Berberine Inhibits Metastasis of Nasopharyngeal Carcinoma 5-8F Cells by Targeting Rho-kinase Mediated Ezrin Phosphorylation at Threonine 567

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Running title: Berberine-mediated reduction of Ezrin phosphorylation
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Ezrin is highly expressed in metastatic tumors and is involved in filopodia formation as well as promotion of tumor metastasis. Thus, Ezrin may serve as a potential target for anti-metastatic therapy. This study demonstrates that berberine reduces filopodia formation of a nasopharyngeal carcinoma (NPC) cell line, 5-8F, at non-cytotoxic concentrations. Furthermore, invasion and motility of 5-8F cells are decreased in a dose-and time-dependent manner resulting in 73.0% invasion and 67.0% motility inhibition at 20 μM. The inhibitory effects of berberine on 5-8F cells metastasis was further confirmed in a mouse model of metastasis. Berberine treatment in vivo resulted in a 51.1% inhibition of tumor metastasis to the lymph nodes and decreased Ezrin phosphorylation at Threonine 567 in metastatic samples. Berberine suppressed the presence of phosphorylated-Ezrin (phospho-Ezrin) in a dose- and time-dependent manner, but had no effect on total Ezrin protein expression at non-cytotoxic concentrations. Furthermore, the inhibitory effects of berberine on phospho-Ezrin were dependent on the suppression of Rho-kinase activity. Reduction of Ezrin phosphorylation at Thr567 by berberine was associated with its inhibitory effect on filopodia formation in 5-8F cells. However, berberine did not effectively inhibit the motility and invasion of NPC cells containing Ezrin Thr567 mutants. These results confirm that berberine inhibits Ezrin phosphorylation at Thr567. Nonetheless, berberine reduces motility and invasion of cells, and inhibits tumor metastasis. The reduction of Rho kinase-mediated Ezrin phosphorylation mediated by berberine may be a novel anti-metastatic pathway in NPC 5-8F cells.

Ezrin, a member of the ezrin-radixin-moesin (ERM) family of cytoskeletal proteins, has been implicated in dynamic membrane-based processes such as the formation and stabilization of filopodia (1). DNA and protein sequencing indicate that human Ezrin is a highly charged protein with an overall pI of 6.1 and a calculated molecular mass of 69 kDa (2,3). It is also evolutionarily conserved among widely divergent organisms. Within its N-terminal domain, Ezrin has high amino acid sequence homology to the erythrocyte cytoskeleton protein band 4.1. Ezrin is involved in a variety of cellular functions including cell
adhesion, migration, and organization of cell surface structures (4,5). It may also contribute to the formation of the scaffolding between the actin cytoskeleton and receptor retention (6), as well as filopodia formation (1). Ezrin is overly expressed in various cancers and associated with cancer metastasis (7-17).

One important mechanism of regulating the function of Ezrin is through phosphorylation at a conserved threonine residue in the C terminus (Thr567) (18-21). Ezrin exists in a folded conformation to mask its binding sites from other molecules, while phosphorylation of this conserved threonine residue causes conformational changes exposing its binding sites (18,21). Therefore, phosphorylation of Ezrin at Thr567 keeps it open and active, and prolongs its life time (18).

Berberine (2, 3–methylenedioxy–9, 10–dimethoxyproto berberine chloride), an isoquinoline alkaloid present in plants of the genera Berberis and Coptis, possesses antimicrobial activity against bacteria, fungi, viruses, Chlamydia, and protozoans (22-25). It also possesses an anti-tumor effect on many types of malignancies, such as esophageal cancer (26), hepatoma, lung carcinoma (27), leukemia (28), uterine cancer, prostate carcinoma (29), and gastric carcinoma (30). Recently, the use of berberine has attracted great attention as an alternative anti-metastasis therapy considering its low toxicity and low cost. The mechanistic studies on berberine antitumor effects showed that berberine inhibits nicotinamide adenine dinucleotide (NADH) oxidase, reverse transcriptase and diamino- oxidase (31), topoisomerase (32), nuclear transcript factor kappa B (NFkB) (33), activator protein 1 (AP-1) (27), cyclooxygenase-2 (34), and N-acetyltransferase activity (35). Berberine induces apoptosis/necrosis in several human cancer cells (28,29,31),(36). Most notably, berberine significantly inhibits the spontaneous mediastinal lymph node metastasis produced by orthotopic implantation of Lewis lung carcinoma (LLC) into the lung parenchyma in vivo (27), and inhibits the motility and invasion of highly metastatic A549 cells at non-cytotoxic concentrations in vitro (33). In a previous study, the Coptis chinensis compound containing berberine was used to treat patients with metastatic nasopharyngeal carcinoma (NPC) and NPC metastasis was inhibited (37). However, little is known about the molecular mechanisms of these berberine anti-metastatic effects. This study demonstrates that Rho-kinase activity is suppressed by berberine, which leads to a reduction in Ezrin phosphorylation at Thr567 in NPC 5-8F cells. Therefore, a novel anti-metastatic mechanism of berberine is identified in this study.

