

Berberine Inhibits HIF-1 α Expression via Enhanced Proteolysis

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ABSTRACT

We have studied the antiangiogenic property of berberine. We showed that berberine could directly inhibit in vitro human umbilical vein endothelial cell (HUVEC) tube formation and migration. In addition, to determine whether berberine could influence the cross-talk between the gastric adenocarcinoma cell line SC-M1 and vascular endothelial cells, we performed modified confrontation culture experiments and showed that berberine (7.5 μ M, 16 h) could inhibit the capacity of hypoxic SC-M1 cells to stimulate HUVEC migration. These results demonstrated berberine's antiangiogenic property and its clinical potential as an inhibitor of tumor angiogenesis. Parallel Western blot analyses revealed that berberine prevented hypoxic SC-M1 cultures from expressing vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF)-1 α , two key factors in mediating tumor angiogenesis. However, overexpression of HIF-1 α in SC-M1 cells dramatically reversed the inhib-

itory effect of berberine on SC-M1-induced in vitro HUVEC migration. These data indicated that HIF-1 α repression is a critical step in the inhibitory effect of berberine on tumor-induced angiogenesis. Northern blot analyses plus pulse-chase assays revealed that berberine did not down-regulate HIF-1 α mRNA but destabilized HIF-1 α protein. We found that berberine-induced HIF-1 α degradation was blocked by a 26S proteasome inhibitor. Moreover, immunoprecipitation and Western blot analyses showed that berberine increased the lysine-acetylated HIF-1 α in hypoxic SC-M1 cultures. These data indicated that a proteasomal proteolytic pathway and lysine acetylation were involved in berberine-triggered HIF-1 α degradation. In conclusion, our data provided molecular evidence to support berberine as a potent antiangiogenic agent in cancer therapy.

Cancer, one of the world's leading causes of death, is a disease of abnormal gene expression, manifested by deregulated DNA synthesis and aberrant proliferation. Studies of tumorigenesis have revealed that as a tumor expands, vigorous proliferation of cancer cells creates a hypoxic microenvironment, which, if not alleviated, may restrict tumor growth or even cause cell death. These phenomena indicate that tumor-induced angiogenesis plays a critical role in tumor growth. Therefore, blockade of tumor-induced angiogenesis represents a potential anticancer strategy (Folkman, 1971; Gullino, 1978). Therefore, efforts have been made to search for antiangiogenic agents, and many potential candidates have been tested in clinical trials (Carmeliet and Jain, 2000).

It is interesting that thalidomide, an old drug known for its teratogenicity, is also under phase III clinical trials in cancer therapy because of its antiangiogenic activity. This example suggests that it could be beneficial for cancer therapy by examining the antiangiogenic activity of chemicals that were not originally intended to kill cancer cells.

Huanglian (*Coptis chinensis*) is a widely used herb in traditional Chinese medicine for the treatment of inflammation-related diseases such as gastroenteritis. In addition, molecular evidence has showed that Huanglian is able to inhibit topoisomerase I activity (Kobayashi et al., 1995) and to inhibit cyclin B1 expression and CDC2 kinase activity in a gastric cancer cell line (Li et al., 2000). These findings indicate that Huanglian contains compound(s) that can inhibit cell cycle progression and may be effective in cancer treatment. To collect more information regarding the anticancer potential of Huanglian, it is worthwhile to determine

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; MEM, minimal essential medium; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; EGM; HPF, high-power field; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; HIF, hypoxia-inducible factor; N-CBZ-LLL-AL, N-carbobenzyloxy-leucine-leucine-leucine-aldehyde; Ac-HIF-1, acetylated HIF-1; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; HRE, hypoxia-response element.

whether Huanglian is also effective in inhibiting tumor angiogenesis and, furthermore, to characterize the antiangiogenic ingredient(s) of this herb for detailed pharmacological studies.

Berberine is one of the major alkaloids in Huanglian. This compound has strong anti-inflammatory and antimicrobial activities. It also demonstrated antiarrhythmic activity and was therefore considered to be useful in treating some cardiovascular diseases such as hypertension and chronic heart failure (Lau et al., 2001; Hong et al., 2002). The information regarding berberine's anticancer activities has been limited. It was reported that berberine could inhibit the transcriptional activity of cyclooxygenase-2, an enzyme that plays a key role in colon tumorigenesis (Fukuda et al., 1999a). Berberine was also found to inhibit the activity of activator protein 1, a transcription factor that plays a critical role in inflammation and carcinogenesis (Fukuda et al., 1999b). In our study, we explored the antiangiogenic property of berberine. We observed that berberine-treated gastric cancer cells (SC-M1) were unable to induce in vitro angiogenesis, which was accompanied by the finding that berberine reduced the expression of vascular endothelial growth factor (VEGF) in both normoxic and hypoxic SC-M1 cultures. Finally, we identified the HIF-1 α as a target of berberine, which revealed an important part of berberine's anticancer mechanisms.

Materials and Methods

Cell Culture. Human lung adenocarcinoma CL1-5 cells (Chang et al., 2003) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA). Human gastric adenocarcinoma SC-M1 cell line was established from primary tumor tissue in a 72-year-old man (Jin et al., 1987) and was maintained in MEM (Invitrogen). These media were supplemented with 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins as described previously (Jaffe et al., 1973), and maintained in EGM medium (Cambrex Bio Science Walkersville, Walkersville, MD). HUVECs grown before the sixth passage were used for studies. To generate cells overexpressing HIF-1 α , SC-M1 cells were transfected with pCPE4/HIF-1 α (Krishnamachary et al., 2003) using the Effectene reagent following the protocol provided by the manufacturer (QIAGEN, Valencia, CA). The transfected cells were selected against the medium containing 120 μ g/ml Hygromycin B. The selected clones were pooled and maintained in the medium containing 100 μ g/ml Hygromycin B. All the cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Antibodies and Chemicals. Antibodies used in this study were anti- α -tubulin (Zymed Laboratories Inc., South San Francisco, CA); anti-HIF-1 α (BD Transduction Laboratories, Lexington, KY); anti-GAPDH (Santa Cruz Biotechnology); and anti-acetyl-lysine (Upstate Biotechnology). Berberine hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan).

