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RESEARCH ARTICLE

Molecular mechanism inhibiting human hepatocarcinoma cell invasion by 6-shogaol and 6-gingerol

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Scope: We previously demonstrated that 6-shogaol and 6-gingerol, two active compounds in ginger (*Zingiber officinale*), possess antiinvasive activity against highly metastatic hepatoma cells. The aims of this study were to evaluate the inhibitory effect and molecular mechanism underlying the transcription and translation of matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA) in Hep3B cells as well as the antiangiogenic activity of 6-gingerol and 6-shogaol.

Methods and results: By gelatin zymography and luciferase reporter gene assays, we found that 6-gingerol and 6-shogaol regulate MMP-2/-9 transcription. Moreover, 6-gingerol directly decreased expression of uPA, but the 6-shogaol-mediated decrease in uPA was accompanied by up-regulation of plasminogen activator inhibitor (PAI)-1. 6-Gingerol and 6-shogaol concentrations of $\geq$10 μM and $\geq$2.5 μM, respectively, significantly inhibited the phosphorylation of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling, the activation of NF-κB, and the translocation of NF-κB and STAT3. Incubation of 6-gingerol or 6-shogaol with human umbilical vein endothelial cells or rat aortas significantly attenuated tube formation.

Conclusion: 6-Shogaol and 6-gingerol effectively inhibit invasion and metastasis of hepatocellular carcinoma through diverse molecular mechanisms, including inhibition of the MAPK and PI3k/Akt pathways and NF-κB and STAT3 activities to suppress expression of MMP-2/-9 and uPA and block angiogenesis.

Keywords:
Angiogenesis / 6-Gingerol / Invasion / Matrix metalloproteinase / 6-Shogaol

1 Introduction

Hepatocellular carcinoma (HCC) is a highly lethal cancer that is currently the seventh most common solid tumor and the third leading cause of malignancy-related death worldwide [1]. HCC is frequently asymptomatic in the early stage and hence is usually not detected at a curable time. Approximately 80% of HCC cases develop to an advanced or unresectable malignancy [2]. Although surgery and percutaneous ablation have been successfully used to control some cases of early HCC, recurrence is still over 50% in 3 years [3]. Moreover, HCC is typically hypervascular, which means that the new blood vessels grow rapidly to support the cells with essential nutrients. The intricate vessels form a pipeline that connects one tissue to another. As such, metastasis is the main cause of mortality in patients with HCC [4]. Accordingly, therapies that aim to increase the survival of patients with HCC should minimize the growth of existing tumors, block cell invasion and metastasis, and inhibit angiogenesis.

Matrix metalloproteinase (MMP)-2 and -9 are proteolytic enzymes that are highly expressed in various malignant

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Abbreviations: **ECM**, extracellular matrix; **HCC**, hepatocellular carcinoma; **HUVEC**, human umbilical vein endothelial cells; **MAPK**, mitogen-activated protein kinase; **MMP**, matrix metalloproteinase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PAI**, plasminogen activator inhibitor; **PI3K**, phosphoinositide-3 kinase; **uPA**, urokinase-type plasminogen activator

tumors. These enzymes function to degrade the environmental extracellular matrix (ECM) and the basement membrane. They are markers associated with the invasion and metastasis of cancer cells [5]. MMP-2 and -9 are activated by plasmin, which is generated from specifically cleaved zymogen plasminogen through the urokinase-type plasminogen activator (uPA) and its receptor (uPAR). Through an enzymatic cascade initiated by uPA, type IV collagen (a major constituent of the basement membrane) is consequently degraded by the activated MMPs, thereby increasing cell mobility. uPA is also involved in the adhesion, migration, proliferation, and angiogenesis of tumors [6]. Blocking uPA or MMPs could be an effective strategy to prevent tumor cell invasion and metastasis. Two endogenous inhibitors of the uPA system, plasminogen activator inhibitor (PAI)-1 and -2, are involved in the regulation of uPA–uPAR function by either directly inhibiting their activity or indirectly regulating their internalization. PAI-1 inhibits invasion and metastasis, and high tumor PAI-2 levels are associated with improved prognosis [7]. As MMPs, uPA, and PAI are important regulators of tumor progression and metastasis, they have been identified as candidate prognostic markers [8]. After tumor cells have successfully broken away from an established solid tumor, invaded into a blood vessel and moved to a distant location, the cells still have to gain access to the essential nutrients on which to subsist at the secondary site. Tumors cannot grow beyond a certain size due to a lack of oxygen and other essential nutrients; however, this problem can be solved by forming new blood vessels. Angiogenesis is therefore a key step for the malignant transformation and metastasis of cancer cells [9].

Long-term therapies for tumors cause many unfavorable side effects. The need to refine chemical therapy for cancer treatment to alleviate the suffering of cancer patients is imperative. Many naturally occurring phytochemicals that are present in the diet have been used for cancer prevention. Ginger (*Zingiber officinale*) is a natural dietary rhizome that is widely used as a flavoring agent and occasionally used as a traditional medicinal herb. Ginger has been shown to have antioxidative, antiinflammatory, and anticarcinogenic properties [10, 11]. Several potent constituents such as gingerols, shogaols, paradols, and gingerdiols have been identified in ginger. Among these, shogaols and gingerols are phenolic substances that contain a volatile oil that can be extracted from ginger root and provides the characteristic odor and flavor of ginger [12]. 6-Shogaol [1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] inhibits the growth of ovarian cancer cells [13], and 6-gingerol [5-Hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)-3-decanone] inhibits the oxidant-induced invasion of AH109A cells in a dose-dependent manner [14]. We previously demonstrated that 6-shogaol and 6-gingerol possess antiinvasive activity against hepatoma cells at concentrations <10 μM and <50 μM, respectively [15]. However, the detailed mechanism underlying the antiinvasive activity of these two compounds has not been thoroughly described.

