Cis-guggulsterone inhibits the IKK/NF-κB pathway, whereas *trans*-guggulsterone inhibits MAPK/AP-1 in MCF-7 breast cancer cells: Guggulsterone regulates MMP-9 expression in an isomer-specific manner

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Abstract. Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are major transcription factors that have been associated with breast cancer metastasis by inducing matrix metalloproteinase-9 (MMP-9) expression. In this study, we investigated the inhibitory effects of guggulsterone isomers (*cis* or *trans*) on 12-*O*-tetradecanoylpho-bol-13-acetate (TPA)-induced MMP-9 expression. *Cis*-guggulsterone inhibited TPA-induced MMP expression by blocking IκB kinase (IKK)/NF-κB signaling, whereas *trans*-guggulsterone blocked mitogen-activated protein kinase (MAPK)/AP-1 signaling. *Cis*-guggulsterone was more potent than *trans*-guggulsterone in the inhibition of TPA-induced MMP-9 expression and invasion of MCF-7 cells. Furthermore, we found that the combination of these isomers exerted an additive effect on the inhibition of MCF-7 cell invasion. These results suggest that guggulsterone isomers downregulate MMP-9 expression and tumor cell invasion through the isomer-specific suppression of IKK/NF-κB and MAPK/AP-1 activation. In addition, the suppression of MMP-9 expression correlated well with the inhibition of cell invasion.

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Abbreviations: TPA, 12-*O*-tetradecanoylpho-bol-13-acetate; MMP, matrix metalloproteinase; IKK, I-κB kinase; AP-1, activator protein-1; MAPK, mitogen-activated protein kinase; ECM, extracellular matrix

Key words: guggulsterone, nuclear factor-κB, activator protein-1, matrix metalloproteinase-9, MCF-7

Introduction

Guggulsterone [4,17(20)-pregnadiene-3,16-dione], is a plant sterol derived from the gum resin (*guggulu*) of the tree *Commiphora mukul*. This gum resin has been used for centuries in Ayurvedic medicine to treat obesity, arthritis and hyperlipidemia (1,2). The anti-arthritic, anti-inflammatory and anti-lipid activities of guggulsterone have also been demonstrated (3). Recently, guggulsterone has been shown to exhibit antitumor activity in a variety of human tumor cell types (4). The active compound in the resin is *cis* and *trans* isomers of guggulsterone (3,5). Of note, *cis*-guggulesterone is more potent than the *trans*-isomer in inducing apoptosis in mature adipocytes (3). Furthermore, guggulesterone isomers have been shown to exhibit isomer-specific antitumor activity (6-8). However, the molecular mechanism of guggulesterone-mediated anticancer activity remains elusive.

Invasion processes require the degradation of the extracellular matrix (ECM), which provides biochemical and mechanical barriers to cell movement in cancer (9). Studies have reported that in the matrix metalloproteinase (MMP) family, gelatinases A (72 kDa gelatinase, type IV collagenase and MMP-2) and B (92 kDa gelatinase, type IV collagenase and MMP-9) play a critical role in ECM degradation and cell migration leading to tumor cell invasion in breast cancer (10,11). Elevated MMP-9 levels have been functionally linked to elevated metastasis in a number of tumor types, such as brain (12), prostate (13), bladder (14) and breast tumors (15,16). Consequently, inhibiting the expression of MMP-9 and/or its upstream regulatory pathways may prove to be effective in treating malignant tumors, including breast cancer.

Protein kinase C (PKC) is involved predominantly in the production of MMP-9 (17-21). A PKC activator, 12-*O*-tetradecanoylpho-bol-13-acetate (TPA), induces MMP-9 synthesis and secretion during various pathological processes, such as tumor invasion (16,22). TPA-mediated MMP-9 expression is regulated by activating transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in cancer cells (23-25). The mitogen-activated protein kinase (MAPK) signaling pathway is important for AP-1 activation and NF-κB activation requires IκB kinase (IKK), phosphoinositide 3 kinase-Akt, or p38 MAPK, depending on the cell type (17-21). Consequently, these findings suggest that the main pathways involved in MMP-9 expression following PKC activation are the MAPK, NF-κB and AP-1 pathways.

