

Decursin prevents TPA-induced invasion through suppression of PKC α /p38/NF- κ B-dependent MMP-9 expression in MCF-7 human breast carcinoma cells

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Abstract. Decursin, a coumarin compound, was first isolated from the roots of *Angelica gigas* almost four decades ago. It was found to exhibit cytotoxicity against various human cancer cells and to possess anti-amnesic activity *in vivo* through the inhibition of AChE activity. However, the effect of decursin on breast cancer invasion is unknown. Matrix metalloproteinase-9 (MMP-9) is known to be an important factor for cancer cell invasion. Therefore, in this study, we investigated the inhibitory effect of decursin on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression and cell invasion, as well as the molecular mechanisms involved in MCF-7 cells. Our results showed that decursin inhibits TPA-induced MMP-9 expression and cell invasion through the suppression of NF- κ B. Furthermore, decursin repressed the TPA-induced phosphorylation of p38 MAPK and inhibited TPA-induced translocation of PKC α from the cytosol to the membrane, but did not affect

the translocation of PKC δ . These results indicate that decursin-mediated inhibition of TPA-induced MMP-9 expression and cell invasion involves the suppression of the PKC α , MAPK and NF- κ B pathways in MCF-7 cells. Thus, decursin may have potential value in restricting breast cancer metastasis.

Introduction

Breast cancer is one of the leading causes of malignancy-related death among females worldwide (1). Most breast cancer-related deaths are caused by distant metastasis from the primary tumor site. Despite successful treatment of the primary malignancy, relapse and subsequent metastatic spread can still occur at other areas of the body through the bloodstream or lymphatic channels. This leads to local, regional or distant metastasis in such tissues including bone, lung, liver, kidney, thyroid and brain (2). Accordingly, the major focus of breast cancer treatment is to identify chemopreventive drugs for the treatment of breast cancer metastasis (3).

Invasion and metastasis are fundamental processes and major causes of morbidity and mortality in breast cancer patients. Molecular mechanisms of cancer cell invasion and metastasis involve a complex series of events. One such early event involves proteolytic degradation of extracellular matrix (ECM) components (4). The ECM provides biochemical and mechanical barriers to cell movement of cancer cells (5). ECM consists of type IV collagen, laminin, heparan sulfate proteoglycans, nidogen and fibronectin (6). Therefore, ECM degradation requires extracellular proteinases, of which the matrix metalloproteinases (MMPs) have been shown to play a crucial role in breast cancer.

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MMPs, a major group of enzymes that regulate cellular matrix composition, are zinc- and calcium-dependent endopeptidases consisting of four subclasses based on substrate, including collagenases, gelatinases and stromelysins. In particular, MMP-9 is considered to be an important MMP involved in cancer invasion and has been found to be directly associated with invasion, metastasis and poor prognosis of breast cancer (7,8). A variety of stimuli exist, including growth factors (e.g. fibroblast growth factor-2, epidermal growth factor and hepatocyte growth factor), cytokines (e.g. tumor necrosis factor- α), oncogenes (e.g. *Ras*), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (9-11). Among these stimulators, TPA is a well-known selective activator of protein kinase C (PKC) (12) and can stimulate MMP-9 synthesis and secretion during breast cancer cell invasion (13). Additionally, TPA treatments can induce MMP-9 expression via activation of transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (14,15). Consequently, inhibition of MMP-9 expression and/or its upstream regulatory pathways may be an important approach to treat malignant tumors, including breast carcinoma. NF- κ B and AP-1 are transcription factors important for regulating MMP-9, as the MMP-9 gene promoter contains binding sites for both factors (16). Mitogen-activated protein kinase (MAPK) signaling pathways are important for AP-1 activation and NF- κ B activation and requires I- κ B kinase, ERK, JNK or p38 MAPK, depending on the cell type (11,17).

Decursin is a coumarin compound found in the roots of *Angelica gigas* Nakai, which has been traditionally used in Korean folk medicine as a tonic and for the treatment of anemia and other diseases (18). Decursin induces cell cycle arrest and apoptosis in human prostate cancer cells, human breast cancer cells, human bladder cancer cells and colon cancer cells (19-21). Recent studies demonstrated that decursin blocks MMP-9 expression through inhibition of NF- κ B activation in macrophages and cancer cells (21-23). However, the mechanism by which decursin mediates anti-invasiveness is not well understood. Therefore, it has been hypothesized that decursin may have anticancer properties through the inhibition of cell invasion. In this study, decursin was examined for its potential effects on TPA-induced cell invasion and MMP-9 expression in MCF-7 cells. Furthermore, the related molecular mechanisms were also investigated.

