines, but there is no available information to show whether BDMC suppresses cell migration and invasion of human cervical cancer cells. Materials and Methods: Wound-healing, migration, invasion, zymography, and western blotting assays were used to investigate the effects of BDMC on HeLa cells in vitro. Results: BDMC reduced the total viable cell number in a dose-dependent manner. The wound-healing assay show BDMC suppressed the movement of HeLa cells. Furthermore, the trans-well chamber assays showed that BDMC suppressed the cell migration and invasion. Gelatin zymograph assay showed that BDMC did not inhibit matrix metalloproteinase-2 (MMP-2) and -9 activities in vitro. However, western blotting assay showed that BDMC significantly reduced protein levels of growth factor receptor-bound protein 2 (GRB2), Ras homolog gene family, member A (Rho A), urokinase-type plasminogen activator (uPA), RAS, MMP-2, and N-cadherin but increased those of phosphor-extracellular-signal related kinase (p-ERK1/2), E-cadherin and nuclear factor-κB (NF-κB) in HeLa cells. Confocal laser microscopy assay was used to further confirm BDMC increased NF-κB when compared to controls. Conclusion: BDMC may have potential as a novel anti-metastasis agent for the treatment of human cervical cancer.*

Uterine cervical cancer is the fourth most common cancer in women (1). In Taiwan, cervical cancer is the eighth most common cancer among females, the animal report by the Department of Health, Taiwan, R.O.C. for 2016 indicated that 54 individuals per 1,000,000 died annually from cervical cancer (2). Compared to other cancer types, screening for cervical cancer is the most effective (3). Currently, surgery, chemotherapy and radiotherapy or their combination are used for clinical patients with cervical cancer but side-effects, resistance and recurrence still occur (4). In particular, approximately 90% of patients with cancer die due to the result of invasive and metastatic growth of cancer (5, 6). Therefore, in order to improve clinical outcomes in cervical cancer, new chemotherapeutics directed towards inhibiting cancer cell metastasis are urgently needed.

The essential features of the metastatic process include tumor cell invasion and migration (7, 8). Matrix metalloproteinases (MMPs) degrade mechanical barrier such as extracellular matrix

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and basement membrane leading to cell movement (9). It is well documented that the proteolytic activities of MMPs are involved in the metastasis process, including enabling cell adhesion, migration, and invasion (10, 11). MMP-2 (gelatinase) plays an important role in degrading basement membranes and is involved in cancer invasion and metastasis (12-14); increased MMP-2 expression is associated with reduced survival and poor prognosis in human malignancies (15, 16). Increased mRNA and protein levels of MMP-2 have been detected in cervical cancer (17). Other studies have shown that MMP inhibitors used in patients with metastatic cancer may adequately treat metastasis (18, 19).

Turmeric, one of the most popular medicinal herbs in India and China, has a wide range of pharmacological activities, for treating ulcers, skin diseases and the symptoms of colds and flu (20) and to cure immune-related, metabolic diseases and cancer with no side-effects (20, 21). The major pharmacological activity of turmeric is attributed to curcuminoids that include curcumin and two related compounds demethoxy curcumin (DMC) and *bis*demethoxycurcumin (BDMC) (22). BDMC induces mitochondrial dysfunction and apoptosis in PANC-1 human pancreatic cancer cells (23). In 95D non-small cell lung cancer cells, Hsieh *et al*. showed the effects of BDMC on transforming growth factor-β1-induced epithelial-to-mesenchymal transition (EMT) are mediated through Wnt inhibitory factor-1, revealing a novel mechanism of EMT regulation (24).

Although studies on BDMC exist, no reports show actual anti-metastatic effects on cancer cells. Therefore, in the present study, we analyzed the effects of BDMC on migration and invasion of HeLa human cervical cancer cells *in vitro*. In addition, we also investigated the role of MMP-2 and -9 and NF-κB signaling pathways in HeLa cells after exposure to BDMC.

Materials and Methods

Chemicals and reagents. BDMC, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin were purchased from GIBCO®/Invitrogen Life Technologies (Grand Island, NY, USA). Polyvinylidene difluoride membrane was obtained from Millipore (Temecula, CA, USA). The following primary antibodies were used: GRB2, RAS and NF-κB p65 all from BD Pharmingen (San Jose, CA, USA); ROHA, p-ERK1/2, ERK1/2, NF-κB p50 and uPA all from Santa-Cruz Biotechnology Inc. (Dallas, TX, USA); SNAIL and E-cadherin from Cell Signaling (St Louis, MO, USA). MMP-2, N-cadherin, β-catenin, vimentin, and β-actin from Sigma Chemical Co. (St. Louis, MO, USA); MMP-9 from Millipore (Temecula, CA, USA); and PCNA from GeneTex Inc. (Irvine, CA, USA).