In the present study, we evaluated the inhibitory effect of 6-gingerol and 6-shogaol on the transcription and translation of MMPs and uPA in Hep3B cells. The molecular signaling and antiangiogenic effects were further explored to clarify the antiinvasive and antimetastatic mechanisms of 6-gingerol and 6-shogaol in human liver cancer cells.

2 Materials and methods

2.1 Materials and reagents

6-Shogaol and 6-gingerol were isolated and purified according to the methods described in our previous study [15]. Both of the compounds were dissolved in dimethyl sulfoxide (DMSO). Type IV gelatin, plasminogen, casein, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, nonessential amino acids, sodium pyruvate, Trizol reagent, M200 medium, LSGS medium supplement, Calcein AM fluorescence stain, and the human VEGF ELISA kit were purchased from Invitrogen (Carlsbad, CA). The human IL-8 ELISA Ready-SET-Go kit was purchased from eBioscience (San Diego, CA). The PCR Master Mix 2X kit, RevertAid First Strand cDNA synthesis kit, and Taq DNA polymerase were purchased from Fermentas International Inc. (Glen Burnie, MD). The Nuclear/Cytosolic Fractionation kit was purchased from BioVision (Mountain View, CA). Antilamin B1, anti-β-actin, antitotal and antiphosphorylated ERK, p38, JNK, PI3K, Akt, and mTOR antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-uPA, anti-PAI-1, anti-IκBα, antiphosphorylated p65, antiphosphorylated-STAT3, antiphosphorylated-IκBα antibodies, and rat tail collagen were purchased from Millipore (Billerica, MA). TransFast™ transfection reagent, Passive Lysis Buffer, the Dual-Luciferase® Report Assay System, and the pGL4.32, pGL4.15, and pGL4.74 vectors were purchased from Promega (Madison, WI). Matrigel was purchased from BD Biosciences (San Jose, CA).

2.2 Cell culture

Human hepatoma Hep3B cells were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were cultured as described in our previous study [15]. Human umbilical vein endothelial cells (HUVECs) were obtained from the Cascade Biologics (Portland, OR) and cultured in M200 medium containing 10 ng/mL epidermal growth factor, 3 ng/mL basic fibroblast growth factor, 10 mg/mL heparin, and 1% streptomycin/penicillin, with LSGS medium supplement, at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3 Cell viability assay

Cell viability was determined with the MTT assay as described in our previous study [15]. The percent viability of the treated cells was calculated as follows:

$$[(A_{570nm} - A_{630nm})_{sample}/(A_{570nm} - A_{630nm})_{control}] \times 100$$

2.4 Gelatin and casein zymography

Hep3B cells were incubated in serum-free DMEM with or without treatment for a given time, and the conditioned medium was collected. Zymography was performed according to the method described in our previous study [15].

2.5 Luciferase reporter gene assays

The TransFast™ transfection reagent and nuclease-free water were mixed and vortexed for 10 s and stored at –20°C overnight. Hep3B cells were cultured in a 24-well plate (3×10^4cells/well) and transfected with the mixture of transfection reagent, DNA-carrying plasmids (pGL4.32/NF-κB-Luc, pGL4.15/MMP-2-Luc, or pGL4.15/MMP-9-Luc), and internal control (pGL4.74) plasmids at 37°C for 1 h. Then, 1 mL of FBS-containing medium was added, and cells were incubated for 18 h. The medium was then aspirated, and fresh medium with chemical treatments was added for another incubation of 12 h. Finally, cells were resuspended in 100 μL Passive Lysis Buffer, and the cell lysates were collected to assay the luciferase activity using the Dual-Luciferase® Report Assay System. The luminescence was recorded with a Fluostar Galaxy plate reader.

2.6 Preparation of nuclear/cytosolic extracts

The treated and control cells were centrifuged (800 rpm) at 4°C for 6 min and were lysed with the Nuclear/Cytosolic Fractionation kit. Briefly, the cell lysate was centrifuged at 16 000×*g* at 4°C for 5 min after the addition of CEB-A and CEB-B. The supernatant was removed to a new tube as the cytosolic extract, and 100 μL NEB mix was added to the pellet and vortexed for 15 s. After centrifuging at 16 000×*g* at 4°C for 10 min, the supernatant was collected as the nuclear extract.

2.7 Western blotting

Western blotting was performed according to the method described in our previous study [15]. The nuclear and cytosolic protein levels were normalized to lamin B1 and β-actin, respectively.

2.8 Reverse transcription-polymerase chain reaction

Total RNA extraction, cDNA translation, and DNA amplification were performed using Trizol reagent, the RevertAid First Strand cDNA synthesis kit, and Taq DNA polymerase, respectively. The conditions for RT were as follows: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 s. The conditions for PCR were as follows: 30 cycles of 95°C for 0.5 min, 65°C for 0.5 min, 72°C for 1 min for uPA and PAI-1 or 25 cycles of 95°C for 1 min, 59°C for 0.5 min, 72°C for 1 min for 18s; these steps were followed by a final incubation of 10 min at 72°C. The primer sequences used in PCR were as follows: uPA, forward: CATCTCCT GTGCATG GGTGAAG, reverse: GCC-CTGAAGTC GTTAGTGTCTC; PAI-1, forward: AAGAGCTG GGCACGCAT CTGAC, reverse: GCAGGT GGCAGAGT-GAATGTCC; 18s, forward: TTGGAG GGCAAGTCT GGTG, reverse: CCGCTC CCAAGAT CCAACTA.

