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Curcumin, demethoxycurcumin and bisdemethoxycurcumin differentially inhibit cancer cell invasion through the down-regulation of MMPs and uPA☆

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Abstract

Curcumin (Cur), a component of turmeric (*Curcuma longa*), has been reported to exhibit antimetastatic activities, but the mechanisms remain unclear. Other curcuminoids present in turmeric, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) have not been investigated whether they exhibit antimetastatic activity to the same extent as curcumin. The regulation of matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) play important role in cancer cell invasion by cleavage of extracellular matrix (ECM). In this line, we comparatively examined the influence of Cur, DMC and BDMC on the expressions of uPA, MMP-2, MMP-9, membrane Type 1 MMP (MT1-MMP), tissue inhibitor of metalloproteinases (TIMP-2), and in vitro invasiveness of human fibrosarcoma cells. The results indicate that the differential potency for inhibition of cancer cell invasion was BDMC≥DMC>Cur, whereas the cell migration was not affected. Zymography analysis exhibited that curcumin, DMC and BDMC significantly decreased uPA, active-MMP-2 and MMP-9 but not pro-MMP-2 secretion from the cells in a dose-dependent manner, in which BDMC and DMC show higher potency than curcumin. The suppression of active MMP-2 level correlated with inhibition of MT1-MMP and TIMP-2 protein levels involved in pro-MMP-2 activation. Importantly, BDMC and DMC at 10 μM reduced MT1-MMP and TIMP-2 protein expression, but curcumin slightly reduced only MT1-MMP but not TIMP-2. In addition, three forms of curcuminoids significantly inhibited collagenase, MMP-2, and MMP-9 but not uPA activity. In summary, these data demonstrated that DMC and BDMC show higher antimetastasis potency than curcumin by the differentially down-regulation of ECM degradation enzymes.

Keywords: Curcuminoids; Matrix metalloproteinases (MMPs); Cancer invasion; Bisdemethoxycurcumin

1. Introduction

Most cancer death is not caused by the growth of the primary tumor but results from its invasive spread (metastasis) to secondary site. The critical step of metastasis is the invasion of cancer cells through the extracellular matrix (ECM) using proteolytic enzymes. The key proteases that are involved in degradation of the ECM are the serine protease (plasmin), urokinase plasminogen activator (uPA) system, cysteine protease such as cathepsin B and L and matrix metalloproteinases (MMPs) [1]. Although several enzymes involved degradation of basement membrane, it appears that MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play a key role in cancer cell invasion and metastasis [2] that can degrade type IV collagen, one of the major components of basement membrane. All MMPs are produced in a latent form (pro-MMP) requiring activation for catalytic activity, a process that is usually accomplished by proteolytic removal of the propeptide domain. Once activated, all MMPs are specifically inhibited by a group of tissue inhibitors of metalloproteinases (TIMPs). Membrane Type 1 MMP (MT1-MMP) (MMP-14) has been shown to be a key enzyme in tumor metastasis and angiogenesis [3,4] and was identified as the first physiological activator of pro-MMP-2 [5]. The role of MT1-MMP in pericellular proteolysis can

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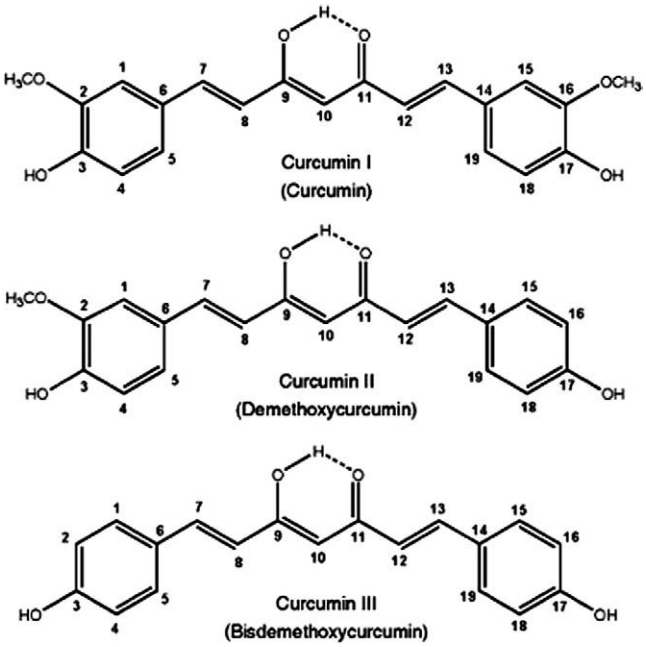

Fig. 1. Structures of curcumin and its derivatives used in the present study.

play a direct role in ECM turnover. When the activity or the expression of ECM degradation enzymes is blocked, their invasion property is abolished. So every level of regulation of MMP-expression, activation and activity can be considered as a target for therapeutic intervention.

Curcuminoids, which are natural phenolic coloring compounds found in the rhizomes of *Curcuma longa* Linn, are commonly known as tumeric. Three main curcuminoids have been isolated from turmeric: curcumin (Cur), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) [6].Their chemical structures are illustrated in Fig. 1. Commercially available curcumin used for research and for clinical trials (curcumin mix) contains ~ 77% pure Cur, 17% DMC and 3% BDMC. Several studies demonstrated that curcuminoids have been shown to display anticarcinogenic [7,8], antioxidant [9,10], anti-inflammation [11], antiangiogenic properties [12,13] and modulation mutidrug-resistance gene and protein function [14,15]. In recent years, it has also been reported that curcumin reduced cancer cell invasion in vitro and in vivo by regulation of invasive gene such as ECM degradation enzymes (MMP-9, MT1-MMP, MMP-2) [16–19]. Whether analogs of Cur, such as DMC and BDMC, modulate cancer cell invasion and metastasis to same extent as Cur remain to be investigated.