Cell culture. HeLa human cervical cancer cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). Cells were cultured in DMEM containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in a humid atmosphere incubator with 5% CO_2 at 37˚C (24).

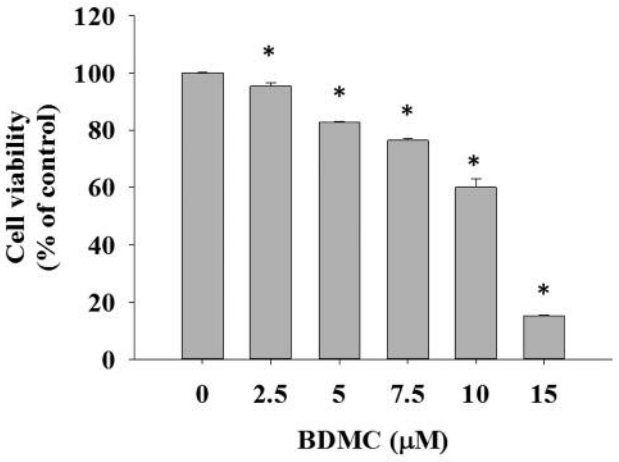

Figure 1. *Bisdemethoxycurcumin (BDMC) reduced the viable cell number of HeLa cells. Cells were kept in 12-well plates with Dulbecco's modified Eagle's medium for 24 h and were incubated with BDMC at final concentrations of 0, 2.5, 5.0, 7.5, 10 and 15 μM, or 0.5% dimethylsulfoxide as a vehicle control for 48 h. After incubation, cells were collected, counted and stained with propidium iodide (5 μg/ml) to measure the total viable cell number by flow cytometry as described in Materials and Methods. *Significantly different from the control at p<0.05.*

Cell viability assays. HeLa cells were seeded in 12-well plates at an initial density of 10^5 cells per well with DMEM for 24 h. They were then incubated with BDMC (0, 2.5, 5.0, 7.5, 10 and 15 μM) or 0.5% DMSO as a vehicle control for 48 h. After incubation, cells were collected, counted and stained with PI (5 μg/ml) to measure the total cell viability by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) as previously described (24).

Wound-healing assay. HeLa cells (5×10^5 cells/well) were seeded in 6-well plate and grown to about 90% confluence after 24 h. The medium was removed and cell monolayers were wounded by manually scraping the cells with a 200 ml plastic pipette tip, and were then washed with phosphate buffered saline (PBS) three times. Cells were then cultured in DMEM containing 0, or 5 μM of BDMC for 24 and 48 h. Cells were examined and photographed using an inverted microscope at different time points as described previously (25, 26).

Invasion and migration assay (Matrigel and invasion assay). The assessment of *in vitro* invasion and migration activities were carried out using Matrigel-coated transwell cell culture chambers (8 μm pore size) as described previously (25). Briefly, HeLa cells (5×10^5 cells/well) in serum-free medium were maintained in 12-well plates and were incubated with 0 or 5 μM of BDMC for 24 and 48 h. For migration assay, cells were placed on the top of the well with membrane coated with collagen and then incubated with BDMC. The cells on the upper surface of the membrane were removed by a cotton swab and invaded cells on the lower surface were fixed with 4% cold formaldehyde, stained with 0.1% crystal violet and then were photographed. The invading cells in the chamber were

