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ARTICLE

Hispolon Suppresses SK-Hep1 Human Hepatoma Cell Metastasis by Inhibiting Matrix Metalloproteinase-2/9 and Urokinase-Plasminogen Activator through the PI3K/Akt and ERK Signaling Pathways

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Cancer metastasis is a primary cause of cancer death. Hispolon is an active phenolic compound of *Phellinus linteus*, a mushroom that has recently been shown to have antioxidant and anticancer activities. In this study, we first observed that hispolon exerted a dose-dependent inhibitory effect on invasion and motility, but not on adhesion, of the highly metastatic SK-Hep1 cells in the absence of cytotoxicity. Mechanistically, hispolon decreased the expression of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), and urokinase-plasminogen activator (uPA) in a concentration-dependent manner. Hispolon also inhibited phosphorylation of extracellular signaling-regulating kinase1/2 (ERK1/2), phosphatidylinositol-3-kinase/serine/threonine protein kinase (or protein kinase B (PI3K/Akt), and focal adhesion kinase (FAK). Furthermore, treatment of SK-Hep1 cells with an inhibitor specific for ERK1/2 (PD98256) decreased the expression of MMP-2, and MMP-9. These results demonstrate that hispolon can inhibit the metastasis of SK-Hep1 cells by reduced expression of MMP-2, MMP-9, and uPA through the suppression of the FAK signaling pathway and of the activity of PI3K/Akt and Ras homologue gene family, member A (RhoA). These findings suggest that hispolon may be used as an antimetastatic agent.

KEYWORDS: Hispolon; *Phellinus linteus*; MMP-2; MMP-9; uPA

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common forms of malignancy in Southeast Asia, including Taiwan. Metastasis is a characteristic of highly malignant cancers with poor clinical outcome. Malignant tumor progression depends upon the capacity to invade, metastasize, and promote the angiogenic host response. One critical characteristic that metastatic cancer cells have acquired is the ability to dissolve basement membranes and the extracellular matrix (ECM). This degradative process is mediated largely by matrix metalloproteinases (MMPs), which are a large family of at least 20 zinc-dependent neutral endopeptidases that together can degrade all known components of ECM (*1*). MMP-9 is abundantly expressed in various malignant tumors and is postulated to play a critical role in tumor invasion and angiogenesis (*2*). Thus, the inhibition of MMP activity, including MMP-9, is important for the prevention of cell invasion. Interestingly, MMP-9 expression levels are especially high in hepatoma cells, such as SK-Hep-1 cells, and the enzyme has been studied in diverse malignant tumor cells because of its inducible character (*3*). In addition, urokinase plasminogen activator (uPA) is a 55 kDa serine protease, which is secreted as an inactive pro-enzyme (pro-uPA). It seems that activation of pro-uPA mostly occurs after binding to its receptor uPAR (uPA receptor). Plasminogen activator inhibitors (PAI-1 and PAI-2) inhibit both receptor-bound and free uPA. uPA may activate a series of protein degradation reaction to regulate or activate MMPs (*4*). Meanwhile, the activity of MMPs is prone to the inhibition of endogenous tissue inhibitor of metalloproteinases (TIMPs), which are specific inhibitors of MMPs, and the imbalance between MMPs and TIMPs may contribute to degradation or deposition of ECM (*5*). The mitogen-activated protein kinases (MAPK) play an important regulatory role in cell growth, differentiation, apoptosis, and metastasis (*6*). In addition, phosphatidylinositol-3-kinase/serine/threonine protein kinase (or protein kinase B) (PI3K/Akt) signal transduction pathway is involved in the development, progression, and metastasis of various tumors (*7*).

Phellinus linteus (Berk. & M.A. Curt.) (PL) is a mushroom that belongs to the genus *Phellinus* and is commonly called "Sangwhang" in Taiwan. It is popular in oriental countries and has been traditionally used as food and medicine. PL contains many bioactive compounds, and is known to improve health and to prevent and remedy various diseases such as gastroenteric disorders, lymphatic

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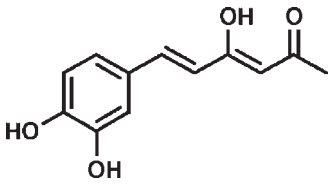

Figure 1. Chemical structure of hispolon.

diseases, and cancer (*8*). Recently, a few pharmacological actions of PL have been elucidated. For instance, the ethanolic extract of PL was shown to have antiproliferative and antimetastatic effects (*9*). In addition, the ethanolic extract of PL was shown to be antimutagenic, partly by inducing quinone oxidoreductase and glutathione S-transferase activities and by increasing glutathione levels (*10*). We recently reported that hispolon, a polyphenol compound isolated from PL, inhibits foot paw edema in mice (*11*). Others have also shown that hispolon has antiproliferative and immunomodulatory activities (*12*, *13*). However, there have been no reports on the antimetastatic effects of hispolon. In the present study, we investigated the antimetastatic effects of hispolon on a highly metastatic human hepatocarcinoma SK-Hep1 cells as well as the underlying mechanistic actions of hispolon in vitro.

MATERIALS AND METHODS

Chemicals. Dulbecco's Modified Eagle's Medium (DMEM), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), RNase A, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin−EDTA, fetal bovine serum (FBS), and penicillin/streptomycin were from Gibco Life Technologies, Inc. (Paisley, UK). Cell culture supplies were purchased from Costar (Corning, Inc., Cypress, CA). The antibody against Akt, RhoA, MAPK/extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). Anti-MMP-2, anti-ERK1/2, anti-PI3K, antifocal adhesion kinase (FAK), anti-pFAK, and antiplasminogen activator inhibitor-1 (PAI)-1 mouse monoclonal antibody and horseradish peroxidase-conjugated goat antimouse IgG antibody were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA). Hispolon (**Figure 1**) was synthesized as described, and its purity was established on the basis of the spectra (^{1}H, ^{13}C NMR and mass) (*14*).

