Research Article

# Resveratrol analog-3,5,4′-trimethoxy-*trans*-stilbene inhibits invasion of human lung adenocarcinoma cells by suppressing the MAPK pathway and decreasing matrix metalloproteinase-2 expression

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Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol that presents various physiological activities. It has been reported that the methylated derivatives of resveratrol show better potential antifungal and antiproliferative activities than resveratrol. In the present study, we investigated the inhibitory effect of 3,5,4′-trimethoxy-*trans*-stilbene (MR-3), a methylated derivative of resveratrol, on the invasion of A549 cells (a human lung adenocarcinoma cell line). We found that treatment with MR-3 at the concentration of 5 μM resulted in antiadhesive, antimigratory, and antiinvasive activities on A549 cells through the suppression of matrix metalloproteinase (MMP)-2 protein expression and transcriptional levels in a time-dependent manner. The suppression of MMP-2 expression by MR-3 led to an inhibition of A549 cell invasion by inactivating phosphorylation of SAPK/c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways. A time-dependent inhibition of protein levels for p65, c-Jun, and c-Fos in the nucleus by MR-3 treatment was also observed. In conclusion, our data demonstrate that the antiinvasive effects of MR-3 on A549 cells are likely mediated through the inhibition of phosphorylation of JNK and p38, as well as a reduction in the protein levels of nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) in the nucleus, ultimately leading to downregulation of MMP-2 expression.

**Keywords:** Invasion / MAPK signaling / MMP-2 / Resveratrol / 3,5,4′-Trimethoxy-*trans*-stilbene

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## 1 Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol present in various plants, including grapes, berries, and peanuts. The physiological activities of polyphenols have been studied extensively, and include cardioprotection [1], inhibition of platelet aggregation, antiinflammatory properties, antioxidant capabilities, and vasorelaxant activities [2]. Recently, resveratrol has been found could shift the physiology of middle-aged mice on a high-calorie diet toward that of mice on a standard diet and significantly increases their survival [3]. According to their structure, polyphenols can be categorized as hydroxybenzoic acids, hydroxycinnamic acids, stilbenes, anthocyanins, flavonols, flavones, flavanones, isoflavones, and monomeric flavanols [4]. Resveratrol belongs to the group of stilbenes. It can suppress proliferation, invasion, and apoptosis of various cancer cells by regulating NF-κB and activator protein-1 (AP-1) activities [5, 6]. In addition, it also exhibits anticancer properties in a wide variety of tumor cell types, including breast, prostate, stomach, colon, pancreas, and thyroid cancers [7, 8]. The highly potent inhibitory effects of resveratrol against tumorigenesis might suggest that it is an efficient cancer chemopreventive agent. There are various natural resveratrol analogs existing in plants. For example, isorhapontigenin which isolated from *Belamcanda chi-*

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**Abbreviations: AP-1**, activator protein-1; **ECM,** extracellular matrix; **JNK**, c-Jun N-terminal kinase; **MMP,** matrix metalloproteinase; **MR-3,** 3,5,4′-trimethoxy-*trans*-stilbene; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

*nensis* is a derivative of stilbene. Its chemical structure is very similar to that of resveratrol, and it has a potent antioxidative effect [9]. Compounds that are closely related to resveratrol structurally and thus might have more efficient biological effects than resveratrol. The antiinvasion research is almost exclusively focused on resveratrol and on its inhibition of angiogenesis in endothelial cells [10–13]. Until now, literature regarding the antiinvasive effects of resveratrol or its analogs on lung cancer cells has been limited.

Lung cancer is the cause of approximately 20% of tumor-related deaths, and in Taiwan it is the first- and the second-most lethal cancer type for females and males, respectively. Approximately 70% of lung cancer patients die from metastasis. Cancer metastasis refers to the spread of cancer cells from the primary neoplasm to distant sites and the growth of secondary tumors at sites distant from the primary tumor. 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. Metastasis is responsible for the majority of failures in cancer treatment, and is the major cause of death in patients with various cancer types [14]. Therefore, in addition to minimizing the growth of existing tumors, treatments that limit spread to new sites and blockade invasion have been pursued to enhance the survival of cancer patients [15]. The invasion and metastasis of cancer cells involves the degradation of environmental barriers such as the extracellular matrix (ECM) and basement membrane by various proteolytic enzymes, including serine proteinase, matrix metalloproteinases (MMPs), membrane type 1-MMP (MT-1 MMP), cathepsins, and plasminogen activator, which results in promoted mobility of cancer cells [16–18]. Among these proteases, MMP-2 and MMP-9 are presumed to be associated with the progression and invasion of different types of cancer cells. Both the MMP-2 and MMP-9 enzymes are capable of degrading type IV collagen, which is a major constituent of the basement membrane. These 2 MMPs are highly expressed in various malignant tumors and closely related to the invasion and metastasis of cancer cells [19–21]. Therefore, several inhibitors against MMPs have been tested in clinical trials for prevention of tumor invasion and metastasis [22, 23]. Resveratrol was found to inhibit MMP-2 activity in human liver myofibroblasts [24].