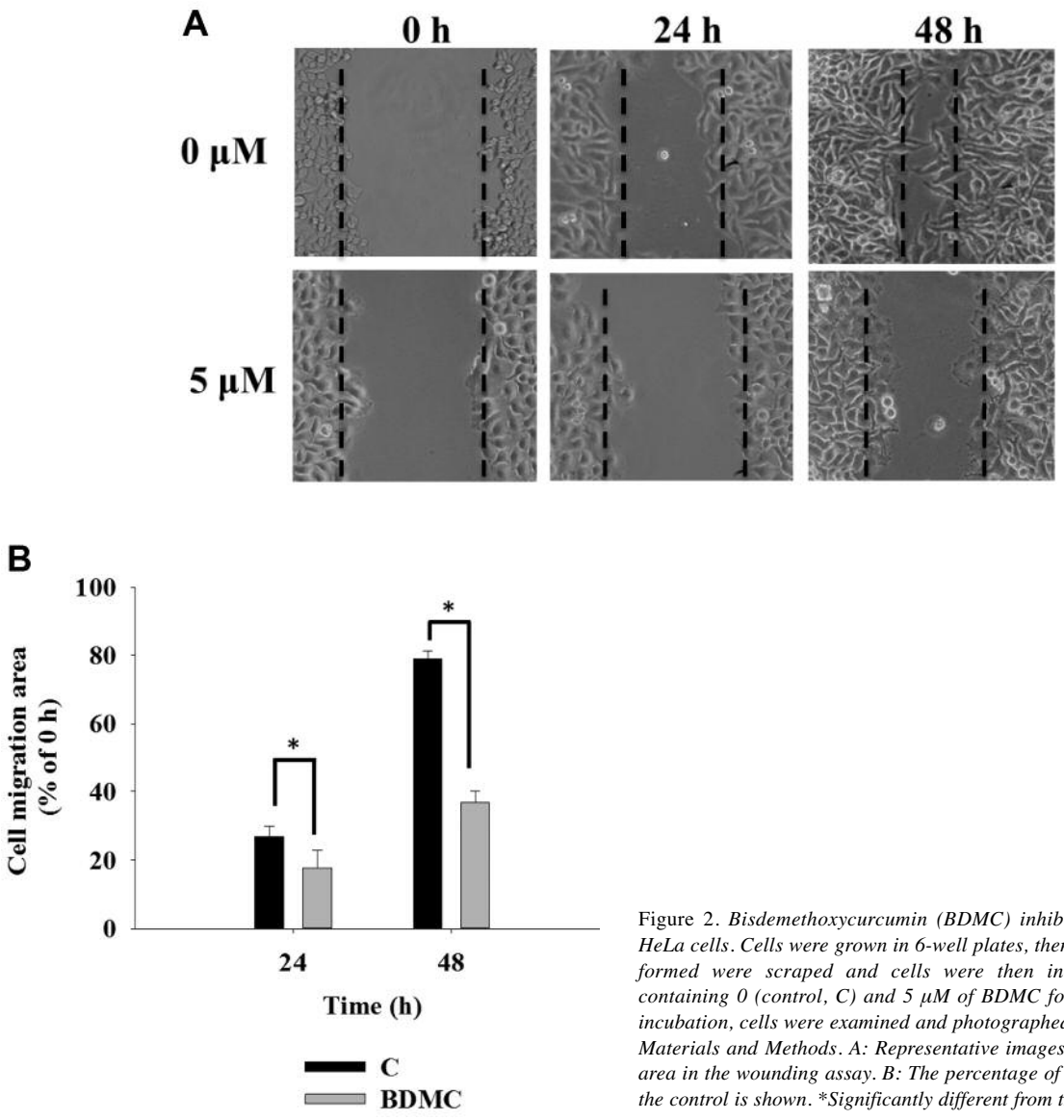

Figure 2. *Bisdemethoxycurcumin (BDMC) inhibited cell mobility of HeLa cells. Cells were grown in 6-well plates, then the cell monolayers formed were scraped and cells were then incubated in medium containing 0 (control, C) and 5 μM of BDMC for 24 and 48 h. After incubation, cells were examined and photographed, as described in the Materials and Methods. A: Representative images of closure of scrape area in the wounding assay. B: The percentage of migration relative to the control is shown. *Significantly different from the control at p<0.05.*

counted under a light microscope at a magnification of ×200. For invasion assay, the same assay was used except the membrane was coated with matrigel as described previously (25, 27, 28).

Gelatin zymography assay. HeLa cells were treated with 5 μM of BDMC at 37˚C for 12, 24 and 48 h and the release of MMP-2 and MMP-9 was assayed using gelatin zymography (8% zymogram gelatin gels) as described previously (25). Briefly, after incubation, the conditioned medium was collected, the total proteins measured were separated by 0.2% gelatin-8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (80 V for 120 min). Afterwards, the gels were washed twice in a wash solution containing 2.5% (v/v) Triton X-100 at room temperature and subsequently incubated in substrate buffer (50 mM Tris HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100, pH 8.0) while shaking for 18 h at 37˚C. The gels were rinsed with distilled water and stained for 30 min with 0.2% Coomassie blue (Bio-Rad, Hercules, CA, USA) in 10% acetic acid and 50% methanol and were photographed on a light box. The gelatinolytic activities of MMP-2 and -9 were densitometrically quantified and analyzed by an image analysis system (Bio-Rad Laboratories, Richmond, CA, USA) as described previously (25).