2.9 Enzyme-linked immunosorbent assay

Hep3B cells were cultured in a 6-well tissue plate and incubated in a serum-free medium with or without treatment for 24 h. The conditioned medium was collected to assay the levels of interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) secretion following the manufacturer's instructions for each ELISA kit.

2.10 Tube formation assay

The tube formation assay was performed according to the method described by Arnaoutova and Kleinman [16]. Briefly, the treated or untreated HUVECs (1.5×10^4 cells/well) were seeded in 96-well culture plates precoated with 50 μL/well of Matrigel and incubated at 37°C for 8 h. The cells were stained using Calcein AM dye at a final concentration of 2 μg/mL and incubated at 37°C for 45 min. Then, tube formation was observed by microscopy.

2.11 Rat aortic rings assay

The rat aortic rings assay was performed according to the method described by Go and Owen [17] with minor modification. Briefly, the aortas were isolated from Sprague–Dawley rats 6–10 weeks of age. Type II collagen medium solution (500 μL, 1.5 mg collagen in 1 mL serum-free DMEM) was added to 24-well culture plates, and the aortic rings were placed in the center of the wells. The plates were incubated at 37°C for 0.5–1.5 h to allow coagulation. Fresh serum-free medium (1 mL) with or without chemicals was added to the well and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was refreshed every 2–3 days. On the eighth day, the aortic rings were stained using Calcein AM dye at a final concentration of 2 μg/mL for

45 min. The length of the microvascular outgrowth was quantified by Image-Pro Plus software.

2.12 Statistical analysis

Data are presented as the mean ± SD for triplicate experiments. Statistical comparisons were performed by one-way analysis of variance followed by a Duncan's multiple-comparison test. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 6-Gingerol and 6-shogaol decrease transcription of MMP-2/-9

Cells were treated with <50 and <10 μM of 6-gingerol and 6-shogaol, respectively, for 24 h, and MTT assays revealed that there was no significant ($p < 0.05$) cytotoxicity (data not shown). Thus, these noncytotoxic conditions were used for subsequent experiments. Hep3B cells were treated with various concentrations of 6-gingerol and 6-shogaol in serum-free medium for 24 h, and then the conditioned media was collected to analyze MMP-2/-9 activity by gelatin zymography. The MMP-2/-9 activities were significantly suppressed with treatment ≥10 μM and ≥2.5 μM 6-gingerol and 6-shogaol, respectively, for 24 h (Fig. 1A). Next, the effects of 6-gingerol and 6-shogaol on the transcription of MMP-2/-9 were assessed by luciferase reporter assays. The levels of luciferase from MMP-9- and MMP-2-reporter constructs were significantly reduced by ≥5 μM and ≥50 μM 6-gingerol, respectively, and ≥2.5 μM and ≥7.5 μM 6-shogaol, respectively (Fig. 1B). These results suggested that the regulation of MMP-2/-9 activity by 6-gingerol and 6-shogaol may be due to the regulation of transcription and that 6-shogaol has a more potent effect than 6-gingerol.

3.2 6-Gingerol decreases uPA directly, but the 6-shogaol-mediated decrease in uPA is accompanied by up-regulation of PAI-1

MMPs are activated by proteolysis of pro-MMPs by uPA. Thus, we next investigated the role of uPA in 6-gingerol- and 6-shogaol-mediated regulation of MMP-2/-9. Casein zymography revealed that 6-gingerol (≥10 μM) and 6-shogaol (≥2.5 μM) significantly decreased uPA activity (Fig. 2A).

