Berberine inhibits Rho GTPases and cell migration at low doses but induces G2 arrest and apoptosis at high doses in human cancer cells

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Abstract. Berberine is an active ingredient extracted from *Coptidis rhizoma* which has been used for centuries as a traditional Chinese medicine for treatment of inflammatory diseases. Recent studies have indicated that berberine has anticancer properties. Berberine arrested cell growth and inhibited cell migration in various cancer cell lines. In this study, we examined the effects of berberine on HONE1 cells, which have been commonly used as a cell model for nasopharyngeal carcinoma. We observed the inhibitory effects of berberine on HONE1 cells at a high dosage (>150 μM). Berberine effectively induced the mitotic arrest of HONE1 cells at 300 μM which was associated with apoptosis. Berberine had differential intracellular localization at low and high doses. At a low dose (50 μM), berberine was localized in the mitochondria while at a high dose (300 μM), berberine was localized in the nucleus which may have induced mitotic arrest. Berberine effectively inhibited cell migration and invasion at low doses. Using a specific GST pull-down assay of activated Rho GTPases, we demonstrated that berberine suppressed the activation of Rho GTPases including RhoA, Cdc42 and Rac1. This indicates a novel function of berberine in the suppression of Rho GTPase signaling to mediate its inhibitory action on cell migration and motility. The potential of berberine to inhibit cancer metastasis in cancer warrants further investigation.

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Introduction

For thousands of years *Coptidis rhizoma* (CR) (*Huanglian* in Chinese) has been used in traditional Chinese medicine (TCM) to treat syndromes incurred by damp-heat, fire or toxicity which are now considered to be inflammatory diseases. Recently, CR has been used for the prevention and treatment of human cancers, such as nasopharyngeal carcinoma (NPC) and hepatoma (1,2). It has been demonstrated that CR extract inhibits cell growth by suppressing the expression of cyclin B1 and by inhibiting CDC2 kinase activity in human cancer cells and induces apoptosis by upregulation of interferon-ß and TNF-α in human breast cancer cells (3,4). We also reported that CR had the strongest cytotoxicity among a selection of sixteen anticancer Chinese herbs in rat leukemia L-1210 cells, and a much higher inhibitory activity for growth of tumour cells was present in the water extract of CR compared to extracts using other solvents for extraction (5). The anticancer activities of berberine, the principal active compound in CR, have been demonstrated in previous studies (6-8). There is considerable interest in the identification of small molecules isolated from natural botanicals with properties to inhibit tumour growth and metastasis. As a candidate, berberine has shown this potential. There are many studies showing that berberine inhibits cell proliferation in leukemia, melanoma, gastric, liver, colon, pancreas, oral, breast, cervical and prostate cancer cell line models and may have potential chemotherapeutic properties against human cancers (9-14). Multiple mechanisms underlying the anticancer action of berberine have been reported and involve the inhibition of NF-κB pathways, induction of cell cycle arrest and apoptosis (15). The anti-metastatic effects of berberine in non-small lung cancer cells have recently been reported, and inhibition of urokinase-plasminogen activator and matrix metalloproteinase-2 was implicated (16). However, studies regarding the role of berberine in the anti-metastasis of human cancer cells are rare.

Perturbation of cell cycle progression by berberine has been reported in different carcinoma cell lines, but with discrepancies. For example, gastrocarcinoma (SNU-5) and leukemia cells were arrested by berberine at the G2/M phase (9,11). In contrast, G1 arrest was reported in epidermal and prostate carcinoma cells treated with berberine (17,18). While the effects of berberine on the cell cycle may be dependent on the cell types used, the different effects of berberine observed may also be attributed to the difference in concentration of berberine used in the studies. A recent study showed that berberine had multi-phasic effects on cancer cells and may be related to the differential intracellular distribution of berberine at different concentrations; berberine was shown to be accumulated in mitochondria at low concentrations (12.5-50 μM) but translocated to nuclei at high concentrations (>50 μM) (19).

In this study, we comprehensively examined the broad spectrum of properties of berberine using a commonly used NPC cell line, HONE1 (20) including its effects on cell proliferation, cell cycle arrest, apoptosis, cell migration and invasion. The biological properties of berberine on nasopharyngeal cancer cells have not been previously reported. In particular, we examined the anti-metastatic ability of berberine at low concentrations to distinguish it from the growth inhibitory effects of berberine commonly observed at high concentrations. The Rho-family of GTPases are known to be involved in the regulation of cell mobility and are commonly activated in human cancer cells (21). We examined the effects of berberine on the activity of Rho GTPase family members, RhoA, Rac1 and Cdc42 using a specific GST pull-down assay for activated Rho GTPases. The elucidation of the mechanisms involved in the anticancer and anti-invasive property of berberine may provide a therapeutic basis for its potential application to inhibit growth and prevent metastasis in human cancers.