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% CO₂ incubator. Decursin, TPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti- β -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against p38, phosphorylated p38 (p-p38), c-Jun N-terminal kinase (JNK), p-JNK, extracellular signal-regulated kinase (ERK), p-ERK, p-c-Jun, p-I κ B α , and p-IKK α were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against MMP-9, p50, p65, I κ B α , IKK α , IKK β , PKC α , PKC δ , proliferating cell nuclear antigen (PCNA), and horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (Santa

Cruz, CA, USA). [α -³²P]dCTP was obtained from Amersham (Buckinghamshire, UK). DMEM containing a high concentration of glucose, FBS, and phosphate-buffered saline (PBS) were obtained from Gibco-BRL (Gaithersburg, ME, USA).

Determination of cell viability. The effect of decursin on MCF-7 cell viability was determined using an established MTT assay. In brief, 3x10⁴ cells were seeded in wells and incubated at 37°C for 24 h to allow attachment. The attached cells were untreated or treated with 1, 5, 10, 25 or 50 μ M decursin for 24 h at 37°C. The cells were washed with PBS prior to adding MTT (0.5 mg/ml PBS) and incubated at 37°C for 30 min. Formazan crystals were dissolved with dimethyl sulfoxide (100 μ l/well) and detected at 570 nm using a model 3550 microplate reader (Bio-Rad, Richmond, CA, USA).

Western blot analysis. MCF-7 cells (5x10⁵) were pre-treated with 25 μ M decursin for 1 h and then incubated with TPA for 24 h at 37°C. Cells were lysed with ice-cold M-PER[®] Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA), and protein concentration was determined using the Bradford method. Samples (20 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% acrylamide and transferred to Hybond[™] polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) using a western blot apparatus. Each membrane was blocked for 2 h with 2% bovine serum albumin or 5% skim milk and then incubated overnight at 4°C with 1 μ g/ml of a 1:2000 dilution of primary antibody. HRP-conjugated IgG (1:2000 dilution) was used as the secondary antibody. Protein expression levels were determined by signal analysis using an image analyzer (Fuji-Film, Tokyo, Japan).

Gelatin zymography assay. Conditioned media was collected after 24 h stimulation, mixed with non-reducing sample buffer, and electrophoresed in a polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed at room temperature for 30 min with 2.5% Triton X-100 solution, and subsequently incubated at 37°C for 16 h in 5 mM CaCl₂, 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 on an image analyzer (Fuji-Film). Proteolysis was visualized as a white zone in a dark blue field. Densitometric analysis was performed using Multi Gauge Image Analysis software (Fuji-Film).

Quantitative real-time polymerase chain reaction. Total RNA was extracted from cells using a FastPure[™] RNA kit (Takara, Shiga, Japan). RNA concentration and purity were determined by absorbance at 260/280 nm. cDNA was synthesized from 1 μ g total RNA using a PrimeScript[™] RT reagent kit (Takara). MMP-9 and glyceraldehyde 3-phosphate dehydrogenase (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). The primers were: MMP-9 (NM 004994) sense, CCTGGAGACCTGAGAACCAATCT, antisense, CCACCC GAGTGTAAACCATAGC; and GAPDH (NM 002046) sense, ATGGAAATCCCATCACCATCTT, antisense, CGCCC CACTTGATTTTGG. To control for variation in mRNA

concentration, all results were normalized to the GAPDH housekeeping gene. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Preparation of nuclear extract. MCF-7 cells (2×10^6) were treated with decursin in the presence or absence of TPA for 4 h. Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at $1,500 \times g$ for 3 min. Cytoplasmic and nuclear extracts were prepared from cells using the NE-PER[®] Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology).

