Berberine Reverses Epithelial-to-Mesenchymal Transition and Inhibits Metastasis and Tumor-Induced Angiogenesis in Human Cervical Cancer Cells

Shu-Chen Chu, Cheng-Chia Yu, Li-Sung Hsu, Kuo-Shuen Chen, Mei-Yu Su, and Pei-Ni Chen

Institute and Department of Food Science, Central Taiwan University of Science and Technology, Taichung, Taiwan (S.-C.C.); Institute of Oral Science, School of Dentistry (C.-C.Y.), Institute of Biochemistry and Biotechnology (L.-S.H., M.-Y.S., P.-N.C.), and Institute of Medicine (K.-S.C.), Chung Shan Medical University, Taichung, Taiwan; Clinical Laboratory (M.-Y.S., P.-N.C.), Department of Dentistry (C.-C.Y.), and Department of Internal Medicine (K.-S.C.), Chung Shan Medical University Hospital, Taichung, Taiwan

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ABSTRACT

Metastasis is the most common cause of cancer-related death in patients, and epithelial-to-mesenchymal transition (EMT) is essential for cancer metastasis, which is a multistep complicated process that includes local invasion, intravasation, extravasation, and proliferation at distant sites. When cancer cells metastasize, angiogenesis is also required for metastatic dissemination, given that an increase in vascular density will allow easier access of tumor cells to circulation, and represents a rational target for therapeutic intervention. Berberine has several anti-inflammation and anticancer biologic effects. In this study, we provided molecular evidence that is associated with the antimetastatic effect of berberine by showing a nearly complete inhibition on invasion (P < 0.001) of highly metastatic SiHa cells via reduced transcriptional activities of matrix metalloproteinase-2 and urokinase-type plasminogen activator. Berberine reversed transforming growth factor-β1-induced EMT and caused upregulation of epithelial markers such as E-cadherin and inhibited mesenchymal markers such as N-cadherin and snail-1. Selective snail-1 inhibition by snail-1-specific small interfering RNA also showed increased E-cadherin expression in SiHa cells. Berberine also reduced tumor-induced angiogenesis in vitro and in vivo. Importantly, an in vivo BALB/c nude mice xenograft model and tail vein injection model showed that berberine treatment reduced tumor growth and lung metastasis by oral gavage, respectively. Taken together, these findings suggested that berberine could reduce metastasis and angiogenesis of cervical cancer cells, thereby constituting an adjuvant treatment of metastasis control.

Introduction

Cervical cancer is the second most common female cancer worldwide, with an estimated 530,000 new cases every year, and the third greatest cause of death from cancer in women. Although cervical cytology screening has helped reduce mortality rates, managing preinvasive and invasive cervical lesions remains a challenge (Smith et al., 2013). Cancer metastasis and resistance to treatment are two major causes for poor survival and prognosis of cervical cancer patients. Most patient deaths from cervical cancer are related to metastasis, which is a complicated and currently uncontrolled process. Therefore, reducing the metastasis of cervical tumor cells is one of the most important research areas in medicine.

Tumor malignancy consists of a series of complicated processes, including invasion, migration, adhesion, angiogenesis, and proliferation. During tumor progression, tumor cells acquire expression of mesenchymal markers, such as vimentin, N-cadherin, and fibronectin, as well as loss of epithelial markers, such as E-cadherin and o-catenin, to result in epithelial–mesenchymal transition (EMT), subsequent tumor metastasis, and proliferation at distant sites. Suppressing
E-cadherin expression by its transcripional suppressor, snail-1, is a key process in EMT (de Herreros et al., 2010). During cancer metastasis, secretion of extracellular proteases is significant in cancer invasion (Rao, 2003). Among these proteases, matrix metalloproteinase-2 (MMP-2, also known as gelatinase A), which belongs to a family of structurally related zinc-dependent extracellular matrix (ECM)-degrading enzymes, is a proteolytic enzyme that is capable of degrading the structural support network for normal and malignant cells, which could serve a crucial role in invasion and angiogenesis of tumor cells (Roomi et al., 2012). In addition to matrix metalloproteinases, serine protease urokinase-type plasminogen activator (u-PA), which is secreted in cervical cancer, is a key factor in initiating a cascade of proteolytic steps that accumulates in degrading the ECM (Roomi et al., 2012; Tee et al., 2012).

Angiogenesis is a physiologic process that generates new blood vessels from pre-existing capillaries, and is critical to growth and metastasis of solid tumors. In normal tissues, the vascular system is regulated by a balance of antiangiogenic and proangiogenic molecules, which ensures that an efficient and orderly network of blood vessels is maintained to meet the metabolic demands of the tissue. Unlike normal tissue vasculature, tumor vessels are generally long and highly chaotic, with numerous abnormalities that result in poor blood flow and high vascular permeability, which may lead to reduced efficiency of chemotherapy in cancer patients and elevated potential for metastasizing to distant organs (Vaupel, 2004). A fundamental step in the transition of tumors from dormant to malignant state is followed by the potential for metastasis. Inhibiting the angiogenesis and metastasis of human cervical cancer cells could serve as an effective strategy against cervical cancer progression (Xie et al., 2013).

