RESEARCH ARTICLE

Anti-invasion effects of 6-shogaol and 6-gingerol, two active components in ginger, on human hepatocarcinoma cells

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Scope: Hepatocellular carcinoma is the most common type of liver cancer and is highly metastatic. Metastasis is considered to be the major cause of death in cancer patients. Ginger is a natural dietary rhizome with anti-oxidative, anti-inflammatory, and anti-carcinogenic activities. The aims of this study were to evaluate the anti-invasion activity of 6-shogaol and 6-gingerol, two compounds found in ginger, on hepatoma cells.

Methods and results: The migratory and invasive abilities of phorbol 12-myristate 13-acetate (PMA)-treated HepG2 and PMA-untreated Hep3B cells were both reduced in a dose-dependent manner by treatment with 6-shogaol and 6-gingerol. Upon incubation of PMA-treated HepG2 cells and PMA-untreated Hep3B cells with 6-shogaol and 6-gingerol, matrix metalloproteinase (MMP)-9 activity decreased, whereas the expression of tissue inhibitor metalloproteinase protein (TIMP)-1 increased in both cell types. Additionally, urokinase-type plasminogen activator activity was dose-dependently decreased in Hep3B cells after incubation with 6-shogaol for 24 h. Analysis with semi-quantitative reverse transcription-PCR showed that the regulation of MMP-9 by 6-shogaol and 6-gingerol and the regulation of TIMP-1 by 6-shogaol in Hep3B cells may on the transcriptional level.

Conclusions: These results suggest that 6-shogaol and 6-gingerol might both exert anti-invasive activity against hepatoma cells through regulation of MMP-9 and TIMP-1 and that 6-shogaol could further regulate urokinase-type plasminogen activity.

Keywords:
6-Gingerol / 6-Shogaol / Invasion / Matrix metalloproteinases / Tissue inhibitor metalloproteinase

1 Introduction

Naturally occurring phytochemicals are present in the human diet from foodstuffs such as garlic, ginger, soy, curcumin, onion, tomatoes, chilies, cruciferous vegetables, and green tea. Many of these compounds are used for the chemoprevention of cancer and contribute to lowering the risk of cancer [1]. Ginger (*Zingiber officinale*) is a natural dietary rhizome that is widely used as a flavoring agent and occasionally used as a traditional medicinal herb. The anti-oxidative, anti-inflammatory, and anti-carcinogenic properties of ginger have been verified [2–4], and treatment with ginger is useful in preventing the development of colorectal cancer [3]. However, the literature concerning the identification of the constituent(s) of ginger active in preventing cancer invasion or metastasis is still limited. Several pungent compounds, such as gingerols, shogaols, paradols,

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Abbreviations: ECM, extracellular matrix; **MMP,** matrix metalloproteinase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PMA,** phorbol 12-myristate 13-acetate; **TIMP,** tissue inhibitor of metalloproteinase protein; **uPA,** urokinase-type plasminogen activator

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and gingerdiols, have been identified in ginger. Among these compounds, shogaols and gingerols are two phenolic substances contained in a volatile oil extracted from ginger root and provide ginger its characteristic odor and flavor [5]. Phenolic substances present in natural dietary foods, such as fruits and vegetables, have been found to protect against cancer both *in vitro* and *in vivo* [6–8]. 6-Shogaol [1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] is a lipid-soluble organic compound that exhibits significant anti-hepatotoxic effect against galactosamine-induced cytotoxicity in primary cultured rat hepatocytes [9] and protects against LPS-induced inflammation in RAW 264.7 macrophage cells [10]. The administration of 6-shogaol prevents secondary pathological events following traumatic spinal cord injuries and promotes the recovery of motor function in an animal model [9]. 6-Gingerol [5-Hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)-3-decanone] is an abundant constituent of ginger and also possesses anti-oxidative and anti-inflammatory activities [11, 12]. It acts to prevent the metastasis of B16 melanoma cells [13] and limits the invasion of the rat ascite hepatoma AH109A cell line [14]. Both 6-shogaol and 6-gingerol can significantly inhibit tumor necrosis factor-α mediated downregulation of adiponectin in 3T3-L1 adipocytes [15]. Additionally, 6-shogaol is more potent than 6-gingerol in inhibiting the proliferation and inducing the apoptosis of COLO 205 cells [16]. The studies mentioned above suggest that 6-shogaol and 6-gingerol are the active components in ginger and that 6-gingerol possesses anti-metastatic and anti-invasive pharmacological activities on cancer cells. However, the effect of 6-shogaol on the invasion of cancer cells has not been verified, and the literature regarding the effects of 6-gingerol on inhibiting the invasion of hepatoma cells is still limited.

Hepatocellular carcinoma is a highly metastatic cancer, representing 83% of all liver cancer cases, and is the third leading cause of cancer deaths worldwide [17]. Metastasis is responsible for the majority of failures of cancer treatment and is the major cause of death in cancer patients. As a result, treatments that can block cancer invasion and metastasis, in addition to minimizing the growth of existing tumors, are being actively pursued to enhance the survival of cancer patients. The invasion and metastasis of cancer cells involves the degradation of the environmental extracellular matrix (ECM) and basement membrane by various proteolytic enzymes and results in the mobility of cancer cells [18–20]. Among these proteases, matrix metalloproteinase (MMP)-2 and MMP-9 are highly expressed in various malignant tumors and are closely related to the invasion and metastasis of cancer cells [21, 22].