In this study, we investigated the roles of guggulsterone isomers in regulating TPA-induced MMP-9 expression, and thus suppressing cell invasion, as well as the mechanisms involved.

Materials and methods

Cells and materials. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics at 37˚C in a 5% CO_2 incubator. Isomers of guggulsterone were obtained from Steraloids Inc. (Newport, RI, USA) and dissolved in dimethyl sulfoxide (DMSO). TPA, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), and anti-β-actin antibodies were from Sigma (St. Louis, MO, USA). Antibodies against p-IκBα, p-IKKαβ, p38, p-p38, JNK, p-JNK, ERK and p-ERK were from Cell Signaling Technology (Beverly, MA, USA). MMP-9, p50, p65, proliferating cell nuclear antigen (PCNA) and horseradish peroxidase (HRP)-conjugated IgG antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). High glucose-containing DMEM, FBS and phosphate-buffered saline (PBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA).

Determination of cell viability. The effect of guggulsterone on MCF-7 cell viability was determined using an MTT assay (26). Briefly, cells were seeded at $3x10^4$ cells/well and allowed to attach. After 24 h, cells were treated with guggulsterone at 5, 10 or 20 μM. After incubation for 24 h, cells were washed with PBS. MTT (0.5 mg/ml PBS) was then added to each well, and the plates were incubated at 37˚C for 30 min. Formazan crystals were dissolved with DMSO (100 μl/well), and an intensity of color was detected at 570 nm using a microplate reader (Model 3550; Bio-Rad, Richmond, CA, USA).

Western blot analysis. MCF-7 cells ($5x10^5$) were pre-treated with guggulsterone (10 and 20 μM) for 1 h and then incubated with TPA for 24 h. Cells were lysed with an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 0.1% SDS). Protein concentration was determined using the Bradford method (27). Samples (20 μg) were separated by SDS-PAGE with 10% acrylamide, and the resolved proteins were transferred onto a hybond-PVDF membrane using a western blotting apparatus. The PVDF membrane was incubated with 2% bovine serum albumin or 5% skim milk to block non-specific sites, and then incubated overnight with 1 μg/ml primary antibody for MMP-9, p38, p-p38, JNK, p-JNK, ERK, p-ERK, β-actin, p50, p65, or PCNA. HRP-conjugated IgG was used as the secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (Fujifilm, Tokyo, Japan).

Gelatin zymography assay. The conditioned medium was collected after 24 h of stimulation with TPA, mixed with non-reducing sample buffer, and separated on a polyacrylamide gel containing 0.1% (w/v) gelatin (25). Gels were washed at room temperature for 30 min with 2.5% Triton X-100 solution, and incubated at 37˚C for 16 h in 5 mM $CaCl_2$, 0.02% Brij and 50 mM Tris-HCl (pH 7.5). The gel was stained for 30 min with 0.25% (w/v) Coomassie brilliant blue in 40% (v/v) methanol/7% (v/v) acetic acid and photographed with an image analyzer (Fujifilm). Proteolysis was imaged as a white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (Fujifilm).

Quantitative real-time PCR assay. Total RNA was extracted from the cells using a FastPure RNA kit (Takara Bio Inc., Shiga, Japan). The RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was then synthesized from 1 μg total RNA using the PrimeScript RT reagent kit (Takara Bio Inc.). MMP-9 and GAPDH mRNA expression were determined by real-time PCR using the ABI PRISM 7900 sequence detection system and SYBR®-Green (Applied Biosystems, Foster City, CA, USA). Primers used were: MMP-9 (NM-004994) sense, CCTGGAGACCTGAGAACC AATCT and antisense, CCACCCGAGTGTAACCATAGC; and GAPDH (NM-002046) sense, ATGGAAATCCCATC ACCATCTT and antisense, CGCCCCACTTGATTTTGG. To control for variation in mRNA concentration, all the results were normalized to the housekeeping gene, GAPDH. Relative quantification was performed using the comparative ΔΔCt method according to the manufacturer's instructions.