Capillary Tube Formation Assay. HUVECs were either treated with berberine for the time indicated or left untreated, then were seeded (5×10^4) on top of ECMatrix gel (Chemicon International, Inc., Temecula, CA) prepared as described previously (Chang et al., 2003) and incubated in 150 μ l of their original media. For tube formation, cells were incubated at 37°C for 16 h under normoxic condition. Three different phase-contrast microscopic high-power fields (HPF, 100 \times) per well were photographed. The total length of capillary tubes in each photograph was measured using a scale ruler.

MTT Assay. CL1-5 or SC-M1 cells (1×10^4) were seeded in 96-well culture plates and maintained in 150 μ l of medium containing a variant dose of berberine as indicated for 72 h at 37°C. Then, 15

μ l of MTT reagent (5 mg/ml; Sigma) was added to each well of the plate and incubated for 4 h at 37°C. After reaction, media were carefully removed by aspiration. Thereafter, 50 μ l of DMSO was added to each well. The plate was gently shaken for 30 min at room temperature. The absorbance of each well was measured at 570 nm.

Preparation of Conditioned Media. The conditioned media were prepared as described previously (Chang et al., 2003). In brief, SC-M1 cells (1.2×10^6 /dish) were seeded in 10-cm culture dishes in 10 ml of whole-serum MEM for 16 h to reach approximately 70% confluence. Cells were either treated with berberine or left untreated for 16 h under normoxic condition. After being washed with phosphate-buffered saline (PBS), cells were then cultured in 6 ml of fresh berberine-free MEM (containing 1% fetal bovine serum) under either normoxic or hypoxic (2% O₂) condition for 4 h at 37°C. Thereafter, the media were collected and subjected to low-speed centrifugation. The supernatants were collected separately as conditioned media and were used immediately in HUVEC migration assays. The remaining cells were used in parallel Western blot and gel-shift analyses.

HUVEC Migration Assay. The migration activity of HUVECs was determined using the growth factor-reduced Matrigel invasion system (BD Biosciences) following the protocol provided by the manufacturer. In brief, HUVECs (5×10^4) were suspended in 0.5 ml of conditioned medium and added to the upper chamber. The upper chamber was lodged into the lower chamber containing 0.75 ml of conditioned medium. After incubating at 37°C for 16 h, the cells in the upper side of the filter membrane were removed with cotton swabs. The membranes were then soaked in methanol for 10 min. Cells that migrated to the lower side of the membranes were stained with Liu stain (Handsel Technologies, Inc., Taipei, Taiwan). The stained cells were counted in three fields under a 200 \times high-power field. Photos were taken with the use of a microscope video system.

Western Blot Analysis. Aliquots (40 μ g) of whole-cell lysates were separated on 10% SDS-polyacrylamide gels, and electrotransferred onto polyvinylidene membranes (Amersham Biosciences, Piscataway, NJ). After blocking with PBS plus 0.1% Tween 20 plus 5% nonfat milk, the blots were incubated with antibodies as indicated, and the signals were obtained by enhanced chemiluminescence (ECL; Amersham Biosciences).

Northern Blot Analysis. Total RNA was isolated from SC-M1 cells using TRIzol (Invitrogen) according to the protocol provided by the manufacturer. Twenty micrograms of total RNA was resolved in a 1% agarose gel containing 6.7% formaldehyde and then transferred to the nylon membrane. DNA probes were labeled by Rediprime II random priming system following the manufacturer's protocol (Amersham Biosciences). Hybridization was performed as described previously (Lin et al., 2000). Signals were visualized and quantitated by filmless autoradiographic analysis using a Typhoon 8600 (Amersham Biosciences).

Gel-Shift Analysis. Electrophoretic mobility-shift assays (EMSA) were performed to detect the binding of cellular proteins to a 26-bp DNA fragment (CCACACTGCATACGTGGGCTCCAACA) derived from the promoter of human *VEGF* gene. SC-M1 cells either treated with berberine or left untreated were fractionated into nuclear and cytoplasmic fractions as described previously (Lin et al., 2000). The DNA was 3'-end-radiolabeled by polynucleotide kinase. Five-microgram aliquots of nuclear fraction were incubated with 50000 cpm of DNA probe at room temperature for 20 min, and then electrophoresed through 6% nondenaturing polyacrylamide gels containing 0.25 \times Tris-borate/EDTA at 150 V for 2 h at 4°C. Gels were dried and radioactive signals were visualized with a PhosphorImager.

Pulse-Chase Assay. SC-M1 cells were pretreated with berberine or left untreated under normoxic condition for 12 h, and washed with PBS. Cells were then incubated in berberine-free, methionine-free MEM containing 10% dialyzed fetal bovine serum for 30 min and then labeled with 100 μ Ci/ml [³⁵S]methionine for 4 h under hypoxic conditions (2% O₂). After labeling, cells were washed with PBS. Berberine was added back to the berberine-pretreated cells; both

untreated and berberine-treated cells were subsequently incubated at 37°C under hypoxic condition for 0, 1, and 3 h. Cells were then washed with PBS for the preparation of whole-cell lysates. Each lysate (4×10^6 cpm) was then immunoprecipitated by anti-HIF-1 α antibody and subjected to electrophoresis as described previously (Lin et al., 2000). Signals were visualized with the use of a PhosphorImager.

Immunoprecipitation Assay. To examine whether berberine increased the acetylation on the lysine residue(s) of HIF-1 α protein, SC-M1 cells (1.2×10^6) were treated with berberine for 16 h or left untreated under normoxic conditions. These cells were then incubated in 6 ml of their original media under hypoxic condition (2% O₂) for 4 h at 37°C. After washing with PBS, cells were lysed and incubated with mouse preimmune serum plus protein A/G-agarose at 4°C for 16 h with gentle shaking. The supernatants were collected. Each lysate (500 μ g) was then immunoprecipitated with anti-acetyl-lysine antibody plus protein A/G-agarose at 4°C for 16 h. After low-speed centrifugation, the supernatants were discarded. The remaining agarose beads were washed 4 times with lysis buffer and then subjected to Western blot analysis using anti-HIF-1 α antibody for Lys-acetylated HIF-1 α .

Statistical Analysis. The data were expressed as mean \pm S.D. Statistical significance was performed with analysis of variance followed by one-way ANOVA test for experiments consisting of more than two groups. A value of $P < 0.05$ was considered to denote statistical significance.