MMP-2 and MMP-9 are activated by plasmin, which is generated from specifically cleaved zymogen plasminogen through the enzyme urokinase-type plasminogen activator (uPA) when it associates with its receptor, uPAR. uPA initiates an enzymatic cascade leading to the activation of both the MMP-2 and the MMP-9 enzymes and making them capable of degrading type IV collagen, which is a major constituent of the basement membrane, making mobility both possible and easy. Therefore, several inhibitors against uPA or MMPs have been tested in clinical trials for prevention of tumor invasion and metastasis [23]. Tissue inhibitor metalloproteinase proteins (TIMPs) are a mammalian protein family composed of TIMP-1, -2, -3, and -4, which together display wide-ranging sequence homology and structural identity. TIMPs have been reported as natural MMP inhibitors that prevent the degradation of the ECM by abolishing the hydrolytic activity of all activated members of the metalloproteinase family, in particular that of membrane type 1-MMP (MT1-MMP), MMP-2, and MMP-9 [24].

Human hepatocarcinoma HepG2 and Hep3B cells are two common cell models for cancer research, which secrete both MMP-2 and MMP-9 simultaneously with or without induction, respectively [25]. The aims of this study were to evaluate the anti-invasion activity of 6-shogaol and 6-gingerol (structures shown in Fig. 1) on inducer-treated HepG2 and inducer-untreated Hep3B cells. To explore the anti-invasive mechanisms involved in human liver cancer cells by 6-shogaol and 6-gingerol, the impacts of these compounds on MMP-2, MMP-9, TIMP-1, and uPA were evaluated.

2 Materials and methods

2.1 Materials and reagents

6-Shogaol and 6-gingerol were isolated and purified through column chromatography; the purity and identity of the isolated compounds were confirmed by HPLC and NMR

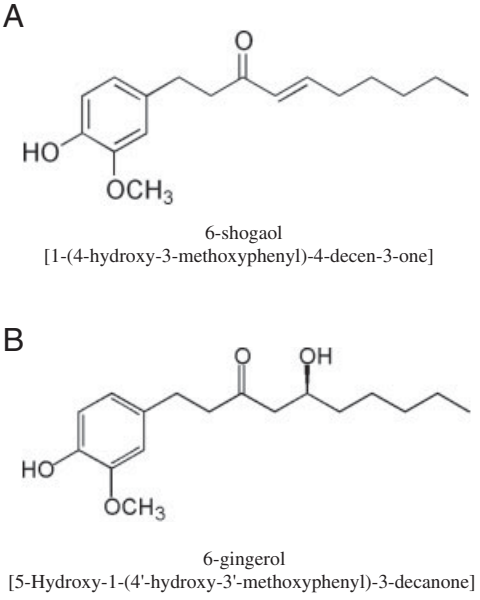

Figure 1. Chemical structures of (A) 6-shogaol and (B) 6-gingerol.

[10, 16]. All chemicals were dissolved in DMSO. Type IV gelatin, phorbol 12-myristate 13-acetate (PMA), DMSO, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). DMEM, fetal bovine serum, and SuperScript® III First-Strand Synthesis SuperMix for quantitative reverse transcription-PCR kit were purchased from Invitrogen (Carlsbad, CA). PCR Master Mix 2X Kit was purchased from Fermentas (Glen Burnie, MD). Transwell® Permeable Support was purchased from Corning (Lowell, MA).

2.2 Cell culture

Human hepatoma HepG2 and Hep3B cells were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown as described in our previous study [25]. Cells were cultured in serum-free medium for the invasive and metastatic experiments.

2.3 Cell viability assay

Cell viability was determined with an MTT assay as described in our previous study [25], and the percent viability of the treated cells was calculated as follows:

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

2.4 Gelatin and casein zymography

HepG2 and Hep3B cells were incubated in the presence and absence, respectively, of the PMA concentrations indicated, and serum-free DMEM with or without compounds (in DMSO) for a given time. The conditioned media were then collected as samples. The zymography was performed according to the method described in our previous study [25].

2.5 Cell migration and invasion assay

The cell invasion assay was performed according to the method described by Repesh [26]. The detailed procedure has been described in our previous study [25].

2.6 Western blotting

Ten micrograms of each of the total cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto a polyvinylidene fluoride membrane using a Bio-Rad Mini Protean electrotransfer system. The probe detection and signal development were performed according to the methods described in our previous study [25].

2.7 Reverse transcription-PCR

Total RNA preparation and reverse transcription-PCR (RT-PCR) were performed according to the methods described in our previous study [25]. Then, the resulting cDNA was amplified by PCR with the following primers: MMP-9 (94 bp), 5′-GGGCTTAGATCATTCCTCAGTG-3′ (sense) and 5′-GCCATTCACGTCGTCCTTAT-3′ (antisense); TIMP-1 (95 bp) 5′-ACTTCCACAGGTCCCACAAC-3′ (sense) and 5′-AGCCACGAAACTGCAGGTAG-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (110 bp), 5′-ATCGACCACTACCTGGGCAA-3′ (sense), and 5′-AGGATAACGCAGGCGATGT-3′ (antisense). PCR amplification was performed under the following conditions: 35 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 2 min (for MMP-9); 28 cycles of 94°C for 1 min, 59°C (for TIMP-1) or 58°C (for glyceraldehyde-3-phosphate dehydrogenase) for 1 min, 72°C for 2 min; then, followed by a final incubation at 72°C for 10 min.

2.8 Protein concentration determination

The protein concentration was determined according to the method described by Bradford [27] using bovine serum albumin as a standard.