Preparation of nuclear extract. The MCF-7 cells ($2x10^6$) were treated with guggulsterone in the presence or absence of TPA for 4 h. Cells were immediately washed twice with PBS (pH 7.5), scraped into 1.5 ml of ice-cold PBS, and pelleted at 1,500 x g for 3 min. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA). The activation of NF-κB and AP-1 was assayed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the κ-chain (κB, 5'-CCGGTTAACAGAGGGGGCTTTCCG AG-3') or the AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') binding site was synthesized and used as a probe for gel retardation assay. Two complementary strands were annealed and labeled with [α-^{32}P]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of nuclear extracts, and a 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 in a final volume of 20 μl. Reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X TBE buffer (final concentrations: 22.5 mM Tris-borate, pH 7.6, 0.5 mM EDTA). Gels were dried and

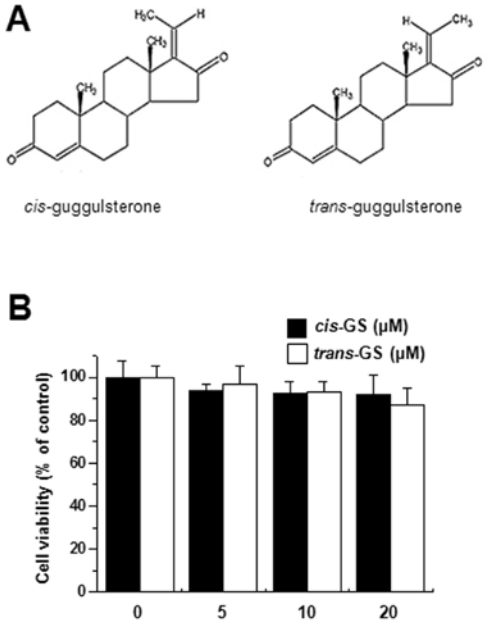

Figure 1. Effect of guggulsterone on the cell viability of MCF-7 cells. (A) Chemical structure of guggulsterone. For the cytotoxicity test, cells were cultured in 96-well plates to 70% confluency and treated with various guggulsterone concentrations (5, 10 and 20 μM) for 24 h. (B) MTT assay was used to detect cell viability. The optical density of the controls was regarded as 100%. Data are presented as the means ± SEM of three independent experiments.

examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB or AP-1 oligonucleotide.

Invasion assay. Invasion assays were carried out in 24-well chambers (8-μm pore size) coated with 20 μl Matrigel diluted in DMEM. The Matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately before the experiments. Cells ($2x10^5$ cells/well) were added to the upper chamber with chemoattractant in the bottom well. Conditioned medium (0.5 ml) was added to the lower compartment of the invasion chamber and chambers were incubated for 24 h. Following incubation, cells on the upper side of the chamber were removed using cotton swabs, and cells that had migrated were fixed and stained with toluidine blue solution. Invaded cells were counted in five random areas of the membrane under a light microscope. Data are presented as the means ± SE from three individual experiments performed in triplicate.

Statistical analysis. Statistical analysis was performed using ANOVA and Duncan's test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of guggulsterone isomers on MCF-7 cell viability. Since the cytotoxicity of guggulsterone *cis*- and *trans*-isomers (Fig. 1A) on MCF-7 cells has not been previously reported, we examined the cytotoxicity to avoid interference from the reagent. The treatment of MCF-7 cells with guggulsterone isomers (5, 10 and 20 μM) for 24 h caused no significant change in cell viability (Fig. 1B).