Hispolon: ^{1}H NMR (DMSO, 400 MHz) δ 2.08 (s, 3 H, CH_3), 5.83 (s, 1H, CH), 6.44 (d, 1 H, J = 16 Hz, CH), 6.75 (d, 1 H, J = 8.2 Hz, ArH), 6.96 (dd, 1 H, J = 8.2, 2.0 Hz, ArH), 7.04 (d, 1 H, J = 2.0, ArH), 7.39 (d, 1 H, J = 16, CH), 9.20 (s, 1 H, OH), 9.62 (s, 1 H, OH). ^{13}C NMR (100 MHz, DMSO) δ 26.5, 100.6, 114.7, 116.0, 119.5, 121.6, 126.5, 140.5, 145.8, 148.4, 178.5, 196.7.

Cell Culture. The hepatocarcinoma SK-Hep-1 cell was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a CO_2 incubator (5% CO_2 in air) at 37 °C and subcultured every 2 days at a dilution of 1:5 using 0.05% trypsin−0.02% EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (DPBS).

Cell Viability. The cells (2×10^5) were cultured in 96-well plate containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultured with hispolon for 24 h. Then, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37 °C, testing for cell viability. The medium was then discarded and 100 μL of dimethylsulfoxide (DMSO) was added. After 30 min incubation, absorbance at 570 nm was read by a microplate reader.

Cell Migration Assay. Tumor cell migration was assayed in transwell chambers (Millipore) according to the method reported by Hung and Chang (2009) (*15*) with some modifications. Briefly, transwell chambers with 6.5 mm polycarbonate filters of 8 μm pore size were used. SK-Hep-1 cells (5×10^5 mL^{-1}) and 0−45.7 μM of hispolon were suspended in DMEM (100 μL, serum free), placed in the upper transwell chamber, and incubated for 24 h at 37 °C. Then, the cells on the upper surface of the filter were completely wiped away with a cotton swab, and the lower surface of the filter was fixed in methanol, stained with Giemsa, and counted under a microscope at a magnification of 200×. For each replicate, the tumor cells in 10 randomly selected fields were determined and the counts were averaged.

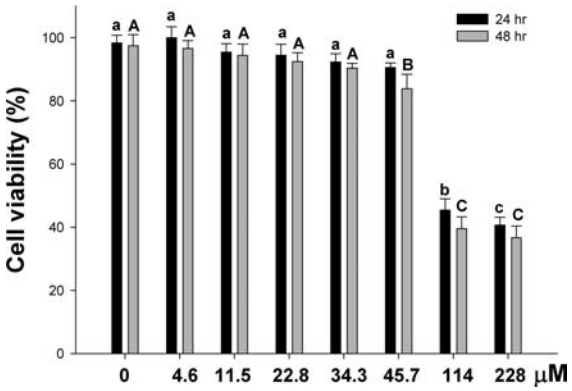

Figure 2. Viability of SK-Hep1 cells incubated with hispolon (4.6, 11.5, 22.8, 34.3, 45.7, 114, and 228 μM) for 24 and 48 h. Cell viability was measured using MTT assay and is expressed as % of cell survival relative to control. Values (means ± SD, n = 3) differ significantly (P < 0.05) when not sharing an alphabetic letter (lower case for 24 h and upper case for 48 h).

Cell Invasion Assay. The invasion of tumor cells was assessed in transwell chambers with a 6.5 mm polyvinyl/pyrrolidone-free polycarbonate filter of 8 μm pore size, as described in the cell migration assay (*15*), except that each filter was coated with 100 μL of a 1:20 diluted matrigel in cold DMEM to form a thin continuous film on the top of the filter. The number of cells was adjusted to 5×10^5 /mL and 100 μL (containing 5×10^4 cells) was transferred to each of triplicate wells in DMEM containing 10% FBS. After incubation for 24 h, the cells were stained and counted as described above, and the number of cells invading the lower side of the filter was measured.

Cell Adhesion Assay. Each well of a 24-well tissue culture plate was coated with 25 g/well Matrigel and left to air-dry for 40 min. SK-Hep1 cells (5×10^4) suspended in DMEM containing 0.5% bovine serum albumin were then dispensed into each well. The plate was incubated in 5% CO_2 at 37 °C for 1 h and then gently washed thrice with PBS to remove unattached cells. Attached cells were then stained with hematoxylin and eosin reagent and counted under a microscope (Eclipse TS100, Nikon, Japan). At least four independent experiments were performed.

Wound-Healing Assay. For cell motility determination, SK-Hep1 cells (5×10^4) were seeded in a 6-well tissue culture plate and grown to 80−90% confluence. After aspiration of the medium, the center of the cell monolayers was scraped with a sterile micropipet tip to create a denuded zone (gap) of constant width. Subsequently, cellular debris was washed with PBS, and SK-Hep1 cells were exposed to various concentrations of hispolon (0, 11.5, 22.8, and 45.7 μM). Wound closure was monitored and photographed at 0, 12, and 24 h with a Nikon inverted microscope. To quantify the migrated cells, pictures of the initial wounded monolayers were compared with the corresponding pictures of cells at the end of the incubation. Artificial lines fitting the cutting edges were drawn on pictures of the original wounds and overlaid on the pictures of cultures after incubation. Cells that had migrated across the white lines were counted in six random fields from each triplicate treatment.