Results

Berberine Directly Decreased HUVEC Migration and Capillary Tube Formation and Also Decreased the Ability of SC-M1 Cells to Induce HUVEC Migration. We used HUVEC and human cancer cell lines to exploit berberine's antiangiogenic potential. First, HUVECs were cultured in whole-serum EGM alone or EGM containing berberine (10 μ M) for 8 h under normoxic condition. These cells were then subjected to in vitro migration and capillary tube formation assays without changing media. We found that berberine caused approximately 72% decrease in the migration activity (Fig. 1A), and approximately 41% decrease in the tube formation capability of HUVECs (Fig. 1B). Next, we examined whether berberine was capable of inhibiting the tumor-in-

duced angiogenesis. We performed MTT and cell proliferation assays to determine proper berberine dose and treatment time. Both of CL1-5 and SC-M1 cells were treated with 1, 10, and 100 μ M berberine for 72 h, and subsequent MTT assays showed that the IC₅₀ for berberine on both cell lines was around 7.5 μ M (Fig. 2A), which was therefore chosen for the cell proliferation assays. We examined the impact of berberine on the proliferation of SC-M1 cells and found that berberine inhibited the proliferation of SC-M1 cells. Data showed that in the initial 24 h, at least, there was no difference in cell number compared with untreated controls (Fig. 2B). Trypan blue exclusion assays showed no significant change in the number of both living and dead cells in the initial 48 h of treatment (data not shown). To avoid the putative cytotoxicity of berberine, we chose 16 h as the treatment time for the following experiments.

We performed modified confrontation culture experiments to examine whether berberine-treated SC-M1 cells could support in vitro HUVEC migration because the migration of vascular endothelial cells plays a key role in tumor angiogenesis. SC-M1 cells were either left untreated or treated with berberine under normoxic condition in whole-serum MEM (containing 10% FBS) and were used to prepare for the serum-reduced, berberine-free conditioned media for the HUVEC migration assays. We prepared three batches of conditioned media for three independent HUVEC migration experiments. As shown in Fig. 3, when cultured in the conditioned media generated from untreated SC-M1 cells, the numbers of HUVEC that migrated through filters were 11 ± 2 , 15 ± 3 , and 13 ± 5 cells per HPF \times 200 for media batches I, II, and III, respectively. In the media from hypoxic SC-M1 cells, the scored HUVEC numbers were 28 ± 4 , 46 ± 6 , and 41 ± 4 cells per HPF \times 200 for media batches I, II, and III, respectively. In the media from berberine-pretreated hypoxic SC-M1 cells, the migrated HUVEC numbers were 13 ± 2 , 18 ± 4 , and 12 ± 2 cells per HPF \times 200 for media batches I, II, and III, respectively. These data indicated that hypoxia (2% O₂, 4 h) augmented the ability of SC-M1 cells to stimulate HUVEC migration (with an average increase of 2.8-fold

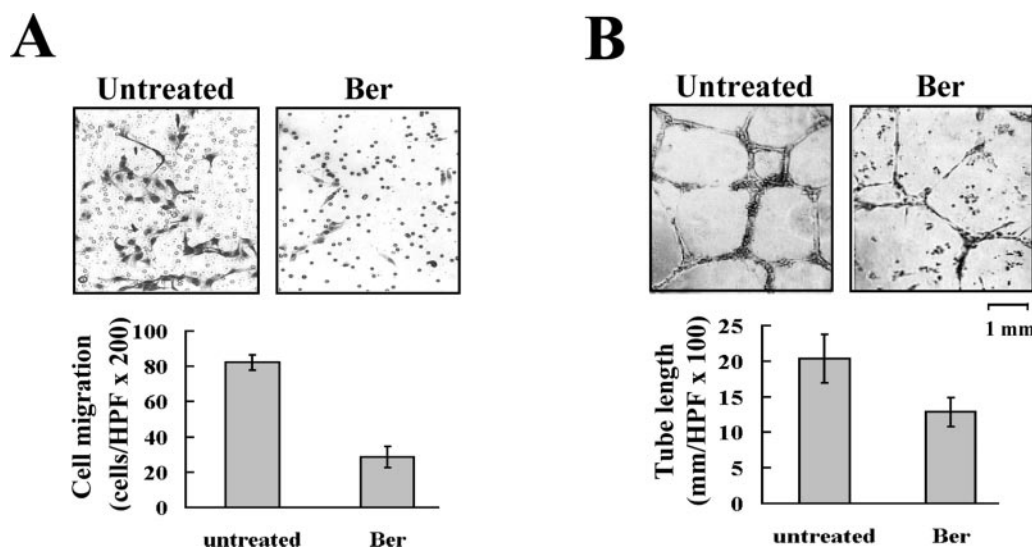


Fig. 1. Berberine inhibited the in vitro capillary tube formation and migration of HUVECs. HUVECs were either left untreated or treated with berberine (Ber, 10 μ M) for 8 h. These cells were then used for migration assays (A) and tube formation assays (B) in their original media, incubated under normoxic condition for 16 h at 37°C. The measurement of HUVEC migration and tube formation activities was as described under *Materials and Methods*. Each bar represents the mean \pm S.D. ($n = 3$).

for the three batches of media) and that this augmentation was completely blocked by berberine.

Berberine Abolished Hypoxia-Induced VEGF Expression in SC-M1 Cells. The results of confrontation culture assays suggested that conditioned medium generated from berberine-treated SC-M1 cells might be short of growth factor(s) required for the induction of HUVEC migration. Therefore, we examined the cellular levels of VEGF in the remaining SC-M1 cells after the collection of conditioned media. VEGF is a critical growth factor in tumor angiogenesis (reviewed by Papetti and Herman, 2002). Although cultured in low-serum media, these cells still responded to hypoxia with a significant increase (approximately 1.8-fold) in VEGF expression, but berberine abolished the induction (Fig. 4A). These results suggested that berberine might down-regulate the regulatory mechanism(s) that mediates hypoxia-induced VEGF expression. Because hypoxia-inducible factor 1 (HIF-1), a transcription factor, plays the key role in mediating hypoxia-induced VEGF transcription, we hypothesized that berberine might down-regulate the transcriptional activity of HIF-1, and decrease the binding of HIF-1 to its response element. Therefore, we synthesized a 26-bp DNA

fragment corresponding to the hypoxia-response element (HRE, from -985 to -960) of VEGF promoter. The HRE fragment contains the HIF-1 binding site 5'-TACGTGGG. We performed EMSA analyses to investigate the protein binding activities on the HRE fragment. After the collection of conditioned media, the remaining SC-M1 cells were fractionated, and the nuclear fractions were incubated with radiolabeled HRE probes and then resolved in native polyacrylamide gels. As shown in Fig. 4B, the lysate from untreated cells readily formed complexes with HRE DNA. Hypoxia induced an increase of approximately 1.7-fold in the formation of protein-DNA complexes, but berberine inhibited hypoxia-induced complex formation. Taken together, these results showed a close link between hypoxia-induced VEGF expression and protein binding on the HRE of VEGF promoter. Therefore, a transcriptional repression mechanism might play an important role in mediating berberine's inhibitory effect on hypoxia-induced VEGF expression.

