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ARTICLE

Evaluation of Anti-invasion Effect of Resveratrol and Related Methoxy Analogues on Human Hepatocarcinoma Cells

Chia-Jui Weng,[†,‡,||] Cheng-Feng Wu,[†,||] Hsiao-Wen Huang,[†] Chi-Hao Wu,[†] Chi-Tang Ho,[§] and Gow-Chin Yen*,[†]

[†]Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, [‡]Graduate Institute of Applied Science of Living, Tainan University of Technology, 529 Jhongjheng Road, Yongkang, Tainan 71002, Taiwan, and [§]Department of Food Science, Rutgers University, New Brunswick, New Jersey 08901. [||]Contributed equally as first authors.

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and is also highly metastatic. Metastasis is considered to be the major cause of death in cancer patients. Resveratrol (3,5,4′-trihydroxystilbene) and related analogues have been reported as candidates to prevent cancer growth and invasion. The bioactivity of resveratrol-related analogues could be altered due to the presence and positioning of methoxy groups on the basic resveratrol chemical structure. This study investigated the effects and mechanism of action of resveratrol and its methoxy analogues on invasion of human hepatocarcinoma cells. 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 resveratrol and 3,5,4′-trimethoxy-*trans*-stilbene (MR-3). Upon incubation of PMA-treated HepG2 cells with resveratrol (0−50 μM) or MR-3 (0−50 μM), the MMP-9 activity decreased but TIMP-1 protein increased in a dose-dependent manner. With resveratrol (0−50 μM) or MR-3 (0−1 μM) treatment on PMA-untreated Hep3B cells, both of the MMP-9 and MMP-2 activities decreased but TIMP-2 protein increased in a dose-dependent manner. These results suggest that resveratrol and its related methoxy analogue MR-3 might exert anti-invasive activity against hepatoma cells through regulation of MMP-2, MMP-9, TIMP-1, and TIMP-2. Further analysis with semiquantitative RT-PCR showed that the regulation of MMP-9 and TIMP-2 expressions by resveratrol and MR-3 in hepatoma cells may be on the transcriptional level but on the translational or post-translational level for TIMP-1.

KEYWORDS: Invasion; MMPs; MR-3; resveratrol; TIMPs; uPA

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly metastatic cancer, representing 83% of all liver cancer cases, and it is the third leading cause of cancer death worldwide (*1*). Metastasis is responsible for the majority of failures in cancer treatment and 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 actively being pursued to enhance the survival of cancer patients. The invasion and metastasis of cancer cells involve degradation of the environmental extracellular matrix (ECM) and basement membrane by various proteolytic enzymes and result in mobility of cancer cells. Among these proteases, MMP-2 and MMP-9 are highly expressed in various malignant tumors and closely related to the invasion and metastasis of cancer cells (*2*).

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) on associating with its receptor (uPAR). By means of the enzymatic cascade initiated by uPA, both the MMP-2 and MMP-9 enzymes are activated and capable of degrading type IV collagen, which is a major constituent of the basement membrane; cell mobility is hence possible and easy. Therefore, several inhibitors against MMPs have been tested in clinical trials for prevention of tumor invasion and metastasis (*3*). Tissue inhibitor metalloproteinase proteins (TIMPs) are a group of mammalian proteins composed of TIMP-1, -2, -3, and -4 that together display wide-ranging sequence homology and structural identity. TIMPs have been reported as natural MMP inhibitors that prevent the degradation of 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 (*4*).

The physiological activities of polyphenols have been studied extensively, particularly for their anticancer activities. Resveratrol (3,5,4′-trihydroxystilbene), which belongs to the group of stilbenes, is a natural polyphenol originally isolated from white hellebore and also present in grapes, berries, and peanuts (*5*). It is well documented that resveratrol can suppress proliferation and

*Author to whom correspondence should be addressed (telephone 886-4-2287-9755; fax 886-4-2285-4378; e-mail gcyen@nchu.edu.tw).

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invasion as well as induce apoptosis in a wide variety of tumor cell types (*6*, *7*). The highly potent inhibitory effects of resveratrol against tumorigenesis suggest that it is an efficient chemopreventive agent for cancer. Furthermore, compounds that are closely structurally related to resveratrol might have similar or more efficient biological effects and a better pharmacokinetic profile than resveratrol. For example, isorhapontigenin is a compound that is very similar to resveratrol in chemical structure and exhibits a potent antioxidant effect (*8*). There are various natural resveratrol-related analogues existing in plants. 3,5,4′-Trimethoxy-*trans*-stilbene (MR-3) is a natural resveratrol analogue that is methoxylated instead of hydroxylated at positions 3, 5, and 4′ in resveratrol. It has been found naturally in the plant *Pterobolium hexapetallum* (*8*) and has been reported to have antineoplastic and antiangiogenic activities (*9*, *10*). 3,5,3′,4′,5′-Pentamethoxystilbene (MR-5) is another resveratrol analogue, also a methoxy derivative of resveratrol, and can be synthesized by artificial means (*11*).

Recent papers indicated that resveratrol can inhibit migration and invasion of human breast cancer MCF-7 and MDA-MB 435 cells (*12*, *13*) as well as human hepatocellular carcinoma HepG2 cells (*14*). A resveratrol methoxy derivative was reported that exhibited a marked inhibitory effect toward COLO 205 cell growth (*15*). Previously, it was found that MR-3 could inhibit invasion of human lung adenocarcinoma A549 cells (*16*). These results indicate that resveratrol and related methoxy analogues may be candidates for preventing cancer growth and invasion. The difference in bioactivity of methoxy derivatives could be related to the presence and positioning of methoxy groups on the basic resveratrol chemical structure. However, the anti-invasive effects of resveratrol and its related methoxy analogues on hepatoma cells have not been widely compared and studied. 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 (*17*). The purpose of this study was to investigate the effects of resveratrol and its methoxy analogues including MR-3 and MR-5 (structures in **Figure 1**) on invasion of inducer-treated HepG2 and inducer-untreated Hep3B cells. To explore the anti-invasive mechanisms engaged in human liver cancer cells by resveratrol and its analogues, the impacts on MMPs, TIMPs, and uPA of these compounds were evaluated.