MR-3 (3,5,4′-trimethoxy-*trans*-stilbene), an analog of resveratrol, is methoxylated instead of hydroxylated at positions 3, 5, and 4′ in resveratrol. It has been naturally found and identified in plant, *Pterobolium hexapetallum* [9]. The purpose of this study was to demonstrate the effect of MR-3 on inhibiting invasion of A549 cells (a highly metastatic human lung cancer cell). Additionally, in order to explore the molecular mechanisms for the involvement of MR-3 in human lung cancer cell invasion and metastasis, we also investigated the activities and expression levels of the MMPs, the activities of the mitogen-activated protein kinase (MAPK) signaling proteins, and the activities of the transcriptional factors NF-κB and AP-1.

## 2 Materials and methods

### 2.1 Materials

MR-3 was synthesized and purified through column chromatography and more than 99% purity verified by HPLC [25]. RPMI 1640 medium was purchased from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Type IV gelatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). DMSO was purchased from Tedia (Fairfield, OH, USA). Millicell culture plate insert and polyvinylidene fluoride (PVDF) membrane were purchased from Millipore (Bedford, MA, USA). One-Step RT-PCR kit was purchased from BD (Biosciences, Bedford, MA). The antibodies of total and phosphorylated MAPK/ERK1/2, p38 MAPK, SAPK/c-Jun N-terminal kinase (JNK), IκBα, c-Fos, c-Jun, c-Rel, NF-κB (p65 and p105), and α-tubulin were purchased from Cell Signaling Technology (Boston, MA, USA). Antilamin B was purchased from Upstate (Lake Placid, NY, USA). SP600125 and SB203580 were purchased from BioSource International (Camarillo, CA, USA).

### 2.2 Cell culture

Human lung adenocarcinoma A549 cells (BCRC No 60074) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in RPMI1640 medium, 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 acids, 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, cells were cultured in a serum-free medium.

### 2.3 Cell viability assay

Cell viability was determined with the MTT assay. A549 cells were seeded onto 96-well plates at a concentration of $1 \times 10^6$ cells/well in RPMI1640 medium without FBS. After 24 h of incubation, the cells were treated with various concentrations of sample in 0.1% DMSO for further incubation. The controls were treated with 0.1% DMSO alone. The dye solution (10 μL; 5 mg/mL, PBS), specific for the MTT assay, was added to each well for an additional 60 min of incubation at 37°C. After the addition of DMSO (100 μL/well), the reaction solution was placed in the dark for 30 min. The absorbance at 570 nm (formation of forma-

 

zan) and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader (BMG LabTechologies, Offenburg, Germany). The percent viability of the treated cells was calculated as follows:

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

### 2.4 Determination of MMP-2 and MMP-9 activities by zymography

A549 cells were incubated in a serum-free RPMI1640 medium in the presence or absence of sample for a given time, and the conditioned media were collected as samples. The unboiled samples were separated by electrophoresis on 8% SDS/polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed twice in a washing buffer (2.5% Triton X-100 in $dH_2O$) at room temperature for 30 min to remove SDS, and were then incubated in a reaction buffer (10 mM $CaCl_2$, 0.01% $NaN_3$, and 40 mM Tris-HCl, pH 8.0) at 37°C for 12 h to allow proteolysis of the gelatin substrate. Bands corresponding to activity were visualized by negative staining using CBB R-250 (BioRad Laboratories, Richmond, CA, USA) and molecular weights were estimated by reference to prestained SDS-PAGE markers.

### 2.5 Cell – matrix adhesion assay

After treatment with various concentrations of sample for 24 h, the cells ($1 \times 10^5$ cells/well) were transferred to a 24-well transwell plate (Costar, Cambridge, MA, USA) that was coated with 20 μg/well type I collagen (Upstate), and were then cultured for 30 min. Nonspecific binding was blocked by incubation with 2% BSA (in PBS) (Sigma) for the next 2 h at room temperature. The cells were washed twice in PBS at room temperature to remove nonadherent cells. The adherent cells on the plate were fixed with methanol for 10 min. After staining with 0.1% crystal violet for 1 h, the cells were lysed in 0.2% Triton X-100. The absorbance was measured with Fluostar Galaxy plate reader at 570 nm. The percentage of adhesive cells was calculated as follows:

$$(Abs_{570\ nm}(sample)/Abs_{570\ nm}\ (control)) \times 100$$

### 2.6 Cell migration and invasion assay

The cell invasion assay was performed according to the method of Repesh [26]. A549 cells were detached from the tissue culture plates, washed with PBS buffer and resuspended in a serum-free RPMI1640 medium ($5 \times 10^4$ cells/200 μL) in the presence or absence of sample. The cells were then seeded onto the upper chambers of Matrigel-coated filter inserts (8 μm pore size; BD Biosciences, San Jose, CA, USA). A serum-containing RPMI1640 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 crystal violet 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 [27].