Chemopreventive approach using nontoxic botanicals could be one of the strategies for cancer management. These natural substances are of interest, as they are potential sources of anticancer compounds with minimal debilitating toxicity and side effects. Berberine, which is a naturally occurring isoquinoline alkaloid, is an active component in the roots, rhizomes, and stem barks of many medicinal plants, including Berberis vulgaris (barberry), Berberis aristata (tree turmeric), Berberis aquifolium (Oregon grape), and Coptis chinensis (Chinese goldthread). Berberine was initially used as an antibiotic because of its potent antimicrobial activity against many organisms, including bacteria, fungi, protozoans, viruses, chlamydia, and helminthes (Yu et al., 2005). Berberine has a wide range of pharmacological and biochemical effects for various clinical conditions, such as diarrhea, hypertension, arrhythmias, and inflammation (Rabbani et al., 1987). Recently, berberine has been demonstrated to possess anticancer activities, including DNA-modified electrodes (Tian et al., 2008), reduction of activator protein 1 activity to induce growth arrest and apoptosis (Mahata et al., 2011), and induction of caspase-dependent apoptosis in cervical cancer (Man tena et al., 2006a, b). Berberine inhibited invasion of human lung carcinoma A549 cells, increased expression of E-cadherin, and repressed expression of vimentin during initiation of transforming growth factor-β1 (TGF-β1)-induced EMT. Berberine inhibited the capacity of hepatocellular carcinoma to stimulate human umbilical vein endothelial cell (HUVEC) proliferation, migration, and endothelial tube formation (Jie et al., 2011). Berberine also inhibited hypoxia-inducible factor-1α (HIF-1α) expression via enhanced proteolysis in gastric adenocarcinoma cell line SC-M1 (Lin et al., 2004). However, the effects of berberine on cancer invasion, angiogenesis, and EMT of human cervical carcinoma and the underlying mechanisms of such effects remain unclear. In the current study, we tested the hypothesis that berberine has antimetastatic and reverse EMT potential for human cervical cells.

Materials and Methods

Materials and Chemicals. Berberine, heparin, Giemsa, gelatin, 4,6-diamidino-2-phenylindole, dimethylsulfoxide (DMSO), 3,4,5-trimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Coomassie Brilliant Blue R-250, and crystal violet were obtained from Sigma-Aldrich (St. Louis, MO). Matrigel was purchased from BD Biosciences (Bedford, MA). The Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate kit was obtained from Millipore (Burlington, MA). Dulbecco’s modified Eagle’s medium (DMEM), medium 199, penicillin, streptomycin, and trypsin-EDTA were obtained from Gibco Invitrogen Corporation (Barcelona, Spain).

Cell Culture. SiHa cells were obtained from the American Type Culture Collection (Manassas, VA), whereas HeLa and CaSki were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. HUVECs were obtained from the Bioresource Collection and Research Center and cultured on gelatin-coated culture dishes in medium 199 with 10% FBS, 25 U/ml of heparin, 30 μg/ml endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO), 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Subcultures were performed with trypsin-EDTA. Cells from passages 5–10 were used. Media were refreshed every other day. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Determination of Cell Viability (MTT Assay). Cells were incubated with 0.5 mg/ml MTT in culture medium for an additional 4 hours; the blue formazan crystals of viable cells were dissolved and measured spectrophotometrically at 570 nm (Chen et al., 2005).

Boyd-Chamber Cell Invasion and Motility Assays. After pretreatment with berberine for 24 hours, cells were harvested and seeded to a Boyden chamber (Neuro Probe, Cabin John, MD) at 1.5 × 10⁴ cells/well in serum-free medium, and then incubated for another 24 hours at 37°C. For the invasion assay, 0.2 μl of Matrigel (0.5 mg/1 ml) was applied to 8-μm pore-sized polycarbonate membrane filters, in which the bottom chamber of the apparatus contained standard medium. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted under a light microscope, whereas a motility assay was carried out as described for the invasion assay without coating with Matrigel (Chen et al., 2011).

Wound-Healing Migration Assay. Cells were seeded into a 12-well culture dish, and then wounds were introduced to the confluent monolayer of cells with a sterile 200-μl plastic pipette tip to create a denuded area. Cell movement into the wound area was photographed at 0 and 24 hours under a microscope (Ho et al., 2011).

Cell Matrix Adhesion Assay. After 24-hour treatment with berberine, cells were placed on 24-well dishes that were coated with type I collagen (10 μg/ml). Nonadherent cells were removed by phosphate-buffered saline washes. After staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100, and the absorbance was measured at 550 nm (Ho et al., 2011).

Determination of MMPs and u-PA by Zymography. In gelatin zymography, collected media were subjected to 0.1% gelatin double gel electrophoresis to determine the MMPs. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in reaction buffer. Gel was then stained with Coomassie Brilliant Blue R-250. The u-PA activity was visualized by casein zymography (Chen et al., 2011).