Western blotting assay. HeLa cells (6×10^6 cells) were harvested after 0 and 5 μM of BDMC treatment for 12, 24 and 48 h. Cells were collected and lysed in a PRO-PREP protein extraction solution [40 mM Tris-HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonide P-40] (iNTRON Biotechnology, FL, USA), and Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA) was used for measuring the total protein (25). Thirty micrograms of protein was electrophoresed loaded on a SDS-PAGE gel and was transferred to a polyvinylidene difluoride membrane.

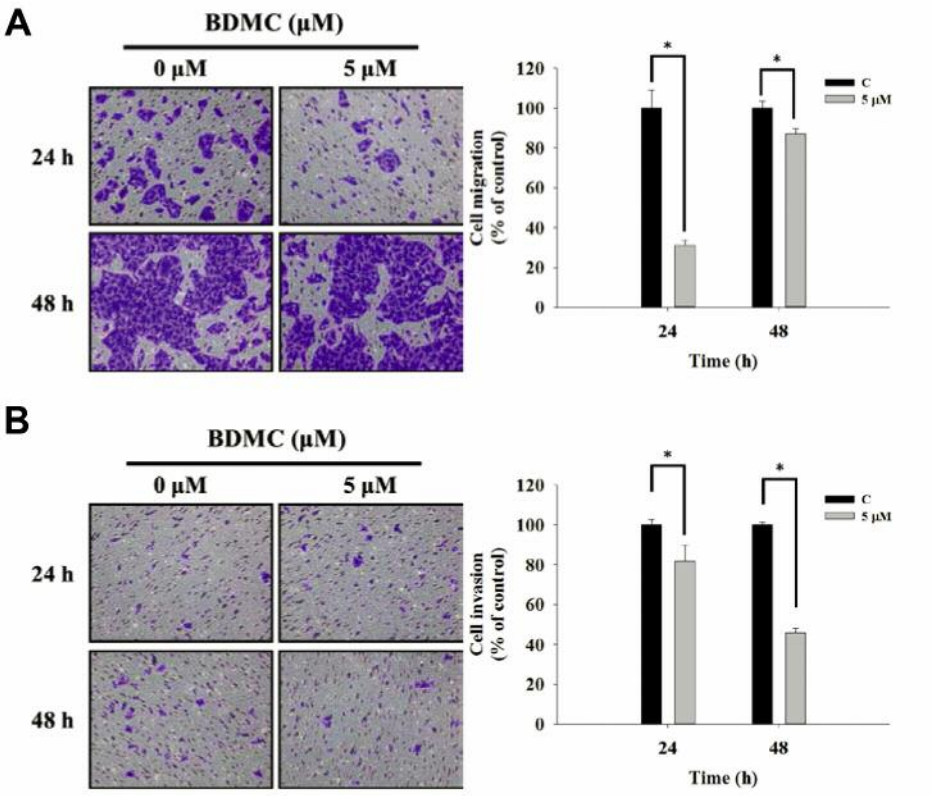

Figure 3. *Bisdemethoxycurcumin (BDMC) inhibited the cell migration and invasion of HeLa cells. The measurement of cell migration (A) and invasion (B) were performed by using Trans-well cell migration and invasion assays as described in the Materials and Methods. *Significantly different from the control (C) at p<0.05.*

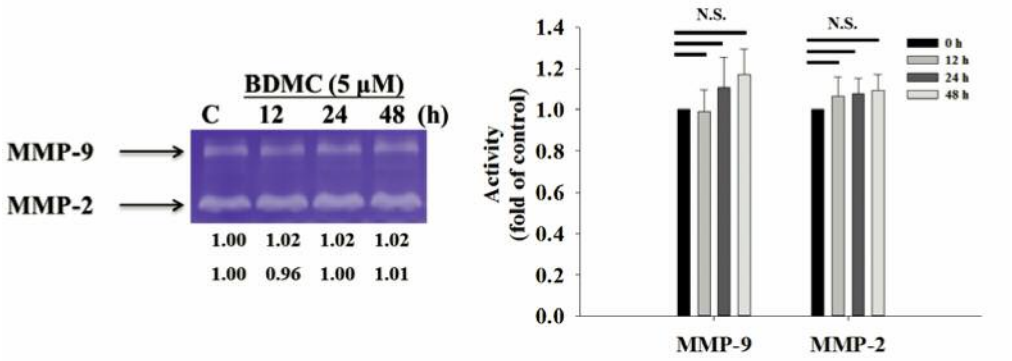

Figure 4. *Bisdemethoxycurcumin (BDMC) did not inhibit the activity of matrix metalloproteinase-2 (MMP-2) and -9 in HeLa cells. HeLa cells were incubated with BDMC (5 μM) for 12, 24 and 48 h, and the gelatinolytic activity of MMP-2/-9 were measured as described in the Materials and Methods. N.S.: Not significantly different from the control (C).*

The membrane was blocked in 5% fat-free dry milk solution in PBS and probed with corresponding (diluted 1:1,000) overnight at 4˚C. After washing, anti-mouse or anti-rabbit IgG (diluted 1:10,000) was added to the membranes for 1 h at room temperature. The proteins were visualized using enhanced chemiluminescence reagents (Millipore, Temecula, CA, USA). The signal was detected using a gel documentation system (Molecular Imager ChemiDoc™ XRS+ Imaging System; Bio-Rad, Hercules, CA, USA) as described previously (25, 29).