MATERIALS AND METHODS

Materials and Reagents. MR-3 and MR-5 were synthesized and purified through column chromatography, and a purity of >99% was verified by HPLC according to the method described by Wang et al. (*18*).

Resveratrol, type IV gelatin, casein, phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit were purchased from Invitrogen Inc. (Carlsbad, CA). The antibodies for TIMP-1 and -2 were purchased from Millipore Co. (Billerica, MA). The β-actin antibody was purchased from BioVision Inc. (Mountain View, CA). Goat anti-rabbit IgG (H&L) horseradish peroxidase conjugated antibody and goat anti-mouse IgG (H&L) horseradish peroxidase conjugated antibody were purchased from Chemicon International Inc. (Billerica, MA). PCR Master Mix 2X Kit was purchased from Fermentas (Glen Burnie, MD). Transwell Permeable Support was purchased from Corning Co. (Lowell, MA).

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 in DMEM, supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM nonessential amino acid (NEAA), and 1 mM sodium pyruvate at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2. In the invasive and metastatic experiments, the cells were cultured in serum-free medium.

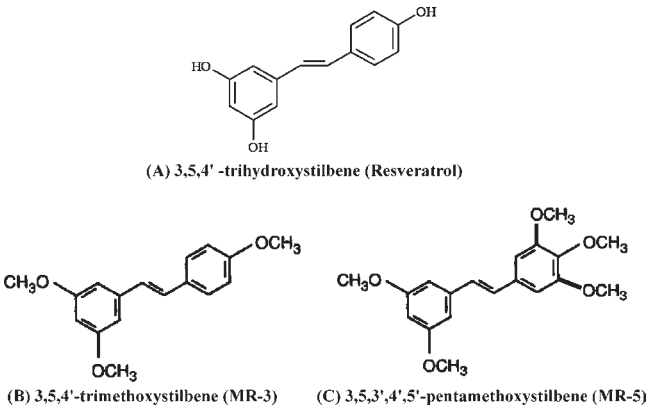

Figure 1. Chemical structures of resveratrol, MR-3, and MR-5.

Cell Viability Assay. Cell viability was determined with an MTT assay. Cells were seeded onto 96-well plates at a concentration of 1×10^4 cells/well in DMEM without FBS. After 24 h of incubation, the cells were treated with various concentrations (0−100 μM) of compounds dissolved in DMSO and further incubated for 24 and 48 h. The controls were treated with 0.1% DMSO alone. The dye solution (10 μL; 5 mg of dye/1 mL phosphate-buffered saline, PBS) was added to each well for an additional 2 h of incubation at 37 °C. After the addition of DMSO (100 μL/well), the reaction solution was incubated for 30 min in the dark. The absorbances at 570 and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader. The percent viability of the treated cells was calculated as follows:

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

Gelatin and Casein Zymography. HepG2 and Hep3B cells were incubated in the presence and absence, respectively, of indicated PMA concentrations and serum-free DMEM with or without compounds (in DMSO) for a given time; then, the conditioned media were collected as samples. The unboiled samples were separated by electrophoresis on 8% sodium dodecyl sulfate (SDS)/polyacrylamide gels containing enzyme substrate (0.1% gelatin in gelatin zymography; 2% casein and 20 μg/mL plasminogen in casein zymography). After electrophoresis, the gels were washed twice in washing buffer (2.5% Triton X-100 in dH_2O) for 30 min at room temperature and were then incubated in reaction buffer (10 mM $CaCl_2$, 0.01% NaN_3, and 40 mM Tris-HCl, pH 8.0) at 37 °C for 12 h. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA).

Cell Migration and Invasion Assay. Cells were detached from the tissue culture plates, washed with PBS buffer, and resuspended in a serum-free DMEM medium (1×10^5 cells/200 μL) in the presence or absence of compound (in DMSO). The cells were then seeded onto the upper chambers of Matrigel-coated filter inserts (8 μm pore size). A serum-containing DMEM medium (500 μL) was added to the lower chambers. After 24 h of incubation, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with Giemsa dye for 1 h. The cells that invaded the lower surface of the filter were counted under a microscope. The migration assay was performed as described for the invasion assay, but without the coating of Matrigel.

Western Blotting. Ten-microgram samples of total cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto a poly(vinylidene fluoride) (PVDF) membrane using a Bio-Rad Mini Protean electrotransfer system. The blot was subsequently blocked with 5% skim milk in PBST for 1 h and probed with their respective specific antibodies overnight at 4 °C. Detection was performed with an appropriate peroxidase-conjugated secondary antibody at room temperature for 1 h. Intensive PBS washing was performed after each incubation. After the final PBS wash, signal was visualized by an enhanced chemiluminescence (ECL) detection system and Kodak X-OMAT Blue Autoradiography Film.