Materials and methods

Chemicals and antibodies. Berberine purchased from Sigma Chemicals (St. Louis, MO, USA) was dissolved in milli-Q water to a stock concentration of 1 mM and stored at -70˚C before use. The primary antibodies used for cleaved-capase 9, cleaved-caspase 3, cleaved-PARP, p-cdc2 (Tyr 15) and ß-actin were purchased from Cell Signaling Technology (Beverly, MA, USA), and the antibody for p-histone 3 and the horseradish peroxidase (HPR)-linked secondary antibodies goat anti-mouse and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. HONE1 cells (a human nasopharyngeal carcinoma cell line) used in this study were kindly provided by Professor R. Glaser, University of Ohio (20). They were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 μg/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) and maintained at 37˚C in a humidified atmosphere of 5% CO_2.

MTT assay. The effect of berberine on cell viability/proliferation was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. MTT, a tetrazolium salt, is reduced to a purple blue formazan product by dehydrogenases in the mitochondria of living cells. The cell viability can thus be determined by quantifying the purple blue formazan product based on UV absorbance. Briefly, 5000 cells/well were plated in 96-well plates and incubated for 24 h. The cells were then treated with different concentrations of berberine (0, 25, 50, 100, 200, 400 and 500 μM) for 24, 48 and 72 h. The cells were then treated with 10 μl of 5 mg/ml MTT (Sigma) and incubated for 4 h at 37˚C. The medium was then discarded, and 200 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the resulting formazan crystals. The absorbance was measured at 570 nm by the Multiskan MS microplate reader (Labsystems, Finland) with a reference at 650 nm serving as blank. The cell viability of berberine-treated cells was calculated as the percentage of cell viability compared to untreated cells, which were arbitrarily assigned 100% viability.

Confocal microscopy for berberine localization. HONE1 cells ($3x10^5$) were seeded onto 35-mm glass-bottom petri dishes (MatTek Inc., Ashland, MA, USA). After overnight incubation, the cells were treated with a series of concentrations of berberine (0, 12.5, 35, 75, 150 and 300 μM) for 2 h. The fluorescence intensity and localization pattern were imaged by a laser-scanning confocal microscope (Carl Zeiss, LSM510 Meta, Germany; excitation wavelength 488 nm, emission wavelength 605 nm). For better illustration of the morphological details of the cells, white pseudo-colour was used to represent the red autofluorescence generated by berberine. All confocal images were acquired under identical microscope settings.

Boyden chamber invasion assay. The anti-invasive ability of berberine on HONE1 cells was investigated by the Boyden Millipore chamber system. Rat tail collagen (type 1) at 4.05 mg/ml (BD Bioscience, Bedford, MA, USA) was diluted in PBS (1:10) and coated onto the upper surface of the 8-μm pore polycarbonate membrane of the upper chamber inserts (12 mm, Millipore, Co. Cork, Ireland). Cells ($2.5x10^4$/well) were seeded onto the membrane of the upper chamber insert and placed in a 6-well plate containing 2 ml of culture medium/well and treated with concentrations of berberine. After a 48-h treatment with berberine, the non-invading cells remaining on the upper surface of the membrane were removed by cotton swabs. The cells that invaded across the collagen to the lower surface of the membrane were fixed by ice-cooled absolute ethanol and stained with 5% Giemsa solution for visualization of cells.

Wound healing assay. HONE1 cells were seeded in 6-well plates and allowed to grow to confluency. A uniform wound was introduced on the monolayers by scraping with a sterile yellow-pipette tip. The culture medium was replaced with fresh medium containing a series of concentrations of berberine (0, 12.5, 35, 75, 150 and 300 μM). The rate of wound closure in berberine-treated and untreated cells was monitored by images captured with a phase-contrast microscope immediately after wound incision (0 h) and after 18, 36 and 48 h.

Cell cycle analysis. Cells in log-phase growth were treated with different concentrations of berberine (0, 12.5, 35, 75, 150 and 300 μM) for 24 and 48 h. The cells were then harvested and fixed with cold (-20˚C) 70% ethanol for 30 min. Cells were subsequently washed in PBS and re-suspended in 1 ml PBS with 50 μg propidium iodide and 20 μg RNase and incubated at 37˚C for 20 min. The cell cycle distribution of treated and untreated cells was determined using a Becton-Dickenson FACScalibur flow cytometer (BD Biosciences, Franklin, NJ, USA). Modfit LT software (Verity Software House, Topsham, ME, USA) was used to evaluate the percentage of cells in G1, S and G2/M fractions.

