Triptolide inhibits matrix metalloproteinase-9 expression and invasion of breast cancer cells through the inhibition of NF-κB and AP-1 signaling pathways

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Abstract. Triptolide is a diterpenoid epoxide that is endogenously produced by the thunder god vine, *Tripterygium wilfordii* Hook F. Triptolide has demonstrated a variety of biological activities, including anticancer activities, in previous studies. Invasion and metastasis are the leading causes of mortality for patients with breast cancer, and the increased expression of matrix metalloproteinase-9 (MMP-9) has been shown to be associated with breast cancer invasion. Therefore, the aim of the present study was to investigate the effect of triptolide on 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced cell invasion and MMP-9 expression in breast cancer cells. The expression of signal molecules was examined by western blotting, zymography and quantitative polymerase chain reaction; an electrophoretic mobility gel shift assay was also used, and cell invasiveness was measured by an *in vitro* Matrigel invasion assay. The MCF-7 human breast cancer cell line was treated with triptolide at the highest concentrations at which no marked cytotoxicity was evident. The results demonstrated that triptolide decreased the expression of MMP-9 through inhibition of the TPA-induced phosphorylation of extracellular signal-regulated kinase (ERK) and the downregulation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) activity. In addition, a Transwell assay revealed that triptolide reduced the ability of MCF-7 cells to invade Matrigel. These data demonstrate that the anti-invasive effect of triptolide is associated with the inhibition of ERK signaling and NF-κB and AP-1 activation, and suggest that triptolide may be a promising drug for breast cancer.

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-9, matrix metalloproteinase-9; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide; PCR, polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, mitogen-activated protein kinase; AP-1, activator protein-1; NF-κB, nuclear factor-κB; p-IKKα/β, phosphorylated IκB kinase α/β; p-IκBα, phosphorylated nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α

Key words: triptolide, matrix metalloproteinase-9, invasion, breast cancer

Introduction

Breast cancer is among the most frequently diagnosed cancers globally and is the most common cancer in women with an estimated 1.67 million new cases of breast cancer being diagnosed in 2012, constituting 25% of all cancers (1). Despite the notable progress that has been made in the treatment of breast cancer over past decades, it remains the leading cause of cancer-associated mortality among women worldwide, resulting in 14% of cancer-associated mortalities (2,3). Survival rates for patients with metastatic breast cancer remain poor (4), and the metastatic spread and invasion of cancer cells are responsible for treatment failure in breast cancer (5).

The metastatic process involves the detachment of cancer cells and their invasion into adjacent normal tissues, penetration of blood vessels and passive transport to distant sites, implantation and the proliferation of metastatic colonies (6). The proteolytic

degradation of parts of the extracellular matrix (ECM), including type IV collagen, laminin, heparin sulfate proteoglycan, nidogens and fibronectin, is required for metastasis to occur (6,7).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that have key functions in remodeling the ECM during development, inflammation and wound repair processes. The degradation of ECM components by MMPs serves a crucial role in the migration and invasion of cancer cells (8,9). Among MMPs, MMP-9 (also known as gelatinase-B) is a key enzyme for the degradation of type IV collagen, which is the main collagen component of the basement membrane. Elevated expression of MMP-9 has been shown to be critical in the invasive process in a number of tumors, particularly breast tumors (7,9,10). Therefore, study of MMP-9 inhibition and the underlying molecular mechanisms has been an important strategy in the search for treatments for potentially invasive tumors, including breast tumors.

The MMP-9 promoter contains various functional regulatory motifs that have the ability to bind with transcription factors, including activator protein-1 (AP-1; at positions -533 and -79 in the MMP-9 promoter), nuclear factor-κB (NF-κB; at -600 bp) and stimulatory protein-1 (Sp1; at -558) (11,12). Through the binding of transcription factors to the specified functional elements in the MMP-9 genes, the expression of MMP-9 is controlled by a variety of stimulatory factors, including growth factors, inflammatory cytokines, tumor necrosis factor (TNF)-α, phorbol ester, epidermal growth factor and 12-O-tetradecanoylphorbol-13-acetate (TPA) (12-16). Cytokines and TPA treatment induce the expression of MMP-9 via the activation of transcription factors, including NF-κB and AP-1 (7,17). Furthermore, phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinases (MAPKs) regulate the predominant cascade participating in MMP-9 expression (11). MAPK signaling involves NF-κB inhibitor (IκB) kinase, p38 MAPK, extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK), according to the type of cell in which the signaling occurs (7,12,18). The MAPK signaling pathway is involved in the activation of transcription factors such as NF-κB and AP-1, which are known regulators of the MMP-9 promoter (7,19).

Triptolide (Fig. 1A) is a biologically active diterpenoid triepoxide that has been isolated from the traditional Chinese herb, the thunder god vine *Tripterygium wilfordii* Hook F (20). This natural product has been demonstrated to have anti-inflammatory, immunosuppressant and antitumor effects *in vivo* and *in vitro* (21,22). Previous studies have attributed the antitumor effects of triptolide to its ability to inhibit the proliferation of tumor cells and induce their apoptosis (23,24). However, the potential inhibitory effect of triptolide on MMP-9 has not yet been evaluated.

Therefore, the present study investigated the effects of triptolide on TPA-induced MMP-9 expression in MCF-7 human breast cancer cells. The molecular mechanisms underlying the inhibition of MMP-9 expression by triptolide were also investigated.