### 2.7 RT-PCR

Total RNA was prepared from A549 cells using the 3-Zol (Trizol) reagent (MDBio, Piscataway, NJ, USA) following the manufacturer's instructions. For RT-PCR, 4 μg of total cellular RNA was used as a template in a 20 μL reaction solution that contained 4 μL dNTPs (2.5 mM), 2.5 μL oligo dT (10 pmole/μL), and RTase (200 U/μL). The reaction was performed at 50°C for 1 h. The cDNA (5 μL) was amplified by PCR with the following primers: MMP-2, 5′-GGCCCTGTCACTCCTGAGAT-3′ (sense) and 5′-GGCATCCAG GTTATCGGGGA-3′ (antisense); GAPDH, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (sense) and 5′-AGCCTTCTCCATGGTTGGTGAAGAC-3′ (antisense). The primers were synthesized by Blossom Biotechnologies (Taipei, Taiwan). PCR amplification was performed under the following conditions: 35 cycles of 94°C for 30 s, 65°C for 30 s, 68°C for 1 min, followed by a final incubation at 68°C for 10 min. PCR products were analyzed by 1.6% agarose gel and visualized by EtBr staining.

### 2.8 Preparation of cell lysates and nuclear fractions

The cell lysates and nuclear fractions were prepared using the Nuclear Extraction Kit (Panomics, Fremont, CA, USA). Briefly, harvested cells ($1 \times 10^6$ cells/6 cm plate) were washed twice with 5 mL cold 1 × PBS. 0.5 mL of Buffer A working reagent (combining 0.5 mL 1 × Buffer A, 5 μL DTT, 5 μL protease inhibitor cocktail and 20 μL 10% IGEPAL) was added to each plate. The plate was transferred to an ice bucket on a rocking platform at 150 rpm for 10 min. A sterilized cell scraper was used to remove the cells followed by pipetting up and down several times to disrupt the cell clumps. Each sample was transferred to a 1.5 mL sterilized eppendorf tube and centrifuge at 14 000 × *g* for 3 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet kept on ice. Seventy-five microliter of Buffer B working reagent (combining 0.5 mL 1 × Buffer B, 5 μL DTT, 5 μL protease inhibitor cocktail) was added to each pellet and vortexed at the highest setting for 10 s. The eppendorf tube was placed horizontally in an ice bucket which was transferred to a rocking platform at 150 rpm for 2 h. After centrifuged at 14 000 × *g* for 5 min at 4°C, the supernatant (nuclear extract) was transferred to a new

 

eppendorf tube for the measurement of the protein concentration of each sample, and was stored at −80°C.

### 2.9 Western blotting

Samples (10 μg) of total cell lysates or nuclear fractions were size fractionated electrophoretically by a 10% polyacrylamide SDS-PAGE gel and transferred onto a PVDF membrane using the BioRad Mini Protean electrotransfer system (Mini-Protean, BioRad Labratories, Richmond, NY, USA). The blots were subsequently incubated with 5% skim milk in PBST for 1 h to block nonspecific binding, and was probed overnight at 4°C with the antibodies against total and phosphorylated MAPK/ERK1/2, p38 MAPK, and SAPK/JNK; IκBα, α-tubulin, NF-κB (p65), c-Rel, c-Jun, c-Fos, and lamin B. The membranes were sequentially detected with an appropriate peroxidase-conjugated secondary antibody incubated at room temperature for 1 h. Intensive PBS washing was performed after each incubation. After the final PBS washing, signals were developed using the ECL (enhanced chemiluminescence) detection system and Kodak X-OMAT Blue Autoradiography Film.

### 2.10 Protein content determination

The protein content was determined by the method of Bradford [28] with BSA as a standard.

### 2.11 Statistical analysis

Data are indicated as mean ± SD for three different determinations. Differences between variants were analyzed by Duncan's multiple range tests for unpaired data. Values of $p < 0.05$ were regarded as statistically significant.

## 3 Results

### 3.1 Effect of MR-3 on the viability of A549 cells

The cytotoxicity of the MR-3 on A549 cells was first determined using the MTT assay. The decrease in absorbance in this assay could either be a consequence of cell death or reduction in cell proliferation. The A549 cells were treated with MR-3 at various concentrations (1, 5, 10, 25, 50, or 100 μM) for 24 h. The results showed that treatment with MR-3 at different concentrations ranging from 0 to 10 μM exhibited no cytotoxic effects on the A549 cells (Fig. 1). Therefore, these concentrations of MR-3 and treatment time without cytotoxicity on A549 cells were used for the subsequent experiments.

### 3.2 MR-3 inhibits adhesion, migration, and invasion of A549 cells

To examine potential antiinvasive effects and cell–matrix adhesion, a migration and invasion assay was performed on

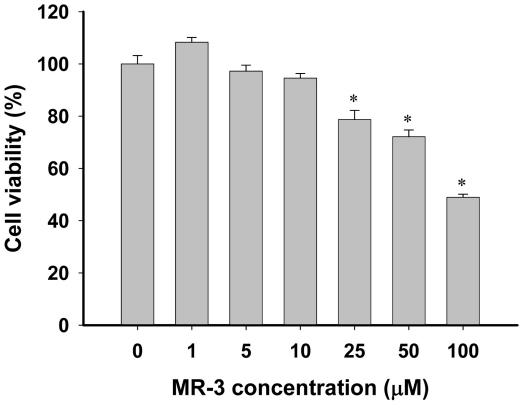


**Figure 1.** Effect of MR-3 on the viability of A549 cells. A549 cells were incubated in a serum-free medium containing various concentrations (1, 5, 10, 25, 50, or 100 μM) of MR-3 for 24 h. Viability of A549 cells was determined by the MTT assay as described in Section 2. Viability of the untreated A549 cells in a serum-free medium was used as the control. Results were statistically analyzed using the Student's *t*-test (*$p < 0.05$ compared with the control). Data (mean ± SD of three independent experiments) are expressed as the percentage of cell viability of the control.