Membrane fractionation. MCF-7 cells (5×10^7) were pre-treated with $25 \mu\text{M}$ decursin for 1 h and then incubated with TPA for 30 min at 37°C . Cells were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.5), and pelleted at 4,000 rpm for 3 min. Cell lysis was carried out in homogenization buffer (20 mM Tris-HCl, 5 mM DTT, 2 mM EDTA, 5 mM EGTA, protease inhibitor, phosphatase inhibitors, pH 7.5) with brief sonication (5 times, 10 sec and 10% amplitude) after incubate on ice for 15-30 min. Cell debris was removed by centrifuging the sample at 3,000 rpm for 10 min at 4°C . Cell lysate was centrifuged at 13,000 rpm for 30 min at 4°C to separate out soluble (cytosolic) and pellet (membrane) fraction. Pellet fraction was incubated in homogenization buffer containing 1% Triton X-100 for 30 min in ice, centrifuged at 50,000 rpm for 1 h and the supernatant was collected as the membrane fraction.

Electrophoretic mobility shift assay (EMSA). Activation of NF- κB and AP-1 was assessed with a gel mobility shift assay using nuclear extracts. An oligonucleotide containing the $\kappa\text{-chain}$ (κB , 5'-CCGGTTAACAGAGGGGGCTTTCCGAG-3') or AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3') binding site was synthesized and used as a probe for the gel retardation assay. The two complementary strands were annealed and labeled with [$\alpha\text{-}^{32}\text{P}$]dCTP. Labeled oligonucleotides (10,000 cpm), 10 μg of 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 dithiothreitol) were then incubated for 30 min at room temperature in a final volume of 20 μl . The reaction mixtures were analyzed by electrophoresis on 4% polyacrylamide gels in 0.5X Tris-borate buffer. The gels were dried and examined by autoradiography. Specific binding was controlled by competition with a 50-fold excess of cold κB oligonucleotide.

Invasion assay. The invasion assay was carried out in 24-well chambers (8- μm pore size) coated with 20 μl matrigel diluted DMEM. The matrigel coating was re-hydrated in 0.5 ml DMEM for 30 min immediately before the experiments. Cells (2×10^5) 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. The 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. Invading cells were counted in five random areas of the membrane using a light microscope. Analyzed data are the means \pm SE from three individual experiments performed in triplicate.

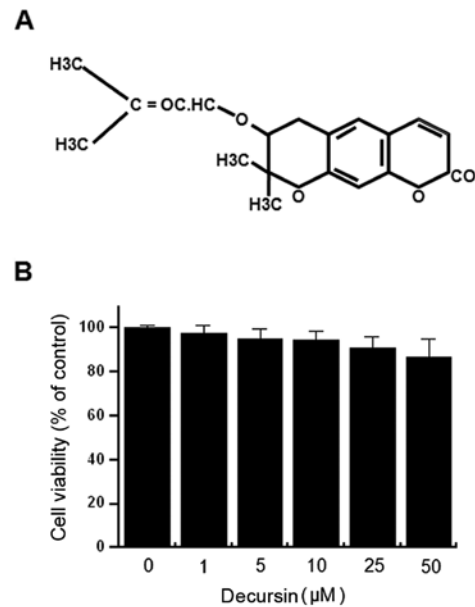


Figure 1. Structure of decursin and its effects on MCF-7 viability. Chemical structure of decursin (A). To test the cytotoxicity of decursin, cells were cultured in 96-well plates until 70% confluence and various concentrations (0, 1, 5, 10, 25 and 50 μM) of decursin were added to cells for 24 h. MTT assay was used to detect the viability of the cells (B). The optical density value of control was regarded as 100%. Data are the mean \pm SEM of three independent experiments.

Statistical analysis. Statistical data analysis was performed using ANOVA and Duncan's test. Differences with $p < 0.05$ were considered statistically significant.

Results

Decursin does not affect MCF-7 cell viability. Fig. 1A is the chemical structure of decursin. In order to investigate the cytotoxicity of decursin on MCF-7 cells, cells were seeded into the wells of 96-well culture plates at a density of 3×10^4 cells/well. The effect of decursin on MCF-7 cellular toxicity was analyzed using the MTT assay. Treatment of MCF-7 cells with all concentrations of decursin for 24 h did not lead to a significant change in cell viability (Fig. 1B) or morphology. Therefore, subsequent experiments utilized the optimal non-toxic concentration (25 μM) of decursin.