Materials and methods

Reagents. Triptolide, TPA and β-actin (cat. no. A3688) antibodies were purchased from Sigma-Aldrich (Merck KGaA). Inhibitors of AP-1 (SR 11302) and NF-κB (Bay 11-7092) were purchased from Santa Cruz Biotechnology, Inc. The MAPK inhibitors SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and PD98059 (ERK inhibitor) were acquired from Merck Millipore. Rabbit antibodies against phosphorylated (p-)c-Fos (cat. no. 5348), p-IκB kinase α/β (p-IκKα/β; cat. no. 2697), stress activated protein kinase (SAPK)/JNK (cat. no. 9258), p-SAPK/JNK (cat. no. 4668), p38 MAPK (cat. no. 8690), p-p38 MAPK (cat. no. 4511), p44/42 MAPK (ERK1/2; cat. no. 4695), p-p44/42 MAPK (p-ERK1/2; cat. no. 4370) and c-Fos (cat. no. 2250) were purchased from Cell Signaling Technology, Inc. Rabbit antibodies against NF-κB p65 (cat. no. ab16502), NF-κB p105/p50 (cat. no. ab32360) and MMP-9 (cat. no. ab76003) were purchased from Abcam. Rabbit antibodies against IκBα (cat. no. sc-371) were purchased from Santa Cruz Biotechnology, Inc. Mouse antibodies against p-IκBα (cat. no. 9246) were purchased from Cell Signaling Technology, Inc. Mouse antibodies against proliferating cell nuclear antigen (PCNA; cat. no. sc-7907), IκKα (cat. no. sc-71333) and IκKβ (cat. no. sc-56918) were purchased from Santa Cruz Biotechnology. The secondary antibodies anti-rabbit IgG HRP-linked antibody (1:1,000 dilution; cat. no. 7074) and anti-mouse IgG HRP-linked antibody (1:1,000 dilution; cat. no. 7076) were purchased from Cell Signaling Technology, Inc.

Cell culture. The MCF-7 human breast cancer cell line was acquired from the American Type Culture Collection. The cells were cultured in high glucose-containing Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% antibiotic (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO_2 incubator at 37˚C.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. MCF-7 cells ($2x10^4$ cells/well) were seeded in a 96-well plate and incubated at 37˚C for 24 h to allow attachment. Cells were either untreated or treated with 1, 5, 10, 25, 50 or 100 nM triptolide at 37˚C for 24 h and then washed with phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.). The MTT assay was then performed using 0.5 mg/ml MTT (Sigma-Aldrich; Merck KGaA). Following the addition of MTT, the cells were incubated at 37˚C for 30 min. Dimethyl sulfoxide was added to dissolve the formazan crystals, and the absorbance at 570 nm was determined using a microplate reader (Bio-Rad Laboratories, Inc.).

Isolation of nuclear and cytoplasmic extracts. Cells were pretreated with 5 or 10 nM triptolide and then treated with 100 nM TPA at 37˚C for 3 h, then washed with PBS and pelleted. Nuclear and cytoplasmic extracts were obtained from the pelleted cells using NE-PER® Cytoplasmic and Nuclear Extraction Reagents, respectively (Pierce; Thermo Fisher Scientific, Inc.).

Western blot analysis. Cells were pretreated with inhibitors of JNK (SP600125; 10 and 20 μM), p38 (SB203580; 10 and 20 μM), ERK (PD98059; 10 and 20 μM), NF-κB (Bay 11-7092; 2.5 and 5 μM) and AP-1 (SR 11302; 2.5 and 5 μM) at 37˚C

for 1 h, and then treated with TPA at 37˚C for 24 h. Proteins were extracted from cells by lysis using M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.) and a proteinase inhibitor. The protein concentration was determined using a Protein Assay Dye Reagent Concentrate (cat. no. 5000006) from Bio-Rad Laboratories, Inc. Cell lysates (10 μg protein/lane) were separated by 10% SDS-PAGE and transferred to Hybond™ polyvinylidene fluoride membranes (Cytiva). Each membrane was blocked at 4˚C for 2 h with skimmed milk or bovine serum albumin (5% in PBS; purchased from MP Biomedicals, LLC) and then incubated overnight at 4˚C with the aforementioned primary antibodies (diluted 1:1,000 in 5% skimmed milk/1X TBS buffer). The corresponding HRP-conjugated anti-IgG antibody (1:1,000 dilution) was used as the secondary antibody and was incubated with the membrane for 1 h at 4˚C. The immunoreactive signals were visualized using an electrochemiluminescent HRP substrate peroxide solution and luminol reagent (Merck Millipore; cat. no. WBKLS0500). Protein levels were measured using an imaging system (Las-4000; FujiFilm Corporation) and an image analyzer software (Multi Gauge v3.0; FujiFilm Corporation). PCNA was used as a loading control for the nucleus, and β-actin was used as an internal control for the cytoplasm.