Measurement of MMP-2 and u-PA Promoter Activity. A 460-bp (–218 to +243) segment from the 5’-promoter region of the
MMP-2 gene and a 644-bp (−562 to +83) segment from the 5′-promoter region of the u-PA gene were cloned. The pGL3–MMP-2 and pGL3–u-PA plasmids were transfected into SiHa cells using PolyJet reagent (SignaGen Laboratories, Gaithersburg, MD) according to the manufacturer’s instructions. After incubation with berberine, cells were collected and disrupted by the Luciferase Assay System (Promega, Madison, WI), and luciferase activities were standardized for 20,000 counts per minute (CPM) by 20 μl of per well. DMSO (control group) and berberine (10 μg/ml) were added into embryo water at 6 hours postfertilization (day 1), mice were randomly divided into three groups (n = 5 for each group) and fed by oral gavage with placebo (control) and berberine (10 and 20 mg/kg per day). Bioluminescence imaging was performed using an IVIS Lumina II animal imaging system (Xenogen Corp., Alameda, CA) (Hu et al., 2012). Tumor growth was monitored by luciferase activity in SiHa cells, and the emitted photons from the target site penetrated through the mammalian tissue and could be externally detected and quantified using a sensitive light imaging system. For lung metastasis assay, SiHa cells (1.5 × 106 cells) that were suspended in 0.1 ml of phosphate-buffered saline were injected into the tail vein of BALB/c nude mice. On the following day (day 1), mice were randomly divided into three groups (n = 5 for each group) and fed placebo (control) or berberine (20 mg/kg of body weight, daily) by oral gavage. Five untreated mice were used as wild-type control. After 21 days, animals were euthanized with CO2. Lungs were counted under a microscope. Lungs were fixed in neutral buffered 5% formalin, and sections were collected and stained with hematoxylin and eosin for morphologic studies (Kim et al., 2009).

Immunohistochemistry Analysis. Paraffin-embedded slides were deparaffinized, and antigen unmasking was carried out by microwave heating in citrate buffer for 20 minutes. Slides were incubated with primary anti-Ki-67, anti–vascular endothelial growth factor (anti-VEGF), and anti-CD31 antibodies, and biotinylated secondary antimouse antibodies were also added.

Statistical Analysis. Statistical significance was analyzed by one-way analysis of variance with post-hoc Dunnett’s test. P < 0.05 was considered statistically significant (Sigma-Stat 2.0; Jandel Scientific, San Rafael, CA).

Results

Berberine Exerts Strong Anti-invasion and Antimigration Efficacy against SiHa Cells. MTT assay results showed that berberine only slightly reduced the viability of HeLa, SiHa, and CaSki cervical cancer cell lines (Fig. 1A). The 20-μM berberine treatment inhibited the invasion potential of CaSki, SiHa, and HeLa cells by 37, 65, and 22%, respectively (Fig. 1B). Among the three cervical cancer cell lines, SiHa cells were affected the most. Berberine exhibited the strongest reduction
effect on invasion and motility of SiHa cells in a dose-dependent manner (Fig. 1C). Quantification analysis indicated that the invasiveness of SiHa cells was reduced by 69% \((P < 0.01)\) when cells were treated with 20 \(\mu\)M berberine. Berberine also significantly reduced the motility \((P < 0.001)\) of SiHa cells in a concentration-dependent manner (Fig. 1D). Wound-healing migration assay was performed to assess if berberine affects cell migration. Incubating SiHa with 1% FBS produced a marked cell migration in the wound area. Berberine was able to significantly inhibit SiHa cell migration by 87.7% in wound-healing assays.
Berberine was then tested to determine its effects on cell–matrix adhesion. The results showed that berberine significantly reduced the cell–collagen interactions of SiHa cells (Fig. 1F). To screen for preventive effectors against migration of other cervical cancer cells, the inhibitory effect of berberine on migration of HeLa and CaSki cells was examined by wound-healing assay. The result showed that berberine significantly reduced the cell migration in both HeLa and CaSki cells (data not shown).

**Berberine Exerts an Inhibitory Effect on MMP-2 and u-PA and Has an Increment Effect on TIMP-2 and PAI-1.**

Given that the expression and activity of u-PA and MMP-2 are critical to cell invasion, the expression and activity of u-PA and MMP-2 of SiHa cells that were treated with different concentrations of berberine were examined by casein zymography and gelatin zymography, respectively. Berberine reduced the activities of both u-PA (Fig. 2A) and MMP-2 (Fig. 2B).

Western blot showed that MMP-2 and u-PA protein expression was significantly decreased along with the concentration of berberine (Fig. 2C). Physiologic activity of MMP-2 is closely related to that of the specific endogenous inhibitor TIMP-2. Therefore, Western blot was performed to determine the effects of berberine on TIMP-2 expression. The results showed that TIMP-2 protein levels were gradually increased along with the concentration of berberine in SiHa (Fig. 2C). To evaluate the effects of berberine on MMP-2 and u-PA promoters, transient transfection was performed with pGL3–u-PA (Fig. 2D) and pGL3–MMP-2 (Fig. 2E) promoters. Luciferase activities of the berberine-treated transfecants were reduced in a dose-dependent manner. To examine whether the inhibitory effect of berberine on MMP-2 expression was linked to NF-κB activities, nuclear extract was analyzed by EMSA for NF-κB DNA binding activity. The result showed that a pretreatment of berberine suppressed NF-κB binding activity (Fig. 2F). Subsequently, Western blot was performed to further confirm these results. The findings indicated that berberine suppressed the nuclear levels of NF-κB with C23 as the internal control. Berberine also significantly inhibited the phosphorylation of NF-κB in the cytoplasm (Fig. 2G). To further delineate whether the inhibition of MMP-2 secretions by berberine was mainly affected by inhibition of the NF-κB signaling pathway, effects of the NF-κB inhibitor on SiHa cells were investigated. The results showed that sole treatment with the 10 μM NF-κB inhibitor led to inhibition of MMP-2 activity similar to that of 10 μM berberine. The combined treatment of inhibitor with 10 μM berberine could also decrease the MMP-2 activity (Fig. 2H).