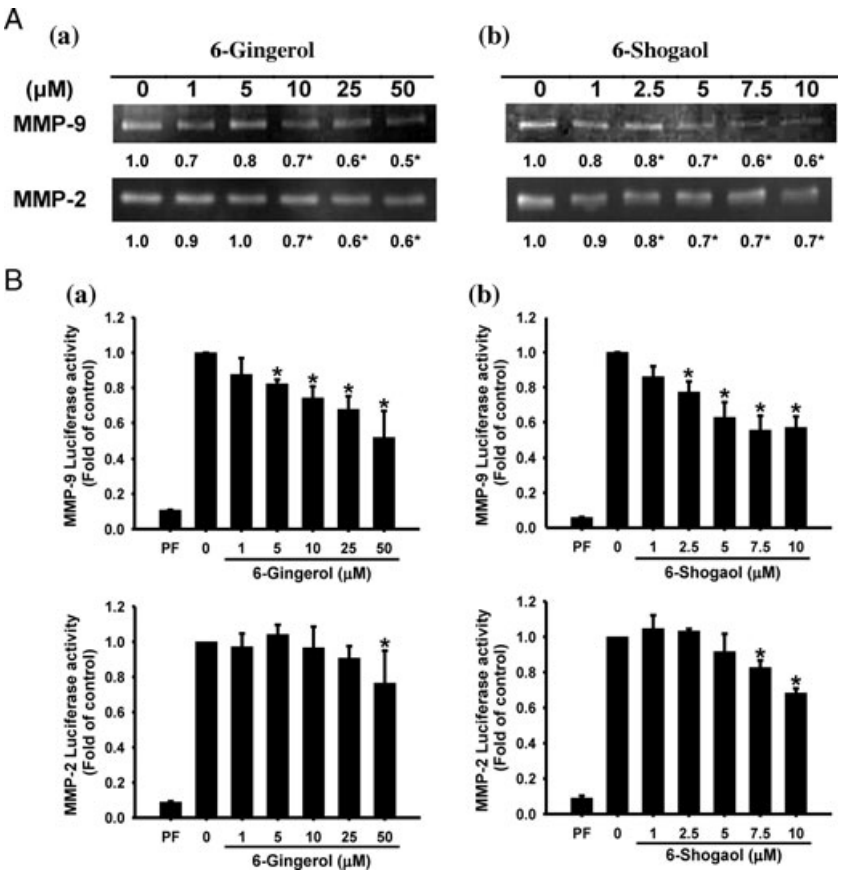

Figure 1. Effects of 6-gingerol (a) and 6-shogaol (b) on the enzyme activity (A, gelatin zymography) and the transcription (B, luciferase reporter gene assay) of MMP-2/-9 in Hep3B cells. Cells were treated with various concentrations of compound for 24 h in a serum-free medium. The bands corresponding to the activity were quantified by densitometric analyses (A). Cells were co-transfected with pGL4.15/MMPs-Luc and pGL4.74 internal control plasmid for 18 h and further treated with the indicated compound for 12 h. PF indicates plasmid-free cells (B). The untreated cells were used as controls. * indicates $p <$ 0.05 compared with the appropriate control.

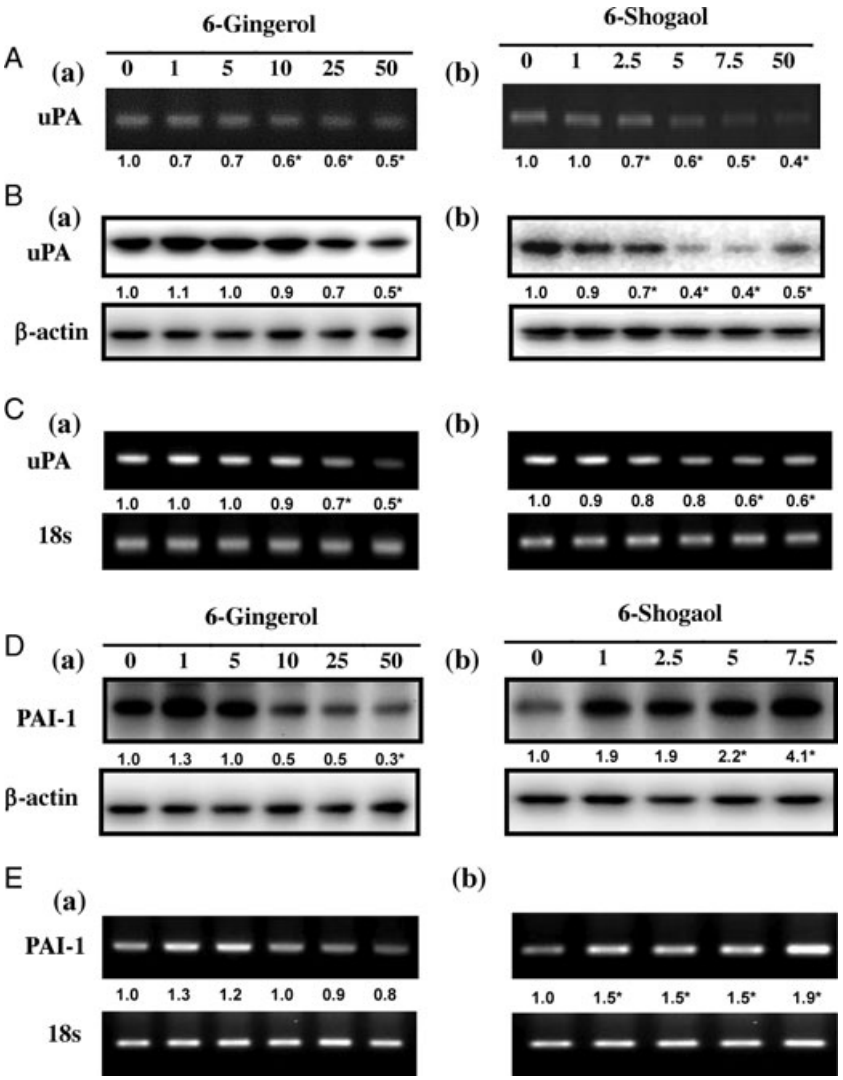

Figure 2. Effects of 6-gingerol (a) and 6-shogaol (b) on enzyme activity (A, casein zymography), protein level (B, Western blots), and mRNA expression (C, semiquantitative RT-PCR) of uPA and on the levels of protein (D) and mRNA (E) of PAI-1 in Hep3B cells. Cells were treated with the indicated concentrations of the indicated compound in serum-free medium for 24 h (A, B, and D) or 12 h (C and E). Untreated cells were used as controls. The signal intensity was quantified by densitometric analyses and compared with that of the control set as 1.0. * indicates $p < 0.05$ compared with each corresponding control.

Western blotting revealed that the levels of uPA protein were significantly decreased by $\geq$50 μM of 6-gingerol and $\geq$2.5 μM of 6-shogaol (Fig. 2B). RT-PCR revealed that the expression of uPA mRNA was significantly suppressed by $\geq$25 μM of 6-gingerol and $\geq$7.5 μM of 6-shogaol (Fig. 2C). Next, we evaluated the expression of the endogenous inhibitor of uPA, PAI-1. The experiments revealed that 6-gingerol decreased but 6-shogaol increased the levels of PAI-1 protein and RNA (Fig. 2D and E). These results suggested that the inhibitory effects of 6-gingerol and 6-shogaol on uPA activity in Hep3B cells might be partly mediated by regulation of transcription or translation. Further, the additional activation of PAI-1 by 6-shogaol but not 6-gingerol might explain the higher efficacy of 6-shogaol on the inhibition of uPA and MMPs.