**Experimental Procedures**

*Reagents and antibodies-* Berberine was purchased from Sigma-Aldrich (St. Louis, MO). The compound was stored at 4°C protected from exposure to light. The stock solution of berberine was dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the medium applied to cells was 0.1% (in both control and treated groups) without affecting cell viability. Antibodies against Ezrin were purchased from Covance (Berkeley, CA). Antibodies against phosphorylated-Ezrin at Thr567 (phospho-Ezrin Thr567) was purchased from Cell Signaling Technology (Danvers, MA). Antibodies against Rho-kinase, PKC, Rac, Cdc42, G protein-coupled receptor kinase 2 (GRK2), myotonic dystrophy kinase-related Cdc42-binding kinase 2 (MRCK), and lymphocyte-oriented kinase (LOK) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against β-actin and normal mouse IgG were purchased
from Upstate Biotechnology, Inc. (Lake Placid, NY). The secondary antibodies, horseradish peroxidase-linked anti-mouse IgG and anti-rabbit IgG, were purchased from Santa Cruz Biotechnology, Inc. GST-Rhotekin-RBD (Rho binding domain of rhotekin, RBD) protein agarose beads were purchased from Cytoskeleton Inc. (Denver, CO). Glutathione-Sepharose 4B was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The protein assay kit was purchased from Bio-Rad (Hermont, VA). Immunoblotting detection reagents were purchased from Amersham Pharmacia Biotech. Chemicals, including DMSO, Tris, HCl, SDS, FITC-phalloidin, DAPI, and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT), inner salt assay, were purchased from Sigma-Aldrich.

Cell culture and berberine treatment-
Human NPC cell lines, 5-8F and 6-10B, were purchased from the Cancer Research Institute of Sun Yatsen University (Guangzhou, China). The 5-8F cell line is highly metastatic, and 6-10B cells are nonmetastatic (8). Cells were cultured as monolayers in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml penicillin, 100 IU/ml streptomycin (Invitrogen, Carlsbad, CA), and maintained in an incubator at 5% CO₂ at 37°C. For berberine treatment, appropriate volumes of berberine stock solution were added to the cell cultures to achieve the indicated concentrations and then incubated for the indicated amount of time. After berberine treatment, cell viability was determined by the MTT assay. The dose-response of berberine inhibited Ezrin phosphorylation was investigated in 5-8F cells treated with 2.5, 5, or 10 μM berberine for 24 h. The time-course of berberine inhibition was also investigated in 5-8F cells treated with 5 μM berberine for 12, 24, and 48 h. After berberine treatments, cells were harvested for protein extraction. The expression of Ezrin and phospho-Ezrin were detected by immunoblotting.

Determination of cell viability (MTT assay)-
Berberine cytotoxicity was evaluated using the MTT assay to determine cell viability. Briefly, 5-8F cells were seeded in 96-well plates at a density of 3.5×10⁵ cells/well and treated with berberine at 0-100 μM concentrations at 37°C for 24 or 48 h. After the exposure period, media was removed, cells were washed with phosphate-buffered saline (PBS), and fresh media was added. Cells were then incubated with 100μl MTT (5mg/ml) in each well for 4 h. The absorbance of formazan produced by production of the MTT compound proportional to the viable cells was recorded at 563 nm with a spectrophotometer.

Detection of lactate dehydrogenase (LDH)-
Cytotoxicity of berberine on NPC cells was evaluated by detecting LDH in cell culture media after berberine treatment. Briefly, 5-8F cells were seeded in 6-well plates at a density of 2×10⁴ cells/well and treated with berberine at 0-100 μM concentrations at 37°C for 48 h. After the exposure period, media was collected for the LDH assay. LDH activity was detected using the LDH assay kit according to the manufacturer’s instructions (Autec Diagnostica Co., Germany).

Immunofluorescence Analysis- The 5-8F cells with or without berberine treatment were fixed with 2.0% formaldehyde in PBS for 30 min, washed with PBS three times, and then treated with PBS containing 0.2% Triton X-100 for 10 min. After being washed with PBS three times, cells were incubated with 0.5% bovine serum albumin (BSA) in PBS. After three PBS washes, cells were stained with 5 μg/ml FITC-Phalloidin (Sigma-Aldrich) for 40 min, and examined using a Zeiss axiophoto microscope (Carl Zeiss, Oberkochen, Germany). Cells stained with
DAPI served as a control. Random fields were counted for cells with filopodia.

**Electron microscopy**- Cultures of 5-8F cells treated with berberine or transfected with plasmids were cultured on CELLocate coverslips (Eppendorf, Hambury, Germany). The cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2 h at room temperature. Samples were then processed conventionally and observed under a scanning electron microscope (SEM) (Hitachi Co., Japan). Cells with filopodia were counted.

**Immunoblotting analysis**- After berberine treatment, cells were harvested and lysed in 1X lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and freshly added 100 µg/ml phenylmethane-sulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 mM sodium orthovanadate). The cell lysates were then pelleted at 10,000×g for 10 min at 4ºC. Resultant protein concentrations of each sample were determined using the Bio-Rad Protein Assay. Equivalent amounts of protein (40 µg/sample) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was blocked with 5% non-fat milk in PBS for 1 h, incubated with specific antibody against Ezrin (Covance) or phospho-Ezrin (Cell Signaling Technology) for 2 h, and then incubated with an appropriate peroxidase-conjugated secondary antibody for 1 h. All incubations were carried out at room temperature, and intensive PBS washing was performed after detection with each antibody. After washing three times with PBS, the signal was developed using 4-chloro-1-napthol/3,3-o-diaminobenzidine, and relative photo-graphic density was quantified by a gel documentation and analysis system. β-actin was used as an internal control to verify basal expression levels and equal protein loading. The ratio of the specific proteins to β-actin was calculated.

**Cell motility and invasion assay**- For cell invasion assays, 5-8F cells were treated with indicated concentrations of berberine for indicated amounts of time. After berberine treatment, the cells were trypsinized, and their invasiveness was tested using the in vitro Boyden chamber invasion assay (38). Matrigel (Collaborative Biomedical Products, Bedford, MA) was diluted to 25 mg/50 ml with cold filtered distilled water and applied to 8-µm pore size polycarbonate membrane filters in the chamber. Treated cells were seeded into Boyden chambers (Neuro Probe Inc., Cabin John, MD) at a density of 1.5×10⁴ cells/well in 50 µl of serum-free media and incubated for 12 h at 37ºC. The bottom well contained standard media with 20% FBS. The cells invading through the filter of the chamber were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted for invading cells under a light microscope.