Western blotting. Cell lysates were collected by scraping the cells with RIPA lysis buffer from the dishes, and the protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Extracted protein (20 μg) was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham, Piscataway, NJ, USA). Chemiluminescent signals were developed by ECL Plus Western blotting detection system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Analysis of the activation of Rho GTPases. The activation of three Rho GTPases was analyzed by the glutathione *S*-transferase (GST) pull-down assay. Details of the procedures have been previously described (22). Activated RhoA was pulled down by GST fusion protein containing the Rho-binding domain (RBD) of Rho effector rhotekin. Activated Cdc42 and Rac1 were pulled down by a GST-p21 binding domain (PBD) of the human p21 activated kinase 1 protein (PAK). The bacterial strain that expresses the GST-RBD-rhotekin and GST-PBD-PAK fusion protein was used in this study. Briefly, HONE1 cells were kept in serum-free medium for 24 h to silent endogenous activation of Rho GTPases. Cells were then treated with or without berberine for 30 min before incubating the cells with serum-supplemented medium to reactivate Rho GTPases. After 30 min of activation, cells were lysed, saturated with fusion protein of GST-PBD or GST-RBD. The binding of reactivated Rho GTPase to the GST fusion protein in the cell lysates was pulled down by glutathione-sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden). Detection of Rho GTPases was performed by Western blotting using anti-RhoA, anti-Cdc42 and anti-Rac1 (Cell Signaling). Total RhoA, Cdc42, Rac1 and actin levels in the primary lysates were detected in parallel.

Staining of stress fibres. Cells ($2x10^3$) were seeded in each well of 8-well chamber slides (Nalge Nunc International, Rochester, NY, USA) and incubated at 37˚C overnight. Cells were then starved by deprivation of serum for 24 h to suppress the basal level of stress fibres. The cells were treated with 0, 25 and 50 μM of berberine for 30 min before stimulation of stress fibre formation by culture medium supplemented with 10% FCS for 30 min. The cells were then fixed with 4% formaldehyde for 20 min, permeabilized by 0.1% Triton X-100 (Sigma) and blocked with 1% BSA (Calbiochem, San Diego, CA, USA) for 30 min. The stress fibres (bundles of F-actin) were stained by incubating with 1:1000 fluorescein phalloidin (Molecular Probes, Carlsbad, CA, USA) for 30 min and then mounted by Dako fluorescent mounting medium (Dako Cytomation, Denmark). The stress fibres were visualized and imaged using a confocal microscope (Carl Zeiss, LSM510 Meta). All images were captured under identical electronic settings so as to facilitate direct comparison among different treatments.

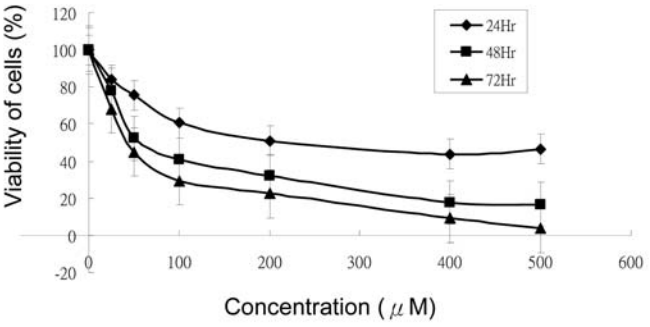

Figure 1. Dose- and time-dependent reduction in the viability of HONE1 cells by berberine. HONE1 cells were plated on 96-well plates and treated with a series of increasing concentrations of berberine (0, 25, 50, 100, 200, 400 and 500 μM) for 24, 48 and 72 h. Cell viability was determined by the MTT assay. Each data point was derived from triplicate wells, and each experiment was repeated five times. The error bars represent the standard deviation.

Results

Berberine inhibited cell proliferation of HONE1 cells in a dose-dependent manner. The effect of berberine on the viability of HONE1 cells was first determined by MTT assay. The HONE1 cells were treated with varying concentrations of berberine (0, 25, 50, 100, 200, 400 and 500 μM) for different time periods (24, 48 and 72 h). Similar to previous studies, a dose-dependent reduction in cell viability was observed with increasing concentrations of berberine and treatment time (Fig. 1). The ID_{50} concentration of berberine on HONE1 cells after a 24-h treatment was ~250 μM. ID_{50} values for longer treatment times at 48 and 72 h were at 50 μM and below 50 μM, respectively.

Differential intracellular distribution of berberine at different concentrations. We examined whether the intracellular location of berberine in HONE1 cells could be influenced by different concentrations of berberine. Since berberine is a fluorescent molecule, intracellular distribution of uptaken berberine can be monitored by fluorescence confocal laser scanning microscopy. Various concentrations of berberine were examined (12.5, 35, 75, 150 and 300 μM), and cells were treated for 2 h (Fig. 2). Notably, the intracellular distribution of berberine was dependent on the concentrations of berberine. No fluorescent signals were detected in control cultures without berberine treatment. Fluorescent signals of berberine were detected in the cytoplasm after a 2-h treatment at a low concentration of berberine (12.5 μM). Localization of berberine in mitochondria was visualized at 35 and 75 μM

