Bufalin-Inhibited Migration and Invasion in Human Osteosarcoma U-2 OS Cells Is Carried Out by Suppression of the Matrix Metalloproteinase-2, ERK, and JNK Signaling Pathways

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Received 7 May 2011; revised 16 August 2011; accepted 18 August 2011

ABSTRACT: Bufalin has been shown to exhibit multiple pharmacological activities, including induction of apoptosis in many types of cancer cell lines. Osteosarcoma is a type of cancer which is difficult to treat and the purpose of this study was to investigate the effects of bufalin on the migration and invasion of human osteosarcoma U-2 OS cells. The wound healing assay and Boyden chamber transwell assay were used for examining the migration of U-2 OS cells. Western blotting and gelatin zymography assays were used for the expression and activities of metalloproteinase (MMP)-2, MMP-7 or MMP-9 levels. Western blotting analysis also was used for measuring the levels of growth factor receptor-bound protein 2 (GRB2), son of sevenless homolog 1 (SOS1), c-Jun N-terminal kinases 1/2 (JNK1/2), extracellular signal-regulated kinase

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Contract grant sponsor: China Medical University, Taichung, Taiwan; Contract grant number: CMU99-ASIA-22

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/tox.20769

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1/2 (ERK1/2), and p38 in bufalin-treated U-2 OS cells. Bufalin inhibited the cell migration and invasion of U-2 OS cells *in vitro*. Moreover, bufalin reduced MMP-2 and MMP-9 enzyme activities of U-2 OS cells. Bufalin also suppressed the protein level of MMP-2 and reduced the levels of mitogen-activated protein kinases (MAPKs) such as JNK1/2 and ERK1/2 signals in U-2 OS cells. Our results suggest that signaling pathways for bufalin-inhibited migration and invasion of U-2 OS cells might be mediated through blocking MAPK signaling and resulting in the inhibition of MMP-2. Bufalin could be a useful agent to develop as a novel antitumor agent by virtue of its ability to inhibit tumor cell migration and invasion. © 2011 Wiley Periodicals, Inc.
Environ Toxicol 00: 000–000, 2011.

Keywords: bufalin; migration and invasion; human osteosarcoma U-2 OS cells; matrix metalloproteinases; traditional Chinese medicine

INTRODUCTION

Cancer is a leading cause of death worldwide. On the basis of the 2009 report from the Department of Health R.O.C. (Taiwan), about 0.3 people per 100,000 die annually from bone cancer in Taiwan (Wu et al., 2011). Osteosarcoma is the most frequent malignant bone tumor in children and adolescents, and it has been characterized by formation of neoplastic bone tissue (Arndt and Crist, 1999; Thompson et al., 2002). The current treatments for bone cancer such as surgery, radiation, chemotherapy, or a combination of radiotherapy and chemotherapy have had mixed success (AbouEl Hassan et al., 2006). There has been growing interest in the use of natural products to inhibit the development of cancer in multiple animal models (Hong and Sporn, 1997) and these compounds also can be used as cancer therapy drugs (AbouEl Hassan et al., 2006).

An important strategy in cancer treatment is to prevent or reduce metastasis of cancer cells. Adhesion ability is associated with invasion and migration of tumor cells (Jung et al., 2008; Lee et al., 2009). The matrix metalloproteinases (MMPs) and urokinase plasminogen activator (u-PA) are over-expressed in cell metastasis, and MMPs contribute to the invasion and metastasis of various tumor cells (Gullu et al., 2000; Kilian et al., 2006; Mizutani et al., 2000). Hence, the inhibition of MMP expression or enzyme activity (Guruvayoorappan and Kuttan, 2008; Okada et al., 2001; Waas et al., 2003) could be used as early targets for preventing cancer metastasis.