The effects of berberine on cell motility were determined using cells seeded into Boyden chambers without a Matrigel coating. Migration of cells in the presence or absence of berberine was measured as described in the motility assay (38). Statistical analysis was corrected with cell viability to clarify the effects of berberine.

**Animals**- Thirty female nude BALB/c mice (5-6 weeks old) were purchased from Animal Center of Central South University (Hunan, China). They were maintained in the Laboratory for Experiments, Central South University under laminar air-flow conditions. The studies were conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals by Central South University.

**Evaluation of anti-metastatic activity of berberine in nude mice**- Analysis of metastatic tumors of 5-8F cells was performed as described previously with modifications.
Briefly, 100 μl aliquots of 5-8F cells, 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin M(A), and 6-10B-pcDNA3.1-Ezrin cell suspensions (1×10^6 cells) mixed with Matrigel were respectively injected into the tail vein of nude mice, 10 mice per group. Two groups of 5-8F cells were given berberine once daily at a concentration of 15 or 30 mg/kg body weight (1X or 2X dosage of human study), respectively. Groups of 6-10B-pcDNA3.1-Ezrin cells were incubated with 15 mg/kg berberine for 30 days starting on day 1 after the injection. Metastasis was evaluated by measuring the weight of the metastasized tumor at mediastinal lymph nodes on day 30 after the injection. Body weight of the nude mice was measured, and other adverse effects of berberine were evaluated. The present study protocols were approved by the ethical committee at Xiangya Hospital of Central South University.

**Immunohistochemistry**- Metastasized tumor samples in the nude mice were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and mounted on slides. Sections were stained with hematoxylin and cosin (H&E) for microscopic examination. Unstained sections were used for staining with antibodies against Ezrin or phospho-Ezrin by immunohistochemistry. Immunohistochemistry followed standard procedures with overnight exposure at room temperature to 1:500 Ezrin antibody or 1:200 phospho-Ezrin antibody diluted in 0.5% non-fat milk. After washing with PBS, the sections were incubated sequentially with a secondary antibody against mouse, peroxidase enzyme label, and diamobenzidine (Sigma), and then stained with hematoxylin (Polysciences, Inc., Warrington PA), dehydrated, and mounted under a glass coverslip. Sections stained with normal mouse IgG served as a negative control.

**Kinase activity assay**- Cultures of 5-8F cells (1.0×10^6) were grown in 100-mm dishes for 12-24 h. When cultures were at 70–80 % confluence, the cells were treated with berberine for 48 h in RPMI 1640 medium containing 10 % FBS. Treated cells were washed once with ice-cold PBS and lysed in 250 μl of kinase lysis buffer (25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM MgCl_2, 1 mM aprotinin, and 1 mM PMSF). The clarified supernatant fractions containing 500 μg protein were subjected to immunoprecipitation using antibodies against Rho, PKC, GRK2, MRCK, or LOK, respectively. The precipitation mediating Ezrin phosphorylation was determined by the kinase assay protocol (Upstate Biotechnology, Inc.). Briefly, 20 μg precipitated complex was added to 2.5 μl of 10X kinase buffer (250 mM Tris-HCl (pH 7.5), 50 mM β-glycerophosphate, 20 mM DL-dithiothreitol (DTT), 1 mM Na_3VO_4, 100 mM MgCl_2), 2.5 μl (2.5 μg) of a GST-Ezrin fusion protein, 10 μl diluted ATP cocktail (Upstate Biotechnology, Inc.), 10 μCi of p^32-ATP, and H_2O added to 25 μl. The reaction was incubated at 30°C for 30 min and then subjected to separation by 12% SDS-PAGE gel. The gels were stained with Coomassie blue and then dried. Phosphorylated GST-Ezrin was analyzed by autoradiography that determines kinase activity.
type 2 were excised, and the radioactivity was measured. The dissociation constant for binding of the inhibitor to the enzyme (Ki) values were calculated from an equation of Ki = IC50 / (1 + S / Km), where S and Km are the concentration of ATP and the Km value for ATP, respectively (39).

Construction of expression vectors— The pcDNA3.1 and pGEX-5x-1 vectors were purchased from Invitrogen (Invitrogen, Carlsbad, CA). A DNA fragment encoding the GTPase-binding domain of p21-activated kinase (PAK-PBD), comprising amino acids 68-166, was generated by PCR and cloned into the BamHI/XhoI sites of pGEX-5x-1 and expressed in E. coli as GST-PAK-PBD fusion protein according to the manufacturer’s protocol. Ezrin and Rho DNA fragments were also generated by PCR and cloned into the BamHI/Xhol sites of pGEX-5x-1 and expressed in E. coli as GST-Rhotekin-RBD (Cytoskeleton Inc.) (40) or GST-PAK-PBD (41) for 12 h at 4°C, respectively. GST fusion proteins were collected by incubation with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 3 h. Precipitates were washed 5 times in the lysis buffer, resuspended in SDS sample buffer, and boiled for 10 min. After centrifugation for 10 min at 10,000×g, the supernatants were subjected to immunoblotting with antibodies against Rho, Rac, or Cdc42, respectively.