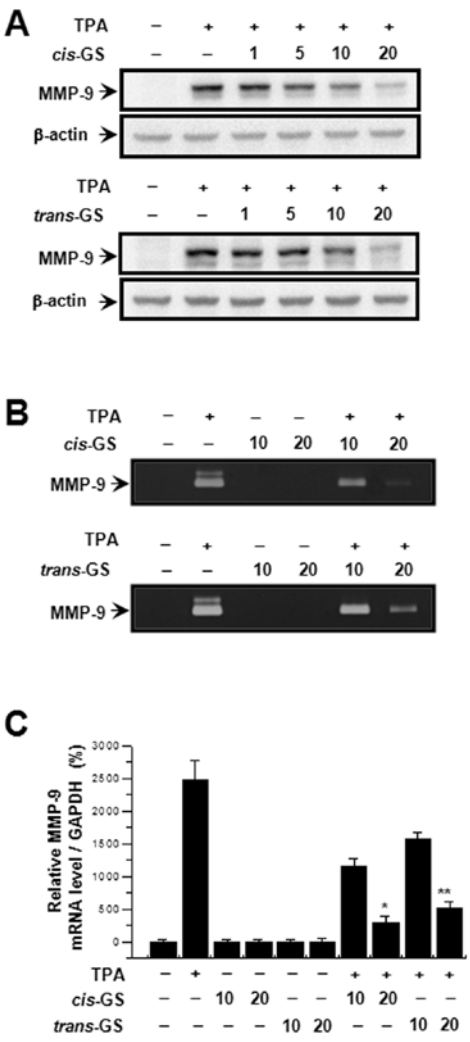

Figure 2. Guggulsterone inhibits TPA-induced MMP-9 expression in MCF-7 cells. MCF-7 cells ($5x10^5$) in monolayer were treated with the indicated guggulsterone concentrations in the presence of 100 nM TPA for 24 h. Cell lysates were analyzed by western blot analysis with anti-MMP-9 antibody. (A) The blot was reprobed with anti-β-actin to confirm equal loading. (B) Conditioned medium was prepared and used for gelatin zymography. (C) MMP-9 mRNA levels were analyzed by real-time PCR and GAPDH was used as the internal control. Each value represents the mean ± SEM of three independent experiments. $^*P<0.01$ vs. TPA; $^{**}P<0.05$ vs. TPA.

Effect of guggulsterone isomers on TPA-induced MMP-9 expression in MCF-7 cells. In order to investigate the effect of guggulsterone on TPA-induced MMP-9 expression, we performed western blot analysis, real-time PCR, and zymography with MCF-7 cells. MCF-7 cells were treated with guggulsterone isomers (1, 5, 10 and 20 μM) in the presence of 100 nM TPA for 24 h. Western blot analysis revealed that the treatment of MCF cells with guggulsterone isomers blocked the upregulation of TPA-induced MMP-9 expression in a concentration-dependent manner (Fig. 2A). Determination of the effects of guggulsterone isomers on TPA-induced MMP-9 secretion revealed that both *cis*- and *trans*-isomers substantially inhibited TPA-induced MMP-9 secretion (Fig. 2B). Real-time PCR also showed that TPA increased the MMP-9 mRNA levels in MCF-7 cells, which was blocked by guggulsterone isomers in a dose-dependent manner (Fig. 2C). Of note, *cis*-gugulsterone decreased MMP-9 expression and secretion to a greater extent