Bufalin is one of the major digoxin-like components in Chan-Su extracts from the venom of *Bufo bufo gargarizan*, and it acts as a Na^{+}-K^{+}-ATPase inhibitor for increasing the intracellular Ca^{2+} concentration (Bagrov et al., 1993; Krenn and Kopp, 1998). In Asian countries, bufalin has long been used as a treatment for heart failure in Chinese medicine (Datta and Dasgupta, 2000; Yu et al., 2008). Bufalin is also known as a topoisomerase II inhibitor (Hashimoto et al., 1997; Pastor et al., 2002), and it induced differentiation (Zhang et al., 1992) and apoptosis (Watabe et al., 1996) in leukemia cells as well as the inhibition of solid tumor growth *in vivo* (Han et al., 2007). Bufalin triggered endothelial cell proliferation and angiogenesis (Lee et al., 1997) which resulted in elevated Ca^{2+} levels and apoptotic death in prostate cancer cells (Yeh et al., 2003). Recently, Jiang et al. reportd that bufalin inhibited human lung cancer cell proliferation via VEGFR1/VEGFR2/EGFR/c-Met–Akt/p44/42/p38-NF-κB signaling pathways (Jiang et al., 2010). In addition, bufalin induced apoptosis of hepatoma HepG2 cells through both Fas- and mitochondria-mediated signaling pathways (Qi et al., 2011).

To date, there is no information showing bufalin-inhibited migration and invasion of human bone cancer cells. In this study, we investigated the effects of bufalin on the migration and invasion of human osteosarcoma U-2 OS cells. This study demonstrated that bufalin suppressed migration and invasion of U-2 OS cells by down-regulating the levels of MMP-2, ERK and JNK signaling pathways *in vitro*.

MATERIALS AND METHODS

Materials and Reagents

Bufalin, dimethyl sulfoxide (DMSO), propidium iodide (PI), crystal violet, Tween-20, and formaldehyde were purchased from Sigma-Aldrich Corp. (St. Louis, MO). McCoy's 5a medium, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from Invitrogen Life Technologies (Carlsbad, CA). Primary antibodies such as MMP-2, MMP-7, GRB2, SOS1, JNK1/2, ERK1/2, p38, and β-actin, and second antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and diluted in PBS Tween-20 before use.

U-2 OS Cell Culture

The human osteosarcoma cell line (U-2 OS) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were plated onto 75 cm^2 tissue culture flasks with 90% McCoy's 5a medium supplemented 2 mM L-glutamine, 10% FBS, and 1% penicillin-streptomycin (100 Units/mL penicillin and 100 μg/mL streptomycin) and grown at 37°C under a humidified 5% CO_2 atmosphere (Huang et al., 2010).

Determination of Percentage of Viable Cells

U-2 OS cells (2×10^5 cells/well) were maintained in 12-well plates for 24 h and then treated with 25, 50, 75, and 100 nM of bufalin for 24 and 48 h, respectively. Cells were harvested, stained with PI (5 μg/mL) (a PI exclusion method) and then analyzed by flow cytometry (BD Biosciences, FACSCalibur Instruction, San Jose, CA) as previously described (Lu et al., 2010; Chiang et al., 2011).

Wound Healing Assay

U-2 OS cells (5×10^5 cells/well) were allowed to form a confluent monolayer in 6-wells plates and then cells were wounded with a 200-μL pipette yellow tip. All cells in the plates were treated with bufalin at final concentrations of 25, 50, and 75 nM and then were incubated in new McCoy's 5a medium with 1% FBS for 12 and 24 h. Photographs were taken using a phase-contrast microscope. The cell-free area of each treatment in the well was measured as previously described (Ho et al., 2009). Cell migration was calculated as the percentage of the remaining cell-free area compared with the area of the initial wound.

Cell Migration Assay

Analysis of migration in U-2 OS cells was assessed using an Oris™ Universal Cell Migration Assembly kit (Platypus Technologies, Madison, WI) following the manufacturer's instructions (Li et al., 2010; Oh and Santy, 2010). Briefly, U-2 OS cells were initially stained with 1 μM of CellTracker™ Green CMFDA (Invitrogen) for 45 min under growth conditions for long-term staining (Barhoumi et al., 1993). Afterward, cells were centrifuged and washed twice with cultured medium. Labeled U-2 OS cells at a density of 1×10^4/100 μL were seeded into each test 96-well of the Oris plate with the well inserts (stoppers) and then incubated at 37°C in a 5% CO_2 humidified chamber to permit cell attachment. After a 12-h incubation, the stoppers of each well were removed and then U-2 OS cells were incubated with 0.5% DMSO (as a control) and bufalin (25, 50, and 75 nM), and migrated cells were determined and images were taken for 0, 12, 24, 36, and 48-h periods of time using a fluorescence microscope (Oh and Santy, 2010; Suh and Han, 2010). Migrated cells were assessed by area closure of the detection zone.