2.9 Statistical analysis

Data are indicated as the mean ± SD for three different determinations. Statistical comparisons were made by means of 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 Effects of 6-shogaol and 6-gingerol on the viability of hepatoma cells

The MTT assay was used to evaluate the cytotoxicity of 6-shogaol and 6-gingerol on hepatoma cells. A range of 0–100 μM of these two compounds was used to treat HepG2 and Hep3B cells for 24 and 48 h. The results showed that the viability of both hepatoma cells was over 80% when treated with 6-shogaol and 6-gingerol at a range of concentrations from 0 to 10 and 0 to 50 μM, respectively, for 24 h (Fig. 2). Hence, the indicated doses and treatment times of 6-shogaol and 6-gingerol for maintaining at least 80% cell viability were used for the subsequent experiments on hepatoma cells.

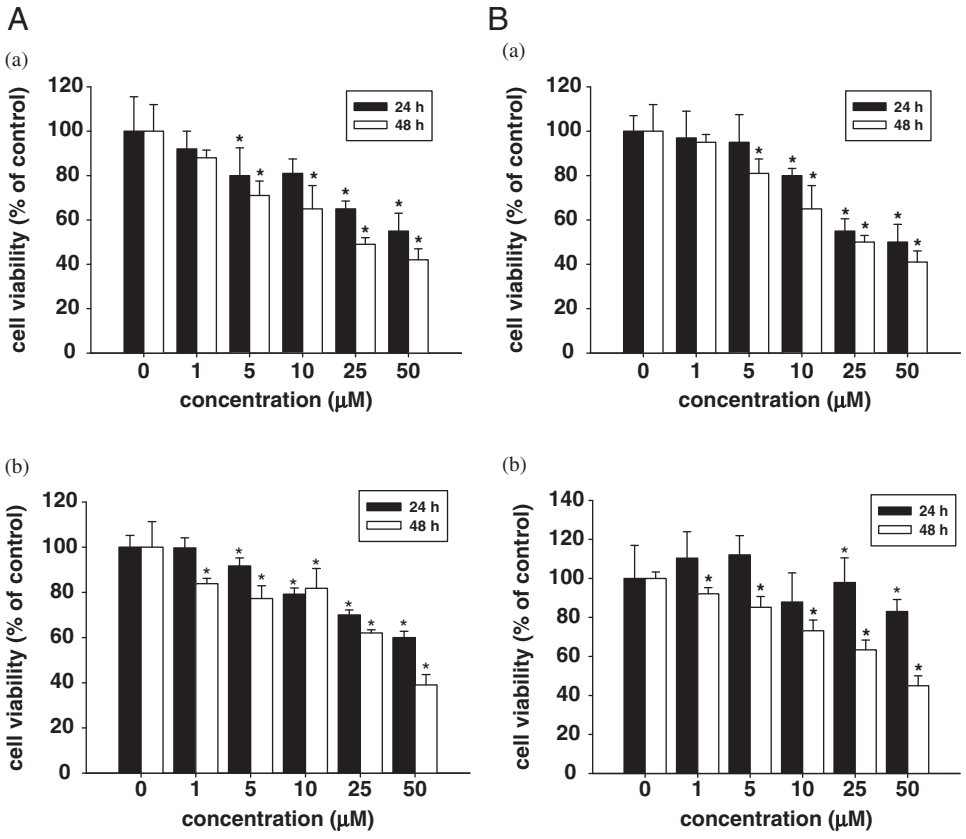

Figure 2. Effects of 6-shogaol (a) and 6-gingerol (b) on the viability of HepG2 (A) and Hep3B (B) cells. Cells were incubated in a serum-free medium with various concentrations of 6-shogaol and 6-gingerol for 24 and 48 h. Cells in a serum-free medium without 6-shogaol and 6-gingerol were used as the control. Data represent the mean ± SD of three independent experiments. ($^*p < 0.05$ compared with the control).

3.2 Effects of 6-shogaol and 6-gingerol on migration and invasion of HepG2 and Hep3B cells

Cell–matrix interaction and cell motility are important for cancer cell invasion. To examine the potential anti-invasive effects of 6-shogaol and 6-gingerol, migration and invasion assays were performed on HepG2 and Hep3B cells. The results indicated that the migratory and invasive activities of HepG2 cells without induction were not affected by treatment with these two compounds (data not shown). We have previously shown [25] that the invasive activity and MMP-9 secretion of HepG2 cells can be induced by 200 nM PMA, so HepG2 cells were treated with PMA first for the subsequent tests. As expected, the migratory and invasive abilities of the HepG2 cells were induced by PMA, and the PMA-induced migration and invasion were further reduced in a dose-dependent manner by a 24-h treatment with 6-shogaol and 6-gingerol at concentrations of >2.5 and $>5\,\mu M$, respectively (Figs. 3A and 4A). After treatment of PMA-treated HepG2 cells with either 2.5 μM 6-shogaol or 5 μM 6-gingerol for 24 h, the migratory abilities were significantly ($p < 0.05$) reduced by 25 and 22% (Fig. 3A), respectively, and the invasive abilities were significantly ($p < 0.05$) reduced by 53 and 52% (Fig. 4A), respectively, relative to PMA treatment alone. When 6-shogaol and 6-gingerol were applied to Hep3B cells, dose-dependent inhibitory effects on migration and invasion were also observed (Figs. 3B and 4B). The migratory abilities were significantly ($p < 0.05$) reduced to 83 and 69% (Fig. 3B), and the invasive activities were significantly ($p < 0.05$) reduced to 53 and 71% (Fig. 4B) by treatment with 1 μM 6-shogaol and 5 μM 6-gingerol on Hep3B cells, respectively. The results suggest that 6-shogaol and 6-gingerol are inhibitors of hepatoma cell migration and invasion and that the effective inhibitory concentration of 6-shogaol on cell invasion is markedly lower than that of 6-gingerol.