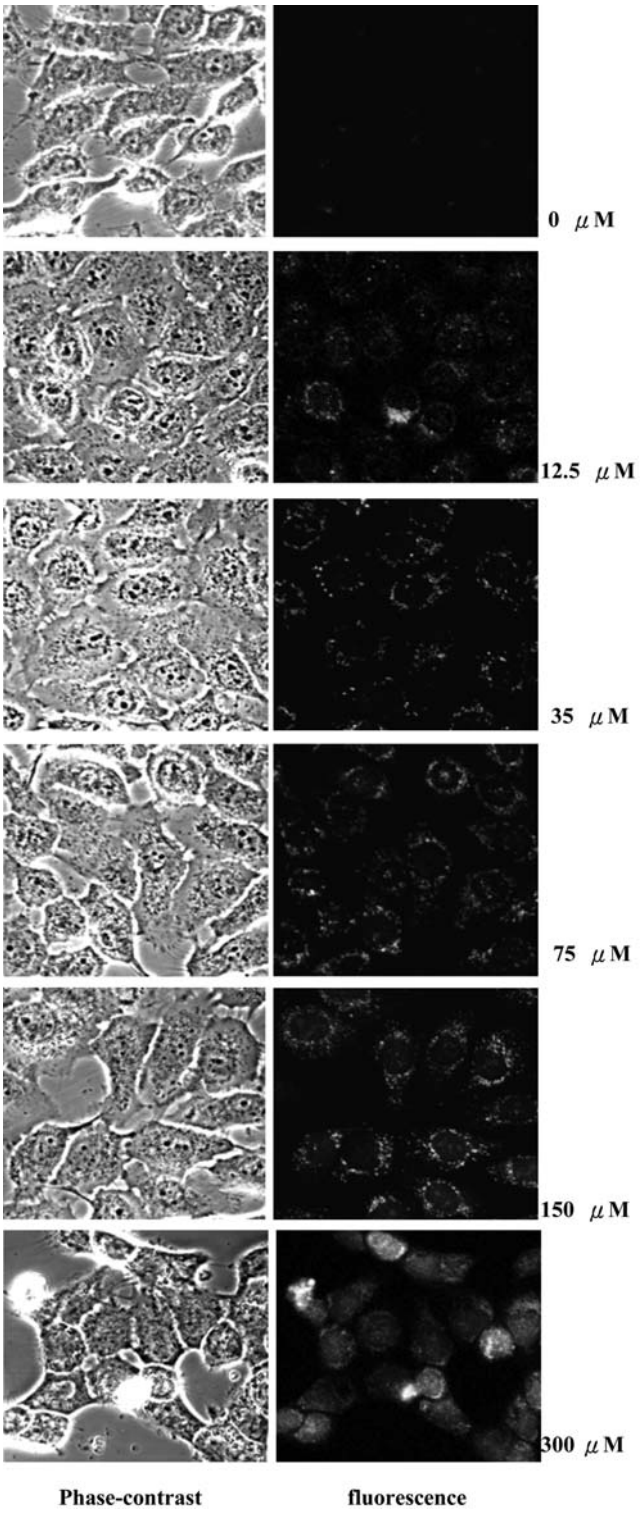

Figure 2. Differential distribution of berberine under different concentrations. HONE1 cells were treated with berberine (0, 12.5, 35, 70, 150 and 300 μM) for 2 h. Phase-contrast (left panel) and fluorescence (right panel) images were captured by confocal microscope under the same instrument settings. Note that different concentrations of berberine caused different intracellular distribution patterns.

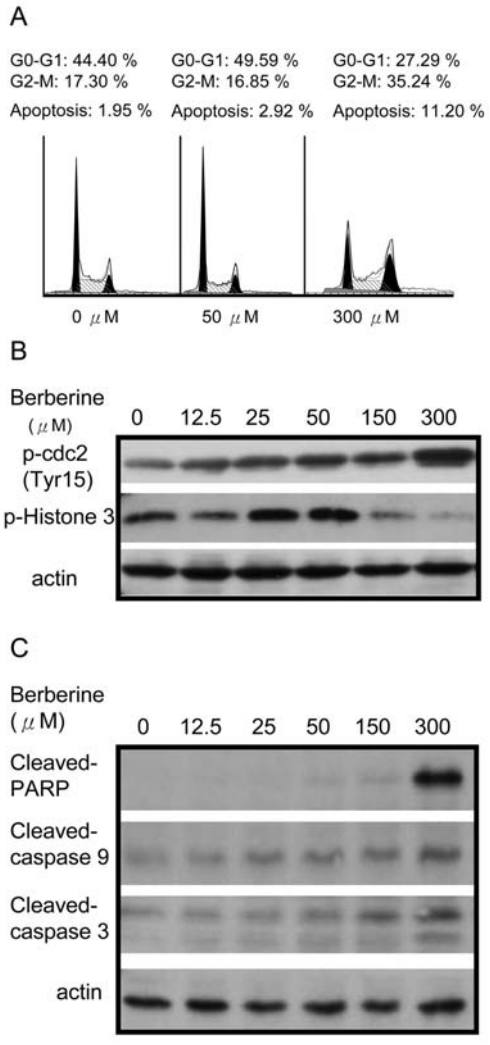

Figure 3. A high dose (300 μM) of berberine induced G2/M phase arrest and apoptosis in HONE1 cells. (A) Histograms of the cell cycle distribution of HONE1 cells after berberine treatment (0, 50 and 300 μM) for 24 h. A marked increase in the percentage of cells in G2/M was induced by 300 μM berberine. (B) Western blots of cell cycle-related proteins. Protein was extracted from HONE1 cells treated with 0, 12.5, 25, 50, 150 and 300 μM berberine for 24 h. Triplicate experiments were conducted with representative blots shown. (C) Western blots of apoptosis-related proteins. Berberine induced apoptosis in a dose-dependent manner (12.5-300 μM) after 24 h of treatment. Triplicate experiments were conducted with representative blots shown.