A549 cells. Treatment of cells with MR-3 at various concentrations (0, 1, 2.5, 5, 7.5, or 10 μM) for 24 h, reduced their adhesive, migratory, and invasive abilities in a dose-dependent manner. After treatment with 5 μM MR-3 for 24 h, the adhesive, migratory, and invasive abilities of A549 cells were significantly ($p < 0.05$) reduced by 34, 39, and 22%, respectively, as compared with that of the control (Fig. 2). These results suggested that MR-3 might be an effective inhibitor for the invasion of A549 cells.

### 3.3 MR-3 inhibits MMP-2 activity and mRNA expression

The effects of MR-3 on the MMP-2 and MMP-9 activities of A549 cells were analyzed to clarify if changes in their activities were involved in MR-3-inhibited invasion of A549 cells. The cells were treated with MR-3 at the concentration of 5 μM for various time intervals (3, 6, 12, and 18 h), and the conditioned media were analyzed using gelatin zymography. It was found that the A549 cells expressed only MMP-2, and the activity of MMP-2 was decreased in a time-dependent manner by the MR-3 treatment (Fig. 3A). The MMP-2 activity was significantly ($p < 0.05$) reduced to 60% after treatment with 5 μM of MR-3 for 12 h. To further investigate if the inhibitory effect of MR-3 on the activity of MMP-2 in A549 cells was at the level of mRNA expression, a semiquantitative RT-PCR analysis was performed. After treatment of the cells with MR-3, the mRNA level of MMP-2 was also reduced in a time-dependent manner while that of the internal control (GAPDH) remained unchanged (Fig. 3B). The MMP-2 mRNA level was significantly ($p < 0.05$)

 