Determination of MMP-2, MMP-9, and uPA by Zymography. MMP in the medium released from SK-Hep-1 cells was assayed using gelatin zymography (7.5% zymogram gelatin gels) according to the methods reported by Liao et al. (2006) (*16*) with some modification. Briefly, the culture medium was electrophoresed (120 V for 90 min) in a 10% SDS-PAGE gel containing 0.1% gelatin. The gel was then washed at room temperature in a solution containing 2.5% (v/v) Triton X-100 with two changes and subsequently transferred to a reaction buffer for enzymatic reaction containing 1% NaN_3, 10 mM $CaCl_2$ and 40 mM Tris−HCl, pH 8.0, at 37 °C with shaking overnight (for 12−15 h). Finally, the MMP gel was stained for 30 min with 0.25% (w/v) Coomassie blue in 10% acetic acid (v/v) and 20% methanol (v/v) and destained in 10% acetic acid (v/v) and 20% methanol (v/v).

Visualization of uPA activity was according to the methods reported by Huang et al. (2006) (*17*). Briefly, 2% w/v casein and 20 mg/mL plasminogen

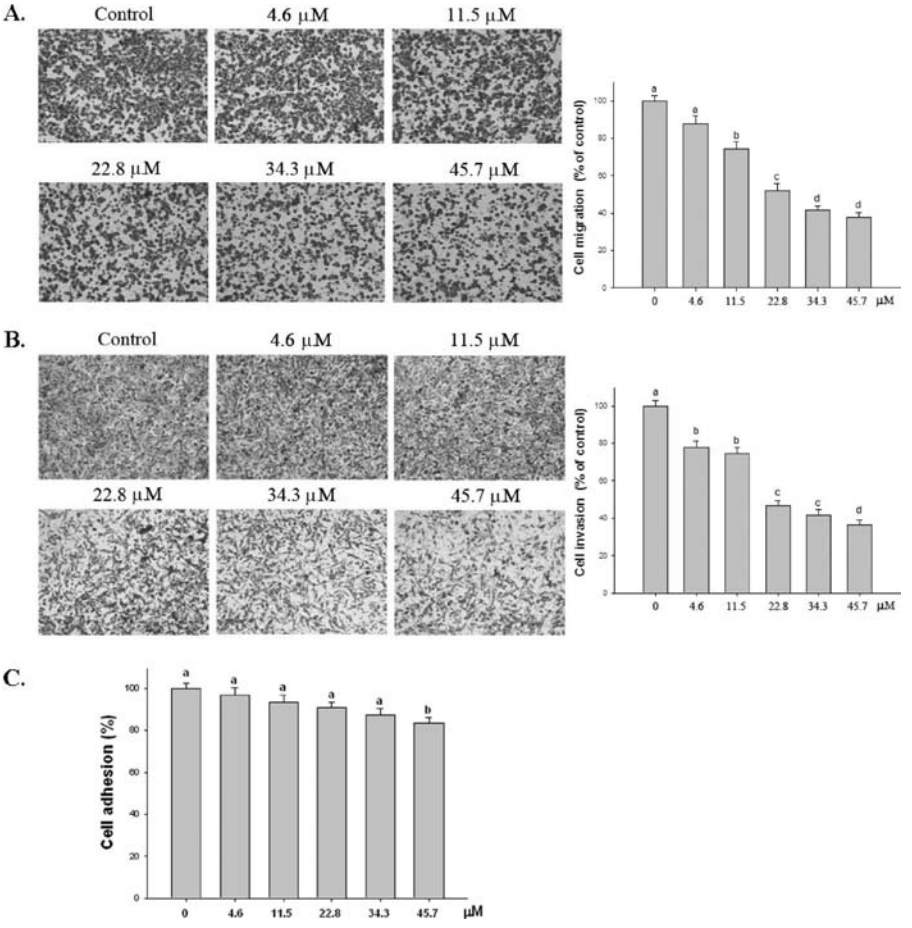

Figure 3. Effects of hispolon on transwell migration (**A**), invasion (**B**), and adhesion (**C**) assay of SK-Hep1 cells. SK-Hep1 cells were incubated with hispolon (4.6, 11.5, 22.8, 34.3, and 45.7 μM) for 24 h, and the transwell migration cells and invasion cells were calculated. Photos of the migration and invasive SK-Hep 1 cells were taken under a microscope (100-fold magnification). Then a portion (100 μL) of the cell suspension (5×10^5 cells/mL) was added to the wells coated with Matrigel, and the plates were incubated at 37 °C for 30 min in the cell−matrix adhesion assay for which the medium was used to determine the number of adherent cells by the MTT assay. Values (means ± SD, $n = 3$) not sharing an alphabetic letter differ significantly ($P < 0.05$).

were added to 8% SDS−PAGE gels. Samples with a total protein of about 20 μg were then loaded onto the gels. The uPA activity of cells treated or untreated with hispolon was measured as described in the gelatin zymography. The relative MMP-2, MMP-9, and uPA activity were quantified by Kodak Molecular Imaging software (version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.

Preparation of Whole-Cell Lysates. SK-Hep1 cells (1×10^5 cells) were plated in a 100 mm Petri dish and were treated with various concentrations of hispolon. SK-Hep1 cells were washed twice with PBS and were scraped into a microcentrifuge tube. The cells were centrifuged at 1250*g* for 5 min, and the pellet was lysed with iced-cold RIPA (radio-immunoprecipitation assay) buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5), to which was added freshly prepared phenylmethylsulfonyl fluoride (10 mg/mL), leupeptin (17 mg/mL), and sodium orthovanadate (10 mg/mL). After incubation for 5 min on ice, the samples were centrifuged at 10000*g* for 10 min, and then the supernatants were collected as whole-cell lysates. The lysates were denatured and subjected to SDS-PAGE and Western blotting. The protein content was determined with Bio-Rad protein assay reagent using BSA as a standard.