concentrations of berberine treatment. No fluorescent signals were detected in the nuclei at these concentrations (12.5, 35 and 75 μM). At the concentration of 150 μM, fluorescent signals of berberine began to be observed at the nuclei. Nuclear localization of berberine was clearly detected in the nuclei of cells treated with 300 μM of berberine. Hence, we were able to confirm the results of a previous study (19) demonstrating that berberine concentrates in the mitochondria in cells at a low concentration but can be translocated to nuclei at high concentrations. The differential distribution of berberine at different concentrations may determine the biological properties exerted by berberine on treated cells.

A high dose of berberine induced a G2 blockage and apoptosis in HONE1 cells. We examined the effects of different concentrations of berberine on cell cycle progression in HONE1 cells. A low dose (50 μM) and a high dose (300 μM) of berberine were chosen, and the cells were treated for 24 h and analysed by flow cytometric analysis for distribution of cells at different phases of the cell cycle. A high dose of berberine (300 μM) induced a marked increase in the

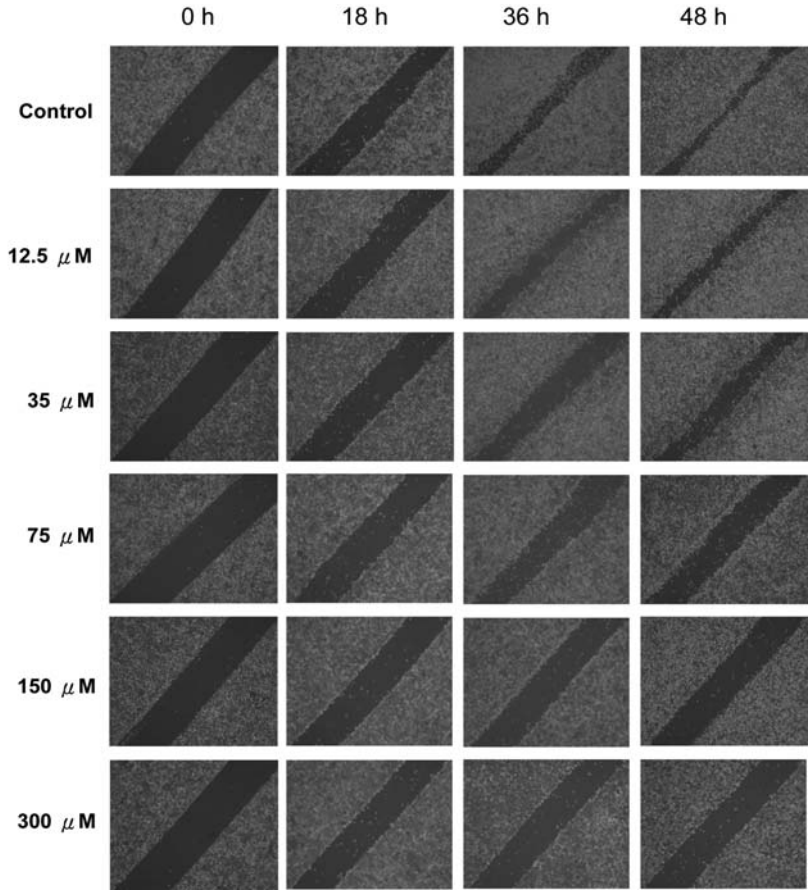

Figure 4. Berberine suppressed the migration ability of HONE1 cells. Similar sized wounds were introduced on confluent monolayer cells, and different concentrations (0, 12.5, 35, 75, 150 and 300 μM) of berberine were added to the culture medium. The rate of wound healing was monitored at the indicated time points (0, 18, 36 and 48 h). Note that the speed of wound healing was suppressed by berberine in HONE1 cells in a time- and dose-dependent manner.

percentage of HONE1 cells of G2/M population (Fig. 3A). An increase in the sub-G1 population was also observed which is indicative of apoptotic cells. In contrast to previous studies, we did not observe a significant alteration in the cell cycle distribution profile (e.g. G1 arrest) in cells treated with a low dose (50 μM) of berberine (Fig. 3A). We further examined the phosphorylation levels of protein involved in mitosis by Western blot analysis to confirm the effect of berberine on mitotic blockage (Fig. 3B). A high level of activated cdc2 (p-cdc2; Tyr15) and decreased expression of p-histone 3 were detected in the mitotically arrested cells. This suggested that the cells were arrested in the G2 phase before entry of mitosis. In addition, the presence of apoptotic cells in cells treated with a high concentration of berberine was confirmed by increased levels of cleaved-PARP, cleaved caspase 3 and cleaved caspase 9 (Fig. 3C).