Confocal laser microscopy assay. Results from western blotting demonstrated that BDMC affect NF-κB protein expression in HeLa cells, thus, for further confirming whether or not BDMC inhibition of cell migration and invasion were involved in the translocation or

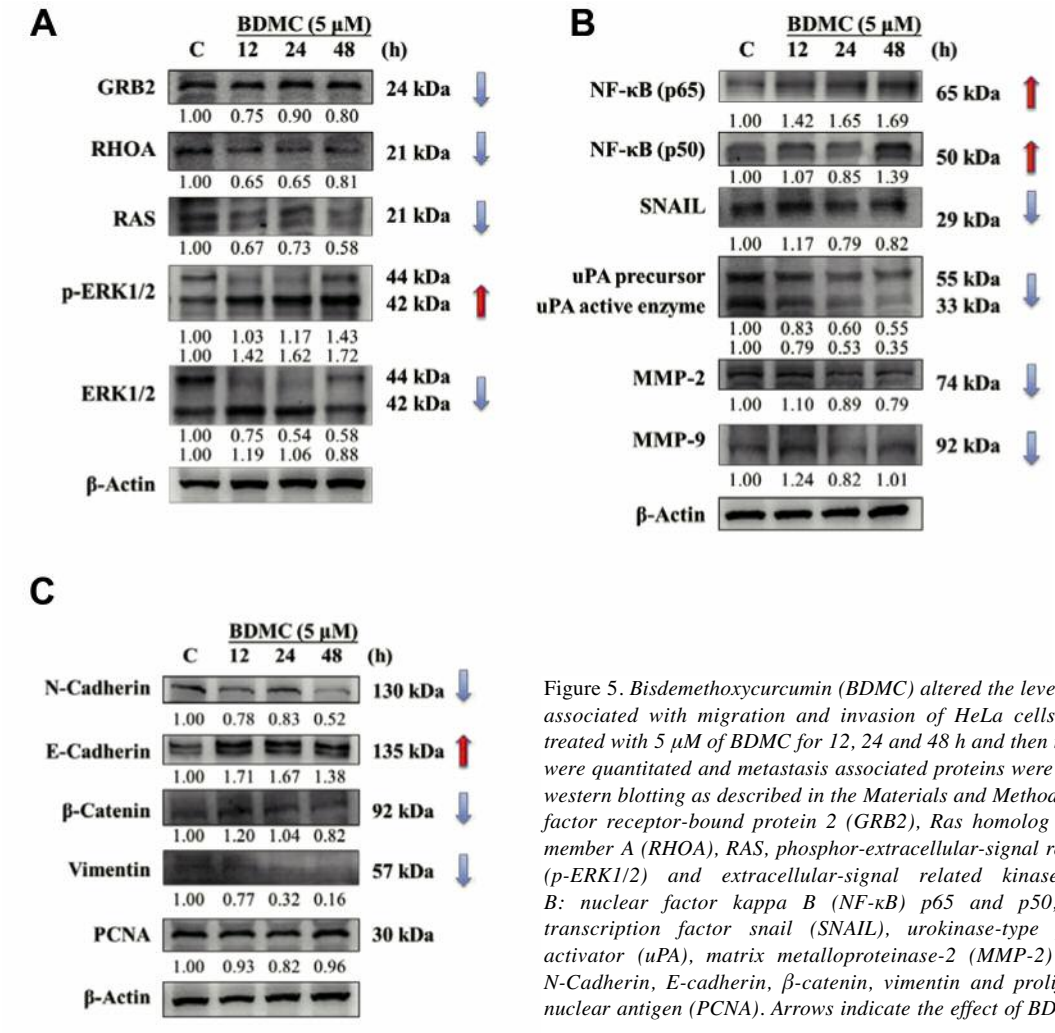

Figure 5. *Bisdemethoxycurcumin (BDMC) altered the levels of proteins associated with migration and invasion of HeLa cells. Cells were treated with 5 μM of BDMC for 12, 24 and 48 h and then total proteins were quantitated and metastasis associated proteins were examined by western blotting as described in the Materials and Methods. A: Growth factor receptor-bound protein 2 (GRB2), Ras homolog gene family, member A (RHOA), RAS, phosphor-extracellular-signal related kinase (p-ERK1/2) and extracellular-signal related kinase (ERK1/2). B: nuclear factor kappa B (NF-κB) p65 and p50, zinc-finger transcription factor snail (SNAIL), urokinase-type plasminogen activator (uPA), matrix metalloproteinase-2 (MMP-2) and -9. C: N-Cadherin, E-cadherin, β-catenin, vimentin and proliferating cell nuclear antigen (PCNA). Arrows indicate the effect of BDMC.*

expression of NF-κB, HeLa cells were treated with 5 μM of BDMC for 24 h and were examined and photographed by confocal laser microscopy systems as described previously (30).

Statistical analysis. All data are expressed as means±SD from at least three experiments. Differences between groups were analyzed by one-way analysis of variance and Dunnett test for multiple comparisons (SigmaPlot for Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). Comparisons were made between groups of BDMC-treated cells and untreated cells (control). Differences with values of $p<0.05$ were considered statistically significant.