In this study, we further explore to investigate the influence of Cur, DMC and BDMC on the expressions of uPA, MMP-2, MMP-9, membrane Type 1 MMP (MT1-MMP), tissue inhibitor of metalloproteinases (TIMP-2) and in vitro invasiveness of human fibrosarcoma cells. We found that Cur, DMC and BDMC differentially regulate the MT1-MMP and TIMP-2 level that has been implicated in MT1-MMP processing and pro-MMP-2 activation. Overall, our data demonstrated that DMC and BDMC show higher anti-metastasis potency than curcumin by the differentially down-regulation of ECM degradation enzymes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) with or without phenol red, penicillin–streptomycin and trysin–EDTA were purchased from GIBCO-BRL (Grand island, NY, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). MMP-2, *Clostridium* collagenase, plasminogen, gelatin, *p*-aminophenyl mercuric acetate (APMA) and casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). MMP-9 was purchased from Roche (Indianapolis, USA). Fluorescein-conjugated gelatin was purchased from Molecular Probe (Eugene, OR, USA). Antibody against MT1-MMP was purchased from Chemicon (Chemicon international, Euromedex, France) and antibody against TIMP-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA).

2.2. Cell lines

HT1080 human fibrosacroma and NIH3T3 fibroblasts cells were grown in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS). Cultures were maintained at 37°C in a 5% CO_2 /95% air atmosphere.

2.3. Extraction and isolation of curcuminoids

Curcuminoids were isolated and purified as described previously [14].Briefly, turmeric powder was extracted with 95% ethanol for 24 h, then curcuminoids mixture was precipitated with petroleum ether that contained 78% curcumin,16% DMC and 5% BDCM. The curcuminoids were further fractionated by silica gel flash column chromatography using first $CHCl_3$ and then $CHCl_3$/methanol with increasing polarity to yield pure fractions of Cur, DMC and BDMC. The identity and purity of each curcuminoid was verified using thin layer chromatography (TLC), high-performance liquid chromatography, IR, mass spectrometry and nuclear magnetic resonance analyses as described previously [20].

2.4. MTT assay for cell viability

Cell viability was measured by the conventional MTT reduction assay as described previously [21]. Briefly, HT1080 cells were inoculated at a density of 3×10^3 cells/well in 96 well plates for 24 h, in 200 μl of DMEM with 10% FCS; after that, culture supernatant was removed and serum-free DMEM containing various concentrations of Cur, DMC and BDMC was added and incubated for 24 h. MTT dye (15 μl, 5 mg/ml) was added and the plate was incubated for

an additional 4 h. The absorbance of MTT-formazan was measured using a microplate reader at 570 nm with a reference wavelength of 630 nm.

2.5. Cell invasion and migration assay

The invasive and migration behavior of HT1080 cells was tested using the modified Boyden chamber assay [22]. Polyvinylpyrrolidone-free polycarbonate filters (Millipore) (8 μM pore size) were coated with gelatin (0.01% w/v) for chemotaxis (cells migration) or with Matrigel (15 μg per filter) for invasion assay. The medium in the lower chamber contained serum-free culture conditioned medium of NIH 3T3 fibroblast cells, which acted as a chemoattractant. HT1080 cells (1×10^5 cells per chamber) were plated onto the upper chamber with or without various concentrations of Cur, DMC and BDMC and incubation for 5 h at 37°C in 5% CO_2. After incubation, the noninvading cells were removed from the upper surface of the membrane. The invading cells on the lower surface of the membrane were fixed with methanol for 1 min and stained with toluidine blue for 5 min. The cells that actively migrate to the under the surface of filter were dissolved with 20% acetic acid and indirectly quantitated by measuring the absorbance at 570 nm. Control experiment was performed in the absence of chemoattractant. The results of three independent experiments were averaged after background substraction.

2.6. Zymography

The secretions of MMP-2 and MMP-9 in culture conditioned medium were assayed by gelatin zymography [23]. HT1080 cells (1×10^5 cells/well) were seeded into 6-well plates and maintained for 24 h in DMEM with 10% FBS. Subconfluent cells cultures were incubated for 24 h in various concentrations of Cur, DMC and BDMC in serum-free DMEM, and the culture supernatants were collected from equal number of cells. Without heating and under nonreducing conditions, the sample underwent electrophoresis in 0.1% w/v gelatin-containing 10% polyacrylamide gels [polyacrylamide gel electrophoresis (PAGE)] in the presence of sodium dodecyl sulfate (SDS). After electrophoresis, gel was washed two times for 30 min in 2.5% Triton X-100 and incubated for 18 h at 37°C in Tris buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). Gels were stained with Coomassie brilliant blue R (0.1% w/v) and destained in 30% methanol and 10% acetic acid. Gelatinolytic activity appeared as a clear band on a blue background. Digestion bands were quantitated using Bio 1 D software (Viber Lourmat).