Berberine Down-Regulated the Expression of HIF-1 α . The EMSA data suggested that berberine might decrease the DNA binding activity and/or the cellular level of HIF-1. Therefore, we examined the levels of HIF-1 α protein in the remaining SC-M1 cells after collecting conditioned media. We found that hypoxia induced an increase in HIF-1 α expression of approximately 2.4-fold, but berberine blocked this induction (Fig. 5). These data might explain the decrease of protein-DNA complexes formation, as described in Fig. 4B, and supported the notion that by blocking hypoxia-induced HIF-1 α expression, berberine abrogated the accumulation of HIF-1 and the transactivation of VEGF gene and subsequently abolished hypoxia-induced VEGF expression. To examine whether HIF-1 α repression is critical for berberine to inhibit SC-M1-induced HUVEC migration, we examined whether overexpression of HIF-1 α reversed berberine's inhibitory effect on SC-M1-induced HUVEC migration. We established an SC-M1-HIF-1 α cell line that overexpresses

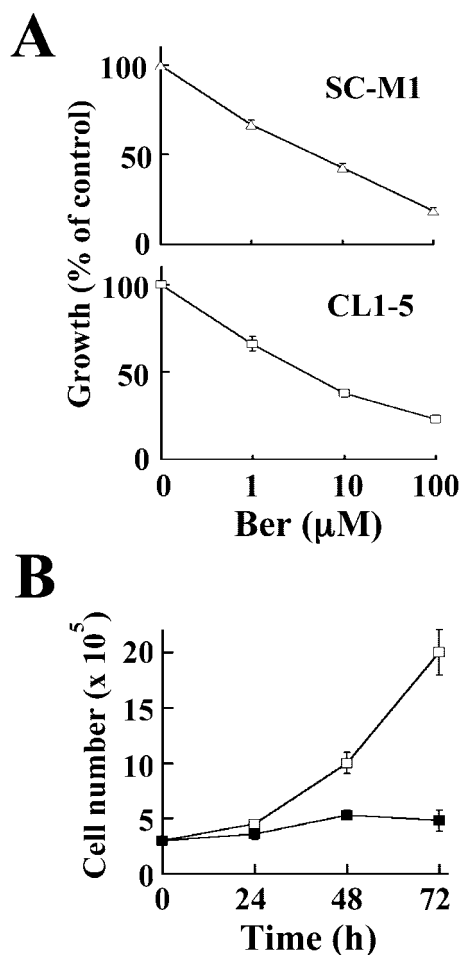


Fig. 2. Berberine inhibited the growth of SC-M1 and CL1-5 cells. A, SC-M1 and CL1-5 cells were treated with different doses of berberine (Ber) for 72 h and subjected to MTT assays to determine the IC_{50} for berberine to these cell lines. B, SC-M1 cells were either treated with 7.5 μ M berberine (■) or left untreated (□). At the times indicated, cells were harvested and counted. Trypan blue exclusion was used to distinguish between living and dead cells. Data represent the mean \pm S.D. of three independent experiments.

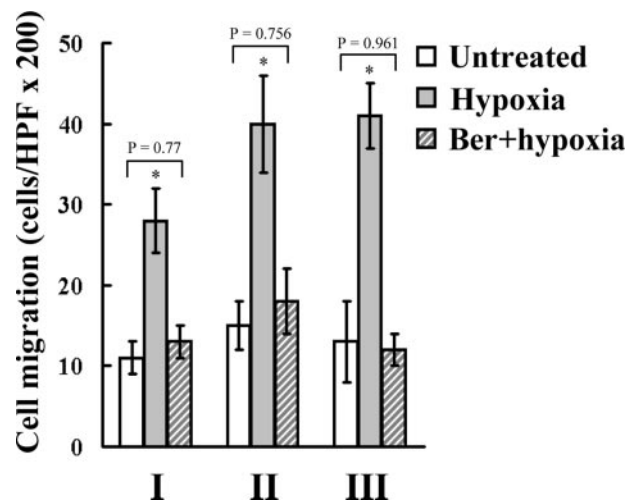


Fig. 3. Berberine inhibited hypoxic induction in SC-M1-induced HUVEC migration. HUVEC migration assays were performed using conditioned media prepared from SC-M1 cells that were either left untreated or exposed to hypoxia for 4 h or from cells pretreated with 7.5 μ M berberine (Ber) for 16 h and then exposed to hypoxia without berberine for 4 h. Three batches of conditioned media were assayed independently. Cell numbers were counted as described under *Materials and Methods* section. Each bar represents the mean \pm S.D. ($n = 3$). I, II, and III represent the batch number of conditioned media. *, $P < 0.01$ versus untreated cells.

HIF-1 α and an SC-M1-mock cell line that served as a control. Western blot analyses showed that under normoxic condition, the HIF-1 α level in SC-M1-HIF-1 α cells was slightly higher than that of parental SC-M1 and SC-M1-mock cells. Under hypoxic conditions (2% O₂, 4 h), however, SC-M1-HIF-1 α cells expressed much more HIF-1 α than the other two cell lines (Fig. 6A). Then, SC-M1-HIF-1 α and SC-M1-mock cells were either left untreated or treated with variant doses of berberine for 72 h and were subjected to MTT assays. The results showed that the IC₅₀ for berberine on both cell lines was around 7.5 μ M (Fig. 6B). Thereafter, both the SC-M1-HIF-1 α and SC-M1-mock cells were subjected to the preparation of conditioned media for HUVEC migration assays. As shown in Fig. 6C, conditioned media generated from normoxic SC-M1-HIF-1 α and SC-M1-mock cells elicited similar capability of supporting HUVEC migration (12.7 ± 2.1 and 12 ± 2.9 cells/HPF \times 200, respectively). The medium from hypoxic SC-M1-mock cells induced a 2.4-fold increase of HUVEC migration (29 ± 5.4 cells/HPF \times 200), but berberine abolished the induction (13.7 ± 5.8 cells/HPF \times 200). However, the medium from hypoxic SC-M1-HIF-1 α cells induced a 4.6-fold increase of HUVEC migration (58 ± 5.9 cells/HPF \times 200), and the medium from berberine-treated hypoxic SC-M1-HIF-1 α cells could still induce a 2.8-fold increase of HUVEC migration (35.3 ± 4.1 cells/HPF \times 200). These results showed that overexpression of HIF-1 α significantly reversed berberine's inhibitory effect on SC-M1-induced HUVEC migration, indicating that inhibition of hypoxia-induced HIF-1 α expression is a critical step for berberine to inhibit SC-M1-induced HUVEC migration.