Gene transfection and generation of stable-transfected cell lines— Cultures of 5-8F cells (5.0×10^6) were treated with berberine at 20 and 40 μM for 48 h as described previously. After being washed with ice-cold PBS, they were lysed in 500 μl of kinase lysis buffer. The clarified supernatant fractions containing 400 μg protein were incubated with 3 μg GST-Rhotekin-RBD (Cytoskeleton Inc.) (40) or GST-PAK-PBD (41) for 12 h at 4°C, respectively. GST fusion proteins were collected by incubation with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) for 3 h. Precipitates were washed 5 times in the lysis buffer, resuspended in SDS sample buffer, and boiled for 10 min. After centrifugation for 10 min at 10,000×g, the supernatants were subjected to immunoblotting with antibodies against Rho, Rac, or Cdc42, respectively.
were transfected with pU6pro-si-Ezrin. The stably-transfected cell lines were obtained by selection for G418 resistance (400 μg/ml) and further confirmed by assessing Ezrin expression. To investigate whether the inhibitory effects of berberine on Rho activity could be reversed with constitutively active Rho-kinase, 5-8F cells were treated with berberine and then transfected with pcDNA3.1-Rho. To confirm that berberine exerts an inhibition on metastasis through Ezrin Thr567 phosphorylation, 5-8F-si-Ezrin cells were transiently transfected with pcDNA3.1, pcDNA3.1-Ezrin, pcDNA3.1-Ezrin-M(A), or pcDNA3.1-Ezrin-M(D), and their motility and invasion were detected using the in vitro Boyden chamber invasion assay (38).

**Results**

*Cytotoxic effects of berberine on NPC cells.* Berberine is an isoquinoline derivative alkaloid, and its chemical structure is shown in Fig. 1A. In this study, the cytotoxicity of berberine was determined by treating 5-8F cells with various concentrations for 24 h and 48 h, followed by an MTT assay. Compared to the control (0.1% DMSO), cell viability was not significantly altered at concentrations of 2.5 to 40 μM berberine (Fig. 1B, *, P<0.05, **, P<0.01). LDH in the cell culture media was also not significantly altered between 2.5 to 40 μM berberine (Fig. 1C, *, P<0.05, **, P<0.01). These results indicate that berberine was nontoxic at concentrations up to 40 μM, which was therefore applied in all subsequent experiments.

**Berberine suppression of filopodia formation by 5-8F cells.** To observe the effective inhibition of filopodia formation by berberine, the highest non-cytotoxic concentration (40 μM) of berberine was used to treat 5-8F cells for 48 h and the filopodia formation was then observed using immunofluorescence detecting F-actin (FITC-Phalloidin). Compared to the control (Fig. 2A-a), the filopodia formation of 5-8F cells decreased after berberine treatment (Fig. 2A-b). Cells with filopodia were significantly reduced by 28.3% after treatment with berberine (Fig. 2A-d, *, P<0.05). To confirm this finding, the filopodia of cells were observed using SEM. The results showed that filopodia formation was inhibited by berberine (Fig. 2B, *, P<0.05). Ezrin is closely associated with filopodia formation, and phosphorylation of Ezrin causes elongation of filopodia (1,42). Therefore, Ezrin and phospho-Ezrin expression in these samples were detected by immunoblotting. The results showed that phospho-Ezrin decreased 9.2-fold after berberine treatment, but total Ezrin protein expression was unaltered (Fig. 2C). Thus, berberine is inhibiting the phosphorylation of Ezrin without affecting the total protein expression.

**Anti-metastatic effects of berberine on 5-8F cells in vitro and in vivo.** Filopodia are associated with cell motility and invasion (43). Therefore, berberine inhibition of cell invasion and motility of 5-8F cells was investigated using a Boyden chamber coated with Matrigel. Results indicate that the number of cells that invaded the lower chamber was significantly reduced by berberine at a dose-dependent manner with a maximal 73.6% inhibition at 20 μM berberine (Fig. 3A, *, P<0.01). Such inhibitory effects were also observed at the motility assay with 67.0% inhibition of motility at 20 μM of berberine (Fig. 3B, *, P<0.01). In a time-course experiment, the lowest non-cytotoxic concentration (5 μM) of berberine was used to treat cells for 24, 48, or 72 h. Our results revealed that berberine significantly inhibited invasion (Fig. 3C) and motility (Fig. 3D) in a time-dependent manner. Anti-metastatic effects of berberine were confirmed in vivo when 5-8F cells were injected into the tail veins of BABL/c mice to
establish a metastatic mouse model. These same mice were treated with berberine and the anti-metastatic effects were assessed. The metastasis of 5-8F cells to the mediastinal lymph nodes was significantly inhibited by berberine (Fig. 3E). Berberine did not cause any reduction of body weight or other adverse effects on the mice (Fig. 3F).

**Berberine suppression of Ezrin phosphorylation.** To determine if the anti-metastatic effects of berberine were associated with Ezrin phosphorylation, phospho-Ezrin was detected in metastatic tumors using immunohistochemistry. Phosphorylation of Ezrin decreased in metastatic tumors from mice treated with berberine, however, total Ezrin expression was not changed (Fig. 4A). The association between berberine treatment and decreased phospho-Ezrin suggests that the reduction of Ezrin phosphorylation may mediate the anti-metastatic effects of berberine. Furthermore, berberine decreased the phosphorylation of Ezrin in a dose (Fig. 4B) and time (Fig. 4C) dependent manner in 5-8F cells.

**Berberine reduces Ezrin phosphorylation through Rho-kinase.** Ezrin is known to be phosphorylated by several protein kinases, including Rho-kinase, PKC, GRK2, MRCK, and LOK (18,44-47). To search for the upstream kinases of Ezrin phosphorylation, we determined whether berberine could inhibit the activities of Rho-kinase, PKC, GRK2, MRCK, or LOK. The highest noncytotoxic concentration of berberine (40 μM) was used to treat 5-8F cells, and Rho, PKC, GRK2, MRCK, and LOK were immunoprecipitated from the treated cultures. An in vitro kinase assay then detected their respective activities. Rho-kinase activity dramatically decreased with berberine treatment, while the activity of PKC, GRK2, MRCK, and LOK remained normal (Fig. 5A, *, P<0.05). In 5-8F cells, berberine treatment reduced Rho-kinase activity as well as the presence of phospho-Ezrin without affecting Rho or Ezrin protein expression (Fig. 5B). These data suggest that berberine inhibits Ezrin phosphorylation through suppression of Rho-kinase activity.