3.3 6-Gingerol and 6-shogaol suppress MAPK and PI3K/Akt signaling

Mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase /protein kinase B (PI3K/Akt) are well-established pathways that modulate MMP expression. As we demonstrated inhibition of MMPs in Hep3B cells by 6-shogaol and 6-gingerol at the transcriptional level, the effects of these two compounds on the MAPK and PI3K/Akt pathways were further investigated to clarify the underlying molecular mechanisms of the effect. Densitometric analyses of Western blots revealed that 6-gingerol and 6-shogaol at concentrations of $\geq$10 μM and $\geq$2.5 μM, respectively, significantly inhibited the phosphorylation of MAPK and PI3K/Akt signaling compared to controls (Fig. 3).

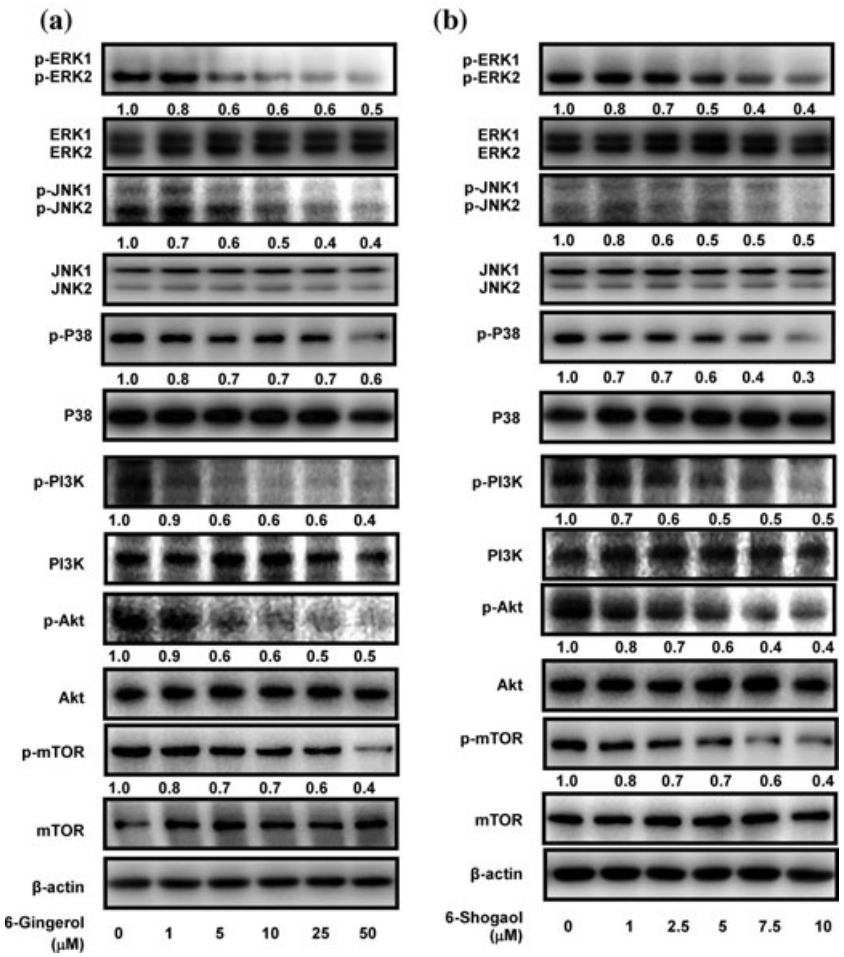

Figure 3. Effects of 6-gingerol (a) and 6-shogaol (b) on the MAPK and PI3K/Akt pathways in Hep3B cells. Cells were treated with the indicated concentrations of the indicated compound for 6 h, and then the cell lysates were subjected to SDS-PAGE followed by Western blotting with the indicated antibodies. The activities of these proteins were quantified by densitometric analyses relative to that of untreated cells set as 1.0.

3.4 6-Gingerol and 6-shogaol inhibit the activation of NF-κB and the translocation of NF-κB and STAT3

NF-κB and STAT3 (signal transducer and activator of transcription 3) are transcription factors localized downstream of the MAPK and PI3K/Akt pathways that are implicated in the regulation of the *MMP-9* and *uPA* genes [18]. We found that luciferase activity from a NF-κB-reporter construct was significantly decreased by 86% and 84% relative to control by 10 μM of 6-gingerol and 2.5 μM of 6-shogaol (Fig. 4A). NF-κB is activated through phosphorylation of IκBα and the subsequent release of the subunits (p65 and p50) from the cytosol to the nucleus to modulate gene expression. Treatment with 6-gingerol and 6-shogaol decreased IκBα phosphorylation, consistent with the decrease in NF-κB-mediated luciferase activity (Fig. 4B). Additionally, the levels of p-p65 and p-STAT3 dose-dependently increased in the cytosol and decreased in the nucleus after treatment with 6-gingerol and 6-shogaol for 6 h (Fig. 4C). Thus, it appears that the translocation of NF-κB and STAT3 from the cytosol to the nucleus is inhibited by 6-gingerol and 6-shogaol.