Suppression of migration of HONE1 cells at low doses of berberine. The ability of berberine to inhibit HONE1 cell migration using wound healing assay was examined. Scratch wounds were inflicted on confluent cell layers before treatment with various concentrations of berberine (12.5, 35, 75, 150 and 300 μM). The migration fronts of HONE1 cells were observed after berberine treatment for 18, 36 and 48 h. Berberine effectively inhibited cell migration at low doses (35 to 75 μM), relatively non-toxic to cells (Fig. 4).

A low dose of berberine suppressed HONE1 cell invasion. We investigated whether berberine inhibits the ability of cancer cells to invade through a collagen-coated Boyden chamber. HONE1 cells were seeded on a Boyden chamber coated with collagen gel and treated with different concentrations of berberine (0, 3.125, 6.25, 12.5, 25 and 50 μM) for 48 h. The post-treated cells at the upper chamber were removed by scraping. The cells which had invaded to the lower chamber were then visualized by staining. Berberine effectively inhibited cell invasion through the collagen-coated Boyden chamber in a dose-dependent manner (Fig. 5). At the low dose of 6.25 μM of berberine, cell invasion ability was reduced by 30%. Strikingly, completely suppressed cell invasion was observed at 50 μM berberine (Fig. 5B).

Berberine suppressed the activation of Rho GTPases at low doses. Since the Rho GTPases are involved in cell migration, we examined whether the activities of Rho GTPases are influenced by berberine. The activities of three members of Rho GTPases commonly involved in cell motility and migration were determined: RhoA, Cdc42 and Rac1. To silent the endogenous activity of Rho GTPases in HONE1 cells, the cells were kept in serum-free medium for 24 h before the assay. The cells were treated with low doses of berberine (25 and 50 μM) before activation of Rho GTPases by the addition of serum. The levels of activated RhoA in the

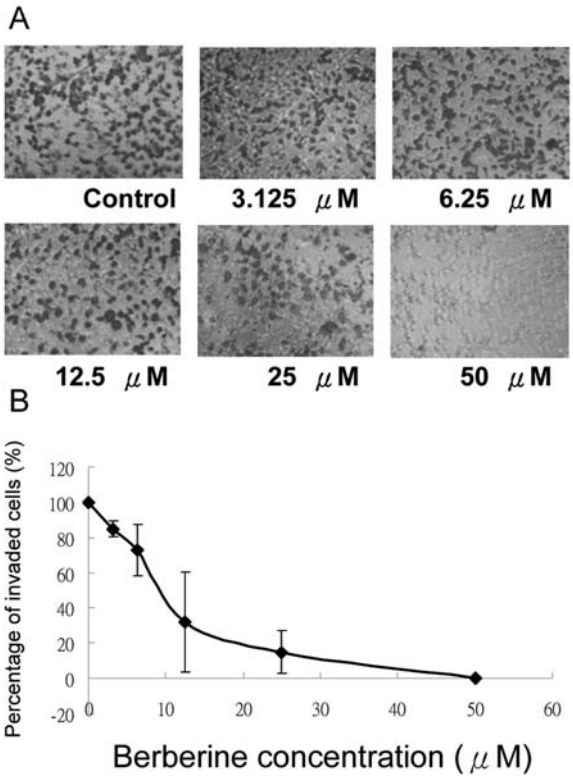

Figure 5. Inhibitory effect of invasion of HONE1 cells by berberine at low doses. A Boyden chamber coated with collagen gel was used to assay the effect of berberine on inhibiting cell invasion. Cells were seeded on the upper chamber and incubated with low concentrations of berberine (0, 3.125, 6.25, 12.5, 25 and 50 μM) for 24 h. (A) Microscopic images of invaded cells. (B) Graphical representation of the percentage of invaded cells under different concentrations of berberine. Images of the stained invaded cells (5 random fields/culture) were captured, and the mean number of cells was recorded. Each experiment was repeated three times. The error bars represent the standard deviation. Note that berberine reduced the invasion ability of HONE1 cells in a dose-dependent manner. The inhibitory effect of invasion by berberine in HONE1 cells was noted at very low doses (3.125 and 6.25 μM), and 50 μM berberine completely suppressed cell invasion.