To calculate the dissociation constant for inhibitor binding (Ki), the concentration of berberine required to inhibit Rho-kinase activity was measured. Rho was precipitated from lysates of 5-8F cells treated with various concentrations of berberine and incubated with 330 mg/ml of histone type 2 and 1 mM ATP at 30°C. The Rho-kinase phosphorylation of histone has been previously reported (39) and was completed here under the standard assay conditions. The phosphorylation proceeded linearly for 45 min and occurred in an enzyme concentration-dependent manner (data not shown). Precipitated Rho-kinase protein was then incubated with various concentrations of ATP at 37°C for 30 min, and the enzyme kinetics were analyzed. This analysis revealed Michaelis-Menten kinetics for this reaction, and the dissociation constant (Km) for ATP was calculated to be 0.20 μM. The K_i value of berberine was estimated to be 0.35 μM. This was higher than the K_i value of a known Rho-kinase inhibitor, Y27632, which is 0.14 μM (39).

Rho GTPases are members of the Ras superfamily of monomeric 20-30-kDa GTP-binding proteins. There are 10 Rho-GTPases in this family, and the most extensively characterized members are Rho, Rac, and Cdc42 (48). To investigate the specificity of berberine on the regulation of GTPases, 5-8F cells were treated with 40 μM berberine. Berberine had no effect on the protein expression of Rho, Rac, or Cdc42 (Fig. 5C). However, the activity of these GTPases was investigated by extracting active Rho, Rac, and Cdc42 from 5-8F cells treated with
berberine, precipitating them with GST-Rhotekin-RBD or GST-PAK-PBD, and then analyzing them by immunoblotting. The results indicated that active Rho significantly decreased after berberine treatment; however, active Rac and Cdk42 were not affected (Fig. 5D).

Active Rho-kinase reverses berberine-suppressed Ezrin phosphorylation-To determine whether the effects of berberine on Ezrin phosphorylation were reversible with constitutively active Rho-kinase, 5-8F cells were treated with berberine and transfected with pcDNA3.1-Rho. As shown in Fig. 6, Rho-kinase activity increased in the berberine-treated and pcDNA3.1-Rho transfected cells and the presence of phospho-Ezrin also increased (Fig. 6). This demonstrates that the inhibitory effects of berberine on Ezrin phosphorylation were reversed with constitutively active Rho-kinase.

Inhibition of filopodia formation by berberine was associated with reduced phospho-Ezrin-To determine whether the inhibitory effects of berberine on filopodia formation was mediated through a decrease in Ezrin phosphorylation, non-metastatic 6-10B cells that only weakly express Ezrin (Fig. 7A) were transfected with pcDNA3.1, pcDNA3.1-Ezrin-M(A), pcDNA3.1-Ezrin-M(D), or pcDNA3.1-Ezrin respectively. Stably-transfected cell lines, 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin-M(A), 6-10B-pcDNA3.1-Ezrin-M(D), or 6-10B-pcDNA3.1-Ezrin were then established. Phospho-Ezrin increased in 6-10B cells transfected with pcDNA3.1-Ezrin compared to cells transfected with pcDNA3.1 or pcDNA3.1-Ezrin-M(A) (Fig. 7B). Filopodia formation increased in 6-10B cells transfected with pcDNA3.1-Ezrin and decreased after berberine treatment (Fig. 7C). This demonstrates that berberine inhibits filopodia formation through a mechanism associated with decreased Ezrin phosphorylation.

Berberine blocks NPC cell metastasis through phospho-Ezrin at Thr567-To confirm the berberine-mediated inhibition of metastasis through regulation of Ezrin phosphorylation at Thr567, the stably-transfected cell lines 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin, 6-10B-pcDNA3.1-Ezrin-M(A), and 6-10B-pcDNA3.1-Ezrin-M(D) were treated with berberine, and their motility and invasiveness were analyzed. The results showed that motility and invasion of 6-10B-pcDNA3.1-Ezrin cells were stronger than that of 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin-M(A), and 6-10B-pcDNA3.1-Ezrin-M(D) cells, and motility and invasion decreased after berberine treatment (Fig. 8A and B). Motility and invasion were not significantly different in 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin-M(A), or 6-10B-pcDNA3.1-Ezrin-M(D) without or with berberine (Fig. 8A and B). To confirm that the inhibitory effects of berberine are mediated through Ezrin phosphorylation, 5-8F-si-Ezrin cells, in which Ezrin expression was blocked, were transiently transfected with pcDNA3.1, pcDNA3.1-Ezrin-M(A), pcDNA3.1-Ezrin-M(D), or pcDNA3.1-Ezrin, and then treated with berberine. The motility and invasion analysis of these cells were similar to 6-10B cells transfected with the same constructs (Fig. 9C and D).

To confirm the inhibitory effects of berberine on metastasis in vivo, 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin, and 6-10B-pcDNA3.1-Ezrin-M(A) cells were injected in the tail vein of BALB/c mice. These mice were also treated with berberine. The tumors that metastasized to the mediastinal lymph nodes were detected. Similar to in vitro mobility and invasion assays, 6-10B-pcDNA3.1 and 6-10B-pcDNA3.1-Ezrin-M(A) cells displayed limited metastatic capabilities. Transfection of 6-10B cells with the pcDNA3.1-Ezrin plasmid restored metastatic capability, which was
effectively inhibited by berberine (Fig. 8C). Levels of phospho-Ezrin were determined from each of the stable-transfected cells with or without berberine treatment. Immunoblot results showed that levels of phospho-Ezrin from 6-10B-pcDNA3.1-Ezrin transfected cells dramatically decreased after berberine treatment; however, the total amount of Ezrin did not change (Fig. 8D). Taken together, berberine exerts an anti-metastatic effect on NPC cells through the reduction of Rho-kinase activity and subsequent Ezrin phosphorylation.