Western Blotting Analysis. Whole-cell lysates proteins (30−50 μg of partially purified protein) were mixed with an equal volume of electrophoresis sample buffer, and the mixture was then boiled for 10 min. Then, an equal protein content of total cell lysate from control, 0.2% DMSO, and hispolon-treated sample were resolved on 10−12% SDS-PAGE gels. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bedford, MA) by electroblotting using an electroblotting apparatus (Bio-Rad). Nonspecific binding of the membranes was blocked with Tris-buffered saline (TBS) containing 1% (w/v) nonfat dry milk and 0.1% (v/v) Tween-20 (TBST) for more than 2 h. Membranes were washed with TBST three times each for 10 min and then incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4 °C. The membranes were washed with TBST and then incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated, goat antimouse, or antirabbit IgG) for 1 h. After washing the membrane three times for 10 min in TBST, the bands were visualized using ECL reagents (Millipore, Billerica, MA). Band intensity on scanned films was quantified using Kodak Molecular Imaging (MI) software and expressed as relative intensity compared with control.

Statistical Analysis. Values are expressed as means ± SD and analyzed using one-way ANOVA followed by LSD test for comparisons of group means. All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a P value < 0.05 is considered statistically significant.

RESULTS

Cytotoxicity of Hispolon to SK-Hep1 Cells. The effect of hispolon (0−228.3 μM) on cell viability was determined by the MTT assay. After incubation for 24 and 48 h, cell viability was not significantly affected by hispolon (4.6−45.7 μM), as compared to the untreated control (**Figure 2**), indicating that hispolon is not toxic to SK-Hep1 cells at these concentrations. When cells were treated with hispolon at 114 and 228 μM for 24 and 48 h, cell viability was significantly decreased. To avoid inhibition of cell viability in the following experiments, we chose to use hispolon concentrations between 4.6 and 45.7 μM and an incubation time of 24 h.

Effect of Hispolon on Migration, Invasion, and Adhesion of SK-Hep1 Cells in Vitro. The transwell assay was used to investigate the migration and invasion of SK-Hep1 cells at 24 h after hispolon

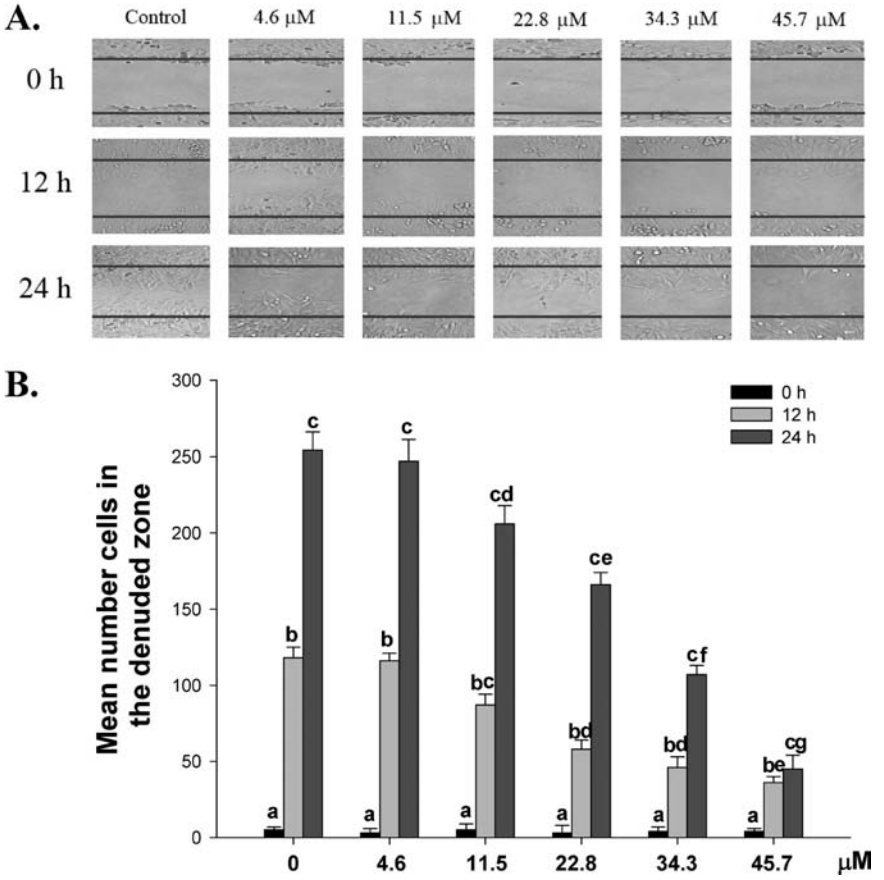

Figure 4. Effects of hispolon on wound healing migration of SK-Hep1 cells. Wound was introduced by scraping confluent cell layers with a pipet tip. SK-Hep1 cells were incubated with hispolon (0, 4.6, 11.5, 22.8, 34.3, and 45.7 μM) for 0, 12, and 24 h, and the migration distances of cells were calculated. (**A**) Representative photographs of invading cells that received either control or hispolon treatment. (**B**) Migrated cells across the black lines were counted in six random fields from each treatment. The mean number of cells in the denuded zone is quantified by three independent experiments. Values (means ± SD n = 3) not sharing an alphabetic letter differ significantly (P < 0.05).