The uPA secretion in culture conditioned medium was examined by casein–plasminogen zymography. The culture supernatant was separated by electrophoresis in 10% PAGE copolymerized with 1 mg/ml of β-casein and 10 μg/ml human plasminogen under nonreducing conditions. After electrophoresis, the gel was washed two times for 30 min in 2.5% Triton X-100 and incubated for 18 h at 37°C in Tris buffer (50 mM Tris–HCl, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.4). Gels were stained and destained as described above.

2.7. Preparation of conditioned media and whole cell lysates

HT1080 cells (1×10^6cells/flask) were seeded into 75-mm^3 T flask and maintained for 24 h in DMEM with 10% FBS. Subconfluent cells cultures were incubated for 24 h in various concentrations of Cur, DMC and BDMC in serum-free DMEM without phenol red. After treatment, the culture supernatant was collected and concentrated with Amicon-Ultra 4 (Millipore) for TIMP-2 analysis, and the cells were washed twice with ice-cold phosphate buffer saline (PBS) and scraped with a cell scraper into ice-cold PBS. The cells were centrifuged at 500×*g* for 10 min, the supernatant was removed and cells pellets were lysed with a lysis buffer containing protease inhibitor (50 mM Tris HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min on ice. The insoluble matter was removed by centrifugation at 12,000×*g*, 15 min and 4°C and the supernatant fraction was collected for MT1-MMP analysis.

2.8. Western blot analysis of TIMP-2 and MT1-MMP

The expressions of MT1-MMP and TIMP-2 proteins were determined by immunoblotting with specific antibodies. Briefly, equal amounts of lysate or concentrated–conditioned media proteins from control and treated cells were resuspended in sample buffer and separated by SDS-PAGE, using 10% acrylamide gels. After electrophoresis, proteins were electroblotted to a Hybond-C Extra nitrocellulose membrane (Amersham). The membrane was blocked at room temperature (RT) with 5% nonfat dry milk in PBS containing 0.3% Tween (PBS-T). Membrane was washed twice with PBS-T and incubated 2 h at RT with the primary antibody, rabbit polyclonal anti-MT1-MMP (1:2,000; Chemicon) or mouse monoclonal anti-TIMP-2 (1:200; Santa Cruz Biotechnology). After washing five times with PBS-T, the membrane was incubated with the second antibodies at RT for 1 h in PBS-T containing 5% nonfat dry milk, and after incubation, the membrane was washed with PBS-T for five times. The second antibodies were horseradish peroxidase-conjugated antirabbit IgG (1:10,000; Amersham) or antimouse IgG (1:10,000; Chemicon). After incubation, the immunoreactive material was visualized by enhanced chemiluminescence and exposure to X-ray film (Kodak) for 5 up to 30 min.

2.9. Substrate degradation by ECM degradation enzymes

Fluorometric assay for the proteolytic activity of collagenase, MMP-2 and MMP-9 was performed using EnzChek Gelatinase/Collagenase Assay kit (Molecular Probe). Briefly, 1 U/ml of collagenase was mixed with 10 μg/ml of fluorescein-conjugated gelatin containing various concentrations of Cur, DMC and BDMC in final volume of 200 μl of reaction buffer in 96-well microplates. The rate of proteolysis was determined