Discussion

Nasopharyngeal carcinoma (NPC) cells are highly metastatic (49). In a previous clinical therapy study, a *Coptic chinensis* compound containing berberine was used to treat patients with NPC (37). A NPC cell line, 5-8F, was used to investigate the anti-metastatic mechanisms of berberine on motility and invasion of NPC cells. Results from that study indicated that berberine can inhibit the invasion and motility of NPC cells in vitro and in vivo. In vivo, berberine can inhibit the metastasis of 5-8F cells into mediastinal lymph nodes, lungs, and liver, with the mediastinal lymph nodes being the main sites of NPC metastasis (49). Therefore, this study focused on the metastasis of NPC cells to mediastinal lymph nodes.

Metastasis is accompanied by various physiological alterations, such as filopodia formation, allowing cancer cells to invade the blood or lymphatic system and to spread to other tissue or organs. Thus, filopodia plays an important role in tumor metastasis (50). The present study demonstrates that noncytotoxic concentrations of berberine exert an inhibitory effect on the filopodia formation of 5-8F cells. Ezrin is a component of the filopodia of epithelial cells that serves as a major cytoplasmic substrate for certain protein-tyrosine kinases. It has been implicated in dynamic membrane-based processes such as the formation and stabilization of filopodia (1). Increased expression of Ezrin is associated with tumor metastasis in NPC (8,15,16,51). In fact, it may be involved in multiple pathways and actually promote tumor metastasis, making it a prospective therapeutic target (17). Therefore, the inhibitory effects of berberine on Ezrin and Ezrin phosphorylation were investigated in the 5-8F NPC cell line. High levels of Ezrin expression were detected in 5-8F cells, contrary to the lower levels of expression detected in non-metastatic 6-10B cells (Fig. 2B). This result is consistent with results from Yu et al. where Ezrin was highly expressed in metastatic murine rhabdomyosarcoma cells (7).

As a cytoskeleton organizer, Ezrin is involved in cell adhesion and migration (4,5), which is also associated with tumor metastasis. The phosphorylation at a conserved threonine residue in the C-terminus (Thr567) is an important mechanism for regulating the function of Ezrin (21). This study demonstrated that berberine effectively inhibited the phosphorylation of Ezrin at Thr567 without affecting Ezrin protein levels. Phosphorylation of Ezrin at Thr567 keeps it open and active and prolongs its lifetime (18). Interestingly, the open conformation of phospho-Ezrin may function as an actin filament/plasma membrane cross-linker (18-20). We hypothesized that the anti-metastatic mechanisms of berberine may be acting through the inhibition of Ezrin phosphorylation at Thr567. To test this hypothesis, plasmids were constructed in which the hydrophilic threonine at position 567 was exchanged with a hydrophobic alanine and stable-transfection of 6-10B cell lines with the construct was achieved. The effects of berberine on the motility and invasion of this cell line were assayed. Interestingly, 6-10B cells expressing wild type...
Ezrin displayed motility, invasion, metastatic capabilities, and filopodia formation. Alternatively, the cells transfected with Ezrin mutants at Thr567 behaved similarly to the controls. To further confirm berberine inhibition through Ezrin phosphorylation at Thr567, we mutated Threonine to aspartic acid and mimic 567 phosphorylation, and observed cell motility and invasion of. Data showed that Asp 567 could not elevate cell motility and invasion, and berberine had no effect on cells with Ezrin at Asp 567. Ezrin Asp567 may not mimic Thr567 phosphorylation. These results suggest that Ezrin phosphorylation at Thr567 plays an important role in cell metastasis. The reduction of Ezrin phosphorylation at Thr567 by berberine may be involved in the metastatic inhibition of NPC 5-8F cells.

Ezrin is a known substrate for Rho-kinase, PKC, GRK2, MRCK, and LOK (18,44,46,47). In this study, only Rho-kinase activity was inhibited by berberine at non-cytotoxic concentration, and repression of Rho-kinase activity by berberine was critical to the inhibition of Ezrin phosphorylation. It is therefore possible that Rho-kinase may be an upstream target of the berberine inhibition of Ezrin phosphorylation. A well-known Rho-kinase inhibitor, Y-27632 [(1)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane-carboxamide dihydrochloride] (39), was used as a comparator to the effects of berberine. Results here demonstrate that the effects of berberine on Rho-kinase were only slightly weaker than Y27632 (Ki berberine = 0.35μM; Ki Y27632 = 0.14 μM (39)).

Rho, Rac, and Cdc42 are the most extensively characterized members of the Rho GTPase family (48). In actin organization, Rho induces the assembly of contractile actin-based filaments such as stress fibers. Rac regulates the formation of lamellipodia and membrane ruffles, while Cdc42 is required for filopodia extension (52). However, our results indicate that berberine had no effects on the protein expression or activity of Rho, Rac, or Cdc42. Therefore, reduction of Rho-kinase activity plays a central role in the anti-metastatic effects of berberine.

In this study, we provided three lines of evidence that berberine inhibits metastasis of NPC 5-8F cells through the suppression of Ezrin phosphorylation. First, berberine inhibited the motility and invasion of 5-8F cells, following the reduction of Ezrin phosphorylated at Thr567 (phospho-Ezrin). Second, phospho-Ezrin was expressed at low levels in lymph metastatic samples after berberine treatment. Finally, reduction of the presence of phospho-Ezrin by berberine was dependent on the repression of Rho-kinase activity. In conclusion, berberine inhibits Ezrin phosphorylation at Thr567 through the reduction of Rho-kinase activity. This effectively reduces filopodia formation, resulting in decreased motility and invasion and inhibition of NPC cell metastasis (Fig. 10).