In Vitro Boyden Chamber Assay For Migration Assay

U-2 OS cell migration was using the chemotactic directional migration assay with a 24-well Transwell insert (Ho et al., 2009). Briefly, 8 μM pore filters (Millipore, Billerica, MA) were coated with 30 μg type I collagen (Millipore) for 1 h. Then, cells at a density of 1×10^4 cells/0.4 mL in 90% McCoy's 5a medium were placed in the upper chamber and treated with 0.5% DMSO (as a control) and with bufalin (25 and 50 nM) before undergoing migration for 24 and 48 h. Nonmigrated cells were removed in the upper chamber by using a cotton swab and the filter from each treatment was individually stained with 2% crystal violet in 2% ethanol after fixing with 3.8% formaldehyde in PBS. Migrated cells adhering to the underside of the filter were examined, photographed, and counted under using a light microscope at 200× magnification. Each treatment including the control condition was assayed twice and three independent experiments were performed as previously described (Chen et al., 2010; Ho et al., 2009).

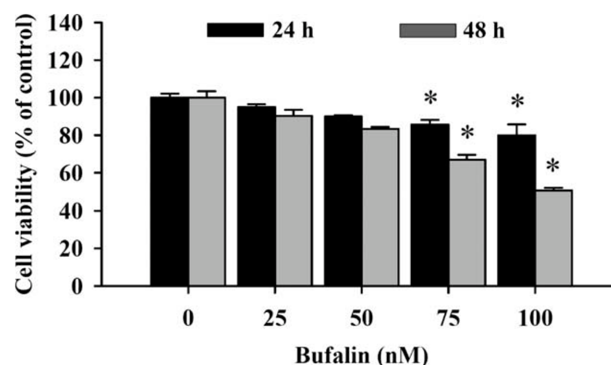

Fig. 1. Bufalin affected the percentage of viable U-2 OS cells *in vitro.* Cells were placed in 90% McCoy's 5a medium + 10% FBS and treated with 25, 50, 75, and 100 nM of bufalin for 24 and 48 h. The cells were collected and analyzed for cell viability by flow cytometry as described in Materials and Methods. Each point is mean ± S.D. of three experiments. $^*p < 0.05$.

In Vitro Boyden Chamber Assay For Invasion Assay

Invasion of U-2 OS cells was determined using Matrigel (BD Biosciences, Franklin Lakes, NJ)-coated transwell cell culture chambers (8 μm pore size, Millipore) as previously described (Ho et al., 2009; Liu et al., 2011). Briefly, cells were cultured for 24 h in serum-free-McCoy's 5a medium, collected and resuspended in serum-free medium. Isolated cells (1×10^4 cells/well) were then placed in the upper chamber of the transwell insert and treated with 0.5% DMSO (as a control) and bufalin (25 and 50 nM). McCoy's 5a medium containing 10% FBS was placed in the lower chamber. All samples were incubated for 24 and 48 h at 37°C in a humidified atmosphere with 95% air and 5% CO_2. Noninvasive cells were then removed in the upper chamber using a cotton swab and the invasive cells were fixed with 3.8% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. Invasive cells in the lower surface of the filter which penetrated through the Matrigel then were counted and photographed using a light microscope at

200× magnification as previously described (Ho et al., 2009; Chen et al., 2010).