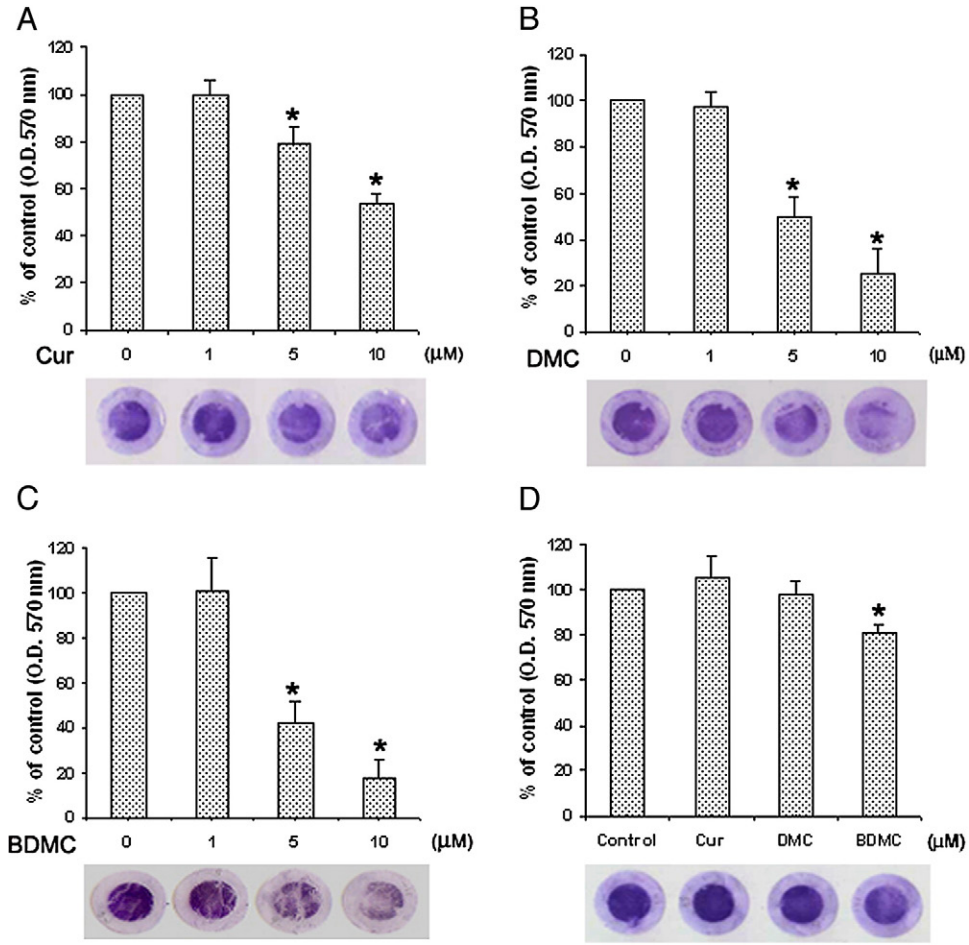

Fig. 2. Effects of curcuminoids on HT1080 cell invasion (A–C) and migration (D). HT1080 cells were seeded onto a Matrigel-coated (A–C) or gelatin-coated (D) filter containing Cur, DMC and BDMC with various indicated concentrations (0–10 μM) for cell invasion assay or at 10 μM for cell migration assay, incubated for 5 h in 37°C. The cells that actively migrated to the lower surface of filters were quantitated as described in Materials and Methods. The data represent the mean±S.D. of three independent experiments. Statistical analyses were performed using one-way ANOVA. *P<.01 was considered statistically significant.

by measuring the fluorescence intensity at 5-min interval for 20 min with a fluorometer. The fluorescence values were measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Enzyme activity was estimated by linear regression of the fluorescent intensity recorded during that time.

For proteolytic activity of MMP-2 and MMP-9, the pro-MMP-2 and pro-MMP-9 were activated to their active form before used. Briefly, 10 μg/ml of pro-MMP-2 was activated with 0.75 mM APMA at 37°C for 1 h. For pro-MMP-9 activation, 0.5 mU of pro-MMP-9 was activated by trypsin (50 μg/ml) at 37°C for 30 min and the reaction was stopped by adding 10 μl aprotinin (1.5 mg/ml). Activated MMP-2 (0.25 μg/ml) and MMP-9 (50μU) were reacted with gelatin fluorescence substrate and Cur, DMC and BDMC. Thereafter, the fluorescence intensity was measured using a fluorometer as described above.

The uPA activity was determined by using an uPA activity kit assay (Chemicon). Following the manufacturer's instruction, dilutions of Cur, DMC and BDMC were premixed with uPA (10 U) in microwell (85 μl final volume), and after 15 min at 4°C, 15 μl of chromogenic uPA substrate was added. The optical densities were measured with a spectrometer at 405 nm at 5-min interval for 60 min.

2.10. Statistical analysis

Statistical analyses were performed using one-way ANOVA and Student's t test. P<.05 or P<.01 was considered statistically significant. All statistical analyses were performed using SPSS 10.0 software.

3. Results

3.1. Effects of curcuminoids on invasion and migration activity

HT1080 cells have an ability to invade through Matrigel. Treatment of Cur, DMC and BDMC for 5 h exhibited a