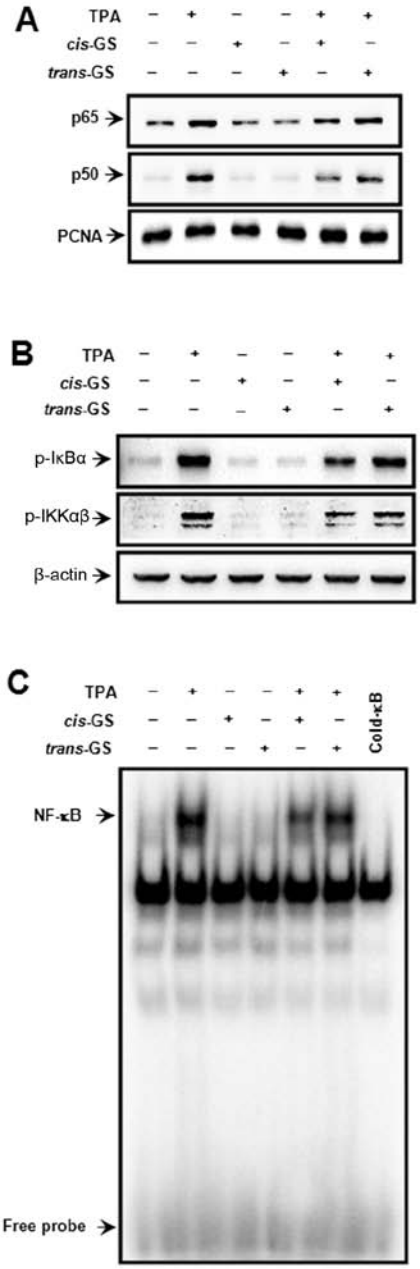

Figure 3. Guggulsterone blocks TPA-induced IKK/NF-κB activation in MCF-7 cells. Cells were treated with 20 μM guggulsterone isomers in the presence of 100 nM TPA. After 3 h of incubation, nuclear extracts were prepared. (A) p65 and p50 translocation to the nucleus and (B) phosphorylation of IκBα and IKKαβ were determined by western blot analysis. (C) NF-κB DNA binding was analyzed by electrophoretic mobility shift analysis as described in Materials and methods.

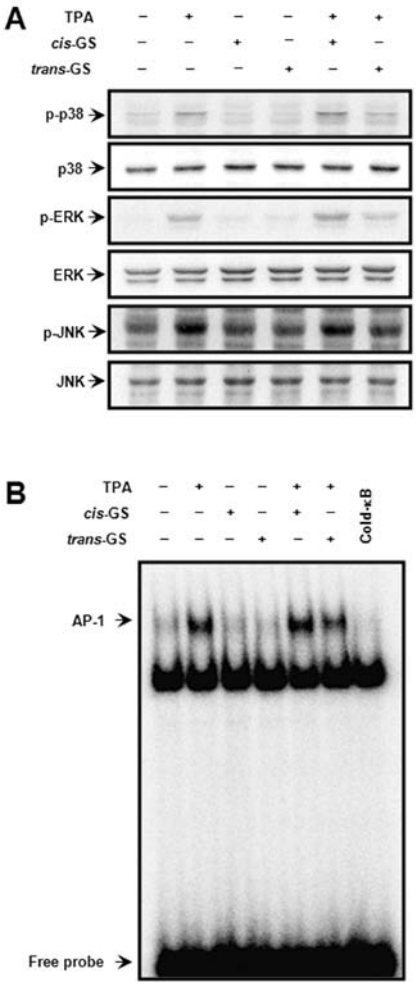

Figure 4. Guggulsterone blocks TPA-induced MAPK/AP-1 activation in MCF-7 cells. Cells were treated with 20 μM guggulsterone isomers in the presence of 100 nM TPA. (A) After 30 min of incubation, cell lysates were prepared for western blot analysis with specific p-p38, p38, p-JNK, JNK, p-ERK and ERK antibodies. Cells were treated with 20 μM guggulsterone isomers in the presence of 100 nM TPA. (B) After 4 h of incubation, nuclear extracts were prepared and examined for AP-1 DNA binding activity by electrophoretic mobility shift analysis as described in Materials and methods.

at 20 μM than *trans*-guggulsterone (Fig. 2A and B). These results indicate that the *cis*-isomer is more potent than the *trans*-isomer in the inhibition of TPA-induced MMP-9 expression and secretion in MCF-7 cells.

Effect of guggulsterone isomers on TPA-induced NF-κB activation. The MMP-9 promoter contains NF-κB and AP-1 binding sites, both of which are centrally involved in MMP-9 gene induction (23,24,28,29). To investigate the transcription factor involved in inhibiting MMP-9 transcription by guggulsterone, we first examined the effect of guggulsterone isomers on the TPA-stimulated NF-κB signaling pathway. The treatment of MCF-7 cells with guggulsterone isomers revealed that *cis*-isomer, but not *trans*-isomer, inhibited the TPA-stimulated nuclear translocation of p65/p50, an NF-κB subunit and NF-κB DNA binding activity (Fig. 3A and C), as well as the TPA-induced phosphorylation of IκBα (Ser-32) and IKKαβ (Ser-176/180) (Fig. 3B). These data indicate that inhibition of the TPA-stimulated activation of the IKK/IκB/NF-κB axis by guggulsterone is stereoisomer-specific and that *cis*-guggulsterone is an inhibitor of IKK.