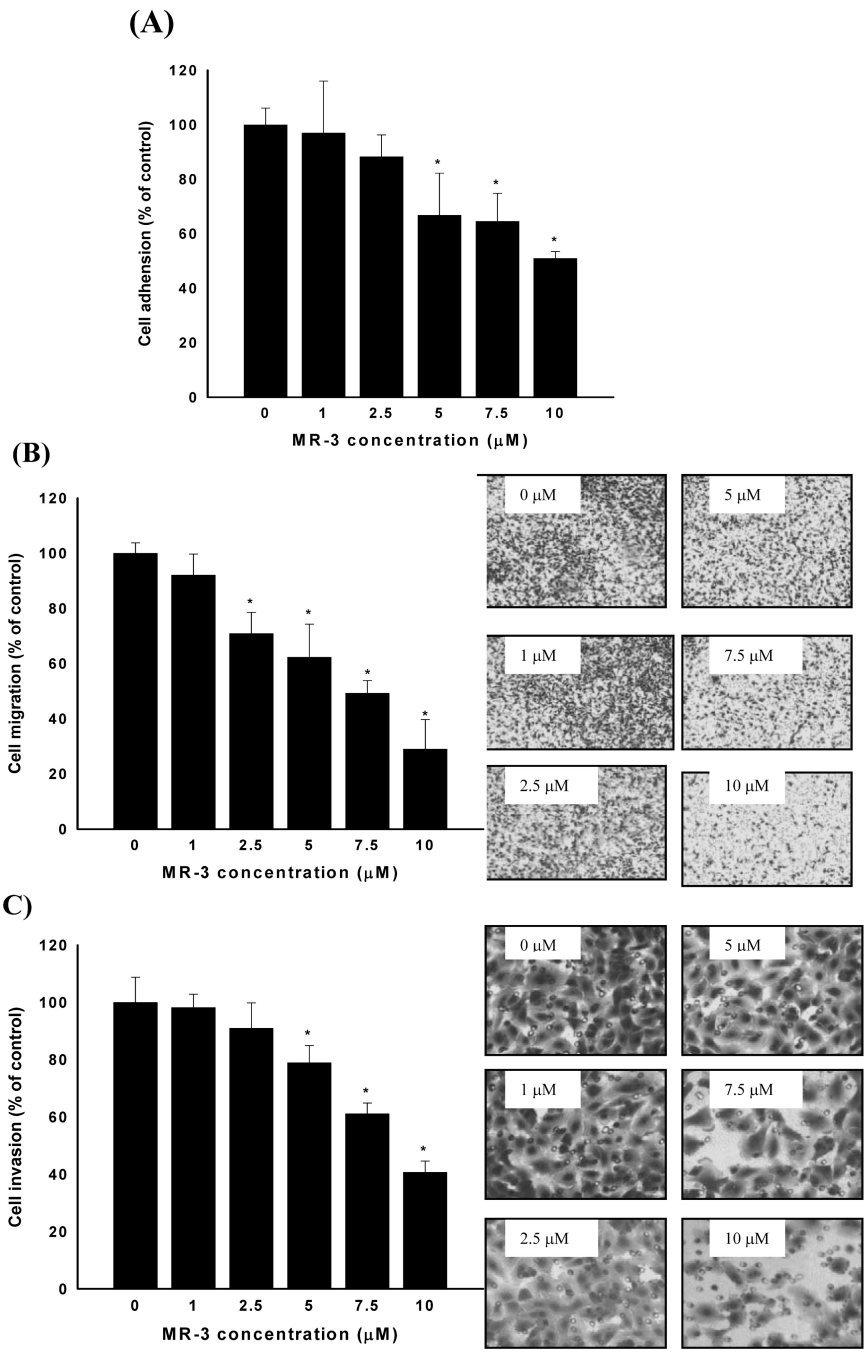


**Figure 2.** Effects of MR-3 on cell–matrix adhension, migration and invasion of human lung adenocarcinoma A549 cells. Cells were treated with the indicated concentrations of MR-3 for 24 h, and were subjected to analyses for cell–matrix adhesion (A), migration (B), and invasion (C) as described in Section 2. Photos of the migratory and invasive A549 cells were taken under a microscope with 100- and 200-fold, respectively. Data are represented as mean ± SD of three independent experiments. *$p < 0.05$ compared with control.

reduced to 20% that of the control after treatment with 5 μM MR-3 for 12 h. Even though both MMP-2 activity and mRNA expression were increased after 18 h of incubation, the effect of MR-3 on the MMP-2 protein level was still consistent with the effect on the mRNA level. These results revealed that MR-3 might regulate the expression of MMP-2 at least in part by altering transcription of the gene.

### 3.4 MR-3 inhibits MMP-2 activity of A549 cells by suppressing phosphorylation of SAPK/JNK and p38

The results of gelatin zymography and Western blotting showed that MR-3 could inhibit the MMP-2 activity and suppress the phosphorylation of JNK and p38 (Fig. 4) in A549 cells. According to the densitometric analyses of

 

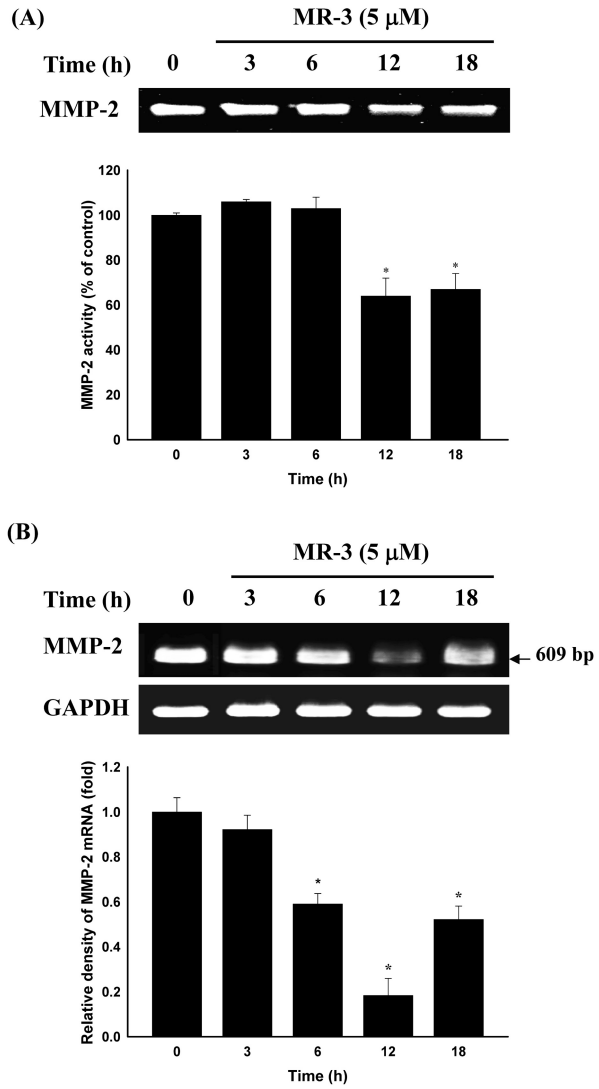


**Figure 3.** Effects of MR-3 on the MMP-2 activity and MMP-2 mRNA expression of human lung adenocarcinoma A549 cells. Cells were treated with 5 μM MR-3 for 3, 6, 12, and 18 h. (A) MMP-2 activity of A549 cells was determined by gelatine zymography. (B) The RNA extracted from A549 cells was subjected to a semiquantitative RT-PCR. GAPDH was used as an internal control. The final PCR products were quantified by densitometric analysis with their control set to 100%. Data are expressed as mean ± SD of three independent experiments. *$p < 0.05$ compared with control.