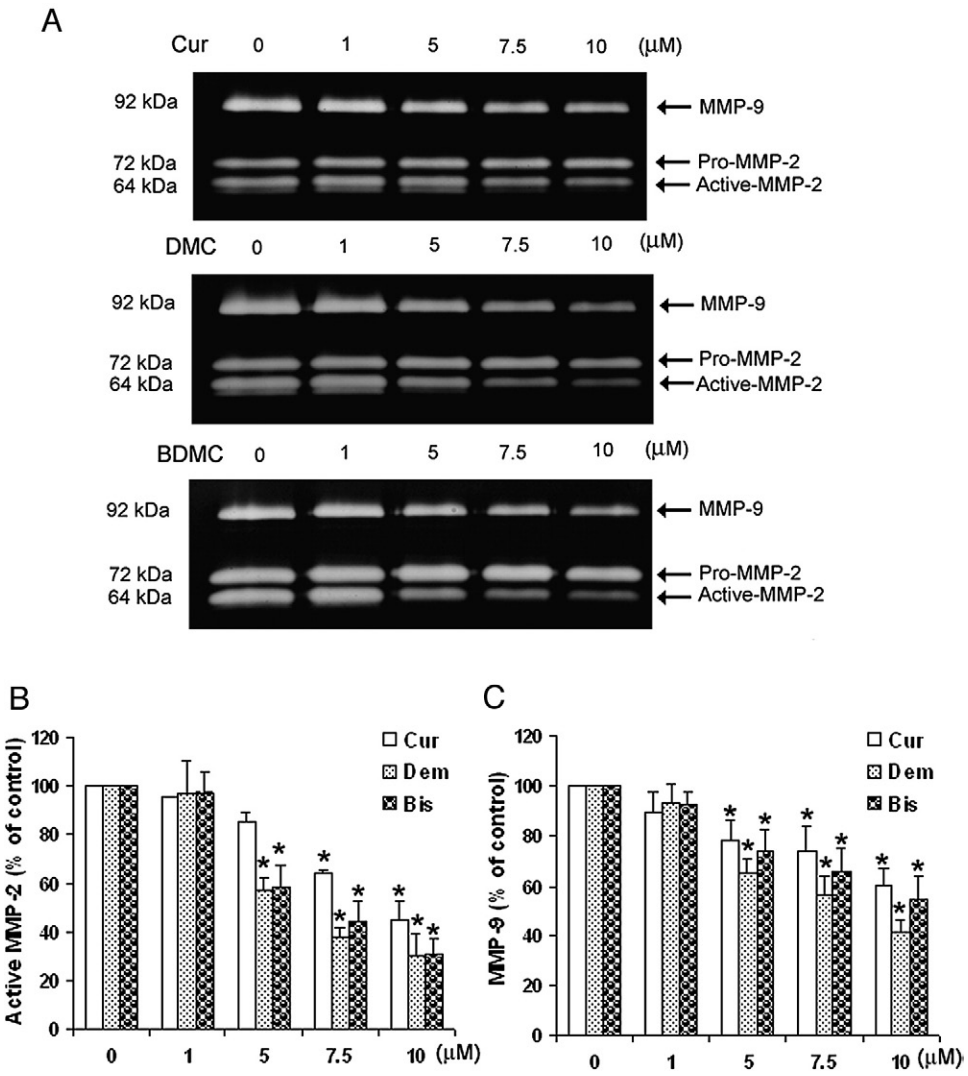

Fig. 3. Effects of curcuminoids on MMP-2 and MMP-9 secretion to conditioned medium. (A) Subconfluent HT1080 cells were incubated (24 h, 37°C) in the absence and presence of various indicated concentrations (1–10 μM) of Cur, DMC and BDMC in serum-free medium. Equal amount of proteins was loaded (5 μg/lane). Gelatinolytic activities of MMP-2 and MMP-9 in the conditioned medium were determined by gelatin zymography. (B and C) The band intensities of the active MMP-2 and MMP-9 were quantitated by densitometry. The data represent the mean±S.D. of three independent experiments. Statistical analyses were performed using one-way ANOVA. *$P<01$ was considered statistically significant.

significant inhibition of cell invasion in a dose-dependent manner with IC_{50} at >10, 5 and 4 μM, respectively (Fig. 2A–C). In contrast, curcumin and DMC did not affect cell migration to chemoattractant (chemotaxis) through gelatin-coated filters, and only BDMC slightly affected cell migration at a high concentration (Fig. 2D). Despite displaying a similar basic chemical structure, the three compounds affected cancer cell invasion and migration in a different fashion. For example, removal of one or two methoxy groups from the molecule of curcumin, as in DMC and BDMC, respectively, strongly inhibited cancer cell invasion. Removal of both methoxy groups, as in BDMC, slightly affected cell motility, whereas there was no effect for Cur and DMC. It has to be noted that cell invasion and migration assay were analyzed in noncytotoxic concentrations of curcuminoids. The effect of curcuminoids on cell viability was measured by the MTT assay. The noncytotoxic concentrations of Cur, DMC and BDMC that caused >80% cell survival are at 19, 12 and 10 μM, respectively (data not shown). These concentrations were used in our experiments.

3.2. Curcuminoids reduced the secretion of active MMP-2, MMP-9 and uPA from HT1080 cells

To investigate whether curcumin, DMC and BDMC can inhibit the secretion of MMP-2, MMP-9 and uPA from HT1080 cells, the cells were treated with various indicated concentration of Cur, DMC and BDMC (1–10 μM) in serum-free medium. The levels of MMP-2 and MMP-9 in conditioned medium were analyzed by gelatin zymography and that of uPA was analyzed by casein-plasminogen

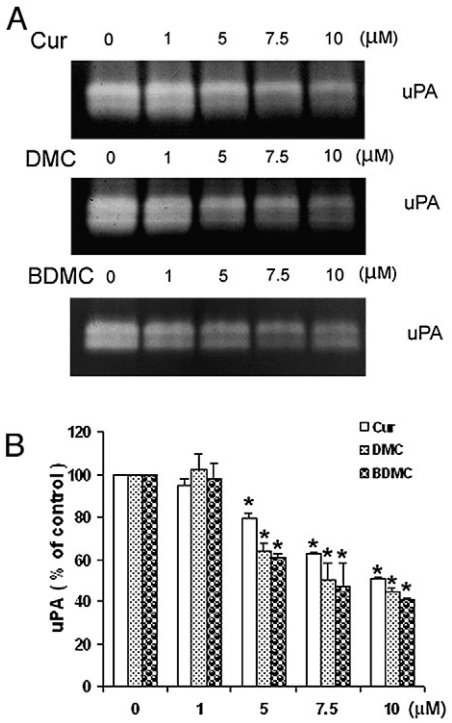

Fig. 4. Effects of curcuminoids on uPA secretion in HT1080 cells. (A) After HT1080 cells were treated with various indicated concentrations (1–10 μM) of Cur, DMC and BDMC for 24 h in serum-free medium, equal amount of proteins was loaded (2.5 μg/lane). Casein–plasminogen zymography was performed for uPA activity in the conditioned medium. (B) The band intensity of uPA activity was quantitated by densitometry. The data represent the mean±S.D. of three independent experiments. Statistical analyses were performed using one-way ANOVA. *$P<.01$ was considered statistically significant.

zymography. As shown in Fig. 3, the secretion of active MMP-2 (62 kDa) was reduced by Cur, DMC and BDMC in a dose-dependent manner, with IC_{50} in the range of 9, 6 and 7 μM, respectively. In contrast, the secretion of pro-MMP-2 (72 kDa) was unaffected by treatment with curcuminoids. On the other hand, MMP-9 secretion was significantly inhibited by DMC with IC_{50} at 8 μM and significantly inhibited by Cur and BDMC with IC_{50} > 10 μM. We next investigated the effect of curcuminoids on uPA secretion from the cells using casein zymography. As shown in Fig. 4, Cur, DMC and BDMC reduced uPA secretion from HT1080 cells in a dose-dependent manner, with IC_{50} at 10, 7.5 and 7 μM, respectively.