Zymography assay. Conditioned medium was collected from the cells, mixed with sample buffer (0.5M Tris-HCI pH 6.8 2.5 ml, Glycerol 2 ml, 10% SDS 4 ml, 0.1% bromopherol blue 0.5 ml and D.W 1 ml) and separated by PAGE containing gelatin (0.1%). The gel was washed for 30 min with Triton X-100 solution (2.5%) at room temperature and then incubated for 16 h in developing buffer (5 mM $CaCl_2$, 0.02% Brij and 50 mM Tris-HCl, pH 7.5) at 37˚C. Afterwards, the gel was stained for 30 min at room temperature with 0.25% Coomassie brilliant blue (40% methanol and 7% acetic acid) and the staining was measured using an image analyzer (FujiFilm Corporation). Densitometric analysis was performed using Multi Gauge image analysis software (Multi Gauge v3.0; FujiFilm Corporation).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The RNA was isolated from cells using RNAiso Plus reagent (Takara Bio, Inc.; cat. no. 9108) and purified using a FastPure RNA kit (Takara Bio, Inc.; cat. no. 9767). cDNA was synthesized from the RNA using a PrimeScript RT reagent kit (Takara Bio, Inc.; cat. no. RR037A) with heating at 37˚C for 15 min and 85˚C for 5 sec. The mRNA levels of MMP-9 and GAPDH were analyzed by qPCR using an ABI PRISM™ 7900 Sequence Detection system and Power SYBR® Green PCR Master mix (both Applied Biosystems; Thermo Fisher Scientific, Inc.; cat. no. 330521). The primers used were as follows: MMP-9 (NM 004994) sense, 5'-CCTGGAGACCTGAGAACCAATCT-3' and antisense, 5'-CCACCCGAGTGTAACCATAGC-3'; and GAPDH (NM002046) sense, 5'-ATGGAAATCCCATCACCATCTT-3' and antisense, 5'-CGCCCCACTTGATTTTGG-3'. qPCR was conducted with 40 cycles of 50˚C for 2 min, 95˚C for 10 min, 95˚C for 15 sec and 60˚C for 1 min. The results for MMP-9 were normalized to those of GAPDH. Relative quantitation was conducted using the comparative Cq ($2^{-\Delta\Delta Cq}$) method (25).

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared using the aforementioned protocol. Oligonucleotides containing AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3'; Promega Corporation; cat. no. E3201) or NF-κB (5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3'; Promega Corporation; cat. no. E3291) binding sites were produced by Promega Corporation and used as probes. Complementary strands were labeled with [α-^{32}P]dCTP (Amersham; Cytiva). The labeled oligonucleotides (10,000 cpm), 10 μg nuclear extracts and binding buffer (10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 50% glycerol, 100 ng poly [dI·dC], 1 mM DTT) were incubated for 30 min at room temperature. Reaction products were analyzed by electrophoresis using 4% PAGE with 0.5X Tris-borate buffer. The gels were then dried and analyzed by autoradiography. A 50-fold excess of cold NF-κB or AP-1 oligonucleotide was used as a control to confirm specific binding.

Luciferase assay. Cells ($3x10^5$ cells/well) were seeded onto 24-well plates and transfected with MMP-9, AP-1 or NF-κB reporter plasmids (provided by Professor Kim Chul Ho, SungKyunKwan University, Suwon, Korea) using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as directed by the manufacturer. The transfected cells were pretreated with 5 or 10 nM triptolide at 37˚C for 1 h and then treated with 100 nM TPA at 37˚C for 3 h. Luciferase reporter assays were implemented using a Dual Luciferase assay kit (Promega Corporation; cat. no. E1910) as recommended by the manufacturer, and the results were collected using a luminometer (Lumat LB 9507; Berthold Technologies GmbH & Co.). The luciferase assay was performed by sequentially measuring the firefly and *Renilla* luciferase activities, with the results expressed as the ratio of firefly to *Renilla* luciferase activity.

Invasion assay. An invasion assay was conducted using 24-well chambers (8-μm pore size) in which the upper side of the Transwell insert was coated at 37˚C for 30 min with Matrigel (BD Biosciences). Cells ($3x10^5$ cells/well) were added to the upper chamber in serum-free DMEM, while the lower compartment contained conditioned DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% antibiotic (10,000 U/ml penicillin and 10,000 μg/ml streptomycin) and 10% FBS. Then, 100 nM TPA with or without 5 or 10 nM triptolide was added to the upper chamber. After incubation at 37˚C for 24 h, the cells in the upper chamber were removed using cotton swabs. The invaded cells on the bottom of the filter were fixed with 3.7-4.0% formalin for 10 min at room temperature and stained with crystal violet for 30 min at room temperature. Invading cells were counted in five random regions of the membrane using light microscopy.

Statistical analysis. Data from three independent experiments are presented as the mean ± standard error of the mean. Statistical analyses were performed by analysis of variance and Tukey's tests using OriginPro 8 (OriginLab Corporation). $P<0.05$ was considered to indicate a statistically significant difference.