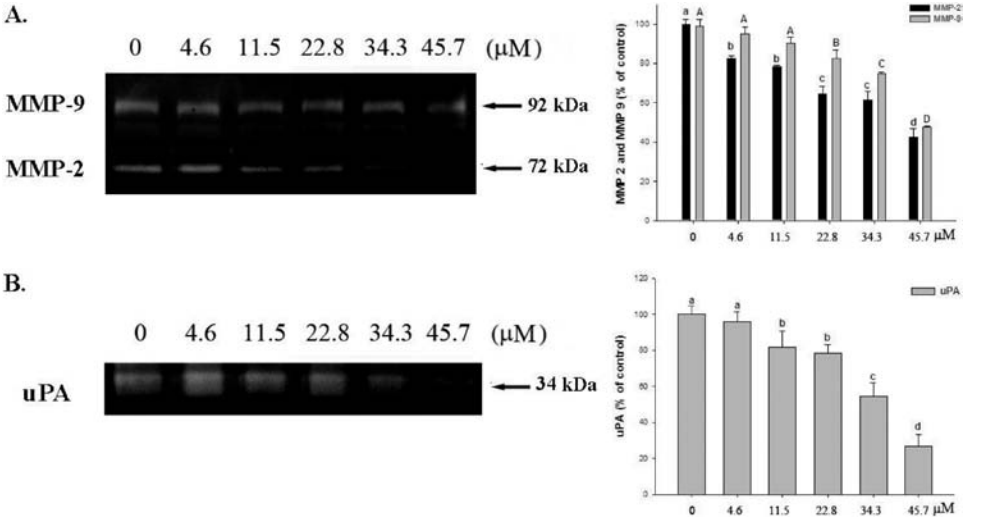

Figure 5. Effects of hispolon on MMP-2, MMP-9 (**A**), and uPA (**B**) activities of SK-Hep 1 cells. Cells were treated with various concentrations (0, 4.6, 11.5, 22.8, 34.3, and 45.7 μM) of hispolon for 24 h. The conditioned media were collected, and MMP-2, MMP-9, and uPA activities were determined by gelatin or casein zymography and quantified by densitometric analysis. Values (means ± SD, n = 3) differ significantly (P < 0.05) when not sharing an alphabetic letter (lower case for MMP-2 and upper case for MMP-9).

treatment. We found that hispolon added at 0−45.7 μM significantly decreased both the migration and invasion (**Figure 3A**,**B**) of SK-Hep-1 cells and that these effects of hispolon were dose-dependent. The IC_{50} values on migration and invasion by hispolon was approximately 23.6 and 21.4 μM, respectively. Because cell−matrix interaction is important for cancer cell invasion, cell−matrix adhesion assay was performed to study the effect of hispolon on cell adhesion. The results showed that hispolon, even added at 45.7 μM, only caused a slight reduction in cell adhesion (**Figure 3C**).