Results

BDMC reduces cell viability of HeLa cells. Data shown in Figure 1 indicate a significant dose-dependent reduction of living HeLa cells after incubated with BDMC for 48 h; therefore, the 20% inhibitory concentration (IC_{20}) of 5 μM was used for cell migration and invasion experiments.

BDMC reduces mobility of HeLa cells. The results of the wound-healing essay indicate that the closure of the wound for BDMC-treated cells was less than that of the control for both treatment durations (Figure 2A). BDMC significantly inhibited cell mobility at 5 μM in 24 and 48 h compared with the control (Figure 2B).

BDMC inhibites migration and invasion of HeLa cells. Transwell cell migration and invasion assays were used for measuring cell migration and invasion in HeLa cells after exposure to BDMC. The results indicated that BDMC significantly ($p<0.05$) inhibited cell migration by 31.24% and 87.07% at 24 and 48 h treatment, respectively (Figure 3A) and inhibited cell invasion by 81.55% and 45.76% at 24 and 48 h treatment, respectively, compared to control cells (Figure 3B). Based on these observations, BDMC significantly inhibited migration and invasion of HeLa cells.

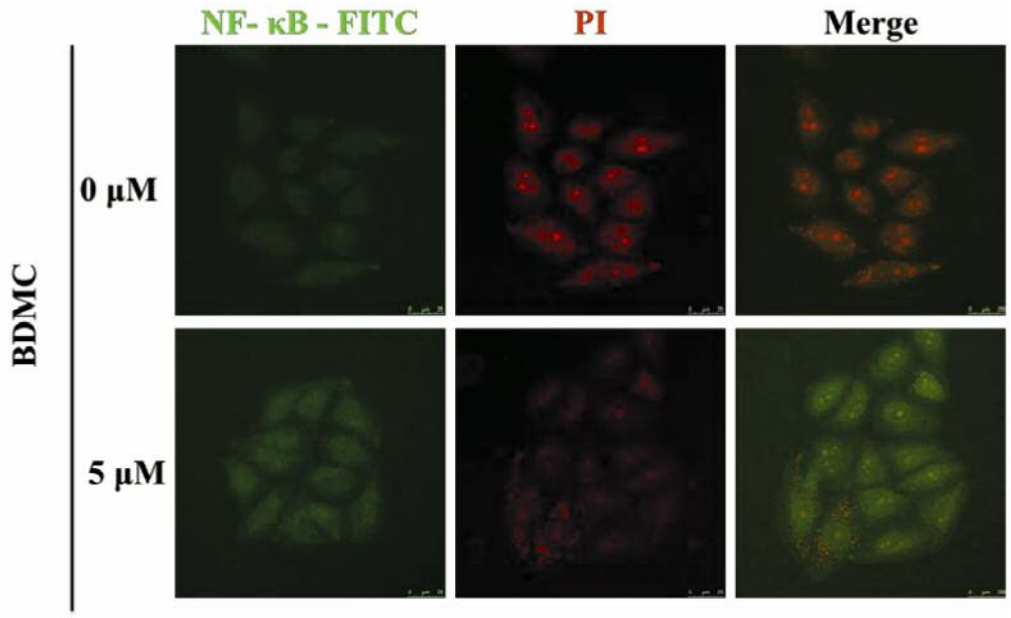

Figure 6. *Bisdemethoxycurcumin (BDMC) affected the translocation of metastasis-associated proteins in HeLa cells. Cells were treated with 5 μM BDMC for 24 h and cells were stained by anti-nuclear factor kappa B (NF-κB) (p65) and then were stained with secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (green fluorescence)] and were examined and photographed using a Leica TCS SP2 confocal laser microscopic systems as described in the Materials and Methods. PI: Propidium iodide.*