**Berberine Targets Signaling Molecules That Regulate EMT in SiHa Cells.** We examined the berberine effect on major regulators and markers of EMT. Berberine significantly elicited the upregulation of epithelial markers, such as E-cadherin. Berberine treatment slightly increased ZO-1 expression but did not affect Claudin-1 expression. Berberine also significantly decreased occludin expression, as well as mesenchymal markers, such as N-cadherin. Berberine treatment slightly decreased fibronectin and vimentin expressions (Fig. 3A). Focal adhesion kinase (FAK) and paxillin are protein tyrosine kinases that are linked to signaling events between cells and the ECM. To understand the possible mechanism underlying berberine antiangiogenic and anti-invasive efficacy on focal adhesion, we examined the effects of berberine on phosphorylated FAK, total FAK, phosphorylated paxillin, and total paxillin expressions. Our results showed that, upon treatment of SiHa cells with berberine, phosphorylation of FAK, paxillin, and Src was noticeably reduced (Fig. 3B). Berberine significantly decreased the transcription factor nuclear protein expression of snail-1 with C23 as the internal control sample in SiHa cells (Fig. 3C). Cadherin-bound β-catenin is required for cell adhesion. Upon activation of wet signaling, β-catenin translocated to the nucleus to induce the expression of EMT-related genes. Western blot analyses clearly revealed elevated β-catenin protein levels in the cytoplasm, whereas nuclear β-catenin was decreased by berberine treatment (Fig. 3D). Quantification of E-cadherin, occludin, ZO-1, phosphorylated FAK, phosphorylated paxillin, and p-Src is shown in Supplemental Fig. 1.

We used snail-1 siRNA to examine further the role of snail-1 in regulating E-cadherin expression. Snail-1 siRNA strongly decreased the level of snail-1 after 48 hours of treatment (Fig. 3E). Knockdown of snail-1 expression by snail-1 siRNA and berberine treatment to determine if berberine increases the E-cadherin level by other pathways independent of snail-1. Western blot results showed that the combined treatment of berberine slightly increased the E-cadherin level when the snail-1 level was selectively inhibited, which suggests that part of the function of snail-1 in berberine inhibition increased the E-cadherin level (Fig. 3E).

To clarify whether berberine could inhibit the expression of mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, Western blot analysis was conducted. Berberine significantly inhibited the phosphorylation of p38 after a treatment of 40 μM berberine, whereas it did not affect phosphorylated extracellular signal-regulated protein kinases 1 and 2 and phosphorylated Akt expression. Berberine treatment slightly decreased phosphorylated c-Jun N-Terminal Protein Kinase 1 and 2 expression. Moreover, no significant change in the total amount of extracellular signal-regulated protein kinases 1 and 2, p38, c-Jun N-Terminal Protein Kinase 1 and 2, PI3K, and Akt proteins were observed (Fig. 3, F and G).

**Berberine Reduced Ability of TGF-β1–Induced EMT, Cell Invasion, and MMP-2 Expression.** TGF-β1–mediated EMT of human cervical cancer cells may contribute to cervical cancer metastasis. To determine whether berberine could affect the TGF-β1–induced scattering, SiHa cells were pretreated with berberine prior to stimulation with TGF-β1. SiHa cells were pretreated with berberine for 2 hours (10 and 20 μM) prior to stimulation with TGF-β1 (5 ng/ml) for 24 hours. After treatment with TGF-β1, the cells adopted a more fibroblast-like morphology and reduced their cell–cell contact. Berberine blocked TGF-β1–induced scattering in a dose-dependent manner (Fig. 4A). To confirm the morphology change in Fig. 4A, immunofluorescence was performed to examine the actin profile. The SiHa cells underwent morphologic and compositional changes consistent with EMT following treatment with TGF-β1. These changes included loss of apical polarity with the acquisition of a more fibroblast-like spindle shape and cytoskeletal remodeling with the appearance of actin stress fibers. Berberine reversed TGF-β1–induced morphologic changes (Fig. 4B). We further examined whether berberine also affected TGF-β1–induced cell invasion. Quantitative analyses by cell invasion assay showed that the invasion of SiHa cells was increased by ~3.0-fold upon TGF-β1 treatment, and TGF-β1–induced invasion
was reduced by berberine treatment in a dose-dependent manner (Fig. 4C). Berberine also reduced TGF-β1–induced MMP-2 activity of SiHa cells in a dose-dependent manner (Fig. 4D). To ensure that berberine-blocked TGF-β1–induced scattering was not caused by cell death or inhibition of proliferation, MTT assay revealed that berberine had no effect on cell viability (Fig. 4E).