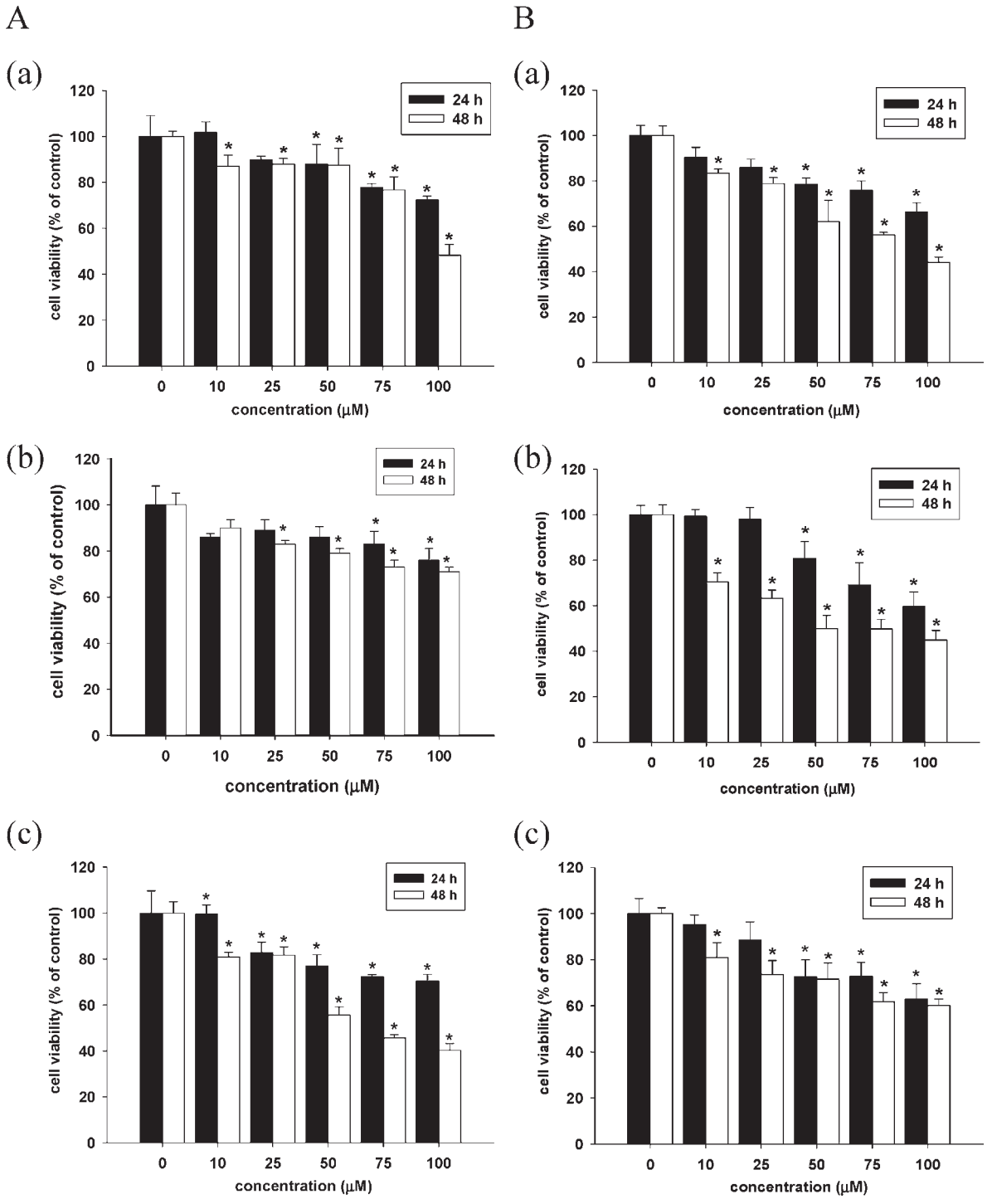

Figure 2. Effects of resveratrol (**a**), MR-3 (**b**), and MR-5 (**c**) on the viability of HepG2 (**A**) and Hep3B (**B**) cells. Cells were incubated in a serum-free medium with various concentrations (0−100 μM) of resveratrol and related analogues for 24 and 48 h. Cells in a serum-free medium without resveratrol and related analogues were used as the control. Data represent the mean ± SD of three independent experiments. ∗, $p < 0.05$ compared with the control.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from cells using the 3-Zol (Trizol) reagent (MoBioPlus Inc., Taipei, Taiwan) and performed by following the manufacturer's instructions. For RT-PCR, total cellular RNA was used as template, and SuperScript III First-Strand Synthesis SuperMix for qRT-PCR kit, which contained RT buffer, dNTP mix, RT random primers, MultiScribe TM reverse transcriptase and nuclease-free H_2O, was used for cDNA synthesis. This reaction was performed under the following conditions: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s. The resulting cDNA was amplified by PCR with the following primers: MMP-9 (94 bp), 5′-GGGCTTAGATCATTCCTCAGTG-3′ (sense) and 5′-GCCATTCACGTCGTCCTTAT-3′ (antisense); MMP-2 (108 bp) 5′-ATCCTGGCTTTCCCAAGCTC-3′ (sense) and 5′-CACCCTTGAAGAAGTAGCTGTG-3′ (antisense); TIMP-1 (95 bp) 5′-ACTTCCACAGGTCCCACAAC-3′ (sense) and 5′-AGCCACGAAACTGCAGGTAG-3′ (antisense); TIMP-2 (90 bp) 5′-CAACCCTATCAAGAGGATCCA-3′ (sense) and 5′-GGGGCCGTGTAGATAAACTCT-3′ (antisense); G6PD (glucose-6-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 MMP-9) or 62 °C (for MMP-2) for 1 min, 72 °C for 2 min; 28 cycles of 94 °C for 1 min, 59 °C (for TIMP-1) or 58 °C (for TIMP-2 and G6PD) for 1 min, 72 °C for 2 min; these steps were followed by a final incubation at 72 °C for 10 min.