3.5 6-Gingerol and 6-shogaol have antiangiogenic activity through inhibition of IL-8 and VEGF, respectively

Angiogenesis is a crucial step to support the survival and development of metastasized tumors at secondary sites. Two common angiogenic factors, VEGF and IL-8, are often used as diagnostic markers for tumor metastasis and proliferation. We treated Hep3B cells with 6-gingerol and 6-shogaol for 24 h and collected the conditioned media to analyze the levels of VEGF and IL-8. 6-Shogaol and 6-gingerol, respectively, reduced VEGF and IL-8 secretion (Fig. 5A and B). Accordingly, 6-gingerol and 6-shogaol might be potential antiangiogenic phytochemicals. Thus, we performed two in vitro experiments to test the antiangiogenic activities of 6-gingerol and 6-shogaol: the tube formation assay and aortic ring assay, which evaluate the number (or area), length, and branching of tube formation. HUVECs were seeded in Matrigel-coated

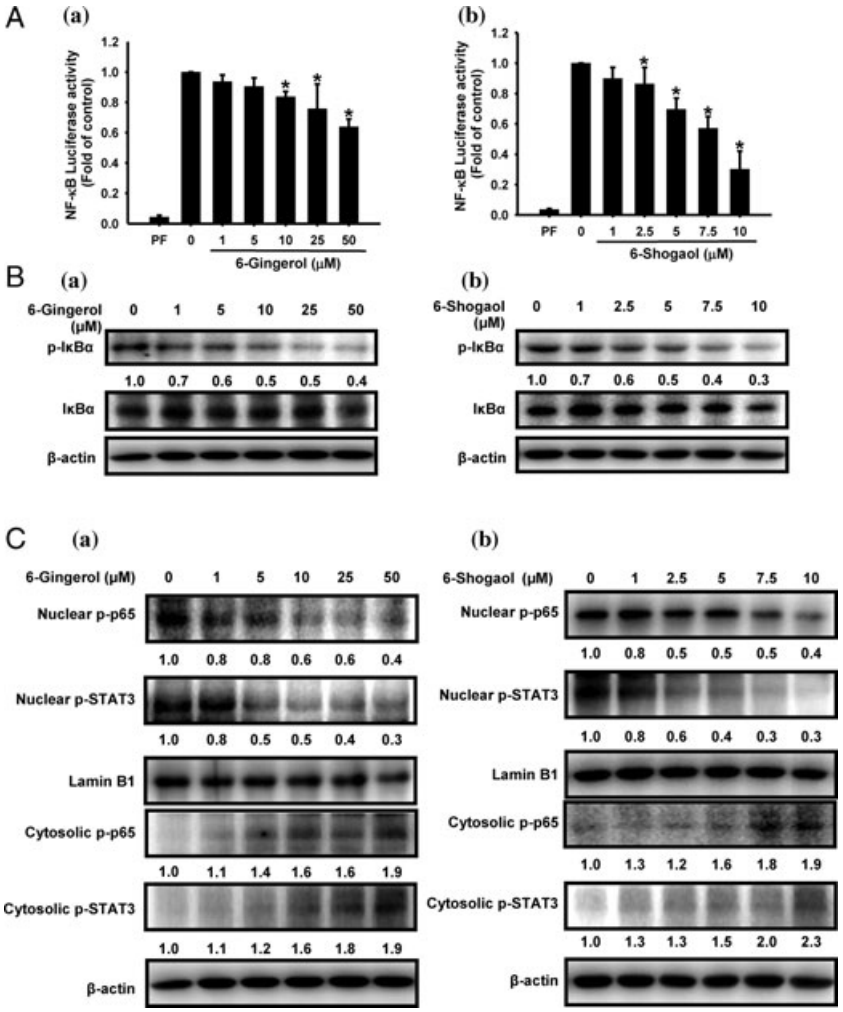

Figure 4. Effects of 6-gingerol (a) and 6-shogaol (b) on the transcription of NF-κB reporter constructs (A, luciferase reporter gene assay), p-IkBα level (B, Western blots), and translocation of NF-κB and STAT3 (B, Western blots) in Hep3B cells. The activities of these proteins were quantified by densitometric analyses relative to that of untreated cells set as 1.0. * indicates $p < 0.05$ compared with each corresponding control.

96-well plates and treated with previously determined nontoxic doses of 6-gingerol or 6-shogaol for 8 h. We observed retardation of tube formation and significantly decreased tube lengths that were 81% and 64% of the control after treatment with 10 μM of 6-gingerol and 5 μM of 6-shogaol (Fig. 5C). Similarly, the outgrowth capillary lengths were significantly reduced to 71% and 63% of the control (Fig. 5D).

4 Discussion

Shogaols are dehydrated products of the structurally related gingerols that are abundant in fresh and thermally dried ginger, respectively. 6-Gingerol and 6-shogaol are potent compounds in ginger that possess several chemopreventive and anticancer properties on various cancer cells, such as B16 melanoma cells [19], gastric cancer cells [20], and breast cancer cells [21, 22]. 6-Gingerol is also an active antiinvasion compound for the hepatoma AH109A cell line [14], and 6-shogaol exerts significant antihepatotoxic effects against galactosamine-induced cytotoxicity in primary cultured rat hepatocytes [23]. Our previous study demonstrated that the antiinvasive activity of 6-gingerol and 6-shogaol on hepatoma cells was mediated by regulation of MMP and tissue inhibitor of metalloproteinase (TIMP)-1 [15]. In the present study, we further revealed that the 6-gingerol and 6-shogaol decrease in MMP activity might be partly mediated by transcriptional regulation (Fig. 1) and involve the inhibition of uPA (Fig. 2). Moreover, 6-gingerol and 6-shogaol, respectively, attenuate and enhance PAI-1 expression (Fig. 2). The mechanisms underlying the toxicity of 6-gingerol and 6-shogaol on colorectal cancer cells have been reported by Ishiguro et al. [20]. The differential effects of 6-gingerol and 6-shogaol on the regulation of PAI in Hep3B cells might explain the higher efficacy of 6-shogaol on the inhibition of uPA and MMPs. The modulation of MMP activity through regulation of uPA, PAI-1, and TIMP-1 may confer the