3.3 6-Shogaol and 6-gingerol inhibit the MMP-9 activities of HepG2 and Hep3B cells

To clarify whether the activity of MMP-2 and MMP-9 is involved in the invasion of hepatoma cells, the effective anti-invasive dosages of 6-shogaol and 6-gingerol were used to analyze the effects of these two compounds on MMP-2 and MMP-9 in hepatoma cells. The PMA-treated HepG2 and PMA-untreated Hep3B cells were incubated in serum-free medium with or without a given compound for 24 h. The conditioned media were then used to analyze MMP-9 and MMP-2 activity by gelatin zymography. The activity of MMP-9 was suppressed in a dose-dependent manner, but the MMP-2 activity was not significantly changed when HepG2 and Hep3B cells were treated with 6-shogaol (1, 2.5, 5, and 10 μM) or 6-gingerol (5, 10, 25, and 50 μM), respectively (Fig. 5). The dose-independent and insignificant decrease of MMP-2 activity might be

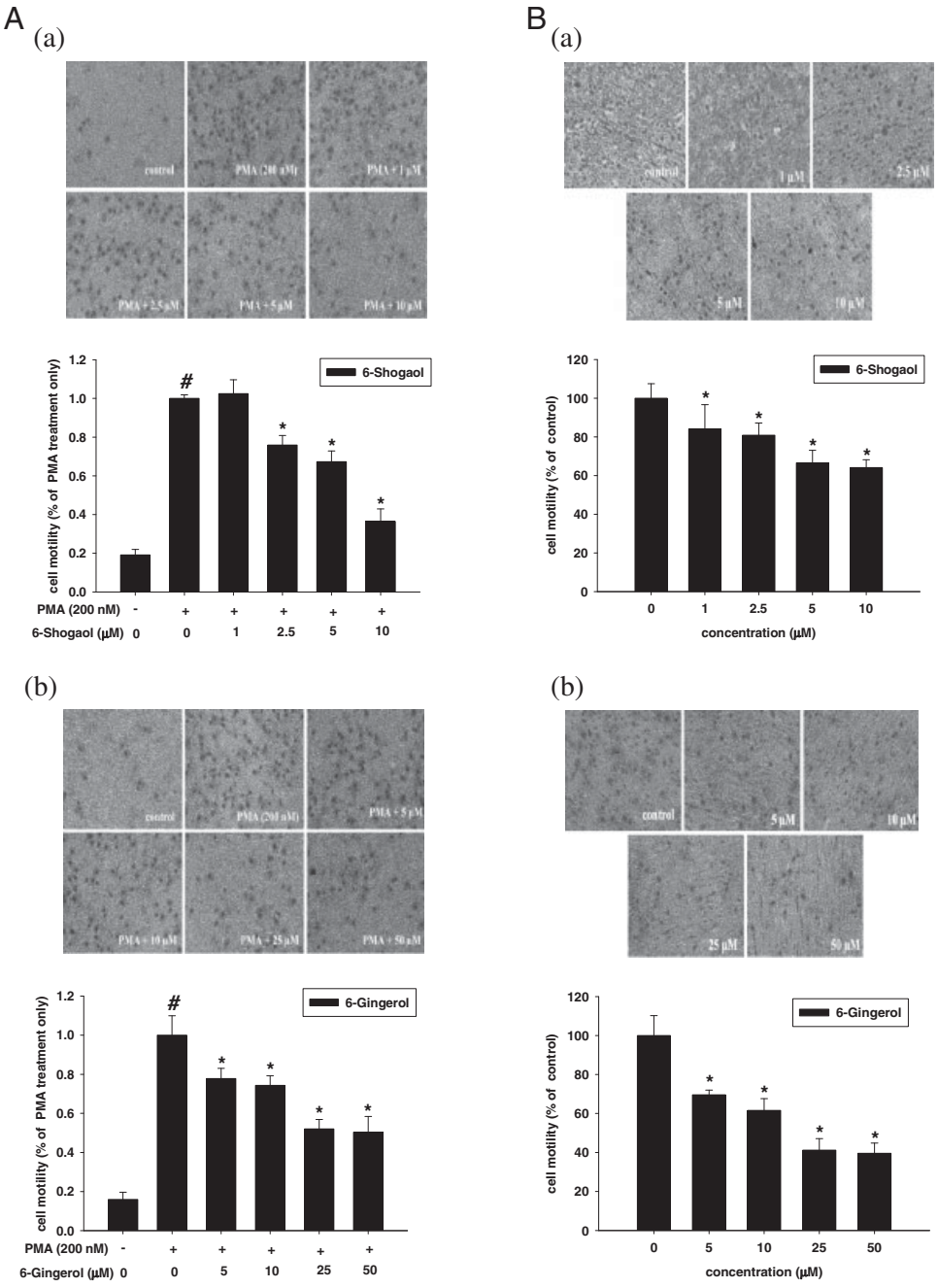

Figure 3. Effects of 6-shogaol (a) and 6-gingerol (b) on the motility of PMA-treated HepG2 (A) and PMA-untreated Hep3B (B) cells. The photographic image (100 ×) represents the cells migrating through PCF membrane. The bar graphs represent the migratory cell numbers from cells treated with various concentrations of 6-shogaol or 6-gingerol for 24 h. Values are reported as mean ± SD, n=3. (In panel (A), # indicates $p<0.05$ compared with the control and *indicates $p<0.05$ compared with the PMA treatment only. In panel (B), * indicates $p<0.05$ compared with the control).

a result of the reduction in cell numbers but not the inhibitory effects of 6-shogaol and 6-gingerol treatment.