Gelatin Zymography For MMP-2 and MMP-9 Activity

U-2 OS Cells were plated in 12-well tissue culture plates at a density of 2×10^6 cells/well and then were incubated in serum-free McCoy's 5a medium in the presence of 0, 10, 20, 40, 60, and 80 nM of bufalin for 24 h. The conditioned medium was then collected and it was separated by electrophoresis on 10% SDS-PAGE containing 0.18% gelatin (Sigma-Aldrich Corp.). After the electrophoresis, the gels were soaked in 2.5% Triton X-100 in dH_2O twice for a total of 60 min at 25°C, and then were incubated in substrate buffer (50 mM Tris base, 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij 35 in distilled water, pH 8.0) at 37°C for 24 h. Bands corresponding to activity of MMP 2 and 9 were visualized by negative staining using 0.3% Coomassie blue (Sigma-Aldrich Corp.) in 50% methanol and 10% acetic acid as previously described (Chen et al., 2010; Liu et al., 2011).

Western Blotting Analysis

U-2 OS cells seeded in 6-well plates at density of 1×10^6 cells/well were incubated with bufalin (25 and 50 nM) for

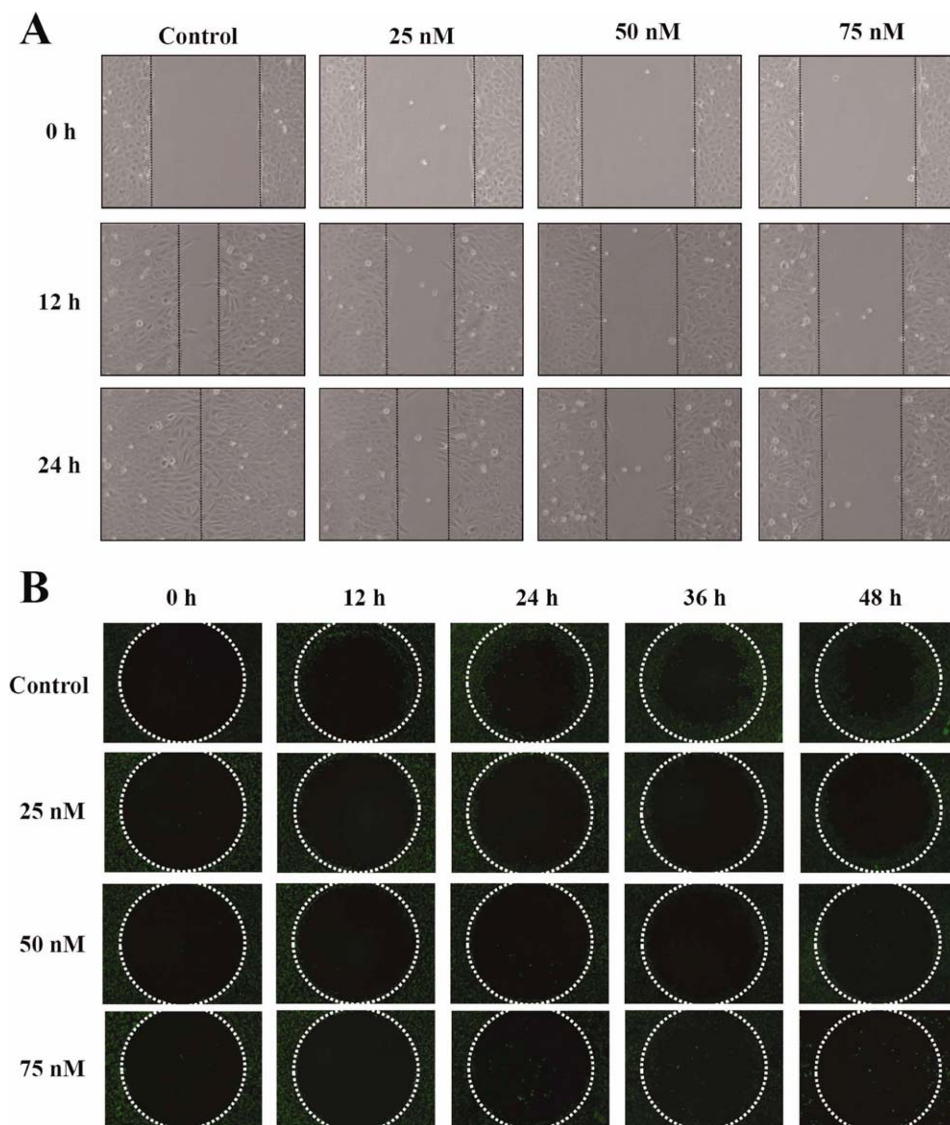

Fig. 2. Bufalin affected the motility and migration of U-2 OS cells. Cells were exposed to 0, 25, 50, and 75 nM of bufalin for various periods of time (12, 24, 36, or 48 h). Cell migration was examined by wound healing assay (A) and Oris™ Universal Cell Migration Assembly kit (B) as described in Materials and Methods. All results represented the average of three experiments and had similar effects.