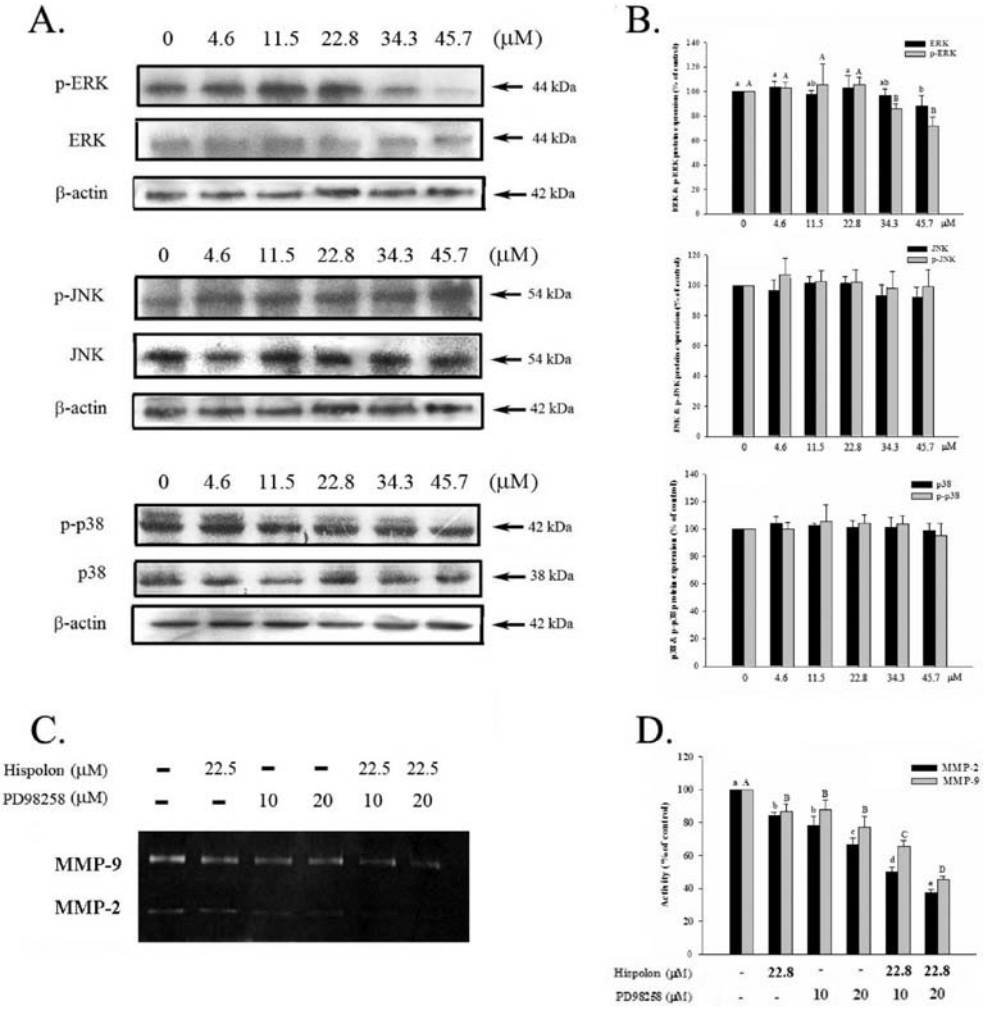

Figure 6. Inhibitory effect of hispolon on MAPK signaling. (**A**) Western blots of cell lysates of SK-Hep1 cells incubated with hispolon (0, 4.6, 11.5, 22.8, 34.3, and 45.7 μM) for 24 h. (**B**) Densitometric analysis of (**A**). (**C**) MMP-9 and MMP-2 activities in cells pretreated with or without 10 or 20 μM ERK inhibitor (PD98256) for 2 h followed by incubation in the presence or absence of 22.8 μM hispolon for 18 h. The culture media were subjected to gelatin zymography for the analysis of MMP-9 and MMP-2 activity. (**D**) Densitometric analysis of **C**. Values (means ± SD, $n = 3$) differ significantly ($P < 0.05$) when not sharing an alphabetic letter (lower case for ERK in (**B**) and for MMP-2 in (**D**); upper case for p-ERK in Figure (**B**) and for MMP-9 in Figure (**D**)).

Effect of Hispolon on Wound Healing Assay. We further assessed the effect of hispolon on the migration of SK-Hep1 cells using the wound healing assay in which the confluent monolayer was scraped with a sterile micropipet tip to create a scratch wound. As shown in **Figure 4A**, hispolon inhibited the migration of SK-Hep1 cells in a dose-dependent manner, with 69.5% and 82.3% inhibition at 45.7 μM after incubation for 12 and 24 h, respectively (**Figure 4B**).

Hispolon Inhibits the Release of MMP-2, MMP-9, and uPA in SK-Hep1 Cells. To examine the possible antimetastatic mechanisms of hispolon, we determined the activity of MMP-2, MMP-9, and uPA in culture media of SK-Hep1 cells by zymographic analysis. In the absence of treatment, SK-Hep1 cells constitutively secreted high levels of MMP-9 and relatively low levels of MMP-2. As shown in **Figure 5A**, hispolon inhibited MMP-9 and MMP-2 activities in a concentration-dependent manner, with 52.6% and 67.6% inhibition at 45.7 μM after incubation for 24 h.

Similarly, uPA activity was also inhibited in a concentration-dependent manner by hispolon treatment, with 76.1% inhibition at 45.7 μM after incubation for 24 h (**Figure 5B**). These results suggest that the antimetastatic effect of hispolon is related to the inhibition of the enzymatically degradative processes of tumor metastasis.

Inhibition by Hispolon of ERK Phosphorylation. We analyzed the phosphorylation of MAPKs in SK-Hep1 cells after treatment with hispolon (0−45.7 μM) for 3 h. Data in **Figure 6A**,**B** showed that hispolon did not significantly affect JNK, p-JNK, or p38 and p-p38 proteins, but it significantly decreased p-ERK and ERK proteins at higher concentrations used (45.7 μM hispolon resulted in a 28% reduction in the amount of phosphorylated ERK1/2).

To further delineate whether the inhibition of cell migration by hispolon occurs mainly through inhibition of ERK1/2 signaling, SK-Hep1 cells were pretreated with a ERK1/2 inhibitor (PD98256; 10 or 20 μM) and then incubated in the presence or absence of hispolon (22.8 μM) for 18 h. The zymographic assays revealed that hispolon or PD98256 (10 μM) alone decreased MMP-2 activity by 16.4 and 19.6%, respectively, and decreased MMP-9 activity by 13.4 and 24.8%, respectively. Furthermore, the combined treatment (10 μM PD98256 plus 22.8 μM hispolon) resulted in enhanced inhibition of MMP-2 and MMP-9 by 34.2% and 52.8%, respectively (**Figure 6C**,**D**).

Effects of Hispolon on the PI3K/Akt Signaling in SK-Hep1 Cells. PI3Ks are a group of ubiquitously expressed lipid kinases which are important players in a major pathway of cell signaling. The PI3K/Akt pathway has been identified as a major regulator of cellular proliferation, differentiation, and death in multiple cell types (*18*). To further investigate the involvement of PI3K/Akt, a series of experiments was performed to measure the expression of

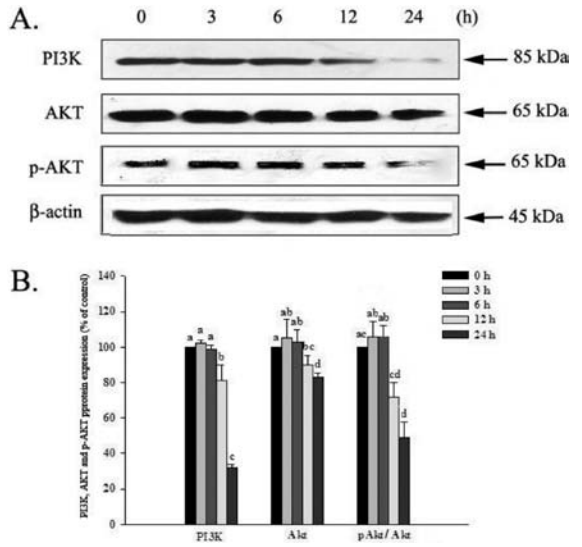

Figure 7. Time-dependent effects of hispolon on the protein expression level of PI3K and the phosphorylated Akt. (**A**) In the time-dependent assay, SK-Hep1 cells were treated with 45.7 μM of hispolon for 0, 3, 6, 12, and 24 h. The expression of PI3K and phosphorylation of Akt were analyzed by Western blotting. β-actin was used as a loading control. (**B**) Densitometric analyses of (**A**) (using control as 100%). Values (means $\pm$ SD, $n = 3$) not sharing an alphabetic letter differ significantly ($P < 0.05$).

candidate signaling molecules upon hispolon stimulation. The results showed that incubation of SK-Hep1 cells with hispolon (45.7 μM) led to a time-dependent decrease of PI3K and pAkt levels (**Figure 7A**). Notably, after incubation for 24 h, hispolon significantly suppressed PI3K and pAkt levels by 75.5% and 50.4%, respectively (**Figure 7B**).