3.4 Both 6-shogaol and 6-gingerol increase protein levels of TIMP-1 but only 6-shogaol suppreses uPA activity

The physiological activity of MMP-9 is significantly related to the activity of uPA and TIMPs (especially TIMP-1). The Western blot method and casein zymography were used to determine the effects of these two compounds on the protein levels of TIMP-1 and the activity of uPA, respectively, in both PMA-treated HepG2 and PMA-untreated Hep3B cells. After quantifying the expression of TIMP-1 protein in each condition and correcting by the corresponding β-actin expression, the data revealed that the protein level of TIMP-1 in HepG2 and Hep3B cells was increased in a dose-dependent manner along with a gradual increase in the concentrations of 6-shogaol and 6-gingerol used for 24 h treatment (Fig. 6A). uPA activity was not detectable in HepG2 cells with or without PMA induction, but it dose-dependently decreased in Hep3B cells after incubation with

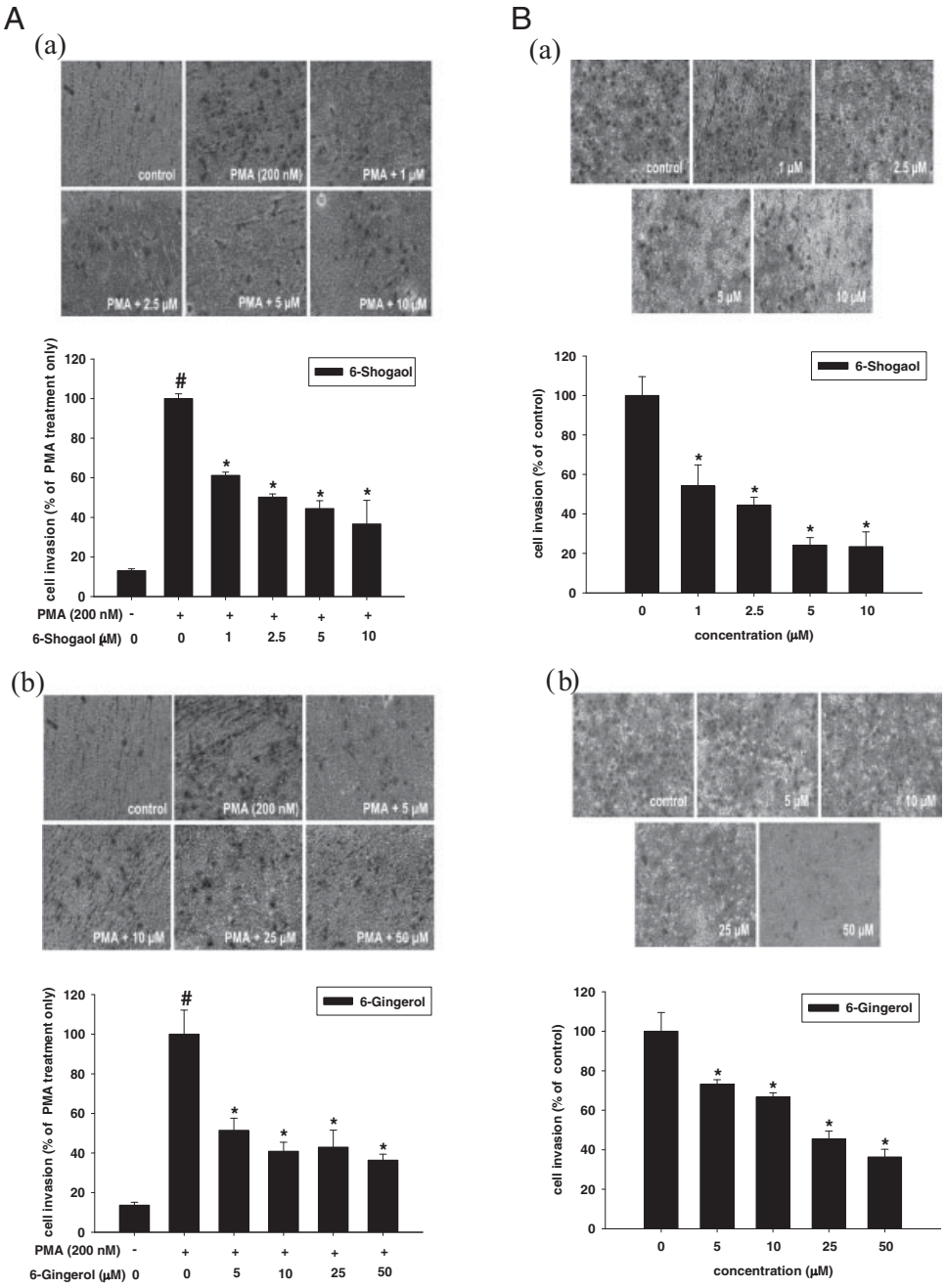

Figure 4. Concentration-dependent inhibitory effects of 6-shogaol (a) and 6-gingerol (b) on the invasion of PMA-treated HepG2 (A) and PMA-untreated Hep3B (B) cells. Photographic images (100 ×) represent the cells invading through Matrigel-coated membrane. The bar graphs represent the invasive cell numbers from 24 h treatments various concentrations of 6-shogaol or 6-gingerol. Values are reported as mean ± SD, n=3. (In panel (A), # indicates $p<0.05$ compared with the control and * indicates $p<0.05$ compared with the PMA treatment only. In panel (B), * indicates $p<0.05$ compared with the control).