**Fig. 2.** Inhibitory effects of berberine (Ber) on the proteinase and transcription activities of MMP-2 and u-PA. SiHa cells were treated with berberine for 24 hours, and then subjected to casein zymography and gelatin zymography to analyze the activities of u-PA (A) and MMP-2 (B), respectively, as described in Materials and Methods. (C) Western blot analysis of MMP-2, u-PA, and TIMP-2 with β-actin being an internal control. Luciferase activity was measured in transiently transfected SiHa cells using pGL3–u-PA (D) and pGL3–MMP-2 (E). (F) Nuclear extracts were analyzed for DNA binding activity of NF-κB using biotin-labeled NF-κB specific oligonucleotide in EMSA. The last lane represents nuclear extracts incubated with unlabeled oligonucleotide (Comp.) to confirm the specificity of binding. (G) Nuclear and cytosol extracts were subjected to SDS-PAGE followed by Western blotting with anti-NF-κB and anti-phosphorylated–NF-κB antibodies, respectively. Signals of proteins were visualized with a chemiluminescence detection system. (H) Cells were treated with NF-κB inhibitor and then incubated in the presence or absence of berberine for 24 hours. Condition media were subjected to gelatin zymography to analyze the activities of MMP-2. Data represent the mean ± S.D., with that of control being 100%, and the statistical significance of results was analyzed using one-way analysis of variance with post-hoc Dunnett’s test (*P < 0.05; **P < 0.01; ***P < 0.001).

**Antiangiogenic Effects of Berberine on HUVECs In Vitro and on Treated Chicken CAM and Transgenic Zebrafish Embryos In Vivo.** MMP-2 and u-PA are key
Fig. 3. The effects of berberine on the cytoskeleton-related protein. (A and B) Western blot analysis of cytoskeleton-related protein with β-actin being an internal control in SiHa cells after 24 hours of treatment with berberine. (C) Nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti–snail-1 antibodies with anti-C23 being an internal control. (D) Cytosol extracts and nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti–β-actin antibodies. (E) The cells were treated with snail-1 siRNA and/or 5 μM berberine. The cell lysates were then subjected to Western blot with anti–snail-1 and anti–E-cadherin antibodies. (F and G) Western blot analysis of mitogen-activated protein kinases and PI3K/Akt pathways with β-actin being an internal control. Similar results were obtained from three repeated and independent experiments. p-Akt, phosphorylated Akt; p-ERK, phosphorylated extracellular signal-regulated protein kinases; p-FAK, phosphorylated focal adhesion kinase; p-JNK, phosphorylated c-Jun N-Terminal Protein Kinase; p-p38, phosphorylated p38; p-paxillin, phosphorylated paxillin; p-Src, phosphorylated p-Src.
Fig. 4. The inhibitory effect of berberine (Ber) on TGF-β1–induced EMT, MMP-2, and cell invasion. SiHa cells were pretreated with berberine for 1 hour and then cultured in the presence of 5 ng/ml TGF-β1 for 24 or 48 hours. (A) Phase image of cells. (B) Immunofluorescence staining of SiHa cells with Texas-568 phalloidin to visualize the actin cytoskeleton. (C) Cells were then subjected to analyses for cell invasion. (D) Condition media were collected for analysis of MMP-2 by gelatin zymography. (E) Cell viability by MTT assay. The quantitative data are presented as means ± S.D. of three independent experiments (*P < 0.001 compared with control; **P < 0.01, and ***P < 0.001 compared with TGF-β1–treated group). DAPI, 4’-6-diamidino-2-phenylindole.
factors in degrading the ECM by invading and proliferating endothelial cells with subsequent invasion of the underlying stroma. Preventing ECM degradation by inhibiting MMP and u-PA activities could be a potential therapeutic approach to block the invasion that occurs during angiogenesis. The present study aimed to confirm whether berberine has antiangiogenic effects on HUVECs. The HUVEC cells were treated with berberine at 0, 10, 20, 30, and 40 μM for proliferation, invasion, and expression of MMP-2 and u-PA. HUVEC cell proliferation was evaluated by MTT assay. The results showed that berberine slightly reduced the viability of HUVEC cells, with 87.6% remaining after a treatment of 40 μM berberine (Fig. 5A). A Boyden chamber Matrigel invasion assay was performed to assess whether berberine affects HUVEC migration. The result showed that berberine significantly reduced the invasion (P < 0.001) (Fig. 5B). Zymography showed a dose-dependent inhibition of MMP-2 and u-PA expression with virtual total inhibition at 10 μM concentration (Fig. 5C). Western blot was performed to determine the effects of berberine on MMP-2, u-PA, TIMP-2, and PAI-1 expression, and the results showed that MMP-2 and u-PA protein expressions were gradually decreased, whereas TIMP-2 and PAI-1 protein levels were gradually increased along with the concentration of berberine in HUVECs (Fig. 5D). Regulatory effects of berberine on proteases and their endogenous inhibitors on mRNA levels were also validated by semiquantitative reverse-transcription PCR analysis. With GAPDH as an internal control, mRNA levels of MMP-2 and u-PA were significantly reduced, whereas mRNA levels of TIMP-2 and PAI-1 were slightly increased in HUVECs (Fig. 5E). CAM assay is an important in vivo model of microvessel formation. Antiangiogenic activities of berberine analogs were investigated using CAM assay. A marked inhibition of angiogenesis was seen upon examination 2 days after berberine (10 μg) was placed at the vascular membrane when
compared with DMSO-treated controls (Fig. 5F). We then investigated the influence of berberine on vascular development in transgenic Tg(βll-1:EGFP) zebrafish. Berberine significantly decreased fluorescent intensities of DLAVs and intersegmental arteries in zebrafish (Fig. 5G). For tube formation, HUVECs were cultured in previously polymerized Matrigel. Berberine inhibited tube formation of HUVECs (Fig. 5H). These results together with our earlier findings suggest that berberine has antiangiogenic effects, such as inhibiting vascular tube formation, endothelial cell invasion, and protease expression.