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**Footnotes**

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ABBREVIATIONS: NPC, nasopharyngeal carcinoma; phospho-Ezrin, phosphorylated-Ezrin; AP-1, activator protein 1; LLC, Lewis lung carcinoma; MTT, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-thoxyphenyl)-2-(4-ulfophenyl) -2H-tetrazolium; LDH, lactate dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; GRK2, G protein-coupled receptor kinase 2; MRCK, myotonic dystrophykinase-related Cdc42-binding kinase 2; LOK, lymphocyte-oriented kinase. RBD, Rho-binding-domain of rhotekin; PAK-PBD, GTPase-binding domain of p21-activated kinase; Ezrin M(A), Ezrin T567A; Ezrin M(D), Ezrin T567D.
FIGURE LEGENDS

Fig. 1. Cytotoxic effects of berberine on 5-8F cells. A. Structure of berberine, an isoquinoline derivative alkaloid. B. Cells were treated with berberine at 2.5, 5, 10, 20, 40, 80, or 100 µM for 24 or 48 h before MTT assay for cell viability. C. After berberine treatment for 48 h, LDH assay was performed. Data are represented as mean ± SD from three independent experiments and statistically analyzed with Student’s t-test (*, #, P<0.05; **, ##, P<0.01). LDH, lactate dehydrogenase; MTT, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-thoxyphenyl) -2-(4-ulfophenyl) -2H-tetrazolium.

Fig. 2. Inhibitory effects of berberine on 5-8F cell filopodia formation and Ezrin expression. Cells were treated with 40 µM berberine for 48 h. A. Cells were stained with FITC-phalloidin and treated with: (a) 0.1% DMSO, (b) 40 µM berberine, and (c) DAPI stain; (d) Average number of cells with filopodia were counted from 10 fields. Scale bar = 20 µm. B. Cells were observed by SEM after treatment with: (a) 0.1% DMSO or (b) 40 µM berberine. (c) Cells with filopodia were counted. Scale bar = 5µm, arrows point to filopodia. C. Ezrin and phosphorylated Ezrin (phospho-Ezrin) expression in 5-8F cells with or without berberine. Cells treated with 0.1% DMSO served as the blank control, and Ezrin and phospho-Ezrin expression were determined by immunoblot. β-actin served as the loading control.

Fig. 3. Berberine inhibition of metastasis in 5-8F cells in vitro and vivo. In the cell motility and invasion assays, 5-8F cells were treated with berberine at 2.5, 5, 10, or 20 µM for 24 h. Cells were treated with berberine at 5 µM for 12, 24, and 48 h for the time-course assay. The treated cells were then assayed for motility (A) and invasion (B) as described. C. Invasion of 5-8F cells at various time points. D. Motility of 5-8F cells at various time points. Results were analyzed using one-way ANOVA with post hoc Dunnett’s test (*, P<0.05; **, P<0.01). In animal experiments, 30 nude BALB/c mice were injected with 5-8F cell suspensions containing Matrigel through tail vein, 1x10⁴ cells/mouse, 10 mice per group. One group treated with the same volume of water served as the control, and two groups were treated with berberine at 15 and 30 mg/kg for 30 days starting on day 1. C. The metastatic tumors from mediastinal lymph node were weighed. D. Body weight of the control group and the groups receiving berberine. (two-sided Welch’s t-test. **, P<0.01).

Fig. 4. Ezrin and phospho-Ezrin expression in metastatic tumors and 5-8F cells with or without berberine treatment. A. Ezrin and phospho-Ezrin expression were detected in metastatic tumor samples from the nude mice using immunohistochemistry. Paraffin sections were H and E stained.
and detected with antibodies against Ezrin or phospho-Ezrin. Sections stained with normal mouse IgG served as the negative control. Arrows, positive cells. Original magnification × 400. Scale bar = 5 μm. In the vitro experiments, assays of berberine inhibition on phospho-Ezrin, (B) 5-8F cells were treated with berberine at 2.5, 5, and 10 μM for 24 h for the dose-course assay, (C) 5-8F cells were treated with berberine at 5 μM for 12, 24, and 48 h for the time-course assay, and then subjected to immunoblotting. Three independent experiments were carried out, abundance ratio to β-actin was counted, and data were represented as mean ± SD from three experiments. *, P<0.05. H&E, hematoxylin and eosin.

Fig. 5. Berberine inhibits Ezrin phosphorylation through Rho-kinase. A. GST-Ezrin phosphorylation mediated by Rho-kinase, PCK, GRK2, MRCK, or LOK was assayed in 5-8F cells with berberine treatment as described. B. Rho-kinase activity was assayed in 5-8F cells with or without berberine treatment by in vitro kinase assay; expression of Rho, Ezrin, and phospho-Ezrin was detected using immunoblotting (IB). C. Detection of Rho, Rac, or Cdc42 expression by immunoblot is shown in 5-8F cells with or without berberine treatment. D. Cells were treated with berberine at 20 or 40 μM for 48h. Active Rho, Rac, or Cdc42 were precipitated with GST-Rhotekin-RBD and GST-PAK-PBD, and were detected with immunoblot using antibodies against Rho, Rac, or Cdc42 respectively. Coomassie blue staining is shown as a loading control. Abundance ratios to β-actin or GST-Ezrin were calculated, and data are represented as mean ± SD from three independent experiments. *, P<0.05. GRK2, G-protein coupled receptor kinase 2; MRCK, myotonic dystrophykinase-related Cdc42-binding kinase 2; LOK, lymphocyte-oriented kinase. IP, immunoprecipitation; IB, immunoblotting.