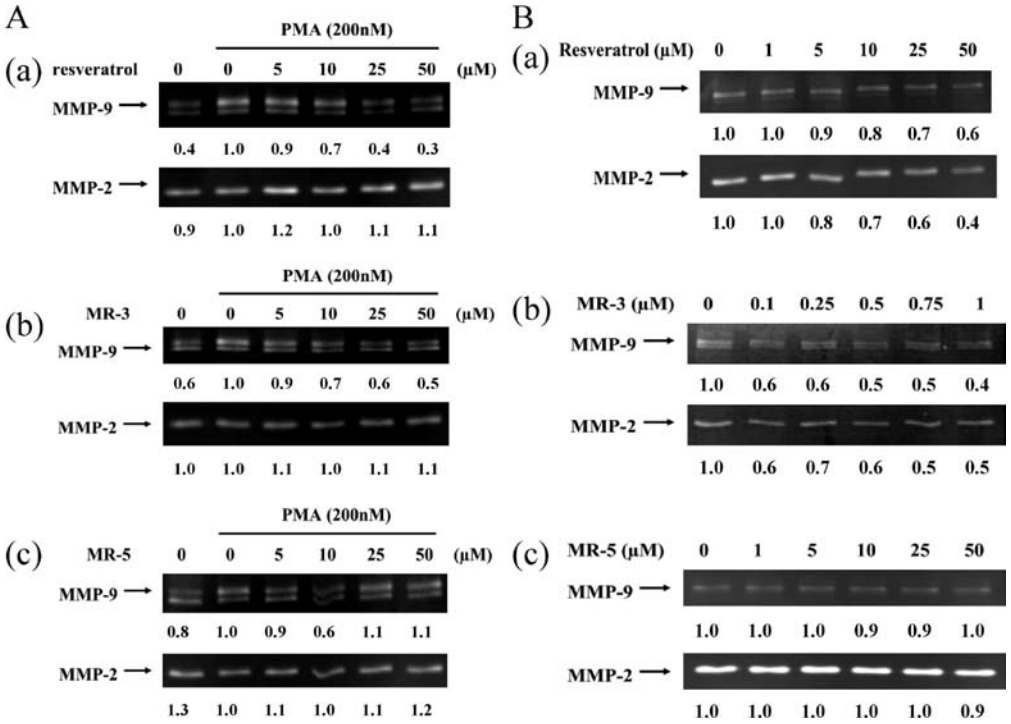

Figure 3. Effects of resveratrol (**a**), MR-3 (**b**), and MR-5 (**c**) on the MMP-9 and MMP-2 activities in PMA-treated HepG2 (**A**) and PMA-untreated Hep3B cells (**B**). Cells were incubated in a serum-free medium with various concentrations of resveratrol and related analogues for 24 h. Activities of these proteins were subsequently quantified by densitometric analyses with that of PMA treatment only or control set to 100%.

Protein Content Determination. The protein content was determined according to the method described by Bradford (*19*) with bovine serum albumin as a standard.

Statistical Analysis. Data are indicated as mean ± SD for three different determinations. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan's multiple-comparison test. Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Effects of Resveratrol and Its Methoxy Analogues on the Viability of Hepatoma Cells. The cytotoxicity of resveratrol and its related methoxy analogues on HepG2 and Hep3B cells was first determined using the MTT assay. The HepG2 and Hep3B cells were treated with resveratrol and its related methoxy analogues at various concentrations (0−100 μM) for 24 and 48 h. The results showed that both hepatoma cells treated with resveratrol, MR-3, and MR-5 at a concentrations lower than 50 μM for 24 h exhibited at least 80% cell viability (**Figure 2**). Hence, the treatment conditions of 24 h and 0−50 μM of each compound were used for the subsequent experiments on hepatoma cells.

Effects of Resveratrol and Its Methoxy Analogues on the MMP-2 and MMP-9 Activities of HepG2 and Hep3B Cells. The variations of MMP-2 and MMP-9 activities upon treatment with resveratrol and its methoxy analogues were used to analyze the potential anti-invasion activity of these compounds on hepatoma cells. We have previously shown (*17*) that HepG2 cells express only MMP-2 and that MMP-9 can be induced by 200 nM PMA; however, Hep3B cells express both MMP-2 and MMP-9 in their normal state. Therefore, the PMA-treated HepG2 and PMA-untreated Hep3B cells were incubated in a serum-free medium with or without a given compound for 24 h. The conditioned media was then used to analyze MMP-9 and MMP-2 activities by gelatin zymography. Resveratrol (5, 10, 25, 50 μM) and MR-3 (5, 10, 25, 50 μM) suppressed PMA-induced MMP-9 activity of HepG2 cells in a dose-dependent manner but did not suppress MMP-2 activity [**Figure 3A(a),A(b)**]. However, both MMP-9 and MMP-2 activities of Hep3B were suppressed by treatment of resveratrol (5, 10, 25, 50 μM) and MR-3 (0.1, 0.25, 0.5, 0.75, 1 μM) in a dose-dependent manner [**Figure 3B(a),B(b)**]. Nevertheless, the MMP-9 and MMP-2 activities in PMA-treated HepG2 and PMA-untreated Hep3B were nearly not to be inhibited by treatment with MR-5 from 0 to 50 μM except 10 μM on MMP-9 of PMA-treated HepG2 [**Figure 3A(c),B(c)**]. This dose-independent MMP-9 suppression by a single dosage of MR-5 on PMA-treated HepG2 cells is strange, and the cause needs to be verified by further specifically designed studies. Hence, we left the insignificant MMP suppressive activity of MR-5 out of consideration and focused on the potential anti-invasive activity of resveratrol and MR-3 against hepatoma cells.

Resveratrol and MR-3 Inhibit 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 resveratrol and MR-3, migration and invasion assays were performed on HepG2 and Hep3B cells. The migratory and invasive abilities of PMA-induced HepG2 cells were reduced in a dose-dependent manner by treatment of resveratrol and MR-3 at a concentration of > 5 μM for 24 h (**Figures 4A** and **5A**). After treatment with 50 μM resveratrol and MR-3 for 24 h, the invasive abilities of HepG2 cells were significantly ($p < 0.05$) reduced by 80 and 60%, respectively, relative to PMA treatment only (**Figure 5A**). The dose-dependent inhibitory effects of resveratrol and MR-3 on migration and invasion were also observed when applied to Hep3B cells (**Figures 4B** and **5B**). The invasive activities of Hep3B cells were significantly ($p < 0.05$) reduced to 27 and 42% by treatment of 50 μM resveratrol and 1 μM MR-3, respectively (**Figure 5B**). These results suggest that resveratrol and MR-3 may be effective inhibitors of invasion of hepatoma cells. The effective inhibitory concentration of MR-3 for Hep3B cells was markedly lower than that for HepG2 cells.