Effect of guggulsterone isomers on TPA-induced AP-1 activation. MAPK signaling pathways are involved in the AP-1 transcriptional activity (29). We investigated the effect of guggulsterone on the TPA-induced activation of MAPK and AP-1 by western blot analysis and EMSA. The TPA-induced ERK, JNK and p38 phosphorylation, and the activation of AP-1 binding activity were detected (Fig. 4). The activation of the signaling molecules by TPA was significantly blocked by *trans*-guggulsterone, but not *cis*-guggulsterone, indicating that the inhibition of TPA-stimulated AP-1 activa-

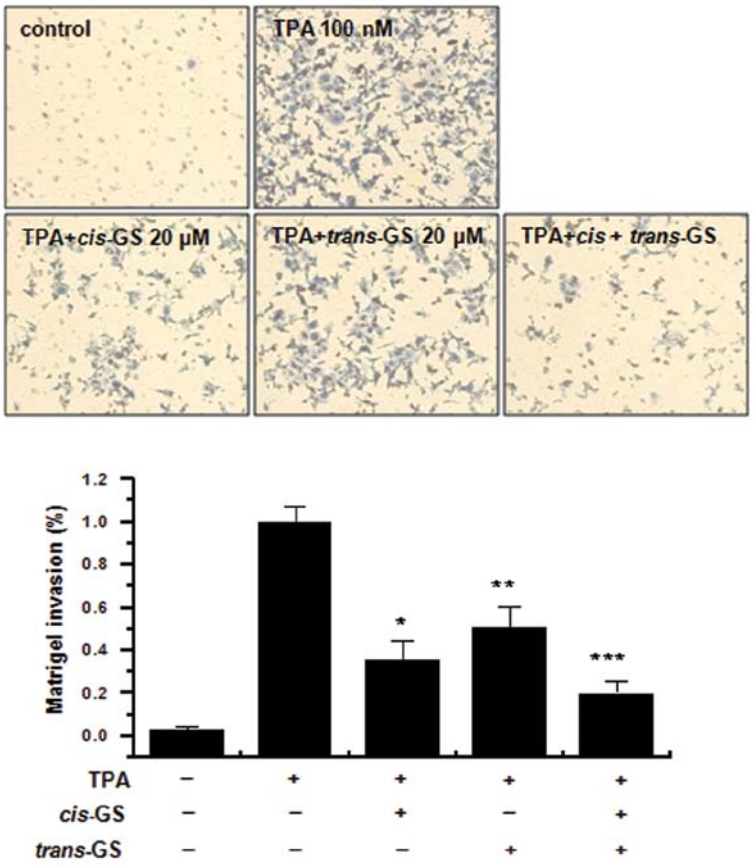

Figure 5. Effect of guggulsterone on TPA-induced Matrigel invasion of MCF-7 cells. Cells were seeded onto the upper chamber and drugs were placed in the well. After 24 h of incubation, cells at the bottom of the filter were fixed, stained and counted. Each value represents the mean ± SEM of three independent experiments. *P<0.01 vs. TPA; **P<0.01 vs. TPA; and ***P<0.01 vs. TPA.

tion by guggulsterone is also stereoisomer-specific; that is, *trans*-guggulsterone regulates TPA-mediated MAPK/AP-1 activation in MCF-7 cells.

Effect of guggulsterone isomers on TPA-induced MCF-7 cell invasion in vitro. The effects of guggulsterone isomers on the invasive potential of MCF-7 breast cancer cells were evaluated using a Matrigel invasion assay. Treatment with TPA increased MCF-7 cell invasion compared to the control MCF-7 cells. The incubation of MCF-7 cells with TPA resulted in a 10-fold increase in the invasion of MCF-7 cells. Treatment with *cis*-guggulesterone decreased the TPA-induced cell invasion by 67%, and the *trans*-isomer reduced the invasion by 51% (Fig. 5). The combination of these isomers exerted an additive effect on the inhibition of MCF-7 cell invasion, showing a 79% inhibition of invasion.