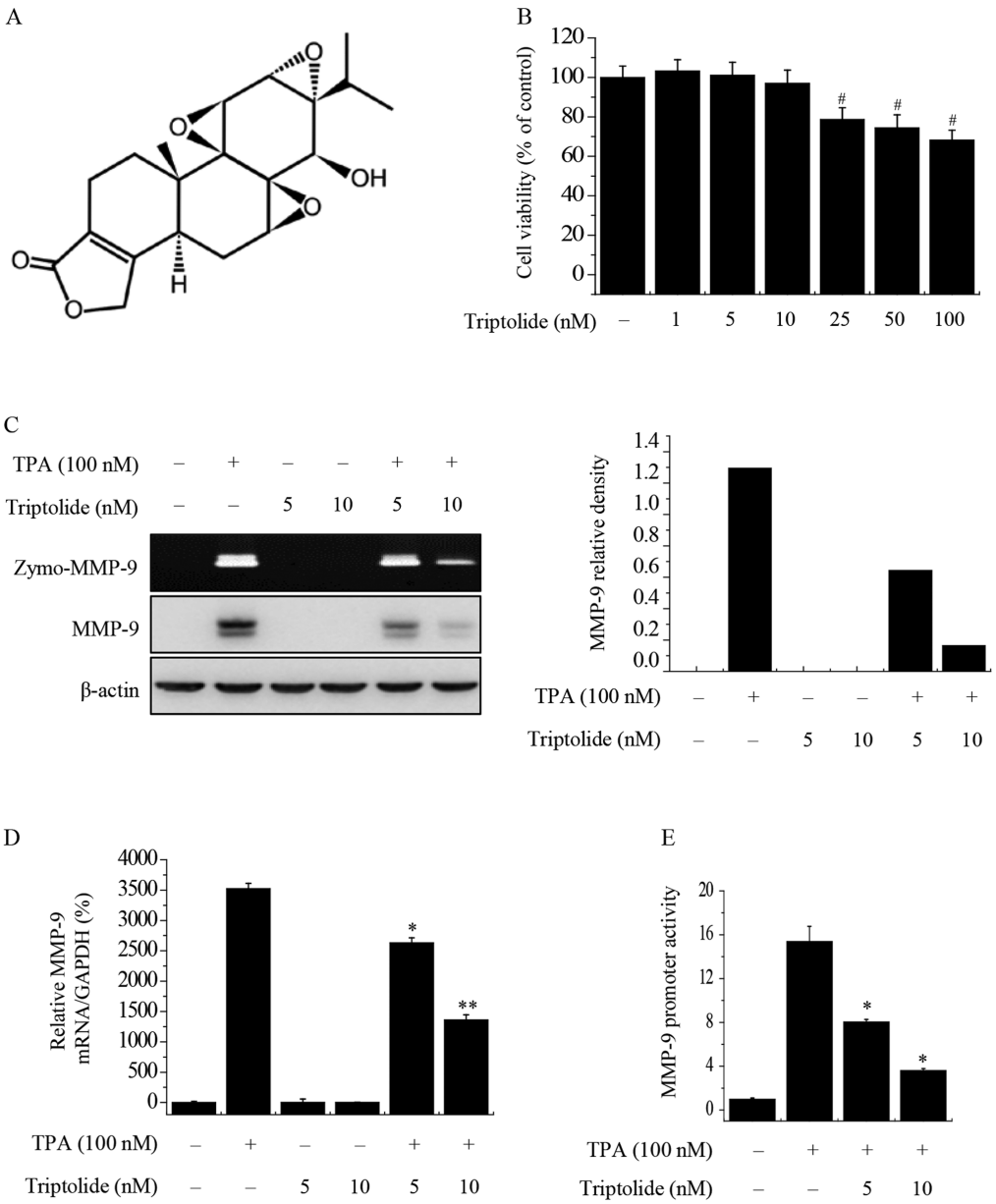

Figure 1. Effects of triptolide on MCF-7 cell viability and MMP-9 expression. (A) Chemical structure of triptolide. (B) Cells were cultured in 96-well plates and treated with different concentrations of triptolide for 24 h. After treatment, cell viability was determined using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide assays. (C) Cells were pretreated with triptolide and TPA was added for 24 h. MMP-9 activity was analyzed by gelatin zymography (top bands). MMP-9 protein expression was analyzed by western blotting with β-actin as an internal control (lower bands). (D) MMP-9 mRNA levels were analyzed by reverse transcription-polymerase chain reaction with GAPDH as an internal control. (E) Wild-type MMP-9-luc reporter and a *Renilla* luciferase thymidine kinase reporter vector were co-transfected into cells. The cells were treated with TPA alone or in combination with triptolide. MMP-9 promoter activity was measured using a dual-luciferase reporter assay. Data are presented as the mean ± SEM of three independent experiments. $^{\#}$P<0.05 vs. untreated cells, *P<0.05 and **P<0.01 vs. TPA alone. MMP-9, matrix metalloproteinase-9; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 12-O-tetradecanoylphorbol-13-acetate; zymo, zymography.

Results

Triptolide affects TPA-induced MMP-9 expression. The cytotoxicity of triptolide in MCF-7 cells was investigated using an MTT assay. Cells were treated with triptolide (0-100 nM) for 24 h and no cytotoxic effects were observed for triptolide at concentrations from 0 to 10 nM (Fig. 1B). Therefore, non-toxic concentrations (5 and 10 nM) of triptolide were selected for use in the subsequent experiments. To investigate the effect of triptolide on TPA-induced MMP-9 expression in MCF-7 cells, zymography, western blot analysis, RT-qPCR and luciferase assays were used. Zymography showed that TPA increased the activity of MMP-9 in MCF-7 cells, and that triptolide blocked the TPA-induced activity of MMP-9 in a concentration-dependent manner. Western blot analysis demonstrated that triptolide suppressed the TPA-induced expression of MMP-9 protein (Fig. 1C). In addition, RT-qPCR showed that triptolide treatment suppressed the TPA-induced expression of MMP-9 at the mRNA level (Fig. 1D). Furthermore, a luciferase reporter assay demonstrated that the treatment of MCF-7 cells with triptolide suppressed TPA-induced MMP-9 promoter activity (Fig. 1E). Together,