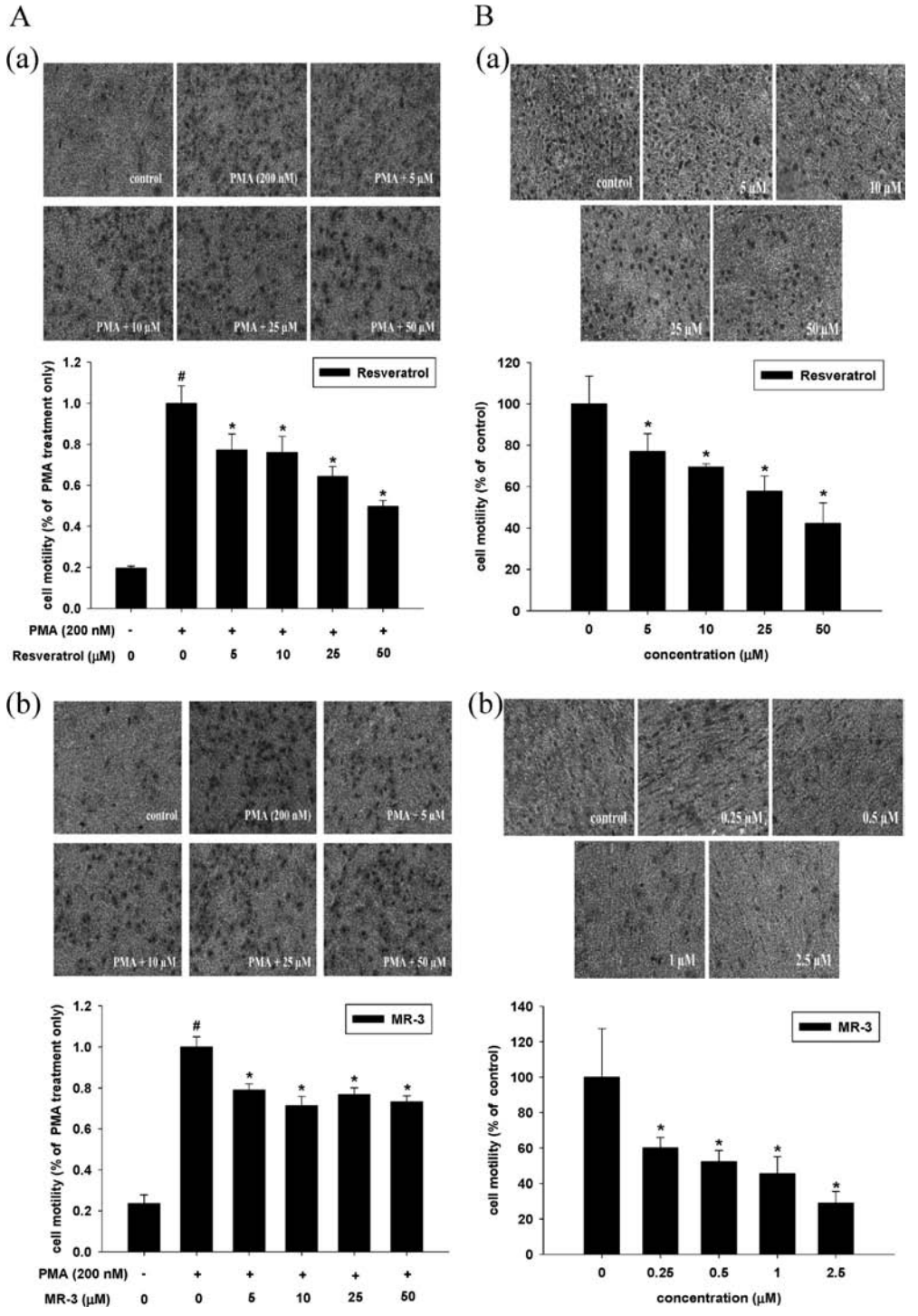

Figure 4. Effects of resveratrol (**a**) and MR-3 (**b**) on mobility of PMA-treated HepG2 (**A**) and PMA-untreated Hep3B (**B**) cells. Photography (100×) represents the cells migrating through PCF membrane. The bar graphs represent the migratory cell numbers that were treated with various concentrations of resveratrol or MR-3 for 24 h. Values are reported as means ± 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.

Resveratrol and MR-3 Increase Protein Levels of TIMP-1 and TIMP-2 but Not uPA Activity. Physiological activities of MMP-2 and MMP-9 are significantly related to that of TIMPs, especially TIMP-1 and TIMP-2. Next, the effective anti-invasive dosages on each hepatoma cell were used, and the Western blot method was employed to determine the effect of resveratrol and MR-3 on the protein levels of TIMP-1 and TIMP-2 in both HepG2 and Hep3B cells. The results showed that the protein levels of TIMP-1 and TIMP-2 in HepG2 and Hep3B, respectively, were increased in a dose-dependent manner along with a gradual increase in treated concentrations of resveratrol and MR-3 for 24 h (**Figure 6**). Although the protein levels of TIMP-2 in HepG2 and TIMP-1 in Hep3B were also increased after treatment with various concentrations of resveratrol and MR-3 as compared to the control, there are no significant differences between control and compound-treated groups as well as dose-independent relation. As uPA activity is involved in the activation of MMPs, we further analyzed the effects of resveratrol and MR-3 on uPA activity in HepG2 and Hep3B cells by casein zymography. uPA activity was not detectable in HepG2 cells and did not change in Hep3B cells