Discussion

This study examined the effects of guggulsterone isomers (*cis* and *trans*) on TPA-induced cell invasion, MMP-9 expression and related molecular mechanisms in MCF-7 cells. We found that *cis*-guggulsterone inhibited TPA-induced MMP expression by blocking IKK/NF-κB signaling, whereas *trans*-guggulsterone by blocking MAPK/AP-1 signaling, suggesting that guggulsterone isomers differ in the modulation of TPA-induced MMP-9 expressions in MCF-7 cells. The combination of isomers exerted additive effects on the inhibition of MCF-7 cell invasion. This study is the first to show that the combination of guggulsterone isomers may be a potential therapeutic strategy for breast cancer.

NF-κB is a transcription factor that plays an important role in the induction of MMP-9 gene expression (30,31). NF-κB comprises a family of inducible transcription factors which regulate host inflammatory and immune responses (32). Diverse signal transduction cascades mediate NF-κB pathway stimulation (32). NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors and consists of two major polypeptides, p65 and p50 (33). NF-κB is initially located in the cytoplasm in an inactive form complexed with IκB, an inhibitory factor of NF-κB. Various inducers such as TPA, cytokines and stress can dissociate this complex, presumably by IκB phosphorylation, resulting in NF-κB being released from the complex. IKKαβ phosphorylates serine residues in the NH_2-terminus of IκB, resulting in NF-κB release and translocation to the nucleus (30). NF-κB then translocates to the nucleus, where it interacts with specific DNA recognition sites to mediate gene transcription. The NF-κB elements are centrally involved in MMP-9 gene induction by TPA (23,34). Shishodia and Aggarwal (6) showed that guggulsterone suppressed NF-κB activation by inhibiting IKK and IκBα degradation in the majority of tumor cells. In support of these observations, we found that the TPA-stimulated phosphorylation of IKKαβ and IκBα and the nuclear translocation of NF-κB were inhibited by treatment with *cis*-guggulsterone,

but not with *trans*-guggulsterone. These findings suggest that *cis*-guggulsterone is a specific inhibitor of the IKK/NF-κB pathway.

AP-1 is a sequence-specific transcriptional factor composed of Jun, Fos and ATF family proteins, which are induced by multiple stimuli such as TPA, cytokines, growth factors and stress (35). The MAPK signaling pathway plays a pivotal role in AP-1 activation (17-21). The activation of ERK results in an increase in AP-1 activity via c-Fos induction, whereas JNK activation leads to the c-Jun phosphorylation (17-21). In this study, the results showed that TPA-induced ERK, JNK and p38 phosphorylation, and AP-1 binding activity via c-Fos induction were blocked by *trans*-guggulsterone, but not by *cis*-guggulsterone. These findings suggest that *trans*-guggulsterone is a specific inhibitor of the MAPK/AP-1 pathway.

MMP-9 activation has been shown to be associated with the progression and invasion of tumors, including mammary tumors (36). Thus, the discovery and development of an agent that inhibits MMP-9 expression are important for the treatment of cancer/tumors. Guggulesterone significantly diminished the TPA-induced cell invasion, and the combination of these isomers exerted additive effects on the inhibition of MCF-7 cell invasion. These results indicate that MMP-9 may be one of the critical molecules involved in processing tumor invasion and metastasis of breast cancer cells.

In conclusion, guggulsterone isomers downregulate MMP-9 expression and tumor cell invasion through the stereoisomer-specific suppression of IKK/NF-κB and MAPK/AP-1 activation. That is, *cis*-guggulsterone regulates the IKK/NF-κB pathway and *trans*-guggulsterone regulates MAPK/AP-1 activation. Therefore, guggulsterone isomers modulate TPA-induced MMP-9 expression in MCF-7 cells in an isomer-specific manner. Moreover, the combination of these isomers exerts an additive effect on inhibition of MCF-7 cell invasion. Based on these observations, we suggest that the combination of *cis*- and *trans*-guggulsterone isomers may be a strong candidate for the prevention of breast tumor invasion and metastasis.

Acknowledgements

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