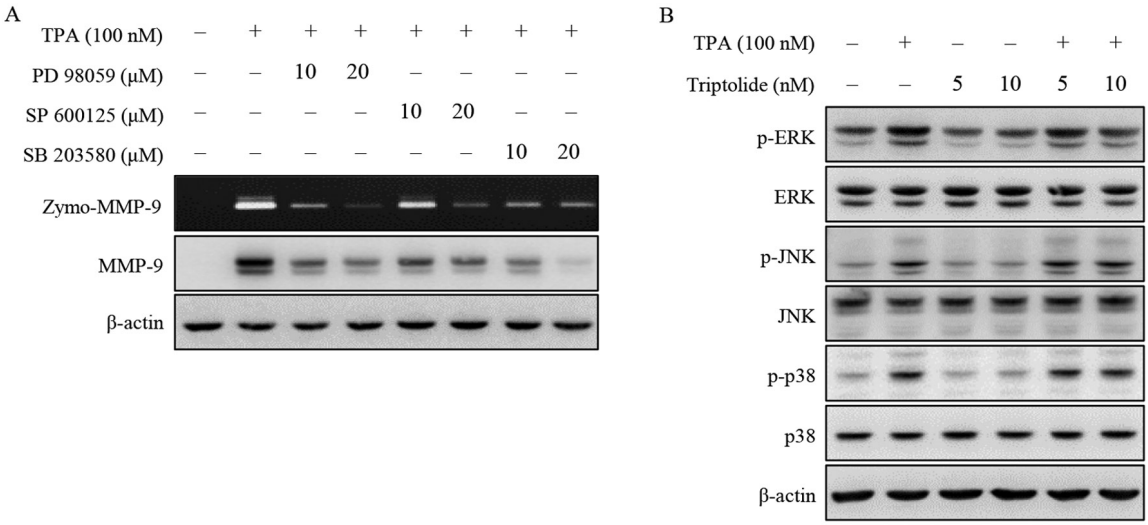

Figure 2. Effect of triptolide on MAPK expression in MCF-7 cells. (A) Cells were pretreated with inhibitors of ERK (PD98059), JNK (SP600125) and p38 MAPK (SB203580), and TPA was added for 24 h. MMP-9 activity was analyzed by gelatin zymography and MMP-9 protein expression was detected by western blotting. (B) Cells were pretreated with triptolide and TPA was added for 24 h. The phosphorylation of ERK, JNK and p38 was analyzed by western blotting. β-actin was used as an internal control. Data presented are the result of three independent experiments. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP-9, matrix metalloproteinase-9; p38 MAPK, mitogen-activated protein kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; p-, phosphorylated; zymo, zymography.

these results demonstrate the inhibitory effects of triptolide on MMP-9 expression.

Triptolide inhibits TPA-induced ERK activation. The mechanism by which triptolide affects signaling was investigated using zymography and western blot analysis. MCF-7 cells were pretreated with inhibitors of JNK (SP600125), p38 (SB203580) and ERK (PD98059) and then treated with TPA. As shown in Fig. 2A, the inhibition of ERK, JNK and p38 suppressed TPA-induced MMP-9 protein expression and activity in MCF-7 cells. In addition, TPA markedly increased the phosphorylation levels of ERK, JNK and p38. Triptolide inhibited the TPA-induced phosphorylation of ERK; however, the total protein level of ERK remained unaltered (Fig. 2B). These results suggest that the inhibition of MMP-9 expression by triptolide is associated with a reduction in the phosphorylation of ERK.

Triptolide inhibits TPA-induced NF-κB and AP-1 activation. To further examine the inhibitory mechanism underlying the transcriptional regulation of MMP-9 by triptolide, western blot analysis was performed to examine the effects of triptolide on NF-κB and AP-1 activation in MCF-7 cells. MCF-7 cells were pretreated with inhibitors of NF-κB (Bay 11-7092) and AP-1 (SR 11302). As shown in Fig. 3A, the inhibition of NF-κB or AP-1 suppressed the TPA-induced expression of MMP-9 protein in MCF-7 cells. The addition of triptolide inhibited the TPA-induced nuclear translocation of NF-κB p65/p50 and phosphorylation of cytoplasmic IκBα and IKKα/β. Total c-Fos expression in the nucleus and total IκBα, IKKα and IKKβ expression in the cytosol did not exhibit any changes. In addition, phosphorylation of the AP-1 subunit c-Fos in the nucleus of the TPA-induced cells was decreased following treatment with triptolide (Fig. 3B). Using luciferase assays, the treatment of MCF-7 cells with triptolide was showed to suppress TPA-induced NF-κB and AP-1 promoter activity (Fig. 3C and D). To investigate DNA-binding activity, EMSAs were performed. The results showed that triptolide markedly inhibited the TPA-induced binding activities of NF-κB and AP-1 (Fig. 3E and F). These results suggest that triptolide inhibits MMP-9 expression via the modulation of its activation by the transcription factors NF-κB and AP-1.