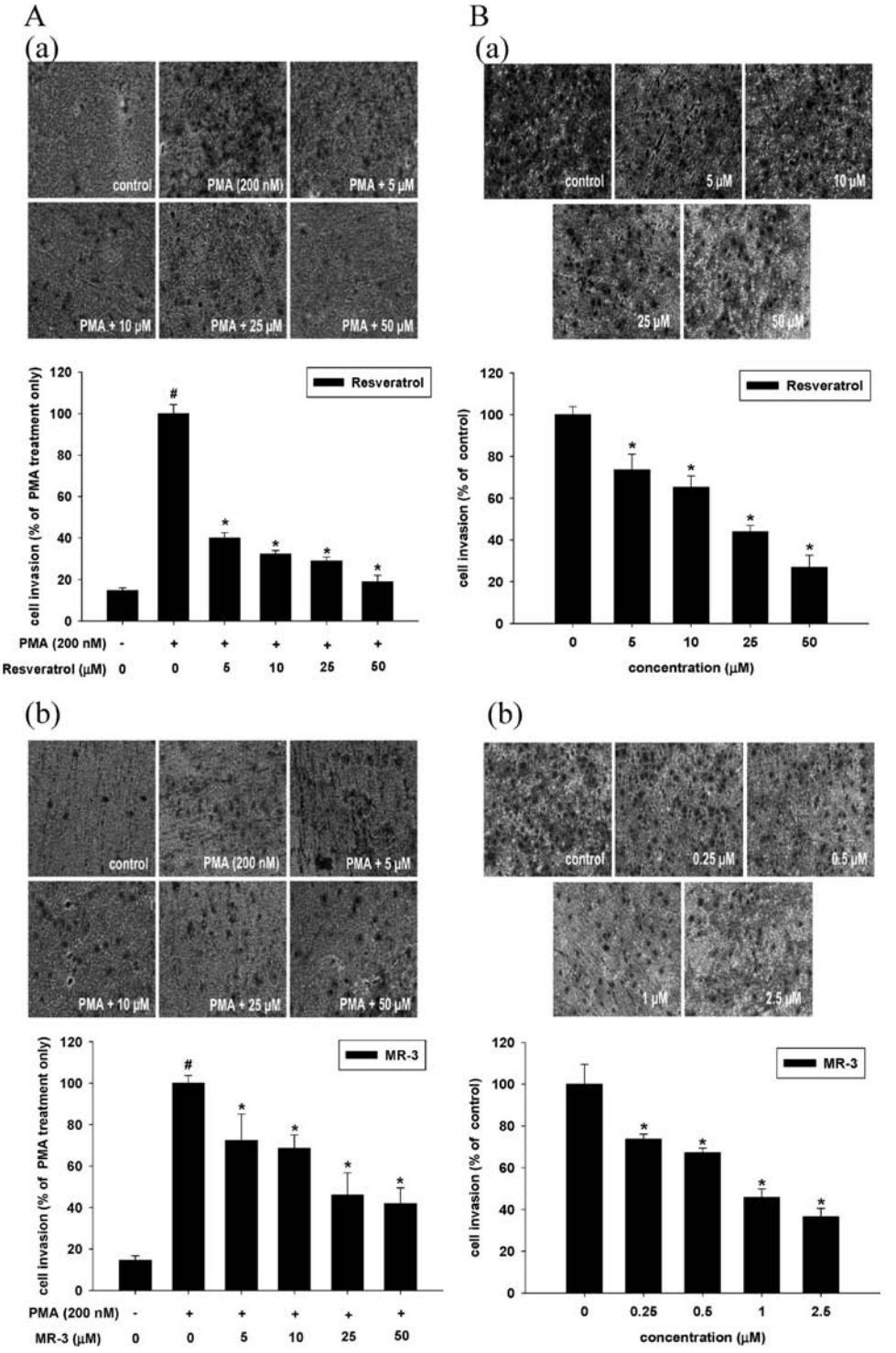

Figure 5. Concentration-dependent inhibitory effects of resveratrol (**a**) and MR-3 (**b**) on invasion of PMA-treated HepG2 (**A**) and PMA-untreated Hep3B (**B**) cells. Photography (100×) represents the cells invading through Matrigel-coated membrane. The bar graphs represent the invasive cell numbers that were treated with various concentrations of resveratrol or MR-3 for 24 h. Values are reported as means ± 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.

after incubation with either of these two compounds for 24 h (data not shown). Given this result, uPA activity may be not involved in the anti-invasive mechanism of resveratrol or MR-3 on hepatoma cells.

Resveratrol and MR-3 Applied a Transcriptional Regulation for MMP-9 and TIMP-2 Expressions but Translational or Post-translational Regulation for TIMP-1 in Hepatoma Cells. According to the results shown in **Figures 3** and **6**, the MMP-9 and TIMP-1 in HepG2 and the MMP-9 and MMP-2 and TIMP-2 in Hep3B were significantly influenced by treatment of resveratrol and MR-3. A semiquantitative RT-PCR was further employed to analyze the effect of resveratrol and MR-3 on the mRNA expressions of these proteins in each hepatoma cells. After individual treatment with these two compounds at a range of concentrations from 0 to 50 μM on the PMA-induced HepG2 cells for 24 h, the mRNA expression of MMP-9 decreased in a dose-dependent manner but MMP-2 and TIMP-1 did not change significantly, whereas that of the internal control (G6PD) remained unchanged