TPA-induced MMP-9 activation was decreased by decursin in MCF-7 cells. To determine the effect of decursin on TPA-induced MMP-9 expression, we pretreated cells with the indicated concentration of decursin prior to 20 nM TPA treatment in MCF-7 cells. After 24 h, western blot analysis, real-time PCR and zymography were performed in MCF-7 cell-containing samples. From our results, western blot analysis showed that the decursin treatment of MCF-7 cells blocked the upregulation of TPA-induced MMP-9 protein expression (Fig. 2A). Real-time PCR revealed a TPA-induced increase in the expression of MMP-9 in MCF-7 cells, and that the addition of decursin blocked this TPA-induced MMP-9 upregulation in a dose-dependent manner (Fig. 2B). In addition, we examined the level of TPA-induced MMP-9 secretion by decursin using zymography analysis. We demonstrated that TPA treatment of

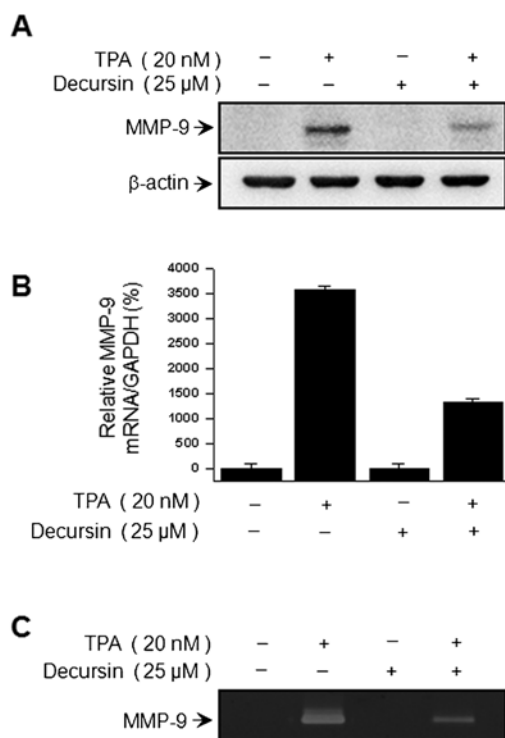


Figure 2. Decursin inhibits TPA-induced MMP-9 expression in MCF-7 cells. MCF-7 cells in a monolayer were treated with the indicated decursin concentrations in the presence of TPA for 24 h. Cell lysates were analyzed by western blotting with anti-MMP-9. The blot was re-probed with anti- β -actin to confirm equal loading (A). MMP-9 mRNA levels were analyzed by real-time PCR and GAPDH was used as an internal control (B). Effects of decursin on the MMP-9 activity in MCF-7 cells. Conditioned medium was prepared and used for gelatin zymography. Cells were pretreated with decursin for 1 h followed by TPA stimulation for 24 h (C). Each value represents the mean \pm SEM of three independent experiments. * $p < 0.01$ vs. TPA.

MCF-7 cells increased MMP-9 secretion, while the addition of decursin significantly diminished TPA-induced MMP-9 secretion (Fig. 2C).

Decursin inhibits membrane localization of TPA-induced PKC α . Activation of PKCs has been shown to correlate with potential tumor metastasis (13,24). TPA has been reported as a PKC activator, and activation of the PKC isoforms, including α , β , δ and ϵ , by TPA has been identified (25). To determine whether decursin activates any PKC isotypes in MCF-7 cells, we analyzed the levels of PKC α and PKC δ in the cytosol and membrane and observed stimulation by TPA, while decursin pretreatment attenuated the translocation of these PKCs. As shown in Fig. 3, TPA-induced membrane localization of PKC α was blocked by pretreatment with decursin for 1 h. These results suggest that TPA-induced MMP-9 expression and invasion may be involved in the activation of PKC α in MCF-7 cells, while the addition of decursin inhibits TPA-induced PKC α activation.