Berberine Increased the Proteolysis of HIF-1 α Protein. To examine whether berberine down-regulated the expression of HIF-1 α mRNA in SC-M1 cells, we treated SC-M1 cells with berberine and then measured their cellular levels of HIF-1 α mRNA. Our data showed that berberine had no inhibitory effect on the HIF-1 α mRNA expression (Fig. 7),

suggesting that berberine might modulate the degradation of HIF-1 α protein in hypoxic SC-M1 cultures. Therefore, we sought to determine whether berberine antagonized the hypoxia-induced stabilization of HIF-1 α protein. First, SC-M1 cells, with or without berberine pretreatment, were pulse-labeled with [³⁵S]methionine and then chased with unlabeled methionine under hypoxia with or without berberine treatment for 0, 1, and 3 h. HIF-1 α proteins were immunoprecipitated, resolved by SDS-polyacrylamide gels, and the signals were detected. As shown in Fig. 8A, the cellular level of ³⁵S-labeled HIF-1 α protein in untreated cells was slightly higher than that of berberine-pretreated cells at 0 h after labeling. One hour after labeling, ³⁵S-labeled HIF-1 α levels were maintained in untreated cells but disappeared in berberine-treated cells. HIF-1 α signals were still detectable in untreated cells 3 h after labeling. These data showed that berberine increased the degradation of HIF-1 α protein. To find out whether proteasomal activity was involved in berberine-mediated HIF-1 α degradation, we examined whether inactivation of proteasomes restored the level of HIF-1 α protein in the berberine-treated hypoxic SC-M1 cells. We found that a proteasome inhibitor, *N*-carbobenzyloxy-leucine-leucine-leucine-aldehyde (*N*-CBZ-LLL-AL), was able to reverse the reduction of HIF-1 α protein in normoxic SC-M1 cells (Fig. 8B, lanes 3, 6, and 7) and in berberine-treated hypoxic cultures (Fig. 8B, lanes 2–5). These data implied that berberine might enhance the ubiquitination/proteasomal degradation of HIF-1 α protein. Thereafter, we searched for the underlying mechanism(s).

It was reported that acetylation on the Lys532 residue in the oxygen-dependent degradation domain of HIF-1 α protein facilitated the degradation of HIF-1 α protein (Jeong et al., 2002). In that report, the authors showed that, under normoxic conditions, a protein acetyltransferase, ARD1, catalyzed the Lys532-acetylation, resulting in enhanced HIF-1 α ubiquitination and degradation. Then, SC-M1 cells were in-

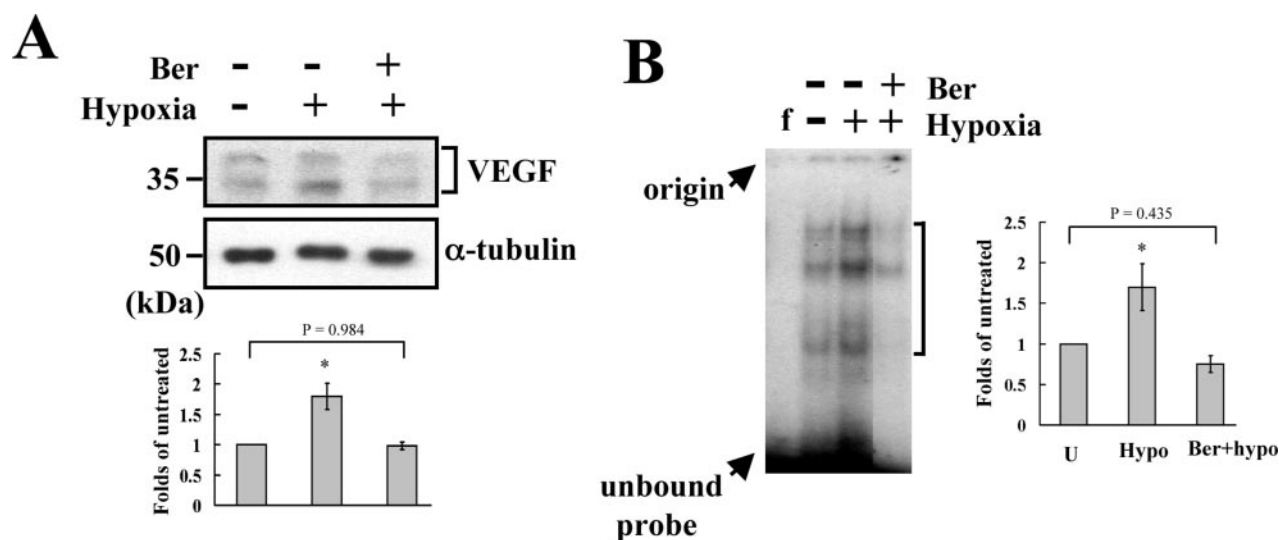


Fig. 4. Berberine decreased both VEGF expression and protein binding on the hypoxia-response element of VEGF. After the collection of conditioned media, the remaining SC-M1 cells were used in the following experiments. A, Western blot analysis. Cellular levels of VEGF and α -tubulin were determined. Signals were quantitated and normalized against α -tubulin measurements. Values, shown as the mean \pm S.D. from three independently performed experiments, represent the fold increase of VEGF in treated cells relative to that measured in untreated cells (to which a value of 1 was assigned). *, $P < 0.01$ versus control (untreated cells). B, EMSA assays. The nuclear fractions of SC-M1 cells were subjected to gel-shift analysis to detect their interactions with HRE. Bracket indicates protein-DNA complex formation. Binding signals were quantitated, and the fold increase relative to that of untreated cells was shown. Values represent the mean \pm S.D. ($n = 3$). f, radiolabeled DNA without incubation with cell lysate. U, untreated. *, $P < 0.01$ versus control.