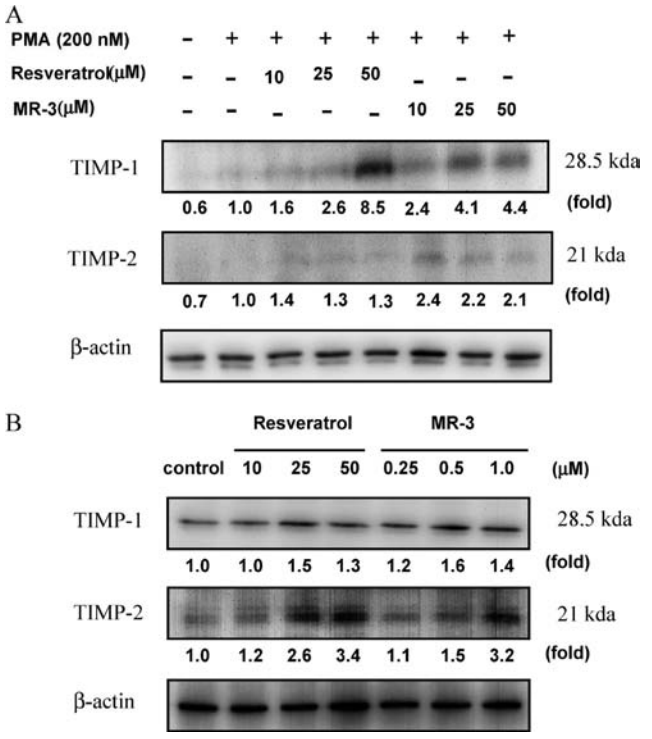

Figure 6. Effects of resveratrol and MR-3 on the expressions of TIMP-1 and TIMP-2 in PMA-treated HepG2 (**A**) and PMA-untreated Hep3B (**B**) cells. Cells were treated with resveratrol and MR-3 at the indicated concentration for 24 h, and the cytosolic extracts were subject to SDS-PAGE followed by Western blotting with TIMP-1 and TIMP-2 antibodies as described under Materials and Methods. Protein levels were subsequently quantified by densitometric analyses, and the relative density was compared with that of PMA treatment only or control being 100%.

(**Figure 7A**). Upon treatment of Hep3B cells with resveratrol (0−50 μM) or MR-3 (0−1 μM) for 24 h, the mRNA expression of MMP-9 decreased but TIMP-2 increased along with an increase in treated concentration (**Figure 7B**). These results indicate that the regulation of MMP-9 expression in HepG2 and MMP-9 and TIMP-2 expressions in Hep3B are, at least partly, on the transcriptional level. The expression of TIMP-1 in HepG2 might be regulated on a translational or post-translational level. It was also suggested that the regulation for MMP-2 in Hep3B was not on a transcriptional level. However, the regulation of MMP-2 activity might result from modified direct activity, cooperative protein interaction and translational or post-translational regulations, and so on. Owing to the complexity, further study on the detailed underlying mechanism is needed.

DISCUSSION

Dietary polyphenol is widely reported as an effective phytochemical for health protection. Resveratrol is one of the polyphenols that has demonstrated chemopreventive potential (*20*). As metastasis is the major cause of death in cancer patients, active compounds demonstrating anti-invasive and antimetastatic properties are defined as a new catalog of chemopreventive agents. The physiological concentration of dietary polyphenols observed in blood was approximated to 0.1−1 μM after the consumption of an ordinary quantity of polyphenol-rich foods or beverages (*21*). However, higher concentration of polyphenols applied to a patient for effective therapeutic purpose should be acceptable. The relationship between MMP expression and the invasive activity of various cancers has been well documented. Resveratrol has been demonstrated to be an inhibitory agent for MMP-9 expression in multiple myeloma and hepatoma SMMC-7721 cells (*22*, *23*) and for the PMA-induced MMP-9 expression in human cervical cancer Caski cells (*24*). Here, we demonstrated that resveratrol and MR-3 at a concentrations lower than 50 μM could effectively inhibit the invasive activity of hepatoma cells by reducing MMP-9 activity with (in HepG2) or without (in Hep3B) PMA induction (**Figure 3**) as well as increasing TIMP-1 and TIMP-2 proteins (**Figure 6**).

PMA, a well-known selective tumor activator, has been used to induce MMP-9 activity and enhance invasion of HepG2 cells (*25*). HepG2 and Hep3B are hepatoma cells with and without tumor suppressor p53 gene, respectively. Wang et al. (*26*) revealed that p53 suppresses cancer cell invasion by inducing the MDM2-mediated degradation of an invasion promoter, Slug. This may provide a reasonable explanation for the migratory and invasive activities conferred by PMA treatment in HepG2 cells, but not in Hep3B cells.

It is well-known that MMP-mediated degradation of the ECM is a crucial 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 (*27*). Davidsen et al. (*28*) showed that TIMP-1 positive or TIMP-1 deficient cancer cell lines displayed significant differences in sensitivity toward chemotherapeutics. TIMP-2 overexpression could decrease invasion of endothelial and tumor cells both in vitro and in vivo (*29*, *30*). The invasion in vitro and metastasis in vivo of the human breast carcinoma cell lines MCF10A and MDA-MB-435 were decreased by retroviral delivery of TIMP-2 into the cells (*31*, *32*). TIMP-1 and TIMP-2 were known to have a particularly high affinity for MMP-9 and MMP-2, respectively (*33*), and TIMP-1 is also the prototype inhibitor present in various types of cells for most MMP family members (*34*). These might provide an explanation for why resveratrol and MR-3 inhibited only MMP-9 via increasing TIMP-1 in PMA-treated HepG2 and inhibited both MMP-2 and -9 via increasing TIMP-2 in PMA-untreated Hep3B. The induction of TIMP-1 and -2 involved in the inhibition of migratory (**Figure 4**) and invasive (**Figure 5**) activities of HepG2 and Hep3B cells was demonstrated in this study. Therefore, the increase of TIMPs protein may be a possible alternative strategy for inhibition of MMPs activity, with the added benefit of anti-invasion activity. The pathway and mechanism regarding the induction of TIMPs in hepatoama cells would be another topic of research in the future.