3.3. Effects of curcuminoids on MT1-MMP and TIMP-2 expression in HT1080 cells

To further explore the modulation of pro-MMP-2 activation mediated by Cur, DMC and BDMC, MT1-MMP and TIMP-2 protein expressions were investigated by Western blot analysis. As shown in Fig. 5A, DMC and BDMC at 10 μM significantly reduced the TIMP-2 protein level; the total MT1-MMP protein expression (active, 60 kDa; inactive form, 45 kDa) was also suppressed by DMC and BDMC (Fig. 5C). Curcumin, at 10 μM, slightly but significantly reduced total MT1-MMP protein expression by suppression of only the inactive form (45kDa) but not the active form (60 kDa) of MT1-MMP (Fig. 5C). Curcumin did not affect TIMP-2 protein expression (Fig. 5A).

3.4. Effects of curcuminoids on collagenase, MMP-2, MMP-9 and uPA activity

To investigate whether curcumin and DMC and BDMC can directly inhibit ECM degradation enzyme activity in vitro, purified enzymes (collagenase, MMP-2, MMP-9 and uPA) were incubated with their fluorogenic substrate and chromogenic substrate. As shown in Fig. 6, Cur, DMC and BDMC significantly inhibited collagenase activity in

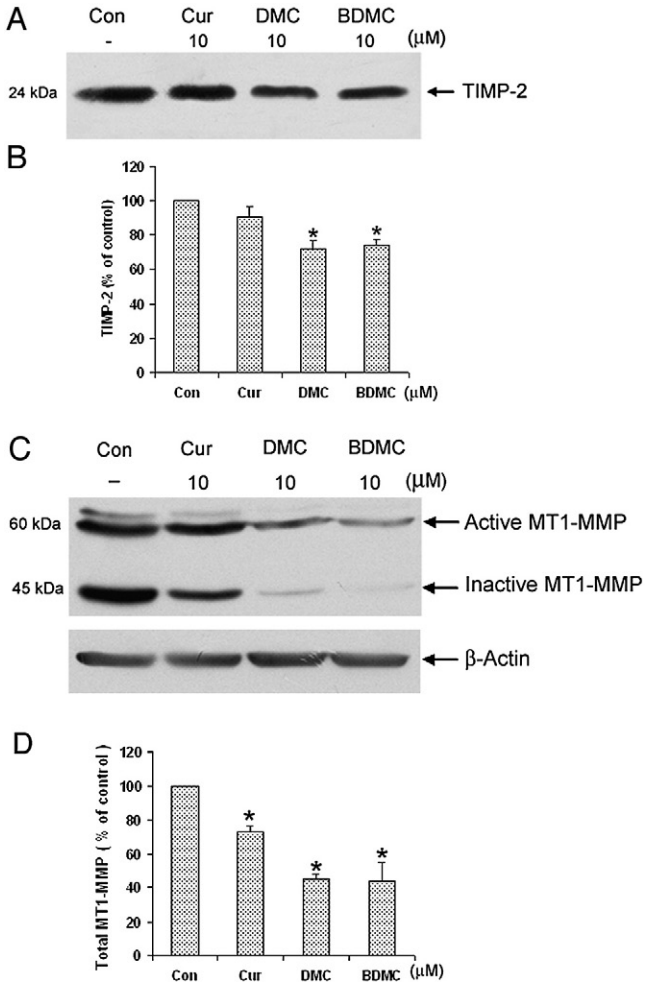

Fig. 5. Analysis of the TIMP-2 (A) and MT1-MMP (C) proteins expressed in HT1080 cells by curcuminoids using Western blot analysis. HT1080 cells were treated with 10 μM of Cur, DMC and BDMC for 24 h; conditioned medium was collected and concentrated for TIMP-2 determination, and the cell lysates were used for MT1-MMP determination. Equal amount of proteins was loaded (40 μg/lane for TIMP-2 and 50 μg/lane for MT1-MMP). The band intensity of TIMP-2 (B) and MT1-MMP (D) was quantitated by densitometry. The data represent the mean±S.D. of three independent experiments. Statistical analyses were performed using one-way ANOVA. *$P<.01$ was considered statistically significant.