Fig. 6. Berberine-suppressed Ezrin phosphorylation can be reversed by active Rho-kinase. Berberine treated 5-8F cells were transfected with pcDNA3.1-Rho. Rho was then precipitated with an anti-Rho antibody (IP). GST-Ezrin was phosphorylated by the precipitated Rho-kinase using an in vitro kinase assay. Phospho-Ezrin was detected by immunoblotting (IB). GST-Ezrin stained with coomassie blue served as the loading control. Three independent experiments were carried out, and densitometric analysis was performed. Abundance ratios to β-actin or GST-Ezrin were calculated, and data are represented as mean ± SD from three independent experiments. *, P<0.05.

Fig. 7. Berberine decreases filopodia formation through inhibition of Ezrin phosphorylation. A. Ezrin and phospho-Ezrin in 6-10B cells were detected using immunoblotting. Positive control was untreated 5-8F cells. 6-10B cells were transfected with pcDNA3.1-Ezrin, pcDNA3.1-Ezrin-M(A), or pcDNA3.1. The stably-transfected cell lines containing pcDNA3.1-Ezrin, pcDNA3.1
-Ezrin-M(A), or pcDNA3.1 were constructed using G418 selection. B. Expression of Ezrin and phospho-Ezrin in these stably-transfected cell lines was detected by immunoblotting. C. Filopodia formation in (a) 6-10B-pcDNA3.1, (b) 6-10B-pcDNA3.1-Ezrin, and (c) 6-10B-pcDNA3.1-Ezrin treated with 5 μM berberine; (d) cells with filopodia were counted. Arrows indicate filopodia; Scale bar = 5 μm. Ezrin-M(A), Ezrin with T567A.

Fig. 8 Berberine inhibits motility and invasion in 6-10B cells through inhibition of phosphorylation of Ezrin at Thr567. A. Motility (A) and invasion (B) of the stably-transfected cell lines 6-10B-pcDNA3.1, 6-10B-pcDNA3.1-Ezrin, 6-10B-pcDNA3.1-Ezrin-M(A), and 6-10B-pcDNA3.1-Ezrin-M(D) with or without berberine treatment. C. Stably-transfected cells were injected into nude mice that were treated with 15 mg/kg berberine for 30 days. The metastasized tumors in the mediastinal lymph nodes were collected as described. Data are represented as mean ± SD from three independent experiments. Results were analyzed by one-way ANOVA with post hoc Dunnett’s test (*, P<0.05). D. Ezrin and phospho-Ezrin expression from stably-transfected cells with or without berberine treatment from three independent experiments. Densitometric analysis was performed, and abundance ratios to β-actin were calculated. Data represent the mean ± SD from three independent experiments. Ezrin-M(A), Ezrin with T567 mutated to Alanine (T567A); Ezrin-M(D), Ezrin with T567 mutated to Aspartic acid (T567D).

Fig. 9 Berberine inhibits motility and invasion in 5-8F-si-Ezrin cells through inhibition of Ezrin phosphorylation at Thr567. A. 5-8F cells were transfected with pU6pro-si-Ezrin, and The 5-8F-si-Ezrin cell line was established by G418 selection. Ezrin expression was effectively blocked in these cells. B. Detection of motility and invasion of 5-8F-si-Ezrin cells. Motility (C) and invastion (D) from 5-8F-si-Ezrin cells transiently transfected with pcDNA3.1, pcDNA3.1-Ezrin-M(A), pcDNA3.1-Ezrin-M(D), or pcDNA3.1-Ezrin with or without berberine treatment. *, P<0.05. Ezrin-M(A), Ezrin with T567 mutated to Alanine (T567A); Ezrin-M(D), Ezrin with T567 mutated to Aspartic acid (T567D).

Fig. 10. Schematic illustration of berberine-inhibited metastasis. Berberine-mediated repression of Ezrin phosphorylation at Thr567 through suppression of Rho-kinase activity, inhibits filopodia formation resulting in the inhibition of motility and invasion, in which leads to decreased metastasis of NPC 5-8F cells.
Figure 2

A

B

C

Berberine (40 μM)  
-  +

Phospho-Ezrin

Ezrin

β-actin
Figure 4

A

Control  Berberine

H&E

Ezrin

Phospho-Ezrin

Negative Control

B

Berberine (μM)  0  2.5  5  10

Phospho-Ezrin

Ezrin

β-actin

Abundance ratio to β-actin

0  1.2  1.4  1.6

0  2.5  5  10 (μM)

C

Berberine (h)  0  12  24  48

Phospho-Ezrin

Ezrin

β-actin

Abundance ratio to β-actin

0  0.2  0.4  0.6  0.8  1.0

0  12  24  48 (h)
Figure 6
Figure 8

A

B

C

D

Figure 8

A

B

C

D

Figure 8

A

B

C

D

Figure 8

A

B

C

D

Figure 8

A

B

C

D

Figure 9

A

B

C

D

Cell motility (cell/field)

Cell invasion (cell/field)

pcDNA3.1

pcDNA3.1-Ezrin-M(A)

pcDNA3.1-Ezrin-M(D)

pcDNA3.1-Ezrin

Berberine (40 μM)

+ + + + - - - - - -

- - + + + + + + + +

- - - - + + + + + +

+ + + + + + + + + +

+ + + + + + + + + +

Motility

Invasion
Figure 10

Berberine

\[ \downarrow \]

Rho-kinase

\[ \downarrow \]

Ezrine phosphorylation at Thr567

\[ \downarrow \]

filopodia

\[ \downarrow \]

mobility

invasion

\[ \downarrow \]

metastasis of 5-8F cell

\[ \downarrow \]
Berberine inhibits metastasis of nasopharyngeal carcinoma 5-8F cells by targeting Rho-kinase mediated Ezrin phosphorylation at threonine 567

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