Triptolide inhibits TPA-induced invasion in vitro. Using a Matrigel invasion assay, the effect of triptolide on the invasive ability of MCF-7 breast cancer cells was investigated. The results showed that a 10 nM concentration of triptolide effectively inhibited the invasion ability of MCF-7 cells by almost 70% compared with that of the untreated control cells (Fig. 4). These results suggest that triptolide suppresses the invasive potential of breast cancer cells.

Discussion

Triptolide has been used in the treatment of autoimmune and inflammatory diseases, such as rheumatoid arthritis (26). Numerous studies have investigated the antitumor effects of triptolide in various types of cancer, and have demonstrated that triptolide can induce apoptosis and inhibit the proliferation of cancer cells *in vitro* and reduce the growth and metastasis of tumors *in vivo* (23,24,26-29). Notably, certain studies have revealed that triptolide inhibits the proliferation of breast cancer cells *in vitro*, induces apoptosis and modulates the expression of several signaling molecules (29,30). Triptolide has been shown to induce apoptosis by increasing caspase-3 activity (30), downregulating estrogen receptor α (31) and the Wnt/β-catenin pathway (32) and regulating DNA repair/damage (33,34) in various subtypes of breast cancer, including basal and triple-negative types. These previous findings suggest that triptolide is a promising treatment for various