cubated under hypoxic conditions with or without concomitant berberine treatment, and the cell lysates were subjected to either Western blot analyses for HIF-1 α , or immunoprecipitation experiments for Lys-acetylated-HIF-1 α . Using anti-acetyl-lysine antibody, we pulled down the cellular proteins whose lysine residues were acetylated. These immunoprecipitates were then subjected to Western blot analysis for HIF-1 α . Our data showed that in normoxic SC-M1 cells, Lys-acetylated HIF-1 α (Ac-HIF-1 α) was readily detected, but HIF-1 α was almost undetectable. Hypoxia increased HIF-1 α but decreased Ac-HIF-1 α . However, berberine inhibited hypoxia-induced HIF-1 α expression and restored Ac-HIF-1 α levels in hypoxic SC-M1 cells (Fig. 8C). Taken together, our data revealed that berberine enhanced the proteolysis of HIF-1 α protein, which was accompanied by the acetylation on lysine(s) of HIF-1 α protein.

Discussion

We have explored the antiangiogenic potential of berberine. Here, based on two lines of evidence, we report that berberine may serve as a potent antiangiogenic agent in cancer therapy. First, berberine inhibits the migration and differentiation of HUVECs in vitro (Fig. 1). The second evidence is from our modified confrontation culture experiments, in which HUVECs are incubated in serum-reduced, berberine-free conditioned media generated from SC-M1 cells. These experimental settings separate HUVECs from berberine's putative cytotoxicity and also minimize the stimulating effects of the serum-born growth factors on both cancer cells and HUVECs. Therefore, we were able to detect the antiangiogenic activity of berberine with improved accuracy. We show that as the conditioned medium from hypoxic SC-M1 cells stimulates HUVEC migration, berberine completely blocks this stimulation (Fig. 3). Taken together, our in vitro studies suggest that in vivo, berberine may abolish the angiogenic function of vascular endothelial cells, preventing

them from responding to the call for angiogenesis, and may also prevent hypoxic tumor cells from inducing angiogenesis. That the VEGF expression of the berberine-treated SC-M1 cells fail to respond to hypoxic induction has led to the iden-

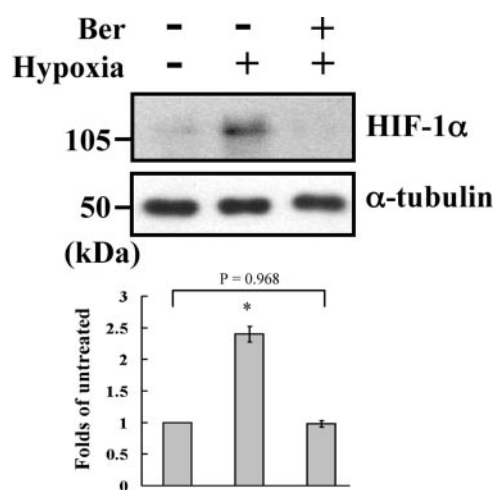


Fig. 5. Berberine inhibited hypoxia-induced HIF-1 α expression. After the collection of conditioned media, the remaining SC-M1 cells were used in Western blot analysis to detect the HIF-1 α protein. Signals were quantitated and normalized against α -tubulin measurements. Values, shown as the mean \pm S.D. from three independently performed experiments, represent the fold increase in treated cells relative to that measured in untreated cells (to which a value of 1 was assigned). Ber, berberine. *, $P < 0.01$ versus untreated cells.