**Berberine Inhibits Angiogenic Potential of SiHa Cells by VEGF Downregulation.** We examined whether berberine was capable of inhibiting tumor-induced tube formation, invasion, and proliferation on HUVECs. HUVECs that were cultured with conditioned media from SiHa cells appeared in a tube-like structure or tube network form. HUVECs that were cultured with conditioned media from SiHa cells with 10 and 20 μM of berberine led to decreases in the tube network compared with the control (Fig. 6A). SiHa-induced proliferation (Fig. 6B) and invasion (Fig. 6C) were reduced by berberine treatment in a dose-dependent manner. VEGF is the most potent angiogenic factor, and is associated with tumor-induced angiogenesis. To determine the effects of berberine on VEGF secretion, VEGF protein level in the conditioned medium was measured by enzyme-linked immunosorbent assay. Berberine reduced the VEGF secretion in the culture media in a dose-dependent manner (Fig. 6D). Western blot was performed to determine the effects of berberine on VEGF expression. The results showed that the VEGF protein expression was significantly decreased along with the concentration of berberine in SiHa cells (Fig. 6E). Considering that HIF-1α, which is a transcription factor, has a primary role in mediating hypoxia-induced VEGF transcription, we hypothesized that berberine might downregulate the expression of HIF-1α. The results showed that HIF-1α expression was significantly decreased in SiHa cells (Fig. 6F).

**Antitumor Effects of Berberine In Vivo.** SiHa-bearing nude mice were treated with placebo or berberine to verify the in vivo antitumor effects of berberine. A 3.8-fold reduction in the berberine-treated (20 mg/kg) animals was observed on day 32 compared with that of the control animals (Fig. 7A). Berberine (20 mg/kg) feeding also induced a 4.1-fold reduction in tumor weight by day 33 (Fig. 7B) without any apparent signs of toxicity, as proven by body-weight monitoring (Fig. 7C) throughout the experiment. Consistent with the profound effect on tumor size, a significant increase in proliferation was determined by Ki-67 stain in tumors (Fig. 7D). Histochemical analysis of the pathologic sections of these tumors showed that berberine-treated tumors had low levels of CD31 (endothelial surface marker) (Fig. 7E) and VEGF (Fig. 7F) compared with control SiHa tumors. These data suggested that berberine treatment reduced angiogenesis and tumor growth properties in vivo.

**Inhibition of Lung Colonization of SiHa by Berberine Treatment.** Nude mice were injected via the tail vein with SiHa cells, and administration of berberine reduced pulmonary metastasis formation of SiHa cells. Within 21 days of injection, the control mice were visibly riddled with metastatic tumor nodules compared with the lungs of SiHa-treated mice (Fig. 8A). Mean lung weights for animals that received 20 mg/kg per day berberine (195 ± 41.3 mg; P < 0.001) were significantly lower than those from control animals (677 ± 119.1 mg; Fig. 8B). Vehicle-treated control animals had massive tumor growth and were given an arbitrary-maximum countable number of about 259 ± 55.6. The number was reduced to 50 ± 67.0 (20 mg/kg per day; P < 0.001) countable colonies by berberine treatment (Fig. 8C). The average body weight of berberine-treated and control mice was not significantly affected (Fig. 8D). Histopathology of the lungs also showed marked reduction in tumor mass in the lungs of berberine-treated animals (Fig. 8E).

Taken together, these findings suggested that berberine is able to transcriptionally regulate MMP-2 expression via the downregulation of the NF-κB pathway, reverse EMT by modulating E-cadherin and snail-1 expression, and inhibit angiogenesis, lung metastasis, and tumor growth (Fig. 9).