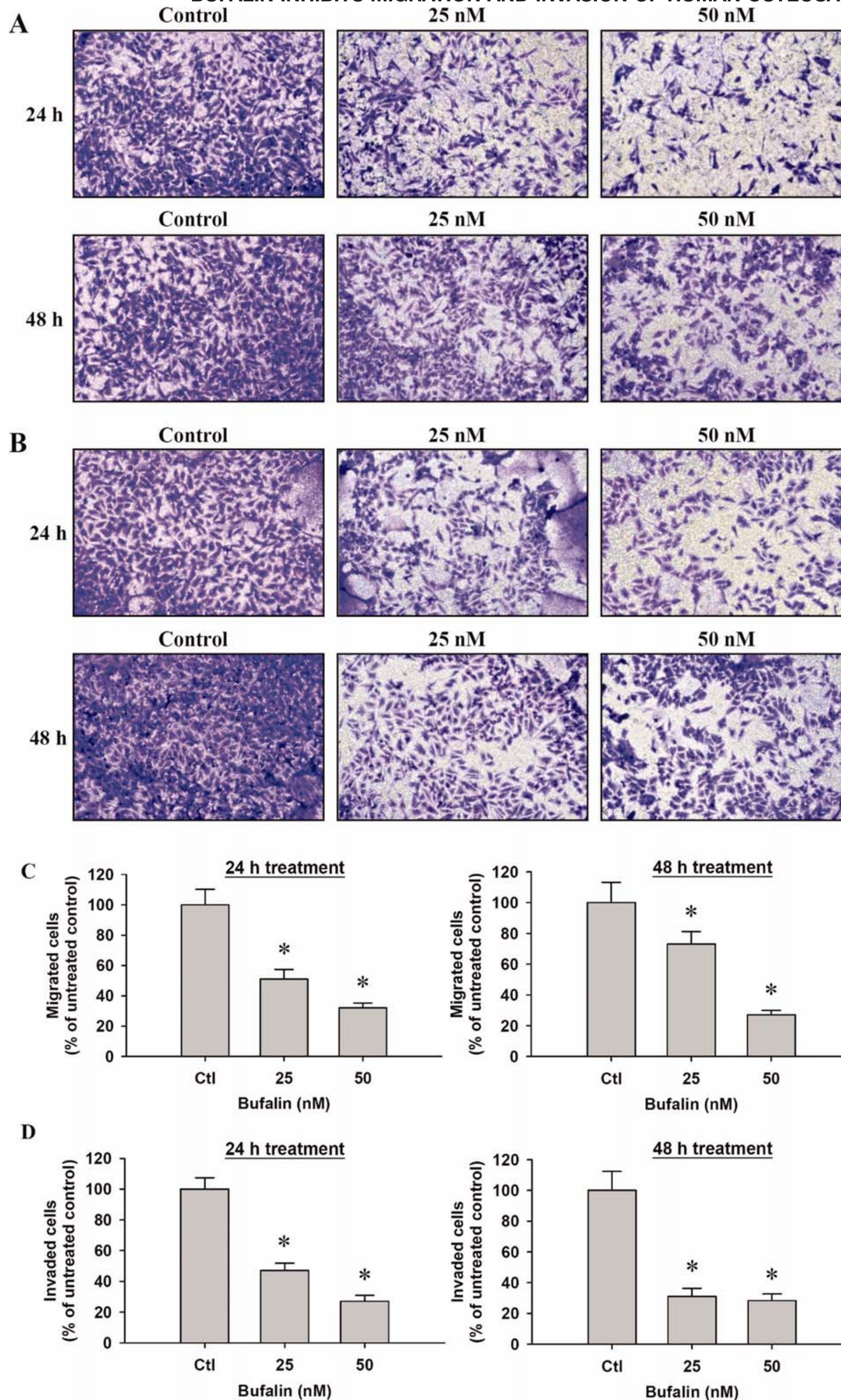

Fig. 3. Bufalin influenced migration and invasion of U-2 OS cells *in vitro*. Cells were treated with 0, 25, and 50 nM of bufalin for 24 and 48 h. A: Cell migration was examined by Boyden chamber assay and type I collagen-coated transwell; B: cell invasion was examined by using Matrigel-coated transwell cell culture chambers. Abilities of migration (C) and invasion (D) in U-2 OS cells were quantified by counting the number of cells that migrated or invaded the underside of the porous polycarbonate membrane under a phase-contrast microscope and represented the average of three experiments. *$p < 0.05$ was compared with the untreated control (Ctl). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

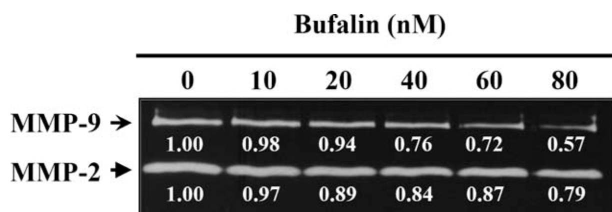

Fig. 4. Bufalin altered MMP-2 and MMP-9 enzyme activities from U-2 OS cells. Cells (2×10^6 cells/well) were incubated with 0, 10, 20, 40, 60, and 80 nM of bufalin for 24 and 48 h. The supernatant were harvested after U-2 OS cells treatment and separated by gelatin zymography as described in Materials and Methods. The ratio of MMP-2 and MMP-9 activities was quantitated by using ImageJ software.

24 and 48 h. Cells from each treatment were isolated and resuspended in ice-cold PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Gyeonggi-Do, Korea) to extract the total protein fractions. Each sample was centrifuged at 13,000 × *g* for 10 min at 4°C to remove cell debris and for collecting the supernatant. The total protein of each sample was determined using a Bio-Rad protein assay kit (Hercules, CA) using bovine serum albumin (BSA) as the standard (Ho et al., 2009). Protein abundance of JNK1/2, ERK1/2, p38, GRB2, SOS1, MMP-2, and MMP-7 was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described (Lu et al., 2010; Chiang et al., 2011).