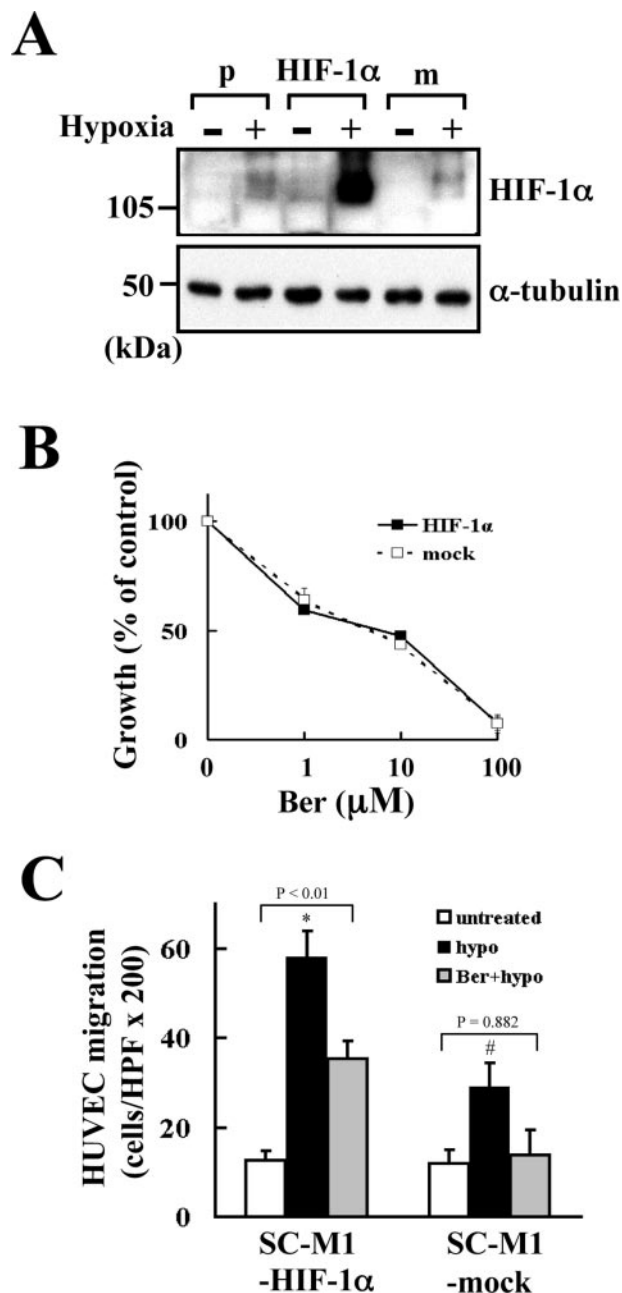


Fig. 6. Overexpression of HIF-1 α in hypoxic SC-M1 cells reversed the inhibitory effect of berberine on HUVEC migration. A, to confirm the transfection of SC-M1 cells by pCEP4/HIF-1 α , normoxic and hypoxic (4 h) cultures of parental (p), HIF-1 α -transfected (HIF-1 α), and mock-transfected (m) SC-M1 cells were subjected to Western blot analyses for HIF-1 α protein. B, HIF-1 α -transfected (HIF-1 α) and mock-transfected (mock) SC-M1 cells were treated with 0, 1, 10, and 100 μ M berberine for 72 h and subjected to MTT assays to determine the IC₅₀ of berberine on these cell lines. C, for in vitro HUVEC migration assays, HIF-1 α -transfected (SC-M1-HIF-1 α) and mock-transfected (SC-M1-mock) SC-M1 cells were either left untreated or treated with 7.5 μ M berberine (Ber) for 16 h and were subjected to the preparation of low-serum, berberine-free conditioned media. These media were examined for the ability to induce in vitro HUVEC migration. HUVECs migrated through filter were stained and counted. Each bar represents the mean \pm S.D. ($n = 3$). *, $P < 0.01$ versus untreated cells; #, $P < 0.05$ versus untreated cells.

tification of HIF-1 α as a pharmacological target of berberine. As shown, although hypoxia increases HIF-1 α expression (2.4-fold) in SC-M1 cells, the conditioned media from these cells stimulate a compatible increase of HUVEC migration (Figs. 3 and 5). The hypoxic induction of HIF-1 α expression and HUVEC migration is completely inhibited by berberine. Under the same experimental settings, overexpression of HIF-1 α enables the hypoxic SC-M1 cells to further stimulate HUVEC migration (4.6-fold; Fig. 6C). However, berberine only partially reverses the induction. To interpret these data, it is noteworthy that ectopic expression of HIF-1 α in SC-M1 cells does not change the IC₅₀ for berberine on the cells (Figs. 2A and 6B). Therefore, these data reveal a critical role of HIF-1 α in SC-M1-induced HUVEC migration; the higher the cellular level of HIF-1 α , the stronger the ability of SC-M1 cells to stimulate HUVEC migration. Based on these findings, we conclude that berberine prevents hypoxic SC-M1 cells from stimulating HUVEC migration, at least through inhibiting the hypoxia-induced HIF-1 α accumulation. Then why can't berberine completely prevent hypoxic SC-M1-HIF-1 α cells from stimulating HUVEC migration as it does to parental SC-M1 cells? One possible reason is that berberine cannot completely eliminate the excessive amount of HIF-1 α within the given period of time. It is possible that prolonged exposure to berberine may inhibit the accumulation of HIF-1 α , and completely block HUVEC migration stimulated by hypoxic SC-M1-HIF-1 α cells. It is well known that HIF-1 α is the regulatory subunit of HIF-1, which is the major transcription factor that mediates the cellular adaptive response

to hypoxia (Wang et al., 1995; Semenza, 2001). HIF-1 can transactivate genes such as VEGF, erythropoietin, glucose transporters 1 and 3, and hexokinases, which are essential for angiogenesis, oxygen transport, and glycolysis, to help cells survive the harsh microenvironment (Semenza, 2000). This information and our findings indicate that inhibition of hypoxia-induced HIF-1 α expression is an important mechanism underlying berberine's inhibitory effect on tumor angiogenesis. In addition, that many genes share the HIF-1 transcriptional activity may explain why in this study hypoxia induces a 2.4-fold increase of HIF-1 α but an incompatible increase (1.8-fold) of VEGF in SC-M1 cells. On the other hand, prior studies have revealed the anti-inflammatory, antiarrhythmic, and antimicrobial effects of berberine, which suggests that berberine may regulate/modify the expression or function of multiple categories of genes or subcellular molecules to exert the corresponding biological effects. So then, it is conceivable that a certain biological effect of berberine, antiangiogenesis for example, may result from the interplay of several pharmacological activities of this compound. In this study, berberine inhibits the proliferation of SC-M1 cells (Fig. 2), suggesting that berberine may inhibit the function of cell cycle machinery. It is possible that by growth arrest of SC-M1 cells, berberine influences their global functions, which may also contribute to berberine's inhibitory effect on tumor angiogenesis.

It was reported that oxygen tensions regulate HIF-1 α expression through modulating the stability of HIF-1 α protein but not the expression of HIF-1 α mRNA. Under normoxic atmosphere, HIF-1 α protein is quickly ubiquitinated and degraded by proteasome, whereas under hypoxic condition, ubiquitination and proteasomal degradation pathways are inhibited, resulting in the accumulation of HIF-1 α protein

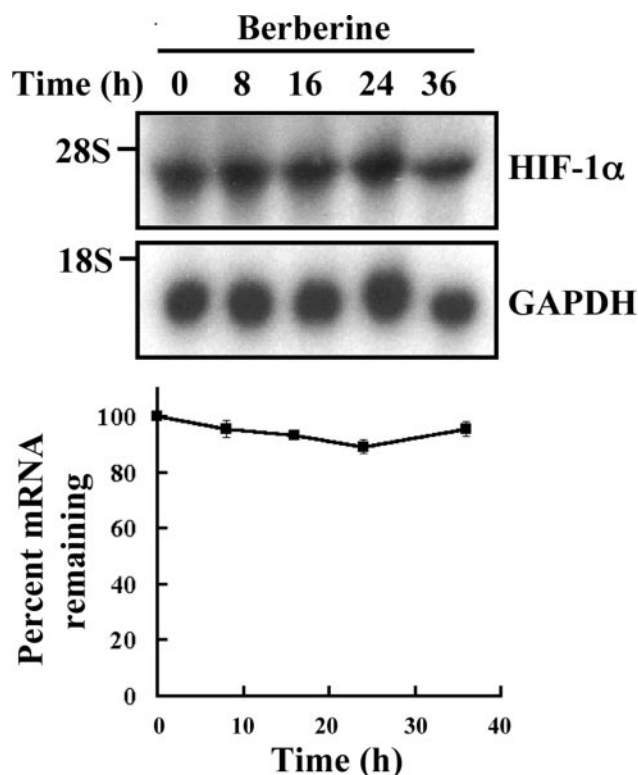


Fig. 7. RNA analysis of HIF-1 α . SC-M1 cells were either left untreated or treated with 7.5 μ M berberine for 8, 16, 24, and 32 h. Northern blot analyses were performed to detect the expression of HIF-1 α mRNA in SC-M1 cells exposed to berberine. The HIF-1 α signals were quantitated and normalized to those of GAPDH. Data represent mean \pm S.D. of three separate experiments.