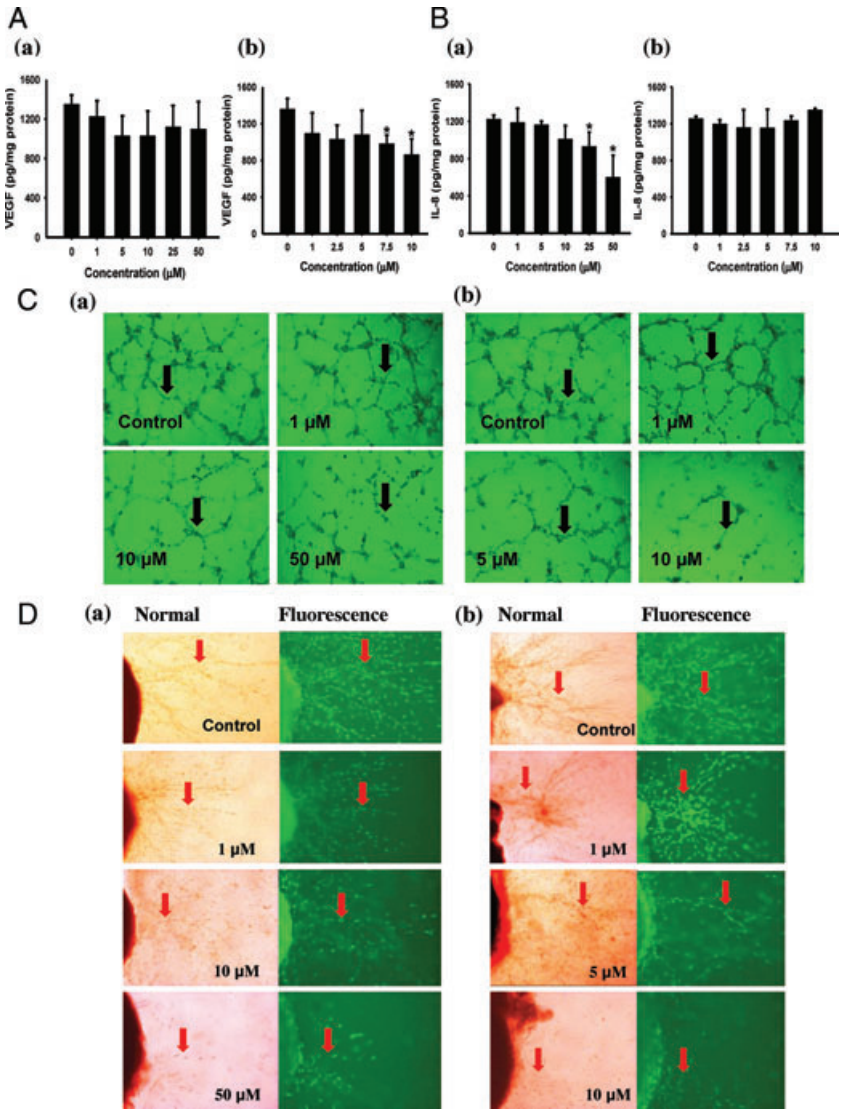

Figure 5. Effects of 6-gingerol (a) and 6-shogaol (b) on VEGF (A, ELISA) and IL-8 (B, ELISA) secretion in Hep3B cells and on the tube (C) and microvessel formation of rat aortic rings (D). Cells were incubated in a serum-free medium containing the indicated concentrations of the indicated compound for 24 h (A and B). HUVECs were treated with containing the indicated concentrations of the indicated compound and seeded on Matrigel-coated 96-well plates for 8 h. The tube formation was observed and photographed under a microscope (40×), and the arrows indicate the forming tubes. Tube length was quantified by Image-Pro Plus software (C). Aortic rings were seeded in collagen-coated 24-well plates and treated with the indicated concentrations of compound. The outgrowth of aortic microvessels was observed (as the arrows indicate) and photographed under a microscope (40×) on the eighth day of treatment. The length of microvascular outgrowth was quantified by Image-Pro Plus software (D). Data represent the mean ± SD of six independent experiments. Untreated cells were used as a control. * indicates $p < 0.05$ compared with each corresponding control.

antiinvasive activity of 6-gingerol and 6-shogaol on Hep3B cells.

The MAPK and PI3K/Akt pathways transduce the effects of several chemokines, growth factors, and cytokines, and the downstream transcription factors NF-κB and STAT3 are activated or inactivated to control numerous processes in cells [24]. The roles of the MAPK and PI3K/Akt pathways and NF-κB and STAT3 transcription factors on the regulation of uPA and MMPs in the invasion and metastasis of cancer cells have been reported in several studies [5, 18]. NF-κB is a well-known carcinogenesis-related transcription factor, and hyperactivation of STAT3 is found in many types of malignancies including hepatoma [25, 26]. The inactivation of STAT3 might reduce the resistance of hepatoma cells to a TRAIL-related anticancer drug [27]. In the present study, 6-shogaol and 6-gingerol not only decreased the signaling of MAPK and PI3K/Akt (Fig. 3) but also inactivated NF-κB and reduced the translocation of NF-κB and STAT3 from the cytosol to the nucleus (Fig. 4). This might contribute to the inhibition of uPA and MMPs by 6-shogaol and 6-gingerol.