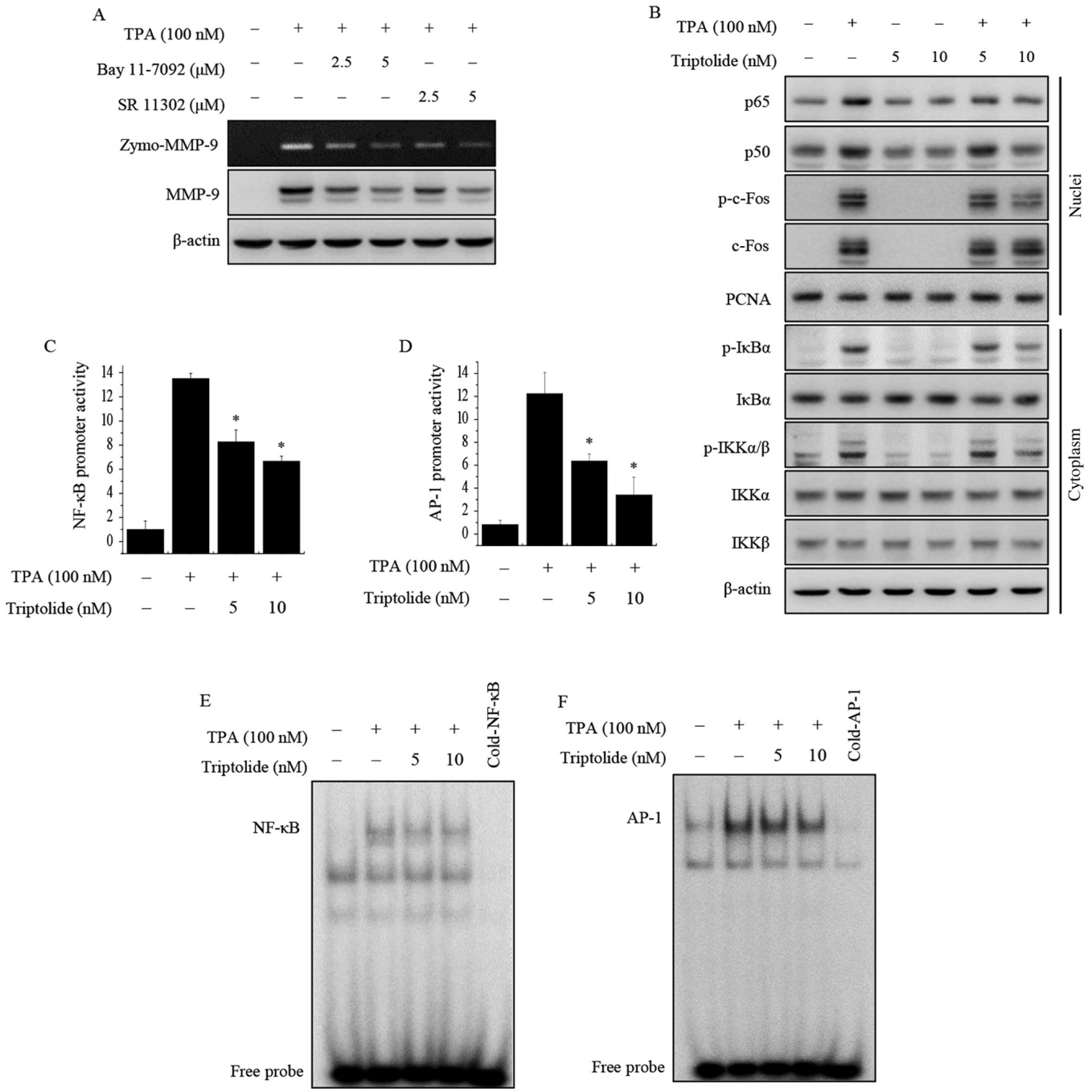

Figure 3. Effects of triptolide on TPA-induced NF-κB and AP-1 activation in MCF-7 cells. (A) MCF-7 cells were pretreated with inhibitors of NF-κB (Bay 11-7092) and AP-1 (SR 11302), and then TPA was added for 24 h. MMP-9 activity was detected by gelatin zymography and MMP-9 protein expression was analyzed by western blotting. (B) Cells were treated with triptolide and/or TPA. After a 3-h incubation, nuclear and cytoplasmic extracts were prepared. Translocation of p65, p50 and p-c-Fos to the nucleus and the levels of p-IκBα, p-IKKα/β, total c-Fos, IκBα, IKKα and IKKβ were determined by western blotting. PCNA was used as a loading control for the nucleus, and β-actin was used as an internal control for the cytoplasm. (C) NF-κB-luc and (D) AP-1-luc reporters were co-transfected with a *Renilla* luciferase thymidine kinase reporter into the cells. The cells were treated with TPA alone or with triptolide, and the NF-κB and AP-1 promoter activities were measured using a dual-luciferase reporter assay. The DNA binding of (E) NF-κB and (F) AP-1 was analyzed using electrophoretic mobility shift assays. Data are presented as the mean ± SEM of three independent experiments. *P<0.05 vs. TPA alone. AP-1, activator protein-1; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-κB; p-, phosphorylated; IKKα/β, IκB kinase α/β; IκBα, NF-κB inhibitor α; TPA, 12-O-tetradecanoylphorbol-13-acetate; zymo, zymography; PCNA, proliferating cell nuclear antigen.

types of breast cancer. Li *et al* (22) revealed that triptolide was cytotoxic to human breast cancer stem cells and primary breast cancer cells *in vitro* and *in vivo*. Furthermore, studies have shown that triptolide is a multi-target anticancer agent that modulates various molecular pathways, for example, by reducing the transcriptional activity of NF-κB and AP-1 and inhibiting the expression of heat shock protein 70 (24,35,36). Triptolide also exerts effects via autophagy and p38/ERK/mTOR phosphorylation (37) and modulates the expression of ERK, NF-κB, focal adhesion kinase, vascular endothelial growth factor, β-catenin and AKT (38). However, no previous studies have investigated whether triptolide inhibits MAPK or

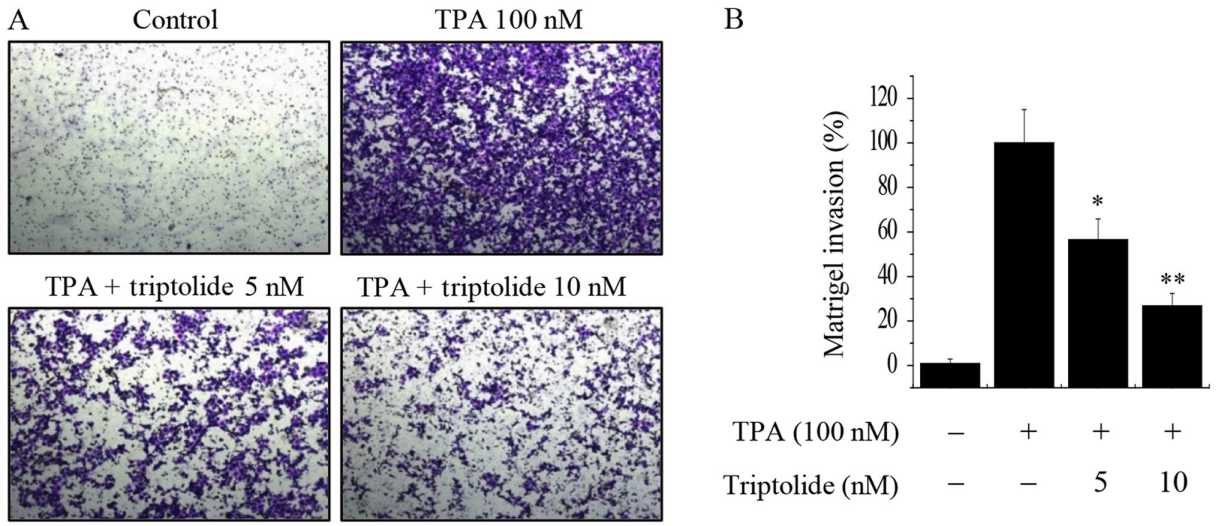

Figure 4. Triptolide inhibits the invasion of Matrigel by MCF-7 cells. (A) Matrigel invasion assays were carried out on cells treated with TPA alone or with triptolide. After 24 h, cells on the bottom of the filter were fixed and stained with crystal violet, and microscopic photography was performed (magnification, x40) (B) Results were quantified by counting the migrated cells in five randomly selected areas. Data are presented as the mean ± SEM of three independent experiments. *P<0.05 and **P<0.01 vs. TPA alone. TPA, 12-O-tetradecanoylphorbol-13-acetate.

transcription factors such as AP-1 and NF-κB. Thus, triptolide has been demonstrated to be an antitumor agent that inhibits proliferation and induces apoptosis in breast cancer cells. However, there is little data available regrating the inhibitory effect of triptolide on the invasion and migration of human breast cancer cells. Therefore, the present study investigated the effects of triptolide on MMP-9 activity in human breast cancer cells and examined the underlying molecular mechanisms of this activity.