Statistical Analysis

All data are presented as means ± S.D. of three experiments. Statistical comparisons were performed using the Student's *t*-test and differences considered significant at the $*p$ less than 0.05 between bufalin-treated and control groups.

RESULTS

Bufalin Affected the Percentage of Viable U-2 OS Cells *In Vitro*

U2 OS cells were treated with various concentrations (25, 50, 75, and 100 nM) of bufalin for 24 and 48 h. Cells from each treatment were isolated to determine viability. Figure 1 shows that there were fewer viable cells at bufalin concentrations of 75 and 100 nM ($p < 0.05$) when compared with the control sample.

Bufalin Inhibited Cell Migration and Invasion in U-2 OS Cells

Cell migration was measured using the wound healing assay and the Oris™ Universal Cell Migration Assembly kit. These results are shown in Figure 2(A,B), which indicated that bufalin inhibited the migration of U-2 OS cells in a dose-dependent manner. These results were confirmed by examining effects of bufalin on migration and invasion of U-2 OS cells *in vitro* by using a 24-well Transwell insert and type I collagen and Matrigel-coated transwell cell culture chambers, respectively. The migration assay showed that bufalin had a significant inhibitory effect on cell migration at concentrations between 25 and 50 nM [Fig. 3(A,C)] and these results are in agreement with the wound healing data (Fig. 2). U-2 OS cells moved from the upper chamber to the lower chamber in the absence of bufalin, but the penetration of the Matrigel-coated filter by U-2 OS cells was inhibited in the presence of bufalin [Fig. 3(B,D)] when cells