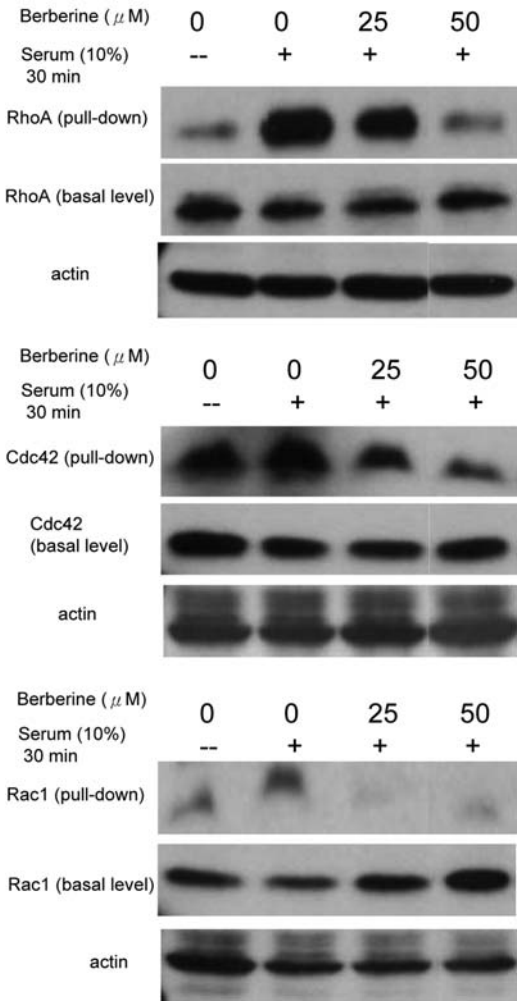

Figure 6. Activation of RhoA, Cdc42 and Rac1 was suppressed by berberine at low doses. Serum-starved cells were pretreated with berberine (0, 20 and 50 μM) for 30 min before the re-addition of serum. After 30 min, cell lysates were prepared. GTP-bound RhoA was enriched by absorbing cell lysates to GST-RBD-rhotekin beads, while GTP-bound Cdc42 and Rac1 were enriched by absorbing cell lysates to GST-PBD-PAK and measured by Western blotting using corresponding specific antibodies. Total RhoA, Cdc42 and Rac1 in th cell lysate were detected and regarded as the basal level. Actin served as the internal control for loading.

presence or absence of berberine treatment were examined by a pull-down assay using GST-rhotekin which binds specifically to activate RhoA. The levels of activated RhoA pulled down were then determined by Western blotting using RhoA-specific antibody. Similarly, a GST-PAK1 pull-down assay was used to quantify activated Cdc42 and Rac1 followed by Western blotting using specific antibodies against Cdc42 and Rac1, respectively. As shown in Fig. 6, activation of RhoA, Cdc42 and Rac1 were all suppressed by berberine in a dose-dependent manner. As far as we are aware, this is the first demonstration of the of berberine on Rho GTPases.

Low doses of berberine suppressed the formation of stress fibres. To confirm the effect of berberine on Rho GTPases, we examined the effects of berberine on stress fibre formation which is mediated by RhoA. The stress fibres were visualized by phalloidin staining. The basal levels of stress fibres were first suppressed by culturing the cells in serum-free medium for 24 h. Activation of stress fibre formation was induced by treating the cells with serum for 30 min which was visualized by the formation of densely packed actin filament bundles around the plasma membrane. The activation of stress fibre formation by serum was effectively suppressed by pre-treating the cells with a low dose of berberine (50 μM) before addition of the serum to the culture medium (Fig. 7).

Discussion

Berberine, an active ingredient extracted from the traditional medicinal herb *Coptidis rhizoma*, has been reported to have anticancer properties in multiple cancer cell lines through induction of apoptosis (23-25). A recent study also revealed that berberine inhibited the invasive growth of lung cancer cells by decreasing the production of urokinase-plasminogen activator and matrix metalloproteinase (16). The ability of berberine to inhibit invasion of cancer cells may prevent cancer metastasis. NPC is a highly invasive head and neck cancer commonly associated with regional spread and systemic dissemination (26,27). Prognosis is poor when NPC

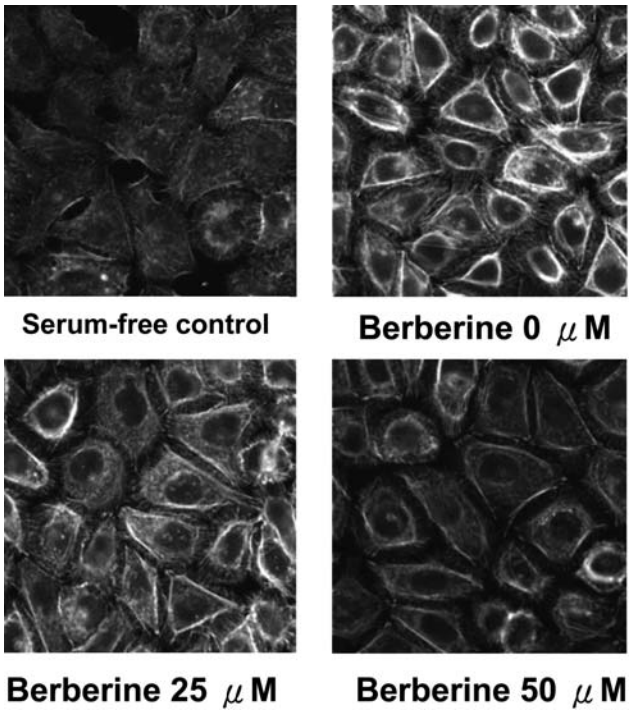

Figure 7. Stress fibre formation was inhibited by low doses of berberine in HONE1 cells. Serum-starved HONE1 cells were treated with low concentrations of berberine (0, 25 and 50 μM) and then subjected to a 30-min incubation in medium with serum. The cells were stained with rhodamine phalloidin to highlight F-actin distribution, and images were captured using fluorescence microscopy. The images are representative of at least three independent experiments performed in duplicate.