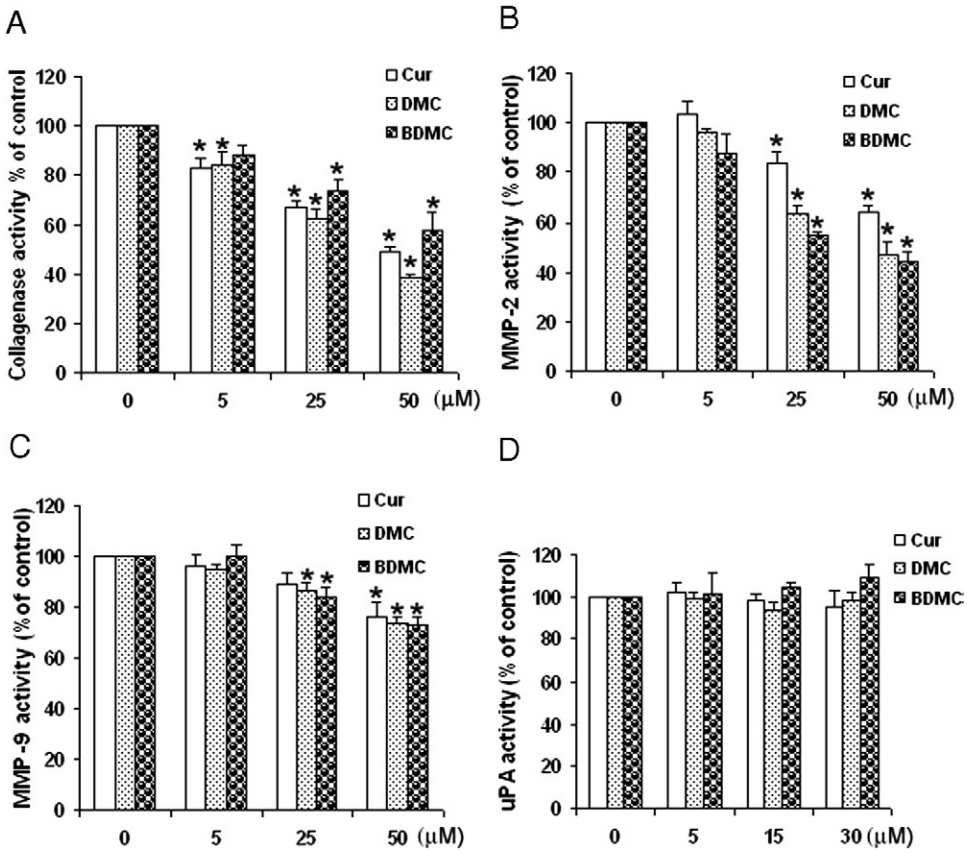

Fig. 6. Effects of curcuminoids on collagenase (A), MMP-2 (B), MMP-9 (C), and uPA (D) enzyme activity. The inhibitory effects of curcuminoids on proteolytic activities of collagenase, MMP-2 and MMP-9 activity were measured using gelatin fluorescence substrate, and uPA activity were measured using a specific chromogenic substrate as described in Materials and Methods. Data represent the mean±S.D. of three independent experiments. Sample groups were significantly different from the control group (∗P<.05) by analysis of Student's t test.

a dose-dependent manner with IC_{50} value of 50, 47 and >50 μM, respectively (Fig. 6A). Cur, DMC and BDMC markedly inhibited MMP-2 activity dose-dependently with IC_{50} value of >50, 45 and 40 μM, respectively (Fig. 6B), but they slightly inhibited MMP-9 activity (Fig. 6C, P<.05). Cur, DMC and BDMC did not affect the catalytic activity of uPA (Fig. 6D).

4. Discussion

Natural curcuminoids isolated from tumeric powder contain 78% Cur, 16% DMC and 5% BDMC. Several studies have shown that Cur, DMC and BDMC have similar biological activity. Interestingly in some cases, DMC or BDMC show higher potency than Cur. For example, DMC demonstrated the highest antiproliferative activity against MCF-7 breast carcinoma cells [24] while BDMC was maximally active in inducing reactive oxygen species [25], and from our findings, we found that BDMC is the most active form for modulation of MDR-1 gene [14]. Other reported on cases where Cur is the most active form to increase the endothelial heme oxygenase-1 expression [26], and we found that curcumin is the most active form as a MDR chemosensitizer [15]. From these data, we suggest that three compounds affected some important biological activities in a different fashion.

Based on the fact that antimetastasis activity of curcumin has been reported, we aimed to investigate whether other curcumin derivatives present in turmeric would also affect cancer cell invasion and MMP gene expression. This is the first report to demonstrate that DMC and BDMC at noncytotoxic doses significantly inhibited HT1080 cell invasion through the basement membrane. The rank order of antimetastasis activity at 10 μM is BDMC, followed by DMC and then Cur (Fig. 2).

Proteolysis in the tumor environment degrades the components of the ECM, allowing invasion of tumor cells to a secondary site. The key proteases that involve in these processes are MMPs and serine protease such as uPA. Although several enzymes are involved in this process, it appears that MMP-2 and MMP-9 play an important role in cell invasion because both enzymes degrade type IV collagen, a major component of the basement membrane. In this study, we investigated the effect of Cur, DMC and BDMC on MMP-2, MMP-9 and uPA secretion from HT1080 cells using zymography assay (Fig. 3). Gelatin zymography revealed that DMC and BDMC comparably

reduced active-MMP-2 and MMP-9 secretion more than Cur, but three forms of curcuminoids did not have an effect on pro-MMP-2 secretion. Cur, DMC and BDMC significant decreased uPA secretion from HT1080 cells, where DMC and BDMC (IC_{50},7.5 and 7 μM, respectively) were found to be comparable while Cur was much less active (IC_{50}, 10 μM). When examined for collagenase, MMP-2 and MMP-9 activity in vitro using fluorescence substrate (Fig. 6), BDMC and DMC were comparable in being maximally active in suppressing MMP-2 activity in a dose-dependent manner; Cur was less active. The inhibition of collagenase and MMP-9 activities by Cur, DMC and BDMC was found to be comparable. All three forms of curcuminoids show no inhibitory effect on uPA activity.