**Discussion**

Metastasis is the spread of cancer cells from the primary site to other parts of the body. This condition is a major cause of cancer-related death, and is a multiple and intricate process that may complicate clinical management and lead to poor prognosis for cancer patients, which has tremendous economical and physical effects on patients. At the advanced stage, tumors also express high levels of proteases, such as MMPs and u-Pas, that degrade tissue ECM and facilitate tumor invasion and metastasis (Pulvea et al., 1997). NF-κB is an important transcription factor involved in the regulation of immune responses as well as in cell proliferation, survival, and metastasis (Park et al., 2007). A recent study has identified that the inhibitor of the NF-κB/NF-κB signaling pathway was involved in the mRNA and protein expression of MMP-2 in human ciliary muscle cells (Lan et al., 2009). The downregulation of the inhibitor of NF-κB or upregulation of phosphorylated NF-κB could induce NF-κB nuclear translocation and consequently promote the increase of MMPs in several types of human cells (Javlau et al., 2002; Park et al., 2007; Zhao et al., 2013). Furthermore, NF-κB regulates the expression of u-PA and its receptor, and expression of both u-PA and the u-PA receptor correlates with invasive cancer cell phenotype and poor prognosis in highly invasive breast cancer cells (Sliva, 2004). Since earlier reports have indicated that NF-κB plays significant roles in the expression of MMP-2 (Lan et al., 2009) and u-PA (Sliva et al., 2002), the impact of berberine on NF-κB activity was examined, and results showed that berberine could reduce the binding of NF-κB to DNA in the DNA-binding domains of MMP-2 and u-PA, which was accompanied by the inhibition of nuclear expression of this factor.

EMT induction in tumor cells has resulted in the acquisition of invasive and metastatic properties. The inhibition of E-cadherin expression is positively correlated with the tumor stage and grade (Tseng et al., 2010). Epithelial molecule E-cadherin connects adjacent cells by homophilic interactions, and is linked to the cytoskeleton by a multcatenin complex attached to their cytoplasmic tails (Inge et al., 2008). In this complex, p120 and β-catenin are directly associated with E-cadherin, whereas α-catenin is the link between β-catenin and the actin microfilament network of the cytoskeleton (Inge et al., 2008). β-Catenin functions in a dual manner in epithelial cells, depending on its intracellular localization, when β-catenin translocates to the nucleus and may induce the expression of EMT-related genes. In the current study, berberine increased the expression of cytosolic β-catenin and
Fig. 6. Inhibitory effects of berberine (Ber) on SiHa-induced tube formation capacity of HUVEC cells and the secretion of VEGF by SiHa cells. HUVECs were cultured in conditioned media from SiHa cells and treated with berberine at the indicated concentration. (A) Phase contrast micrographs illustrating the arrangement of HUVECs into a rich meshwork of capillary-like tubular structures when cultured on Matrigel for 8 hours. (B) Cell viability of HUVECs by MTT assay. (C) Cell invasion of HUVECs by invasion assay (#P < 0.01 compared with control; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with CM-treated group). (D) SiHa cells were treated with various concentrations of berberine for 24 hours, and then conditioned media were subjected to enzyme-linked immunosorbent assay. SiHa cells were treated with various concentrations of berberine for 24 hours, and then cell lysates and nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti-VEGF (E) and anti–HIF1α antibodies (F), respectively. Data represent the mean ± S.D. of at least three independent experiments. Results were statistically evaluated using one-way analysis of variance with post-hoc Dunnett’s test (*P < 0.05; **P < 0.01; ***P < 0.001).
Fig. 7. The in vivo antitumor effects of berberine (Ber). After subcutaneous implantation of SiHa cells, BALB/c nude mice ($n = 5$ for each group) were treated with placebo or berberine and then analyzed for tumor growth. (A) Bioluminescence over time after subcutaneous inoculation of SiHa cells. (B) Average tumor weight. (C) Average mouse body weight. Immunohistochemistry for Ki-67 (cell proliferation marker; $200 \times$) (D), CD31 (the endothelial surface marker; $200 \times$) (E), and VEGF in SiHa tumors ($200 \times$) (F). Comparisons were performed using one-way analysis of variance with post-hoc Dunnett’s test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).
Fig. 8. Suppression of lung metastasis of SiHa cells by berberine (Ber). SiHa cells were injected into the tail veins of 6-week-old female BALB/c nude mice. After injection of cells, berberine (20 mg/kg per day) and vehicle alone were administered by oral gavage for 21 days. Mice were sacrificed and then analyzed for representative photographs of lungs (A), the weight of lung (B), the number of lung metastatic nodules (C), and the body weight of mice (D). (E) Histopathology of lung of metastasis-bearing animals. Lungs of the metastasis-induced animals were fixed in neutral buffered formalin and stained with hematoxylin and eosin. Arrows show areas of metastatic nodules (tumor). Results were statistically evaluated using one-way analysis of variance with post-hoc Dunnett’s test (**P < 0.001).
inhibited the expression of nuclear β-catenin in SiHa cells. A recent study showed that the absence of E-cadherin led to an accumulation of free β-catenin and its association with lymphoid enhancer-binding factor, thereby mimicking Wnt signaling. β-Catenin/lymphoid enhancer-binding factor–mediated transactivation in these cells was antagonized by transient expression of wild-type E-cadherin, but not E-cadherin lacking the β-catenin binding site. The binding of E-cadherin could recruit β-catenin to the cell membrane and prevent its nuclear localization and transactivation, which was also demonstrated in SW480 colon carcinoma cells (Orsulic et al., 1999). Other reports in epidermoid cancer cells have indicated that phosphorylation of β-catenin by Akt increases its transcriptional activity and promotes cancer cell invasion and development (Fang et al., 2007). In the present study, berberine significantly increased the expression of E-cadherin in SiHa cells in a dose-dependent manner, whereas it had no significant effect on phosphorylated Akt and glycogen synthase kinase-3β (data not shown) expression. Therefore, the inhibition of β-catenin from cytosol to nuclear may result in increased expression of E-cadherin, which binds with β-catenin, recruits β-catenin to the cell membrane, and prevents its nuclear localization.