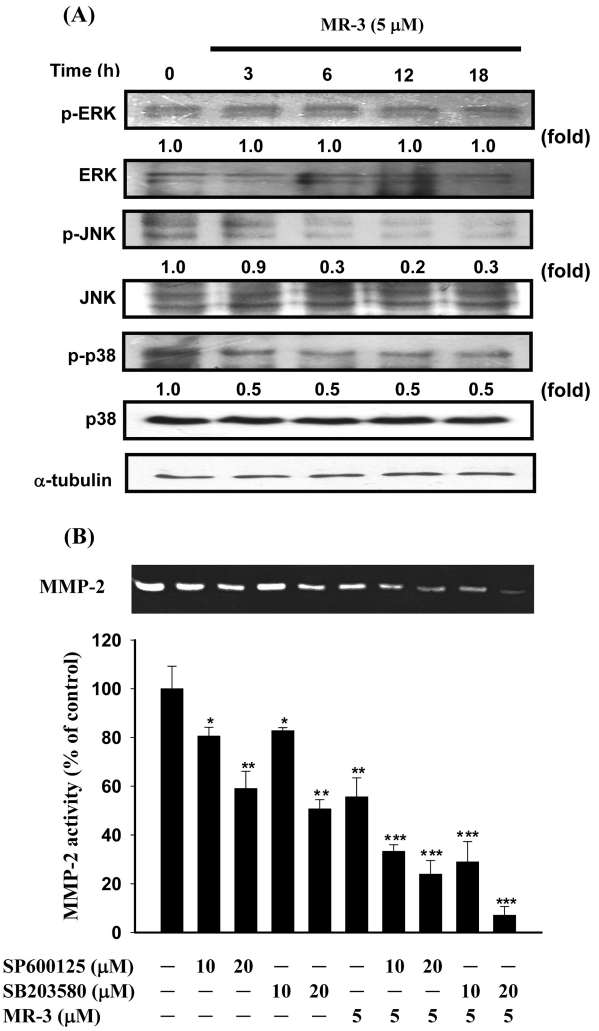


**Figure 4.** Inhibitory effect of MR-3 on MAPK signaling. (A) A549 cells were cultured in serum-free media containing 5 μM MR-3 for 3, 6, 12, and 18 h, and the cell lysates were subjected to SDS-PAGE followed by Western blotting as described in Section 2. Activities of these proteins were subsequently quantified by densitometric analyses, with the control set to 100%. (B) Cells were cultured in 24 well plates and pretreated with or without 10 or 20 μM JNK inhibitor (SP600125) and p38 inhibitor (SB203580) for 2 h. The cells were then incubated in the presence or absence of 5 μM MR-3 for 18 h. The culture media were subjected to gelatin zymography for the analysis of MMP-2 activity as described in Section 2. Activities of these proteins were subsequently quantified by densitometric analyses with of the control set at 100%. Data are represented as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with control.

each blot *versus* the control, treatment with MR-3 (5 μM) for 12 h decreased the phosphorylation of JNK and p38 by 0.8- and 0.5-fold, respectively (Fig. 4A). To further confirm whether the inhibition of MMP-2 expression by MR-3 in A549 cells was mainly carried out through the JNK and p38 signaling pathways, the cells were pretreated with JNK (SP600125; 10 or 20 μM) or p38 inhibitors (SB203580; 10 or 20 μM) for 2 h and then incubated with MR-3 (5 μM) for 18 h. The gelatin zymography analyses showed that treatment with SP600125 (10 or 20 μM), SB203580 (10 or 20 μM), or MR-3 (5 μM) alone reduced the MMP-2 activity by 19, 41, 17, 49, and 44%, respectively, as compared with that of the control. In the combination treatment of SP600125 (10 or 20 μM) or SB203580 (10 or 20 μM) with MR-3 (5 μM), the MMP-2 activity was reduced by 67, 76, 71, and 93%, respectively, as compared with that of the con-

 