cells metastasize to distant organs (28,29), and there are limited drugs available for the prevention of cancer metastasis. The present study aimed to examine the potential application of berberine in the treatment of NPC, particularly in the prevention of metastatic spread, using a commonly used NPC cell line (HONE1). The findings obtained in this study may also be applicable to other human cancer cells and support the possible potential of *Coptidis rhizoma* in the therapy of human cancers (1).

The cytotoxic dose of berberine in NPC cells was initially determined (Fig. 1). We demonstrated that berberine at a high dose (300 μM) induced G2/M blockage and apoptosis as evidenced by flow cytometry with the presence of cleaved-PARP, cleaved-caspase 3 and cleaved-caspase 9 (Fig. 3). These findings are in keeping with previous studies which demonstrated that high doses of berberine induced mitotic arrest and apoptosis in gastric cancer and leukemia cells (9,10). We further demonstrated that the mitotic arrest was associated with an increase in p-cdc2 and a decrease in p-histone 3 which indicated that mitotic cells were arrested at the G2 phase before entering into M phase (Fig. 3). Prolonged arrest of cells at the G2 phase by berberine apparently triggered apoptosis in NPC cells. Notably, the dose of berberine required to induce mitotic arrest coincided with the dose for its localization in the nucleus (Fig. 2). Berberine was shown to be concentrated in mitochondria at a low dose ($\leq$50 μM) and in the nucleus at high doses (>150 μM) (Fig. 2). However, the mechanisms involved in the differential intracellular localization of berberine are still not fully understood at this stage. As berberine is a DNA-binding molecule (19), translocation of berberine to the nucleus may interfere with DNA and block G2 cells from entering the M phase. A previous study revealed that different concentrations of berberine resulted in distinct cellular responses which correlated with its intracellular distribution in melanoma cell lines (19). This also suggested that a low concentration of berberine may alter mitochondrial function, thus activating an 'energy checkpoint' and leading to a slowing of the cell cycle that may be reflected as G1 arrest in prolonged exposure which has been reported in previous studies. In our study, we did not observe significant arrest of HONE1 cells at the G1 phase under a low dose of berberine (50 μM) indicating that the susceptibility of cells to G1 arrest of berberine may be cell type dependent or a longer treatment time may be required to arrest the cells at G1.

Nevertheless, we found that berberine at low concentrations effectively suppressed cell invasion and migration suggesting a potential use of berberine in the prevention of metastasis of cancer cells (Figs. 4 and 5). Abilities to migrate and invade are two major properties involved in tumour cell metastasis (21). Rho GTPases are known to be involved in tumour cell migration and invasion by regulating actin cytoskeleton, cellular-extracellular matrix adhesion and cell-cell communication (30). In addition, berberine was also reported to downregulate expression of PAK1 (p21 activated kinase 1) which is an effector molecule of activated Rho GTPases (7). This prompted us to investigate whether berberine directly inhibits Rho GTPases in NPC cells. Rho GTPases are small proteins that act as binary molecular switches which cycle between an active conformation (GTP-bound) and an inactive conformation (GDP-bound). Once activated, they bind to a spectrum of effector molecules and trigger multiple downstream signaling events (31,32). In this study, we examined the effects of berberine on three best-characterized Rho GTPases, RhoA, Cdc42 and Rac1. We found that low doses of berberine (25 and 50 μM) effectively suppressed the activation of these Rho GTPases (Fig. 6). Activation of RhoA, Cdc42 and Rac1 is known to play important roles in organizing the actin filament involved in changes in cell shape, cell adhesion, cell polarity and cell migration (30,32). RhoA regulates the formation of stress fibres and focal adhesion, Cdc42 regulates the formation of filopodia and Rac1 regulates the formation of lamellipodia. The ability of berberine to inhibit the anti-migration and anti-invasion properties of cancer cells may be related to the effective inactivation of these Rho GTPases by berberine. The effect of berberine in interrupting cytoskeletal dynamics was further demonstrated by the suppressed stress fibre formation in cells treated with berberine (Fig. 7). To the best of our knowedge, this is the first study demonstrating the ability of berberine to inhibit activation of RhoA, Cdc42 and Rac1. The effective concentrations of berberine used to suppress cell invasion were low compared to the concentrations used to suppress cell growth and cell cycle progression. Our study indicates that berberine may have the potential application to prevent cancer metastasis. Its potential use with other chemotherapeutic agents in adjuvant treatment of cancer to prevent cancer growth and metastasis warrants further investigation.

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