Studies on the inhibition of P450 1A1 enzymes by two stilbene analogues, rhapontigenin (3,3′,5-trihydroxy-4′-methoxystilbene) and 2,4,3′,5′-tetramethoxystilbene, showed that the IC_{50} values were 0.4 and 0.3 μM (*35*, *36*), respectively, meaning that the enzyme activity inhibition was sensitive to the substitution patterns on the *trans*-stilbene. The methylated derivatives of flavonoids have also been shown to have a higher antiproliferative potency on cancer cells than their hydroxylated counterparts (*37*). Resveratrol, a *trans*-stilbene, suffers from extensive phase II metabolism (glucuronidation and sulfation on the hydroxyl groups), leading to short half-life and limited bioavailability (*5*). MR-3 may be more favorable than resveratrol because all of its hydroxyl groups, which are subject to extensive glucuronide or sulfate conjugation in the metabolic pathways of resveratrol, are protected by methoxylation. The methoxylated substitution might prolong the eliminated half-life and improve intestinal absorption and metabolic stability (*38*). Moreover, methoxylation

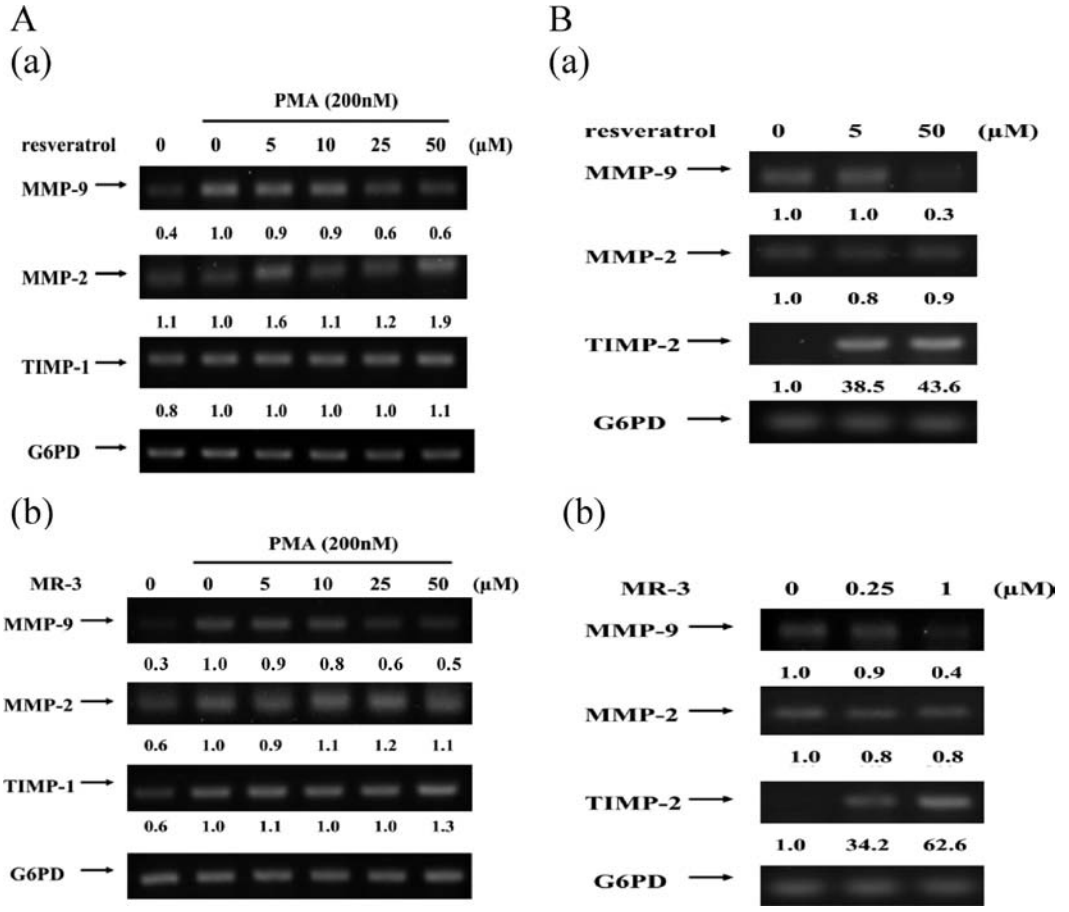

Figure 7. Effects of resveratrol (**a**) and MR-3 (**b**) on MMPs and TIMPs mRNA expression in PMA-treated HepG2 (**A**) and PMA-untreated Hep3B (**B**) cells. HepG2 and Hep3B cells were treated with various concentrations (0−50 μM) of resveratrol or MR-3 for 24 h in the presence or absence of 200 nM PMA. The RNA was extracted from cells and subjected to a semiquantitative RT-PCR. G6PD was used as an internal control. The final PCR products were quantified by densitometric analyses with that of PMA treatment only or control being 100%.

increases the lipophilic properties and cell membrane permeability of MR-3 to enhance its bioavailability. On the basis of its chemical structure, MR-3 could have better pharmacokinetic profiles than resveratrol. In a previous study (*16*), it was observed that MR-3 inhibits invasion of human lung adenocarcinoma A549 cells more efficiently than resveratrol. Although MR-5 is also a methoxylated substitution analogue of resveratrol, the overmethoxylation at positions 3, 5, 3′, 4′, and 5′ in resveratrol might lead to reduction of solubility and increase in molecular weight, which impedes its bioavailability to cells.

In conclusion, resveratrol and its methoxy analogue MR-3 might possess potential anti-invasive activity, and MR-3 is more effective than resveratrol against hepatoma cells. The proposed anti-invasion mechanisms for these two compounds might be mediated through the inhibition of MMP-2 and MMP-9 as well as induction of TIMP-1 and TIMP-2. Further analysis with semiquantitative RT-PCR showed that the regulation of MMP-9 and TIMP-2 expressions by resveratrol and MR-3 may on the transcriptional level. From the results, resveratrol and MR-3 could be used to further test their signal transduction pathways on MMP-9 suppression and TIMP-2 induction for prevention of hepatoma invasion or metastasis.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; MMP, matrix metalloproteinase; MR-3, 3,5,4′-trimethoxy-*trans*-stilbene; MR-5, 3,5,3′,4′,5′-pentamethoxystilbene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMA, phorbol 12-myristate 13-acetate; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase-type plasminogen activator.

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