Decursin inhibits TPA-induced NF- κ B DNA binding activity and the MAPK pathway, but not AP-1 DNA binding activity. To elucidate the mechanism governing decursin-mediated inhibition of MMP-9 expression, the effect of decursin on TPA-induced activation of NF- κ B and AP-1 was confirmed using EMSA. We investigated the NF- κ B and AP-1 signaling

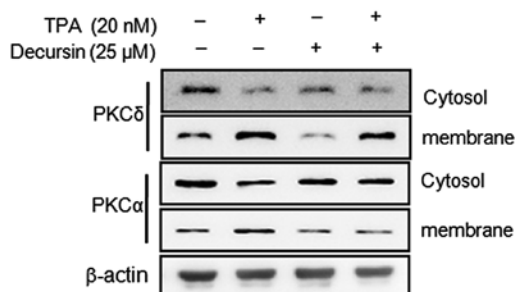


Figure 3. Decursin inhibits TPA-induced PKC α activation. Cells were pretreated with decursin for 1 h and then stimulated with TPA for 30 min. Western blot analysis was performed to detect the PKC isozyme level in cytosol and membrane fractions.

pathways in nuclear extracts prepared 4 h after TPA stimulation. As shown in Fig. 4A and B, TPA substantially increased NF- κ B and AP-1 binding activity. Of note, pre-treatment with decursin inhibited TPA-stimulated NF- κ B binding activity, but not the AP-1 pathway. These results were consistent with the view that decursin specifically blocks NF- κ B activation in MCF-7 cells. In the western blot analysis, TPA stimulated the phosphorylation of I- κ B α and I- κ B β in the cytoplasm and, accordingly, the nuclear translocation of NF- κ B subunits p50 and p65. In the case of AP-1, c-Jun expression was considerably augmented, but c-Fos expression was only negligibly induced in TPA-treated cells. Therefore, from our results we observed that an increase in p-I- κ B α and translocation of p65 and p50 as a result of TPA stimulation was significantly suppressed by decursin (Fig. 4D and E). Furthermore, we confirmed that TPA-induced phosphorylation of c-Jun, a major subunit of AP-1, and decursin have no effect on the phosphorylation of c-Jun (Fig. 4E). To investigate the inhibitory effect of decursin on the MAPK pathway, which is the so-called upstream modulator of NF- κ B and AP-1, TPA-induced phosphorylation and activation of MAPKs was first confirmed. We observed that decursin inhibited the phosphorylation of p38, but not ERK and JNK 30 min after TPA treatment (Fig. 4C). These results suggest that decursin inhibits TPA-induced MMP-9 expression through the regulation of the p38/MAPK-NF- κ B pathway in MCF-7 cells.

Decursin decreases TPA-induced MCF-7 cell invasion in vitro. It has been reported that the upregulation of MMP-9 expression contributes to the invasion of cancer cells (4). Therefore, an *in vitro* invasion assay was used to investigate the inhibitory effects of decursin on the invasive potency of MCF-7 breast adenocarcinoma cells. Treatment with TPA increased MCF-7 cell invasion compared with untreated control cells, as determined by a Matrigel invasion assay. Incubation of MCF-7 cells with TPA resulted in a 10-fold increase in cell invasiveness. However, we observed that treatment with decursin diminished TPA-induced cell invasion by 79% (Fig. 5).

Discussion

Current studies involved in developing effective anti-invasion strategies have focused mainly on the use of natural bioactive agents in MCF-7 cells. In this study, we isolated decursin from the roots of *Angelica gigas* Nakai and examined its