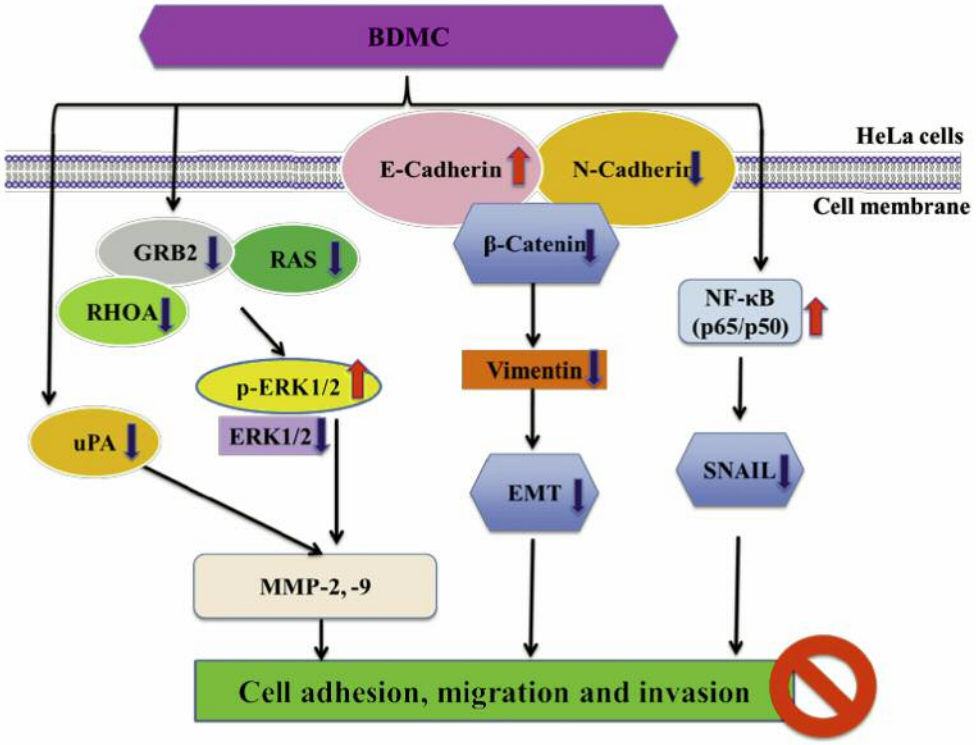

Figure 7. *The possible signaling pathways for bisdemethoxycurcumin (BDMC) inhibition of cell migration and invasion of HeLa human cervical cancer cells. GRB2: Growth factor receptor-bound protein 2; RHOA: Ras homolog gene family, member A; p-ERK1/2: phosphor-extracellular-signal related kinase; ERK1/2: extracellular-signal related kinase; NF-κB: nuclear factor kappa B; SNAIL: zinc-finger transcription factor snail; uPA: urokinase-type plasminogen activator; MMP-2: matrix metalloproteinase-2; MMP-9: matrix metalloproteinase-9; PCNA: proliferating cell nuclear antigen; EMT: epithelial–mesenchymal transition.*

BDMC regulates the activities of MMP-2 and -9 in HeLa cells. Gelatin zymography for the gelatinolytic activity of MMP-2 and -9 indicated that BDMC did not significantly affect the gelatinolytic activity of MMP-2 or -9 when compared to the control groups (Figure 4).

BDMC alters levels of proteins associated with migration and invasion of HeLa cells. In order to explore the link between upstream regulated proteins associated with cell migration and invasion, after exposure to BDMC at 5 μM, HeLa cells were collected for western blotting and the results are presented in Figure 5. BDMC did not significantly affected PCNA, however, it significantly reduced the levels of GRB2, MMP-2, MMP-9, ERK1/2, RAS, RHOA, SNAIL, N-cadherin, vimentin, β-catenin and uPA but increased those of p-ERK1/2, E-cadherin and NF-κB (p65/p50) in HeLa cells *in vitro*.