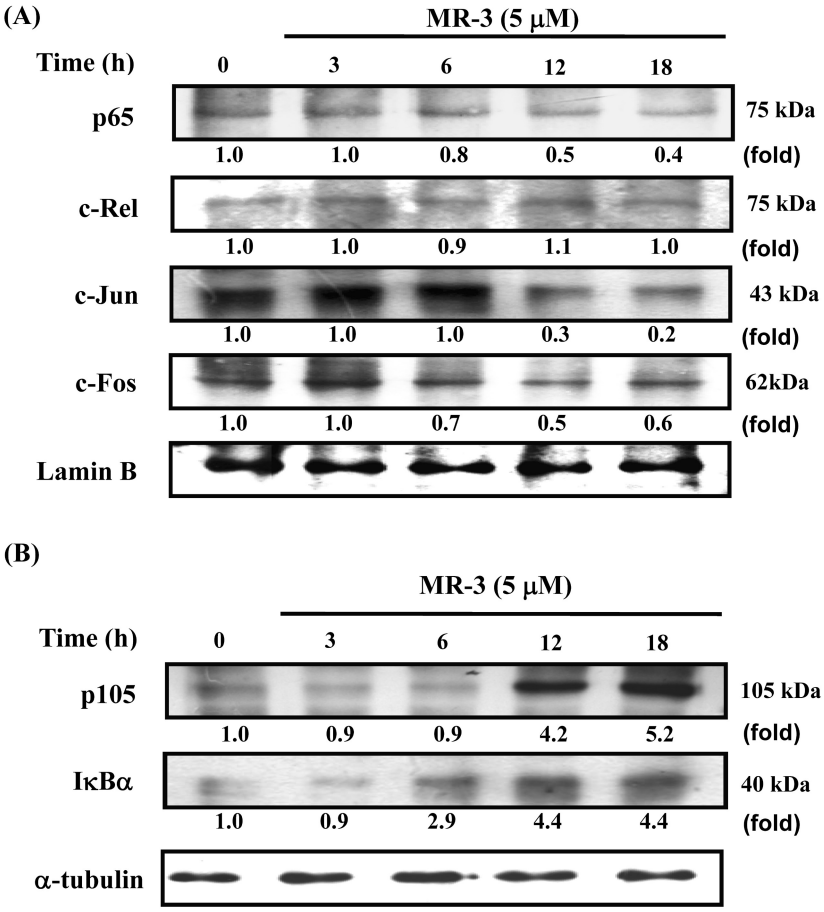


**Figure 5.** Effect of MR-3 on the expressions of NF-κB and AP-1 in nuclear fractions. A549 cells were treated with MR-3 at a concentration of 5 μM for 3, 6, 12, and 18 h, and the nuclear extracts subjected to SDS-PAGE followed by western blotting with anti-p-65, anti-c-Rel, anti-c-Jun, and anti-c-Fos antibodies (A); the cytosolic fractions were subjected to SDS-PAGE followed by Western blotting with anti-p105 and anti-IκBα antibodies (B) as described in Section 2. Activities of these proteins were subsequently quantified by densitometric analyses with the control set to 100%.

trol (Fig. 4B). The synergistic suppressive effects suggested that inhibition of MMP-2 expression in A549 cells by MR-3 could be mediated through the inactivation of JNK and p38.

### 3.5 MR-3 inhibits transcriptional activity of MMP-2 through suppression of NF-κB and AP-1 activities

In the present study, we examined the effects of MR-3 on the expression of NF-κB and AP-1 in A549 cells. The cells were treated with 5 μM MR-3 for various time intervals (3, 6, 12, and 18 h), and the cytosolic and nuclear extracts analyzed by Western blotting. The results showed that the protein levels of p65, c-Jun, and c-Fos, but not c-Rel, in nucleus were decreased by the MR-3 treatment in a time-dependent manner (Fig. 5A). It has been shown that NF-κB is activated through the phosphorylation of IκBα, which releases the NF-κB subunits (p65 and p50) into the cytosol while the activated NF-κB protein translocates into nucleus to regulate gene expression. As the level of nonphosphorylated IκBα in the cytosol increases, in the meantime, translocation of the p65 and p50 subunits into nucleus decreases. Cytosolic extracts of the cells in these experiments were subjected to SDS-PAGE followed by Western blotting with antibodies specific to nonphosphorylated IκBα and p105 to examine the involvement of NF-κB on MMP-2 expression. The intensity of Western blots showed a negative correlation between p105 and IκBα in the cytosol and p65 in nucleus (Fig. 5B). These results indicated that the protein levels of NF-κB and AP-1 in the nucleus were decreased in a time-dependent manner by MR-3 (5 μM) treatment.

## 4 Discussion

Active compounds having antiinvasive and antimetastatic properties have been defined as a new catalog of chemopreventive agents [29]. Cancer invasion and metastasis often accompany various physiological alterations such as overexpression of MMPs. MMPs are a group of proteolytic enzymes involved in the degradation of the ECM, and are capable of promoting the invasion of tumor cells into the blood or lymphatic systems, ultimately resulting in the spread of the tumor to other tissues and organs [16]. The relationship between MMP expression and the invasive activity of various cancers has been well documented. The effects of resveratrol and its analog, MR-3, on the MMP-2

 

activity of human lung adenocarcinoma A549 cells had been firstly evaluated, and only MR-3 could inhibit the MMP-2 activity of A549 cells (data not shown). In the present study, we demonstrated that the MR-3 could effectively inhibit the invasive activity of A549 cells. Identification of the mechanism for the antiinvasion properties of MR-3 might help to prevent invasion and metastasis for a wide range of tumors.