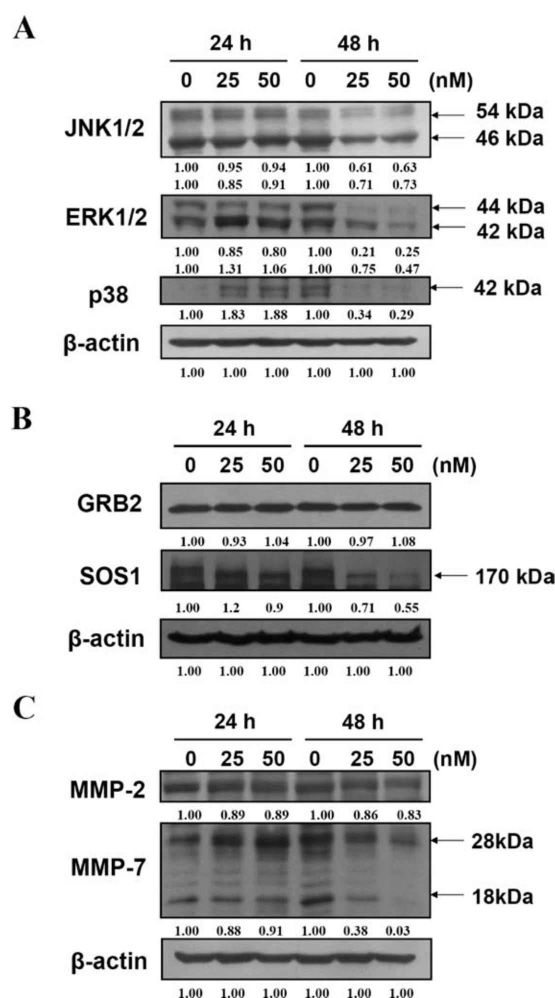

Fig. 5. Bufalin affected on the protein levels associated with migration and invasion in U-2 OS cells. Cells were treated with 0, 25 and 50 nM of bufalin for 24 and 48 h. The total proteins were collected and the protein levels of JNK1/2, ERK1/2, p38 (A), GRB2, SOS1 (B), MMP-2, and MMP-7 (C) were examined by SDS-PAGE and Western blotting as described in Materials and Methods. Direct reprobing with anti-β-actin antibody as an internal control.

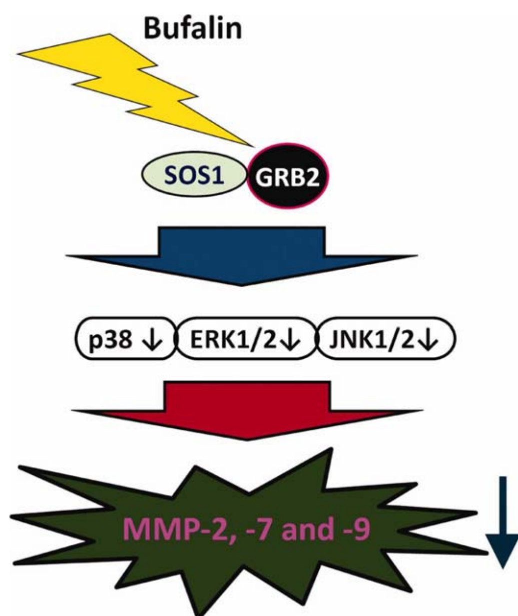

Fig. 6. The proposed model for a schematic presentation of the mechanisms in bufalin-suppressed migration and invasion in human osteosarcoma U-2 OS cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were incubated with bufalin for 24 and 48-h exposure, respectively.

Bufalin Attenuated the Activities of MMP-2 and MMP-9 in U-2 OS Cells

It is well documented that MMP-2 and -9 play important roles in invasion of cancer cells (Gullu et al., 2000; Kilian et al., 2006). We determined if bufalin could inhibit the secretion of MMP-2 and -9 in U2 OS cells. Gelatin zymography was used to perform bufalin-affected MMP-2 and MMP-9 activities for 24 and 48-h treatment. Figure 4 indicates that bufalin inhibited MMP-2 and MMP-9 activities in U-2 OS cells.