FAK is a focal adhesion–associated protein tyrosine kinase which participates in many principal cellular processes, including increase of cell migration, formation of focal adhesion, and cell spreading (Chen et al., 2010). Paxillin is one of the major binding partners of FAK, and the paxillin signaling pathway is a principal component of adhesion changes that are associated with EMT in various human carcinomas (Shah et al., 2012). Paxillin is a focal adhesion–associated adaptor protein known to participate in cell migration and adhesion (Chiu et al., 2012). Results from the present study showed that berberine could decrease the phosphorylation of both FAK and paxillin. Further studies are needed to understand whether the effects of berberine on FAK activation are direct or occur by targeting the molecules that are upstream of FAK, such as integrins or epidermal growth factor receptor, which are also activated in response to E-cadherin loss (Garouniatis et al., 2013).

TGF-β1 is a multifunctional cytokine that is involved in the regulation of many biologic processes. In epithelial cells, TGF-β1 is a potent growth inhibitor, and this function contributes to its role in tumor suppression (Roberts and Wakefield, 2003). Paradoxically, TGF-β1 is overexpressed in many malignant human carcinomas, including cervical cancer cells. Recent studies have shown that overexpression of TGF-β1 at early stages of tumorigenesis provided tumor-suppressive effects primarily via growth inhibition, whereas overexpression of TGF-β1 at late stages promoted tumor progression, metastasis, and EMT (Roberts and Wakefield, 2003). The present study showed that SiHa cells underwent morphologic and compositional changes that are consistent with EMT following treatment with TGF-β1. These changes included cytoskeletal remodeling with the appearance of actin stress fibers and the loss of apical polarity with the acquisition of a more fibroblast-like spindle shape. Berberine also reversed TGF-β1–induced EMT, cell invasion, and MMP-2 expression in SiHa cells.

Angiogenesis is a fundamental step in the transition of tumors from dormant to malignant state, being considered one of the hallmarks of cancer, and having a critical role in tumor progression, invasion, and metastasis; therefore, angiogenesis represents a rational target for therapeutic intervention (Hanahan and Weinberg, 2000). Different strategies for angiogenesis intervention are based on modulating any of the key steps of the angiogenic process, including endothelial cell proliferation, protease secretion, cell-matrix adhesion, migration, and invasion. The present study demonstrated that berberine inhibited the capacity of SiHa to induce proliferation, invasion, and endothelial tube formation of HUVECs, suggesting that berberine could influence the cross-talk between the vascular endothelial cells and SiHa cancer cells. Subsequent analyses revealed that berberine prevented secretion of VEGF from SiHa cells and downregulated HIF-1α expression. Daily oral gavage administration of berberine at doses of 20 mg/kg in mice resulted in a potent inhibition of tumor-induced angiogenesis of SiHa cervical cancer cells. In conclusion, berberine effectively inhibited tumor growth of SiHa cells partly by its antiangiogenic properties. These findings suggested that MMP-2 inhibition correlated well with the antiangiogenic and antimetastatic efficacy, and berberine has the therapeutic potential to inhibit angiogenesis and metastasis in vivo and in vitro.

The major disadvantages of many effective cancer chemotherapeutic agents are drug resistance and systemic toxicity. To overcome such problems, studies of combination chemotherapy have focused on finding nature compounds with a known action mechanism that could elevate the therapeutic index of clinical
anticanic drugs (Chen et al., 2005). One recent study indicated a significant benefit of using adjuvant chemotherapy following chemoradiation. Paclitaxel (also known as taxol) and doxorubicin are also clinical chemotherapeutic agents with broad therapeutic activity against various cancers, including cervical cancer. Phytotherapeutic agents with high anticancer activity and less toxicity to normal tissues have been suggested as possible candidates for their ability to augment the effectiveness of clinical anticancer drugs. In this study, accumulating evidence indicated that treatment with a low concentration of 5 μM berberine did not affect the viability of SiHa cells, but improved the efficacy of taxol and doxorubicin (Supplemental Fig. 2) and possessed a strong anticancer activity on cervical cancer cells. The analysis of cell growth showed that berberine could improve the inhibitory effects of doxorubicin and taxol on cell growth. In conclusion, this study was the first to show that berberine could inhibit cervical cancer cell invasion and reverse EMT, not only by E-cadherin induction, but also by suppressing MMP-2 and u-PA transcription and enzyme activities. Berberine also inhibited angiogenic potential of SiHa cells by VEGF downregulation. This study further suggests that berberine may be a template for new anticancer drug developments that can reverse EMT. These results demonstrate antiangiogenic properties of berberine and its clinical potential as an inhibitor of tumor angiogenesis and cancer metastasis in cervical cancer cells.

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Authorship Contributions
Participated in research design: P.-N. Chen.
Conducted experiments: P.-N. Chen, Chu.
Contributed new reagents or analytic tools: Yu.
Performed data analysis: Yu, K.-S. Chen, Su.
Wrote or contributed to the writing of the manuscript: P.-N. Chen, Hsu.

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