6-shogaol for 24 h (Fig. 6B). Taken together, we conclude that the inhibitory effect on MMP-9 in hepatoma cells by 6-shogaol may be through the regulation of both uPA and TIMP-1 but that the effect of 6-gingerol may be through TIMP-1 only.

3.5 6-Shogaol transcriptionally regulates MMP-9 and TIMP-1, but 6-gingerol regulates only MMP-9

According to the results shown in Figs. 5 and 6, the expression of MMP-9 and TIMP-1 in PMA-treated HepG2 and PMA-untreated Hep3B cells was significantly influenced by 6-shogaol and 6-gingerol treatment. Semi-quantitative RT-PCR was further employed to analyze the effects of 6-shogaol and 6-gingerol on the mRNA expression of MMP-9 and TIMP-1 in each hepatoma cell line. After treatment of PMA-treated HepG2 cells with 0–10 μM 6-shogaol or 0–50 μM 6-gingerol for 24 h, the mRNA expression of MMP-9 and TIMP-1 was nearly unchanged, except in the case of 1 μM 6-shogaol treatment, which led to dramatically decreased levels of MMP-9 mRNA (Fig. 7A). The dose-independent reduction of MMP-9 mRNA by treatment of PMA-treated HEPG2 cells with a single dosage of 6-shogaol

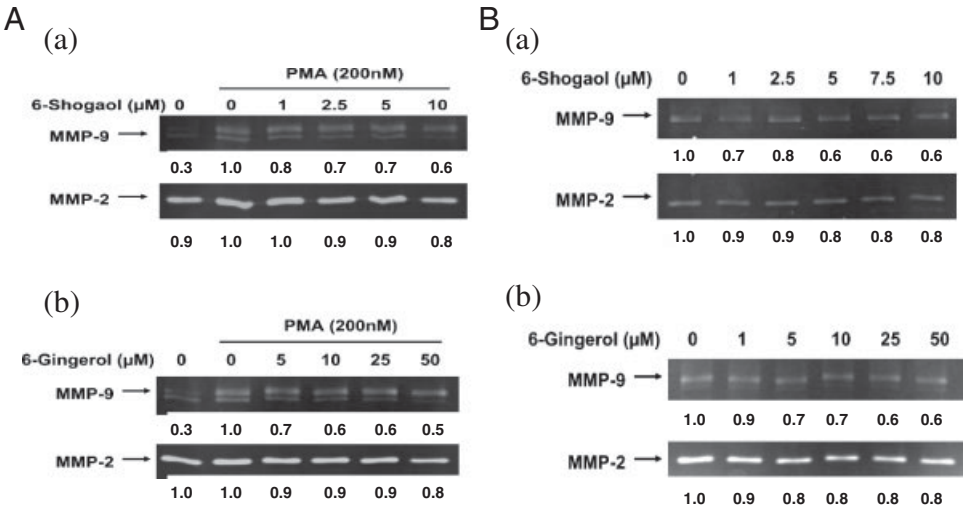

Figure 5. Effects of 6-shogaol (a) and 6-gingerol (b) on activity of both MMP-9 and MMP-2 in PMA-treated HepG2 (A) and PMA-untreated Hep3B cells. Cells were incubated in a serum-free medium with various concentrations of 6-shogaol and 6-gingerol for 24 h. Activities of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only or control set to 100%.

is unexpected, and the cause needs to be verified in further specifically designed studies. Therefore, we ignored this phenomenon in HepG2 cells and paid more attention to the effects of these two compounds on Hep3B cells. Upon treatment of the Hep3B cells with 6-shogaol or 6-gingerol for 24 h, the mRNA expression of MMP-9 decreased but the expression of TIMP-1 increased along with an increased concentration of 6-shogaol, whereas that of the internal control (G6PD) remained unchanged. However, a decrease in the levels of MMP-9 mRNA was only seen after treatment with 6-gingerol (Fig. 7B). Given these results, both 6-shogaol and 6-gingerol affected MMP-9 and TIMP-1 in PMA-treated HepG2 cells *via* a method other than transcriptional regulation; however, transcriptional regulation was at least partly responsible for the regulation of MMP-9 and TIMP-1 in Hep3B cells by 6-shogaol and MMP-9 by 6-gingerol.

4 Discussion

Metastasis is the major cause of death in cancer patients. Thus, active compounds demonstrating anti-invasive and anti-metastatic properties are defined as a new catalog of chemopreventive agents. 6-Shogaol is the most effective individual component of ginger for the inhibition of growth of ovarian cancer cells [28]. 6-Gingerol, the most abundant component in ginger, can inhibit the oxidant-induced invasion of AH109A, a hepatoma cell line that does not produce MMPs, in a dose-dependent manner (50–200 μM) by means of its anti-oxidative activity [14]. Metastasis occurs through a complex multistep process consisting of invasion of cells from a primary tumor into the circulation, immigration of these cells to distant organs, adhesion to endothelial cells and infiltration into tissue. The compounds which are effective to suppress cell migration could be contributed to the inhibition of cell invasion. In this study, 6-shogaol and 6-gingerol at concentrations of <10 and <50 μM, respectively, could effectively inhibit both the migratory and the invasive activities of PMA-treated HepG2 and PMA-untreated Hep3B cells (Figs. 3 and 4). Although the anti-invasive activity of 6-gingerol on hepatoma cells has been tested and verified in the AH109A cell model, the underlying mechanism of 6-gingerol action on invasion of HepG2 and Hep3B cells was supposed to be different from that of AH109A cells due to the different MMP-producing properties of these cells. 6-Gingerol can inhibit cell adhesion, invasion, motility, and MMP-2 and -9 activities in MDA-MB-231 human breast cancer cells [29]. It also inhibits angiogenesis both *in vitro* and *in vivo*, and suppresses the formation of lung metastases of B16F10 melanoma in an experimental tumor-bearing mouse model [30]. Therefore, the anti-MMP-2 and -9 and anti-angiogenesis effects could be the anti-invasive strategies of 6-gingerol.