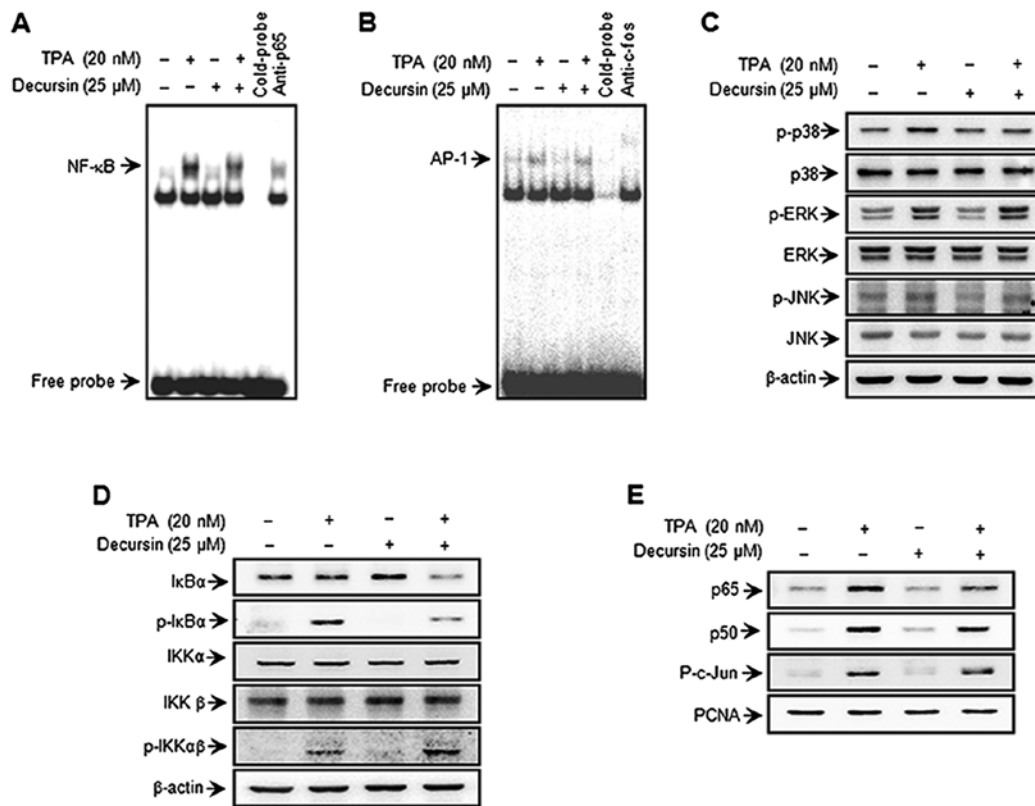


Figure 4. Decursin inhibits TPA-induced transcriptional activation of MMP-9 and phosphorylation of p38 MAPK in MCF-7 cells. Cells were treated with decursin in the presence of TPA. Following incubation for 4 h, nuclear extracts were prepared. DNA binding of NF- κ B and AP-1 was analyzed by EMSA (A and B). Western blotting was performed to determine the cytoplasmic levels of p-IKK $\alpha\beta$, IKK α , IKK β , p-I κ B α , and I κ B α , as well as the nuclear levels of the subunits of NF- κ B (p50 and p65) and AP-1 (p-c-Jun) (D and E). Cells were pretreated with TPA for 30 min in the presence or absence of decursin. Cell lysates were prepared for western blot analysis with specific p-p38, p38, p-JNK, JNK, p-ERK and ERK antibodies (C).

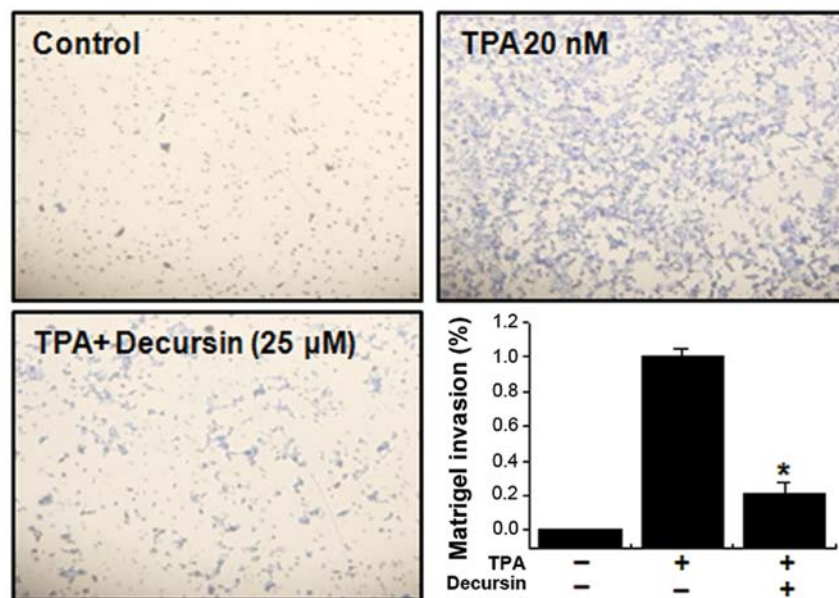


Figure 5. Decursin inhibits TPA-induced Matrigel invasion in MCF-7 cells. Cells were seeded onto the upper chamber, and drugs were placed in the well. After a 24-h incubation, cells on the bottom of the filter were fixed, stained, and counted. Each value represents the mean \pm SEM of three independent experiments. *p < 0.01 vs. TPA.

effects on TPA-induced MMP-9 expression and invasion in MCF-7 cells. In recent studies, decursin was found to prevent MMP-9 expression by suppression of the NF- κ B pathway in cancer cells and macrophages (22,26). Additionally, our

previous study showed that decursin inhibits UVB-induced MMP expression in human dermal fibroblasts via regulation of NF- κ B (27). However, to date, no report exists on the anti-invasion effects of decursin in MCF-7 cells. In this study, we

examined the anti-invasive potential of decursin and explored the molecular mechanisms underlying its activity.