The results of gelatin zymography showed that A549 cells secreted MMP-2 but not MMP-9 (data not shown). Secretion of MMP-9 has been reported in lung, colon, and breast cancers [30] and MMP-2 expression has previously been found in A549 cells [31]. It was reported that even when A549 cells were treated with fibronectin (a high-molecular-weight glycoprotein having the ability to stabilize the attachment of ECM to cells) and laminin-10/11 (a major noncollagenous component of the basal lamina), the activity of MMP-9 still was not significantly enhanced [32]. Hence, the literature regarding the invasive and metastatic activity of A549 cells has focused almost exclusively on the activity of MMP-2 [33, 34]. In this study, we first demonstrated that treatment with MR-3 exerted an inhibitory effect on the adhesive, migratory, and invasive activities of highly metastatic A549 cells in a dose-dependent manner (Fig. 2), and also inhibited the activity of MMP-2 (Fig. 3A) in a time-dependent manner without inducing cytotoxicity (97% cell viability remaining). The RT-PCR results further revealed that the reduction in MMP-2 activity was a result of changes at the level of transcription (rather than translation or posttranslational levels) (Fig. 3B). These results suggested that MR-3, a methylated derivative of resveratrol, might be an effective antiinvasive compound for human lung adenocarcinoma by suppressing MMP-2 expression. Pterostilbene, a derivative of resveratrol with methylation at positions 3 and 5, has been shown to exhibit greater antifungal activity than resveratrol [35, 36]. The methylated derivatives of flavonoids have also been shown to exhibit a higher antiproliferative potency on cancer cells than their hydroxylated counterparts [37]. It was inferred that the superior ability of methylated derivatives of polyphenols such as methoxyflavonoids in antiinvasion, antifungal, and antiproliferation might be related to their lipophilic properties and increased uptake through the cell membrane. In addition, the MMP-2 activity and mRNA expression were increased after 18 h of incubation. A similar phenomenon was also observed in phosphorylated JNK detection (Fig. 4A). Therefore, 18 h would be the maximum incubation time for maintaining the efficiency of MR-3 on signaling proteins, but the effect on antiinvasion of cells was observed to last out to at least 24 h.

Although the promoter of the MMP-2 gene is without any binding sites for NF-κB, c-Jun, or c-Fos, the expression of MMP-2 in cancer cells through an NF-κB or AP-1-dependent pathway has been observed in recent reports [38, 39]. Activation of NF-κB and AP-1 was found to be

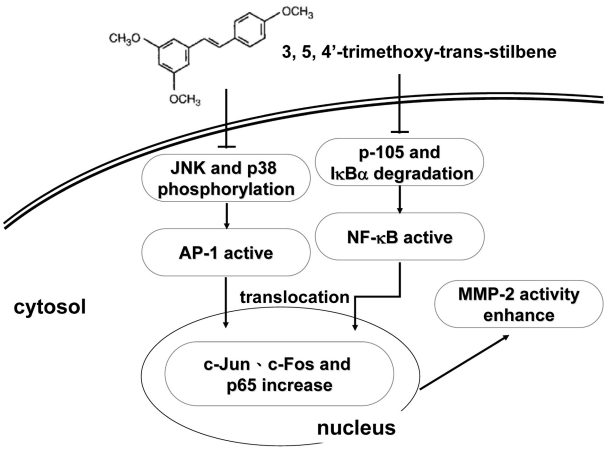


**Figure 6.** Schematic representation of the signaling pathways involved in the inhibition of invasion of human lung adenocarcinoma A549 cells by MR-3.

involved in many pathological processes such as inflammation, cancer cell adhesion, invasion, metastasis, and angiogenesis [40 – 42]. Thus, the suppression of AP-1 and NF-κB downstream of the MAPK pathways might inhibit MMP-2 expression and enable a potential reduction in tumor invasion and metastasis. Resveratrol has been shown to be an inhibitor of NF-κB [43] and AP-1 [44]. Therefore, the resveratrol anoalog-MR-3 would be presumed to have similar effects on NF-κB and AP-1. It was reported that flavanone and silibinin could inhibit MMP-2 expression in A549 cells through inactivation of the MAPK/ERK, p38, and PI3K/Akt signal transduction pathways [45, 34] while berberine could inhibit metastatic activity by decreasing the quantities of NF-κB, c-Jun, and c-Fos in the nuclei of A549 cells [33]. Here, treatment of A549 cells with MR-3 (5 μM) inhibited MMP-2 expression (Fig. 3) as well as p38 and JNK phosphorylation (Fig. 4). The involvement of p38 and JNK were further confirmed by the use of p38 and JNK inhibitors (SP600125 and SB203580, respectively) (Fig. 4B). We have also demonstrated that treating A549 cells with MR-3 (5 μM) resulted in an inhibition of the nuclear translocation of c-Jun, c-Fos, and p65 proteins (Fig. 5). Taken together, the inhibitory effect of MR-3 on MMP-2 expression might be carried out through the inactivation of the phosphorylation of p38 and JNK in the cytosol, as well as through a decrease in the translocation of NF-κB and AP-1 into the nucleus. Therefore, the effect of MR-3 on reducing MMP-2 expression might contribute to the inhibition of invasion in A549 cells. Figure 6 shows the proposed mechanisms for the MR-3-inhibited invasion of A549 cells. The antiinvasive effect of MR-3 on A549 cells might be mediated through the inhibition of the phosphorylation of p38 and JNK, as well as through a reduction in the protein levels of AP-1 and NF-κB in the nucleus, leading to the downregulation of MMP-2 expression. Combined with the recent findings on methylated resveratrol, which increased

 

antiangiogenesis activity 30-fold [12], MR-3 could be a potential candidate for the prevention of human lung adenocarcinoma invasion and metastasis.

In conclusion, it was demonstrated that MR-3 could effectively inhibit the invasion of A549 cells.

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