Shogaol is a dehydrated product of the structurally similar gingerols. As a large quantity of gingerols is found in fresh ginger, shogaols are abundant in dried and thermally treated ginger. The only structural difference between 6-shogaol and 6-gingerol is the double bond on the carbon side chain forming α,β-unsaturated ketone moiety in 6-shogaol or the hydroxyl moiety in 6-gingerol. Previous studies have suggested that the α,β-unsaturated ketone moiety is very susceptible to nucleophilic addition reactions with thiols [31] and is essential for exerting cytotoxic activity *via* this susceptibility [32]. The α,β-unsaturated carbonyl group in 6-shogaol might influence the conformation of the compound and modulate its inhibitory effect. Additionally, the hydroxyl group might reduce the lipophilic property and cell membrane permeability of 6-gingerol and impede its bioavailability to cells. In other words, the difference in chemical structural of 6-shogaol and 6-gingerol might be an influential factor of their bioactivity. Several studies have illustrated a consistent difference in the bioactivity of 6-shogaol and 6-gingerol. For example, 6-shogaol is far more potent than 6-gingerol in inhibiting the proliferation of and

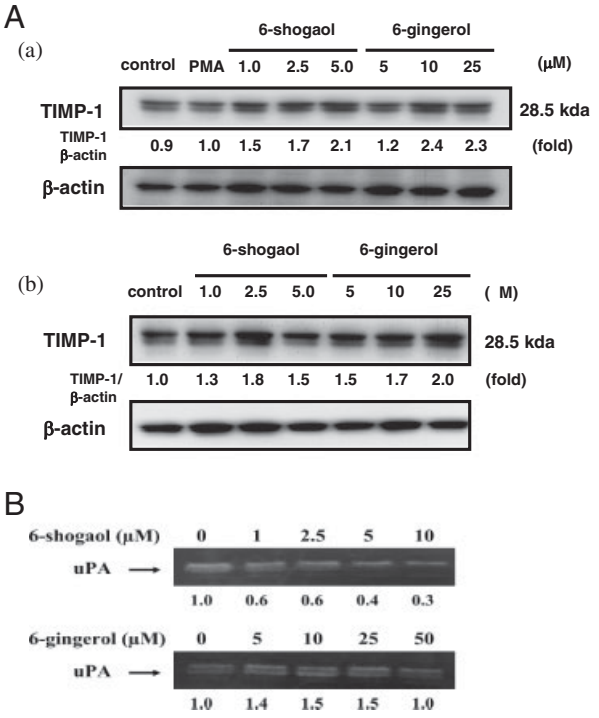

Figure 6. Effects of 6-shogaol and 6-gingerol on the protein level of TIMP-1 in PMA-treated HepG2 [A(a)] and PMA-untreated Hep3B [A(b)] cells, and on the activity of uPA in PMA-untreated Hep3B cells (B). Cells were treated with 6-shogaol and 6-gingerol at the indicated concentrations for 24 h; the conditioned medium was subjected to casein zymography for uPA assay, whereas cytosolic extracts were subjected to SDS-PAGE followed by Western blotting with TIMP-1 antibody, as described in Section 2. Determined activities or protein levels were subsequently quantified by densitometric analyses and the relative density was compared with that of PMA treatment alone or control, which was set as 100%.