Effect of Hispolon on TIMPs, PAI-1, FAK, and RhoA Expressions in SK-Hep1 Cells. To further explore the modulation of pro-MMP activation by hispolon, we determined TIMP-1/2 protein expression levels. As shown in **Figure 8A**, hispolon strongly increased TIMP-1 and TIMP-2 activity in a concentration-dependent manner. In addition, hispolon increased protein expression of PAI-1, and the effect was concentration-dependent (**Figure 8B**).

To evaluate the effect of hispolon on FAK and RhoA protein expression, SK-Hep1 was treated with hispolon at 0−45.7 μM for 24 h. As shown in **Figure 8C**, hispolon suppressed FAK expression, when hispolon concentration was higher than 22.8 μM. In addition, hispolon significantly suppressed the phosphorylation of FAK (pFAK) in SK-Hep1 cells. Similarly, hispolon (0−45.7 μM, 24 h) suppressed RhoA protein expression in a concentration-dependent manner, and the effect became statistically significant when hispolon concentration was higher than 22.8 μM, (**Figure 8D**).

DISCUSSION

In this study, we explored the antimetastatic effects and mechanistic actions of hispolon in human hepatoma SK-Hep1 cells. We found that hispolon significantly inhibited the invasion (assessed using the transwell assay) and migration (assessed using the transwell assay and the wound-healing assay) of SK-Hep1 cells. Then, we showed that hispolon notably inhibited the activities of MMP-2, MMP-9, and uPA. These results demonstrated that the antimetastastic effect of hispolon was associated with the inhibition of enzymatically degradative processes of tumor metastasis. To our knowledge, it is the first study to demonstrate that hispolon reduces the biochemical mechanism(s) of the metastasis in SK-Hep-1 cells.

FAK, a cytoplasmic kinase that is involved in ECM and integrin-mediated signaling pathways, has been suggested to have an essential role in metastasis through the modulation of tumor cell migration and invasion (*19*). Activated FAK in cancer cells relays signals through multiple downstream targets. For example, activated FAK binds the Src-homology domain 2 (SH2) of PI3K, thereby transporting the catalytic subunit of PI3K to the membrane, where it catalyzes the phosphorylation of inositol lipids (*20*). The residues surrounding Tyr^{397} can also constitute a sequence that binds to the Ras signaling pathway. The downstream targets of the Ras signaling pathway include ERK1/2 (*4*). Indeed, these pathways are activated during integrin binding to the ECM, resulting in the transduction of external stimuli from the ECM to the nucleus (*21*). In this study, we found that hispolon inhibited the activation of FAK, as evidenced by reduced phosphorylation of FAK. We also demonstrated that treatment with hispolon inhibited phosphorylation of ERK1/2, whereas hispolon did not significantly affect phospho-p38 and JNK1/2 activity. The involvement of the MAPK pathway was supported by the use of PD98256, a specific ERK1/2 inhibitor, which inhibited MMP-2 and MMP-9 secretion. In addition, we showed that hispolon inhibited PI3K/Akt in SK-Hep1 cells. Thus, it seems that FAK promotes SK-Hep1 cancer cell migration and invasion in concert with the activation of the ERK and PI3K/Akt signaling pathways. Studies in ovarian carcinoma cells have shown that FAK and ERK1/2 are important for fibronectin stimulated invasiveness and MMP-9 secretion by these cells (*22*). Moreover, it was found that the MMP-9 gene promoter is partially regulated through activation of the ERK1/2 pathway (*23*). Therefore, it appears that FAK can promote SK-Hep1 cancer cell invasion through the ERK1/2 and MMP-9 pathway.

RhoA has been implicated in the regulation of the contraction and retraction forces that are required for cell migration (*18*). Increased RhoA expression enhances the phosphorylatioin of myosin, which cross-links actin filaments and generates contractile forces, promoting movement of the cell body and facilitating cell rear detachment. RhoA also is implicated in the invasion of human microvascular endothelial cells. Active-RhoA could induce the expression of MMP-9 metalloproteinase and promote migration of endothelial cells (*24*). RhoA may exert this activity through its ability to stimulate ERK/stress-activated protein kinase pathways and, subsequently, to upregulate transcription factors such as AP-1 or NF-κB known to be involved in the expression of certain metalloproteinases including MMP-9. The main role of RhoA in cell migration is suggested by its ability to regulate actin dynamics and, in particular, actin-myosin-dependent cell contraction (*25*). Our finding that hispolon concentration-dependently decreased RhoA protein expression suggests that hispolon may also inhibit the metastasis of SK-Hep1 cells through the PI3K/Akt−RhoA and RhoA−MMP-9 pathways.

In conclusion, we have demonstrated that hispolon inhibits the migration and invasion of carcinoma cancer SK-Hep1 cells. Mechanistically, we show that this effect of hispolon may occur through inactivation of the ERK1/2 signaling pathway, exerting inhibitory effects on FAK, pFAK, and RhoA protein expressions and inhibiting PI3K, and phospho-Akt levels, thereby decreasing the activities of MMP-2, MMP-9, and uPA leading to inhibition of metastasis of SK-Hep1 cells (**Figure 9**). Further preclinical and clinical studies are required to demonstrate the potential of hispolon as an anticancer agent.