Angiogenesis is an essential and vital process for a metastasized tumor cell to survive and develop at a secondary site. HCC is typically hypervascular, in which a high density of microvessels surrounds the cells, resulting in poor prognosis. Both 6-gingerol and 6-shogaol have been revealed that possessing the capability on suppression of angiogenesis [28]. However, we found that the angiogenesis of Hep3B cells was attenuated and the secretion of IL-8 and VEGF was decreased by 6-gingerol and 6-shogaol, respectively (Fig. 5). A variety of growth factors and cytokines induce blood vessel growth in hepatoma tumors. Of these, IL-8 and VEGF are detectable in the initial stage of the invasion and metastasis of HCC [4]. Blocking IL-8 and VEGF secretion reduces the formation of vessels and inhibits the proliferation of primary and metastatic tumors [29, 30]. It has become clear that MMPs contribute to angiogenesis by mechanisms other than

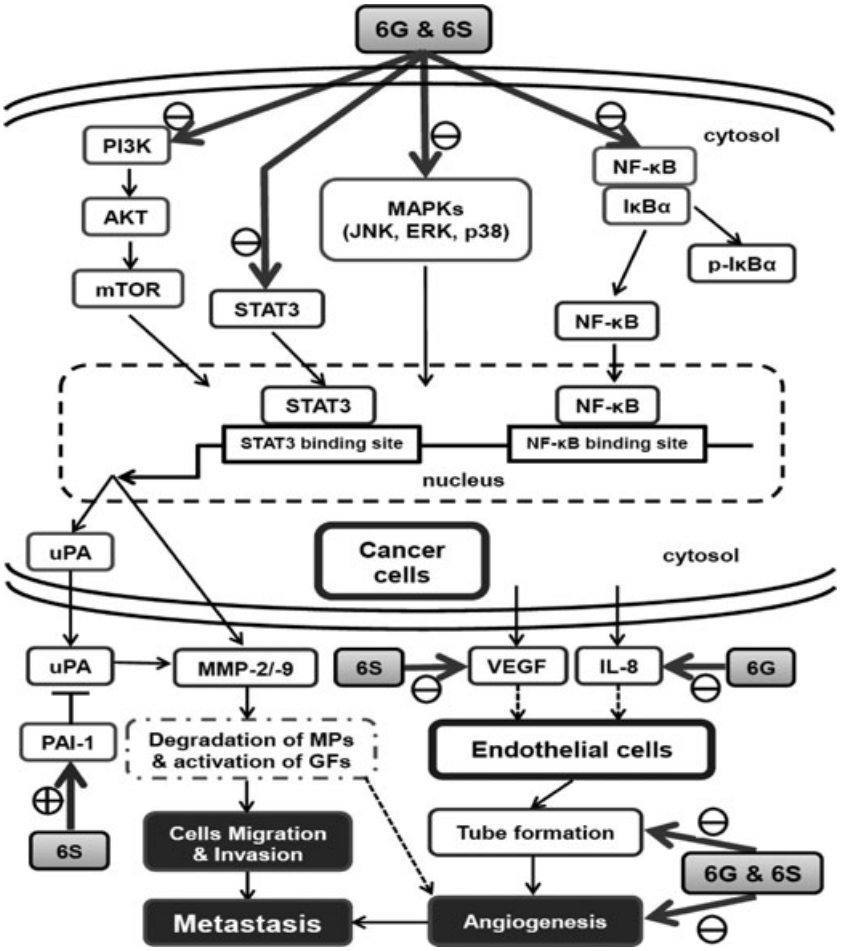

Figure 6. The proposed molecular mechanisms for the antiinvasive effects of 6-gingerol and 6-shogaol on human hepatoma cells. ⊖ indicates inhibition. ⊕ indicates induction. 6S, 6-shogaol; 6G, 6-gingerol; MPs, matrix proteins; GFs, growth factors.

degradation of the ECM [31]. The suppression of uPA–uPAR inhibits angiogenesis, invasion, and in vivo tumor development in pancreatic cancer cells [32]. The roles of MAPKs, PI3K/Akt, NF-κB, and STAT3 in angiogenesis have also been revealed [33]. Thus, we hypothesized that the target proteins that regulate 6-shogaol- and 6-gingerol-mediated inhibition of invasion and metastasis might also prevent angiogenesis in HCC. However, the antiangiogenic potential of 6-shogaol and 6-gingerol also contributes to their antiinvasive activities. The inhibitory effect on angiogenesis might be due to other antiinvasive mechanisms of 6-gingerol and 6-shogaol on Hep3B cells.

6-Shogaol more potently inhibited invasion and migration and the suppression of MMPs and uPA in HCC cells than 6-gingerol. The only structural difference between these two compounds is the double bond on the carbon side chain forming an α,β-unsaturated ketone moiety in 6-shogaol and a hydroxyl moiety in 6-gingerol. The differences in these two compounds in cell susceptibility, cell membrane permeability, and several biological activities caused by their structural variances were discussed in our previous study [15]. Here, we focused on the characteristics of α,β-unsaturated carbonyls to that might confer the potent antiinvasion activity of 6-shogaol. A phytochemical with an α,β-unsaturated carbonyl group might inhibit the phosphorylation of STAT3 through the modification of *S*-glutathionylation in STAT3 [34]. The natural and synthetic α,β-unsaturated carbonyls inhibit the activation of NF-κB [35], and the effect might be due to the differences in IκKβ, which has the same thiol moiety in the phosphorylated site as STAT3 [36]. The α,β-unsaturated carbonyl group might interact specifically with the thiol group in tubulin to interfere with the normal function of tubulin, such as in angiogenesis [37, 38].

The daily human consumption of approximately 250 mg–1 g of ginger contains 1.0–3.0% of 6-gingerol and its derivatives [12], and the rationality of the dosages of 6-gingerol and 6-shogaol used in this study was discussed in our previous study [15]. Taking the results of these two studies together, we conclude that 6-shogaol and 6-gingerol effectively inhibit the invasion and metastasis of HCC by diverse molecular mechanisms including inhibiting the MAPK and PI3k/Akt pathways and decreasing NF-κB and STAT3 activity to decrease MMP-2/-9 and uPA and suppress angiogenesis. The proposed mechanisms for the antiinvasive functions of these two compounds are represented schematically in Fig. 6.

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