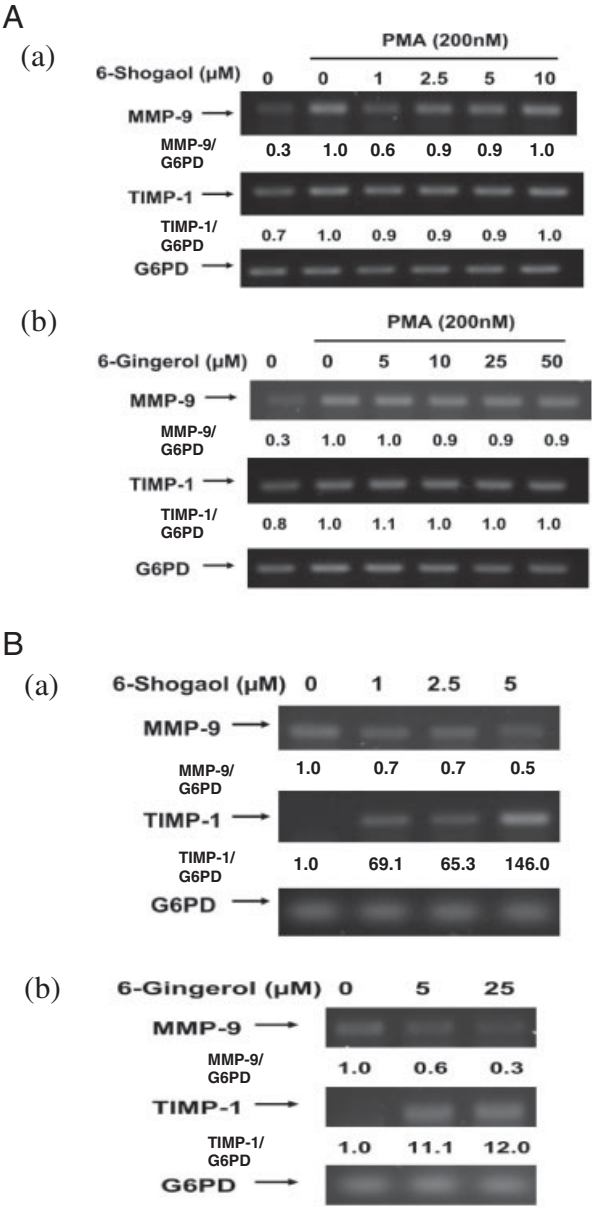

Figure 7. Effects of 6-shogaol and 6-gingerol on MMP-9 and TIMP-1 mRNA expression in PMA-treated HepG2 (A) and PMA-untreated Hep3B (B) cells. HepG2 and Hep3B cells in the presence or absence of 200 nM PMA, respectively, were incubated with the indicated concentrations of 6-shogaol (a) or 6-gingerol (b) for 24 h. The RNA was extracted from cells and subjected to a semi-quantitative RT-PCR. G6PD was used as an internal control. The final PCR products were quantified by densitometric analyses with that of PMA treatment alone or control, which was set as 100%.

inducing apoptosis in COLO 205 cells [16] and in inhibiting iNOS and COX-2 expressions in LPS-activated macrophages [10]. In this study, we also found that 6-shogaol is more effective than 6-gingerol in inhibiting the migration and invasion of hepatoma cells. Taking into account the compounds' dietary availability, the ordinary daily human consumption of ginger has approximately 250 mg–1 g and 1.0–3.0% 6-gingerol and its derivatives [5]. The concentrations of 6-gingerol (5–50 μM (~1.4–14 mg/mL)) and 6-shogaol (1–10 μM (~0.28–2.8 mg/mL)) used in this study should be similar to what is present *in vivo*.

The MMP-mediated degradation of the ECM is a well-known factor in tumor invasion and metastasis. TIMPs are endogenous inhibitors that can block the hydrolytic activities of MMPs. The balance between the levels of activated MMPs and free TIMPs determines overall MMP activity and contributes to tumor invasion and metastasis. The overexpression of TIMPs has been demonstrated to reduce experimental metastasis. TIMP-1 has been shown to have a statistically significant association with the response to chemotherapy in metastatic breast cancer [33]. Davidsen *et al.* [34] showed that TIMP-1-positive or TIMP-1-deficient cancer cell lines displayed significant differences in their sensitivity toward chemotherapeutics. TIMP-1 is a general prototype inhibitor for most MMP family members and is

present in various cell types [35]; however, TIMP-1 has a particularly high affinity for MMP-9 [36]. This study indicated that 6-shogaol and 6-gingerol reduced MMP-9 activity (Fig. 5), partially through the increase of TIMP-1 protein level (Fig. 6A), and further led to the inhibition of migratory (Fig. 3) and invasive (Fig. 4) activities of PMA-treated HepG2 and PMA-untreated Hep3B cells. Moreover, the suppression of uPA activity by 6-shogaol was also seen in Hep3B cells. Therefore, the increase of TIMP protein levels and the reduction of uPA activity may be a possible alternative strategy for the inhibition of MMP activity, with the added benefit of anti-invasion activity. We further demonstrated that the regulation of MMP-9 and TIMP-1 expression by 6-shogaol and 6-gingerol in PMA-treated HepG2 cells and the regulation of TIMP-1 expression by 6-gingerol in PMA-untreated Hep3B cells was not done through transcriptional regulation (Fig. 7), but rather on the translational or post-translational level. Owing to this complexity, further study of the detailed underlying mechanism is needed.

HepG2 and Hep3B are hepatoma cells with and without the tumor suppressor p53, respectively, while PMA is a well-known selective tumor activator that is often used to induce MMP-9 activity and enhance the invasion of HepG2 cells [37]. Wang *et al.* [38] indicated that p53 suppresses cancer cell invasion by inducing the MDM2-mediated degradation of an invasion promoter, Slug. This mechanism may provide a reasonable explanation for the migratory and invasive activities conferred by PMA treatment in HepG2 but not Hep3B cells. The different responses (*e.g.* mRNA expression of MMP-9 and TIMP-1) of HepG2 and Hep3B cells when treated with 6-shogaol and 6-gingerol are to be expected.

5 Concluding remarks

The results in this study suggest that 6-shogaol and 6-gingerol could possess potential anti-invasive activity against hepatoma cells, with 6-shogaol being more effective than 6-gingerol. The proposed anti-invasion mechanisms for these two compounds on hepatoma cells might be mediated through the inhibition of MMP-9 and the induction of TIMP-1, but only 6-shogaol decreased uPA activity in Hep3B cells. Further analysis with semi-quantitative RT-PCR showed that the regulation of MMP-9 by 6-shogaol and 6-gingerol and the regulation of TIMP-1 by 6-shogaol in Hep3B cells may on the transcriptional level. From the results presented here, 6-shogaol and 6-gingerol could be used to further test the effect of their signal transduction pathways on MMP-9 suppression and TIMP-1 induction for the prevention of hepatoma invasion or metastasis.

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