Bufalin Altered Levels of Proteins Associated With Migration and Invasion in U-2 OS Cells

Effects of bufalin on levels of proteins associated with migration and invasion in U-2 OS cells were examined. Results are shown in Figure 5, which indicated that bufalin decreased protein levels of JNK1/2, ERK1/2, p38 [Fig. 5(A)], GRB2, SOS1 [Fig. 5(B)], MMP-2, and MMP-9 [Fig. 5(C)] in U-2 OS cells. The extracellular signal-regulated kinase (ERK) signaling pathway has been reported to up-regulate the expression of MMPs (Chakraborti et al., 2003). Hence, we investigated the effects of bufalin on the ERK and JNK signaling pathways. Results indicated that bufalin inhibited the ERK1/2 and JNK1/2 [Fig. 5(A)] in U-2 OS cells, suggesting that bufalin suppressed migration and invasion of U-2 OS cells by suppressing the ERK and JNK signaling pathways.

DISCUSSION

Cancer cells interact with their surrounding cells and the matrix proteins allowing cancer cells to replicate, gain nourishment, and migrate to other locations. The interaction of cancer cells with extracellular matrices (ECM) is essential for metastasis which is the principle cause of death in cancer patients (Tsuji et al., 2002). Tumor metastasis (the movement of tumor cells from a primary site to a secondary site) is done via a series of steps such as cell attachment, invasion, migration, vessel formation and cell proliferation, and these steps are regulated by an extremely complex mechanism (Fidler 2002; Fidler et al., 2007).

Many investigators are focused on the development of new agents for blocking cancer cell metastasis (Chakraborti et al., 2003). However, the efficacy of agents is remains unsatisfactory. In normal bone tissue, matrix is constantly degraded and replaced by new matrix (Duffy et al., 2008), and the proteolytic enzymes (matrix metalloproteinases; MMPs) are required in this process (Everts et al., 1992; Ortega et al., 2003). MMPs play an important role in tumor angiogenesis, metastasis and stimulation of growth factor release from the ECM (Coussens and Werb, 1996). Other studies have shown that bufalin inhibited the growth of and induced apoptosis in human osteosarcoma U-2 OS cells (Yin et al., 2007). There is however, no available report on bufalin-inhibiting migration and invasion of U-2 OS cells. We investigated the antimetastatic functions of bufalin on the invasion/migration of human osteosarcoma U-2 OS cells. Our results indicated that bufalin can inhibit *in vitro* migration and invasion ability of U-2 OS cells [Fig. 3(A,B)]. Our results also showed that bufalin inhibited the MMP-2 and -9 activities (Fig. 4). Bufalin inhibited the protein levels of GRB2, SOS1, ERK1/2, JNK1/2, p38, MMP-2, and -7 (Fig. 5) in U-2 OS cells.

MMP-2 and -9 are involved in the invasive metastatic potential of tumor cells (Zhang et al., 2004) and both enzymes (MMP-2 and -9) were present in human osteosarcoma cells (Meikle et al., 1992). Thus, the inhibition of MMPs expression or enzyme activity can be early targets for preventing cancer metastasis (Coussens and Werb, 1996; Waas et al., 2003; Guruvayoorappan and Kuttan, 2008). It was reported that ERK1/2 is the key molecule of the ERK signaling pathway (Mendes et al., 2007), which promotes tumor invasion and metastasis (Peng et al., 2009). We have used an inhibitor of JNK (SP600125) to pretreat U-2 OS cells which were then exposed to bufalin causing a reduction in MMP-2 and MMP-9 levels (data not shown).

Taken together, our findings suggest that bufalin has multiple anti-metastatic activities in U-2 OS cells. The

possible signaling pathways for bufalin-inhibited migration and invasion in U-2 OS cells may occur by blocking MAPKs (JNK and ERK) resulting in inhibition of MMP-2. Future in vivo efficacy studies with bufalin should be done in animal models.

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