Breast cancer is the most commonly detected cancer and one of the leading causes of death in women worldwide. Metastasis is the primary cause of breast cancer mortality. Tumor metastasis is a complex, multistep process that includes cell proliferation, ECM degradation, cell migration and tumor growth at metastatic sites (28). Morphologically, tumor invasion is associated with the presence of a distorted edge of the primary tumor, where individual or cohorts of tumor cells actively invade the tissue ECM surrounding the primary tumor (29).

MMP-9 is a critical molecule in tumor invasion and metastasis. MMP-9 activation has been shown to be associated with tumor progression and invasion, including mammary tumors (30). In previous studies, inflammatory cytokines, growth factors and TPA were shown to stimulate MMP-9 by activating different intracellular-signaling pathways in breast cancer cells (31). TPA increases the invasiveness of MCF-7 cells by increasing transcription of MMP-9 and activating the PKC, MAPK and phosphoinositide 3 kinase (PI3K)/Akt pathways. Activation of PKC by TPA involves the translocation of PKC isoforms to the plasma membrane, resulting in proliferation, differentiation, malignant transformation, and tumor promotion and progression in cancer cells. Our previous studies demonstrated that PKC α and PKC δ play critical roles in MMP-9 induction and invasion in MCF-7 cells. In our previous data, TPA stimulation resulted in the translocation of PKC α and PKC δ from the cytosol to the cell membrane, although translocation of PKC β was not observed. Treatment with a non-cytotoxic dose of a PKC δ inhibitor (rottlerin), a broad PKC inhibitor (GF109203X), and a PKC α inhibitor (Gö6976) caused marked inhibition in TPA-induced MMP-9 expression and secretion (32). In this study, we observed that decursin inhibited TPA-induced membrane localization of PKC α , but not of PKC δ .

Additionally, to understand the TPA-induced signaling cascade underlying MMP-9 expression in MCF-7 cells, we assessed the effects of three MAPKs and the DNA binding abilities of transcription factors. The three major MAPK families, JNK, ERK and p38 kinase, are expressed in MCF-7 cells, and their active phosphorylated forms can be detected (28). MAPK signaling pathways are important for NF- κ B and AP-1 activation, which require I κ B kinase, PI3K-Akt or p38 MAPK, depending on the cell type (11,17,33). Our results show that decursin displayed inhibitory effects on the phosphorylation of p38 MAPK. In addition, it has been established that TPA induces the nuclear transcription factors NF- κ B and AP-1 in MCF-7 cells. NF- κ B and AP-1 are transcription factors that are important in regulating MMP-9, as the MMP-9 gene promoter contains NF- κ B and AP-1 binding sites (16). The NF- κ B and AP-1 elements are centrally involved in TPA-mediated MMP-9 gene induction (14,34). The present results show that decursin inhibits activation of NF- κ B but not AP-1 in MCF-7 cells. Previous results confirm that TPA-induced MMP-9 expression can be significantly inhibited by selective inhibitors of p38 (SP600125) (35). The present results show that decursin significantly inhibits TPA-activated p38 MAPK. Together, these results confirm that TPA-stimulated cell invasion can be suppressed by the inhibition of the MAPK/NF- κ B pathways.

Finally, these experiments confirmed that TPA-stimulated cell invasion was suppressed by decursin. The data obtained from our Matrigel invasion assay showed that decursin inhibits the TPA-induced invasion potential of MCF-7 cells (Fig. 5). In conclusion, decursin inhibited TPA-induced invasion by reducing MMP-9 activation mainly through the PKC α , MAPK, and NF- κ B pathways in MCF-7 cells. This is the first study demonstrating that decursin can suppress TPA-stimulated cancer cell invasion by inhibiting MMP-9 expression through the suppression of PKC α . Furthermore, our results present, for the first time, details of the molecular mechanisms in MCF-7 cells responsible for this inhibitory effect.

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