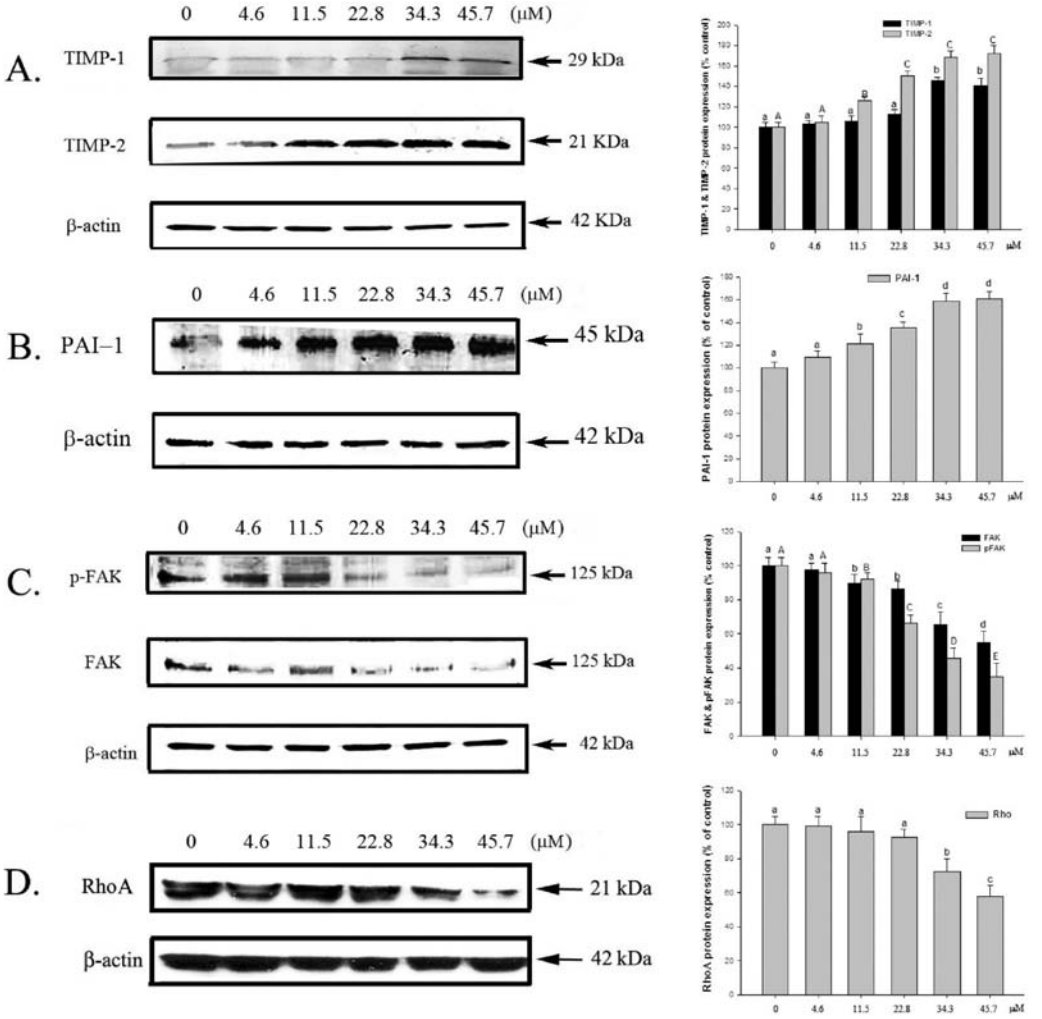

Figure 8. Effects of hispolon on TIMP (**A**), uPA inhibitor (**B**), FAK (**C**), and RhoA (**D**) protein expression. SK-Hep1 cells were treated with 0, 4.6, 11.5, 22.8, 34.3, and 45.7 μM for 24 h, and cell lysates were subjected to SDS-PAGE followed by Western blotting and subsequently quantified by densitometric analyses (using control as 100%) Values (means $\pm$ SD, $n = 3$) not sharing an alphabetic letter differ significantly ($P < 0.05$).

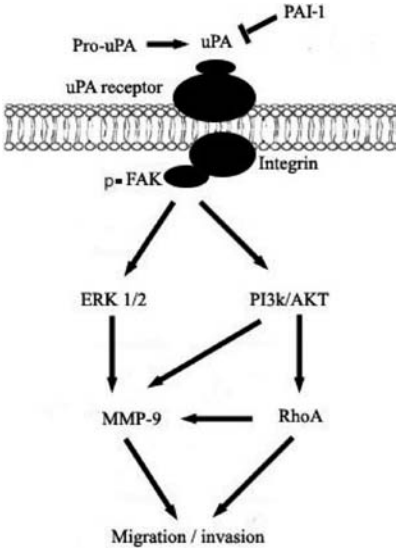

Figure 9. Proposed signaling pathways for hispolon-mediated inhibition against migration and invasion of SK-Hep1 cells. The effect of hispolon is achieved likely through the inhibition of FAK, which regulates RhoA/MMP9 expression through ERK1/2 and PI3K/Akt signaling pathways.

ABBREVIATIONS USED

MMPs, matrix metalloproteinases; uPA, urokinase-type plasminogen activator; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; ERK, extracellular signaling-regulating kinase; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; PI3K, phosphoinositide 3-kinase; FAK, focal adhesion kinase; RhoA, Ras homologue gene family, member A.

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