Because the three curcuminoids reduced active MMP-2 secretion from the cells, this result led us to further investigate the effect of curcuminoids on MT1-MMP and TIMP-2 protein expression using Western blot analysis. MMP-2 is constitutively expressed and secreted as a latent zymogen, pro-MMP-2, and its main activation takes place on the cell surface; this process is mediated by MT1-MMP and TIMP-2 [27,28]. Pro-MMP-2 activation occurs in a ternary complex with TIMP-2 and MT1-MMP [29]; complex formation between MT1-MMP and TIMP-2 seem to be an initial step, which is followed by binding to pro-MMP-2. Then, pro-MMP-2 is activated by a TIMP-2-free active MT1-MMP molecule.

The balance between MT1-MMP and TIMP-2 level is important for pro-MMP-2 activation [30] — pro-MMP-2 can be activated by highly expressed MT1-MMP and low TIMP-2 concentration relative to MT1-MMP, which promote the binding of pro-MMP-2 to MT1-MMP, whereas higher concentration of TIMP-2 prevents the activation process by compete binding to the free MT1-MMP. After activation, MT1-MMP proteolytic processing transforms it to its 45-kDa inactive form [31,32]. The appearance of this inactive MT1-MMP form was strongly correlated with MMP-2 activation [33]. In this study, we found that BDMC and DMC strongly down-regulated total MT1-MMP protein (active and inactive form; reduced to 55% when compared with the control) and also slightly reduced TIMP-2 protein (reduced to 25% when compared with the control), whereas Cur slightly down-regulated total MT1-MMP (reduced to 25% compared with the control) but with no effect on TIMP-2 level (Fig. 5). With these data taken together, curcuminoids relatively reduced the balance ratio of MT1-MMP and TIMP2, leading to higher concentration of TIMP2 and prevention of the pro-MMP-2 activation process in the following order: BDMC≥DMC>Cur.

With these results taken together, Cur, DMC and BDMC decreased active MMP-2, MMP-9, uPA, MT1-MMP and TIMP-2 protein level in HT1080 cells and also inhibited collagenase, MMP-2 and MMP-9 activity, which gives a reasonable explanation for the inhibition of cancer cell invasion. Among three forms of curcuminoids, BDMC and DMC show higher potency than Cur, suggesting that loosing a methoxy group on each side of Cur might promote antimetastasis activity. In comparison to the preclinical work, comprehensive pharmacokinetic data in humans do not exist. From the data of pilot and Phase I clinical studies performed with curcumin, it appears that low systemic bioavailability follow oral dosing. Efficient first-pass and some degree of intestinal metabolism of Cur, particularly glucuronidation and sulfation, might explain its poor systemic availability when administered via the oral route [34]. Tetrahydrocurcumin and hexahydrocurcumin are the major reductive metabolites observed in most studies. Tetrahydro and hexahydro metabolites were predominantly present as glucuronides, but a significant proportion of sulfate conjugates was also observed [35]. Although DMC is one of the major bioactive constituents (17%) of curcuminoids, knowledge about its metabolite fate is scant. Results of enzymatic hydrolysis studies conducted under various conditions revealed that Cur and DMC are chemically less stable than BDMC, whereas the reductive metabolites of all three curcuminoids are stable compounds [35]. The chemical instability of the parent curcuminoids might be an explanation for the difficulties in detecting systemic bioavailability of these compounds. Nevertheless, several observations in volunteers and patients suggest that curcumin might possess systemic biological activity at low oral doses. A single oral dose of 20 mg of curcumin appeared to induce concentration in the gallbladder in human volunteers compared to an amylum placebo [36]. Phase I clinical data have confirmed that the low systematic bioavailability of curcumin following oral dosing limits the tissues that the parent compound can reach at efficacious concentration to exert beneficial effects. Nevertheless, the attainment of physiologically active levels of curcumin in the gut, particularly the colon and rectum, has been demonstrated in animals and humans. Healthy volunteers who ingested 2 g pure curcumin powder after fasting showed less than 10 ng/ml curcumin in their plasma 1 h postdose. In the same study, coingestion of curcumin with 20 mg of piperine appeared to increase the bioavailability of curcumin by 2000% [37]. Modification of curcumin in micronized form or nanocapsulation might substantially increase the availability of the parent compound in the biophase. In view of the pharmacological properties of curcumin, in the future, Phase II clinical evaluation of individuals at risk of developing cancers appears to be appropriate.

In conclusion, we report, for the first time, that the absence of one or two methoxy groups in the ortho position on the aromatic ring of curcuminoids (see structure in Fig. 1) is essential to differentially enhance antimetastasis activity; the order being BDMC≥DMC>Cur. The inhibition was regulated to decrease ECM degradation enzyme secretion from invasive cells and the pro-MMP-2 activation process via MT1-MMP and TIMP-2; however, its mechanism of action still remains elusive. This study provides an additional activity of antimetastasis potential mediated by DMC and BDMC.

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