MMP-9 is a key enzyme in tumor metastasis and invasion, and its activation is associated with the progression of breast tumors (7). In the present study, the results revealed that triptolide inhibited the TPA-induced expression of MMP-9 in MCF-7 human breast cancer cells at the protein and mRNA levels, which suggests that triptolide may have potent anti-metastatic activity. In addition, the present study demonstrated that triptolide inhibited TPA-induced MMP-9 expression by suppressing ERK pathways and, subsequently, NF-κB and AP-1 activity in human breast cancer cells.

MMPs are involved in numerous signaling pathways, including pathways involving NF-κB and AP-1, as well as MAPK, PI3K and protein kinase C. However, no systemic research focusing on NF-κB and AP-1 in a triptolide-treated MCF-7 model has previously been reported. In the present study, the results indicate that NF-κB and AP-1 are important factors associated with MMP-9. A previous study examined the effects of TPA on the expression of MMP-2 and MMP-9 in MCF-7 and MDA-MB-231 cell lines, and revealed that TPA induced MMP-9 enzyme activity and protein expression but had no effect on MMP-2 expression (39). MAPK pathways, the predominant cascade modulating MMP-9 expression, are involved in cellular proliferation and survival (12). Tang *et al* (40) reported that the activation of ERK mediated apoptosis and cell cycle arrest after DNA damage, independent of p53. Tan *et al* (36) demonstrated that the treatment of breast cancer cells with triptolide activated ERK in a dose- and time-dependent manner. These studies indicate that ERK activation is crucial in the mediation of triptolide-induced caspase-dependent apoptosis. The present study revealed that the inhibition of MMP-9 expression by triptolide is associated with reduced phosphorylation of ERK. Triptolide has been shown to exert an anti-invasive effect in breast cancer cells via inhibition of the ERK pathway (36).

The present study investigated the effects of triptolide on the DNA-binding activity of TPA-induced NF-κB and AP-1 to determine the molecular signaling pathways by which triptolide influences the migration and invasion of breast cancer cells. The NF-κB and AP-1 elements of the MMP-9 promoter have been demonstrated to serve a prominent role in the TPA- and cytokine-induced expression of the MMP-9 gene and the associated invasion of tumor cells (7,13,17). Chung *et al* (41) reported that the anti-metastatic and anti-tumor effects of caffeic acid and its phenyl ester are mediated through the selective suppression of MMP-9 activity and the inhibition of NF-κB and MMP-9 transcriptional activities. In addition, Weng *et al* (13) reported that the anti-invasive effects of lucidenic acid against the TPA-induced invasion of human hepatoma cells proceeded via inactivation of the MAPK signal transduction pathway and attenuation of the binding activities of NF-κB and AP-1. Furthermore, another study revealed that triptolide affected the proliferation and metastasis of melanoma cells via the inhibition of NF-κB expression, which consequently suppressed MMP-9 and MMP-2 expression (42). These signaling patterns have also been reported for other metalloproteinases. For example, triptolide was demonstrated to regulate the MAPK/ERK/JNK/AP-1 signaling pathway and directly affect the activation of MMP-1/3/13 in rheumatoid arthritis (43). However, no studies have explored these pathways in breast cancer models, and therefore further investigation is required. In the present study, triptolide inhibited the transcriptional activity of MMP-9 in TPA-induced MCF-7 breast cancer cells by suppressing NF-κB and AP-1 DNA-binding activities.

TPA has been well identified as a tumor promotor in a variety of human cell lines and has demonstrated the ability to increase the expression of nuclear factors associated with metastasis in selected tumor cell lines. TPA signaling has an association with AP-1 as well as MMP-1, -3 and -9, which have TPA-responsive elements, and TPA-sensitive MMPs are stimulated by cytokines including IL-1 and TNF-α (39). Therefore, the results of the present suggest that the effects of TPA, as a promoter of ERK signaling and NF-κB and AP-1 activation-mediated breast cancer metastasis, were effectively inhibited by triptolide administration in the MCF-7 breast cancer cell line. MMP-9 expression was not evident in MCF-7 cells in the normal state in the present study or our previous study (9). Thus, TPA is a selective agent for increasing the expression of MMP-9, and served to establish a model for examining the ability of triptolide to suppress the metastasis of MCF-7 cells. Triptolide was selected for evaluation due to its ability to inhibit TPA-induced MMP-9 expression in MCF-7 cells (9). However, systemic research with various transcription factors, such as NF-κB and AP-1 and the associated signaling pathways, in an MCF-7 cell model is lacking. Although the present study suggests the potential of triptolide as a potential anticancer drug candidate, the mechanism requires further investigation; for example, the involvement of MMP-2 (44), MMP-3 and MMP-5 could be examined.

In conclusion, the present study demonstrated that triptolide effectively decreased the expression of MMP-9 and cell invasion through inhibition of the TPA-induced phosphorylation of ERK and the downregulation of NF-κB and AP-1 activity. These findings suggest that triptolide is a potent inhibitor of TPA-induced MMP-9 expression and shows promise as a potential therapeutic agent for preventing the metastasis and invasion of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HSC and OYH conceived and designed the study and were major contributors to writing the manuscript. OYH performed the experiments and analyzed the data. JSK and YJJ contributed to conception and design, and acquisition of funding. KHP and HYJ were involved in the additional experiments and revision process. JSK and HSC confirm the authenticity of all the raw data. All authors read and approved the final manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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