BDMC alters the translocation of migration- and invasion-associated proteins in HeLa cells. In order to further confirm whether BDMC affects the translocation of NF-κB, which was involved in cell migration and invasion of HeLa cells, cells were treated with or without 5 μM of BDMC for 24 h, and were stained with anti-NF-κB and then were examined and photographed by confocal laser microscopy system. The results in Figure 6 show that BDMC increased NF-κB expression in the cytoplasm and nucleus compared to the control (0 μM) and the results were consistent with that of western blotting.

Discussion

Cancer cell metastasis involves a series of complicated processes including cell movement, adhesion, migration, invasion and angiogenesis to form new tumors in other sites of the body (8, 31). It is well known that during tumor progression, tumor cells acquire the expression of mesenchymal markers (vimentin, N-cadherin, and fibronectin) but lose that of epithelial markers (E-cadherin) through EMT, enabling tumor metastasis (32). The molecular mechanisms underlying cancer invasion and metastasis are still not completely clear (33). Many studies have investigated natural products for inhibiting cancer cell metastasis. In India and China, the curcuminoids are commonly used as coloring agent as well as food additives. The World Health Organization stated the acceptable daily intake of curcuminoids to be in the range of 0-3 mg/kg as a food additive (34). Other studies have shown that curcuminoids inhibit cancer cell migration and invasion (35-38). BDMC, which is a curcuminoid, was shown to reduce cancer cell numbers through induction of cell-cycle arrest and apoptosis in human cancer cell lines (23, 24), however, its effects on cell migration and invasion of human cervical cancer cells have not been reported to our knowledge. Herein, we investigated the effects of BDMC on the HeLa cell migration and invasion *in vitro*.

The wound-healing assay protocol is acceptable for examining cancer cell mobility (39, 40). Results from Figures 2 indicated that BDMC significantly inhibited cell mobility of HeLa cells *in vitro*. Transwell-assay methods have been recognized as measuring the inhibition of cancer cell migration and invasion (25, 41). Herein, the Transwell-assay was used to investigate HeLa cell migration and invasion after treatment with BDMC (5 μM) for 24 and 48 h and results indicated that BDMC suppressed cell migration and invasion *in vitro* (Figure 3A). Based on these observations, we further investigated the molecular effects of BDMC inhibited cell migration and invasion of HeLa cells and possible alterations in the expression of several molecules. At first, we examined the gelatinolytic activity of MMP-2 and -9 treatment with BDMC. MMP-2 and MMP-9 are the gelatinase for degradation of the extracellular matrix (6, 42) and are considered to be valid drug targets (43). It was reported that BDMC inhibits cancer cell invasion through the down-regulation of MMPs and uPA (38). Herein, we demonstrated that BDMC inhibited invasion and motility of HeLa cells but did not reduce the activity of MMP-2 and -9 *in vitro* (Figure 4).

In the present study, BDMC reduced protein expression of GRB2, MMP-2, MMP-9, RAS, RhoA, SNAIL, N-cadherin, vimentin, β-catenin, uPA and ERK1/2, but increased the p-ERK1/2, E-cadherin and NF-κB (p65/p50) (Figure 5) in HeLa cells examined by western blotting assay. GRB2 has been reported to be involved in cell proliferation and migration of various types of cancer cell (44). BDMC significantly inhibited ERK1/2 but increased p-ERK1/2 in Hela cells (Figure 5A). ERK1/2 have been shown to contribute to cancer cell proliferation, migration and metastasis in some cell lines (45, 46) and increased p-ERK1/2 activity promotes cancer cell proliferation and metastasis in various cancer cell lines (47-49). Our findings indicate that BDMC reduced MMP-2 and -9 activities and protein expression, which may be *via* ERK1/2 phosphorylation, and it also inhibited the vimentin pathway in HeLa cells. BDMC increased E-cadherin but decreased N-cadherin in HeLa cells. It is reported that during cancer cell metastatic process, polarization of epithelial cells converted into mesenchymal cells was characterized by the loss of E-cadherin-mediated cell–cell contacts, as well as the acquisition of increased migratory and invasive potential (50-54). We found that BDMC significantly inhibited NF-κB expression (Figure 6). NF-κB is a transcription factor closely linked to cell survival, proliferation and metastasis (55) and the NF-κB pathway has been recognized to be a target to treat inflammation and cancer (56).

In conclusion, in the present study, we found that BDMC induced cytotoxic effects on HeLa cells, thus, we selected the dose of BDMC that did not kill cells for inhibiting cell migration and invasion *in vitro*. BDMC suppressed cell

mobility, migration and invasion in of HeLa cells through inhibition of MMP-2 and -9 signaling pathway by mechanisms that are summarized in Figure 7. Thus, we suggest that BDMC may be a potential candidate for developing preventive agents against human cervical cancer metastasis.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Acknowledgements

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