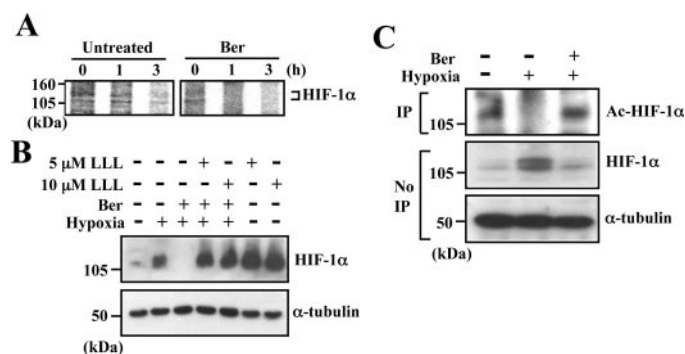


Fig. 8. Berberine enhanced the proteolysis of HIF-1 α . **A**, pulse-chase assays. SC-M1 cells were either treated with 7.5 μ M berberine (Ber) or left untreated for 12 h and then subjected to methionine starvation as described under *Materials and Methods* section. After being labeled with [³⁵S]methionine, berberine (7.5 μ M) was added back to the berberine-pretreated cells, and both untreated and berberine-treated cells were incubated under hypoxic conditions for 0, 1, and 3 h. Cells were then lysed, immunoprecipitated with anti-HIF-1 α antibody, and separated on polyacrylamide gels. Signals were detected with a PhosphorImager. **B**, cells were either left untreated, treated with hypoxia alone for 4 h, or pretreated with N-CBZ-LLL-AL (LLL, 5 or 10 μ M), berberine (Ber, 7.5 μ M), or berberine plus N-CBZ-LLL-AL for 16 h and followed by 4 h of hypoxia. These cells were then subjected to Western blot analyses for HIF-1 α protein. **C**, SC-M1 cells were either left untreated or treated with hypoxia for 4 h or pretreated with 7.5 μ M berberine for 16 h plus 4 h of hypoxia. Cell lysates (500 μ g) were immunoprecipitated with anti-acetyl lysine antibody, and the precipitates were subjected to Western blot analyses for Ac-HIF-1 α protein. Parallel Western blot analyses using 40 μ g of lysate were performed to show the cellular level of HIF-1 α protein. IP, immunoprecipitation.

(Cockman et al., 2000; Semenza, 2001). In this study, we show that berberine may not down-regulate the transcription of HIF-1 α gene. Instead, berberine decreases the half-life of HIF-1 α protein by facilitating the ubiquitination/proteasomal degradation process. So far, phosphorylation-, hydroxylation-, and acetylation-dependent ubiquitination have been found to play a role in oxygen-triggered HIF-1 α degradation (Laney and Hochstrasser, 1999; Jeong et al., 2002; Metzen et al., 2003; Linke et al., 2004). The acetylation pathway per se requires the acetylation of the Lys532 in the oxygen-dependent degradation domain of HIF-1 α protein, which is catalyzed by ARD1. In this study, we show that hypoxia induces HIF-1 α and reduces the cellular level of Lys-acetylated HIF-1 α , but berberine inhibits the accumulation of HIF-1 α and concomitantly increases the Lys-acetylated HIF-1 α protein (Fig. 8C). Whether berberine induces Lys532 acetylation is currently unknown. However, our data indicate that acetylation of lysine residue(s) may play an important role in berberine-triggered HIF-1 α degradation. Therefore, it is possible that berberine may stimulate ARD1 activity to cause Lys532 acetylation in HIF-1 α protein during hypoxia, and hence the facilitated ubiquitination/degradation of HIF-1 α . This hypothesis has yet to be confirmed.

As far as the signaling pathway(s) is concerned, prior studies have implicated phosphatidylinositol 3-kinase (PI3K)/Akt, extracellular signal-regulated kinase (ERK), and epidermal growth factor receptor signaling in the regulation of HIF-1 α expression. Activation of the PI3K/Akt pathway results in the stabilization of HIF-1 α protein, and activation of ERK signaling increases HIF-1 transcriptional activity (Richard et al., 1999; Jiang et al., 2001). EGF induces HIF-1 α expression via PI3K activation (Jiang et al., 2001), whereas inhibition of epidermal growth factor receptor tyrosine kinase activity inhibits tumor angiogenesis (Woodburn, 1999; Raymond et al., 2000; Hirata et al., 2002). Consistent with these findings, our preliminary data show that berberine-triggered HIF-1 α degradation is accompanied by the blockade of both ERK and PI3K/Akt signaling (data not shown), suggesting their involvement in berberine's antiangiogenic activity. And because PI3K plays a key role in EGF-induced HIF-1 α expression, by blocking PI3K/Akt signaling and HIF-1 α expression, berberine may block EGFR signaling. Therefore, berberine's inhibitory effect on tumor angiogenesis involves multiple genes and signaling pathways.

In summary, we have revealed the antiangiogenic potential of berberine using angiogenic cell models. Although the ability to inhibit endothelial cell tube formation and migration in vitro may not predict in vivo response, our studies disclose that berberine can retard cell proliferation, target HIF-1 α for degradation, and inhibit hypoxia-induced VEGF expression, which indicate that berberine may act as a chemotherapeutic agent and a potent inhibitor of tumor angiogenesis. Based on our findings, we suggest further verification of berberine's antiangiogenic activity as well as its ultimate inhibitory effects on tumor growth and metastasis using animal models. Given that HIF-1 α is a critical regulator of hypoxic adaptive response, our findings point out that administration of berberine may benefit cancer treatment but may on the other hand contradict the treatment of ischemic lesions. We found that lysine acetylation is involved in berberine-triggered HIF-1 α degradation. This finding suggests that acetyltransferases, such as ARD1, and their reg-

ulatory molecules may play a role in carrying out the inhibitory effect of berberine on tumor angiogenesis.

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