RESEARCH ARTICLE

Suppression of phorbol-12-myristate-13-acetate-induced tumor cell invasion by bergamottin *via* the inhibition of protein kinase Cδ/p38 mitogen-activated protein kinase and JNK/nuclear factor-κB-dependent matrix metalloproteinase-9 expression

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Matrix metalloproteinase (MMP) plays an important role in the invasion and metastasis of cancer cells. The inhibitory effects of bergamottin, a cytochrome P450 inhibitor from *Citrus paradis* (grapefruit), on tumor invasion and migration and the possible mechanisms involved in this inhibition were investigated in human fibrosarcoma HT-1080 cells. Bergamottin reduced phorbol-12-myristate-13-acetate (PMA)-induced activation of MMP-9 and MMP-2 and further inhibited cell invasion and migration. Bergamottin suppressed PMA-enhanced expression of MMP-9 protein, mRNA and transcription activity levels through suppression of nuclear factor-κB (NF-κB) activation without changing the tissue inhibitor of metalloproteinase 1 level. Bergamottin also reduced PMA-enhanced MMP-2 expression through suppression of membrane-type 1 MMP, but did not alter tissue inhibitor of metalloproteinase 2 levels. Bergamottin inhibited PMA-induced NF-κB nuclear translocation and IκBα degradation, which are upstream of PMA-induced MMP-9 expression and invasion. Furthermore, bergamottin strongly repressed the PMA-induced phosphorylation of p38 mitogen-activated protein kinase and c-Jun N-terminal kinase (JNK), which are dependent on the protein kinase C-δ pathway. In conclusion, we demonstrated that the anti-invasive effects of bergamottin might occur through inhibition of protein kinase C-δ, p38 mitogen-activated protein kinase, and JNK phosphorylation and reduction of NF-κB activation, leading to downregulation of MMP-9 expression. These results suggest that the suppression of MMP expression contributes, at least in part, to the antitumor activity of bergamottin.

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1 Introduction

Evidence from epidemiological studies suggests that diets rich in fruits and vegetables are protective against a number of different cancers [1]. Grapefruits are a rich source of bioactive compounds that may serve as cancer chemopreventive agents [2]. Grapefruit and grapefruit-based products are rich in flavonoids [3], which have been inversely correlated to the

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Abbreviations: AP-1, activator protein-1; **CYPs,** cytochrome P450s; **ECM,** extracellular matrix; **FBS,** fetal bovine serum; **JNK,** c-Jun N-terminal kinase; **MAPK,** mitogen-activated protein kinase; **MMP,** matrix metalloproteinase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; **NF-κB,** nuclear factor-κB; **PMA,** phorbol-12-myristate-13-acetate; **PKC,** protein kinase C; **TIMP,** tissue inhibitor of metalloproteinase; **TNF-α,** tumor necrosis factor-α

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occurrence of certain chronic diseases, such as several types of cancer and cardiovascular disease as demonstrated in several cohort and case control studies [1, 4]. Overall, scientists continuously survey antioxidant- and phytochemical-rich fruits and vegetables for their complex spectrum of biological and medicinal activities, which range from antibiotic to antitumor properties. Furanocoumarins, a class of aromatic compounds, are produced by plants. Because of their important biological activities and clinical applications, such as the inhibition of cytochrome P450s (CYPs) and plant defense mechanisms, furanocoumarins have been studied in a number of biological systems including plants, humans, fungi and insects. Recent *in vitro* studies have characterized two furanocoumarin compounds in grapefruit (bergamottin and 6′,7′-dihydroxybergamottin) as potent CYPs inhibitors that reduce microsomal CYP3A4 activity [5–7]. Bergamottin has also been described to inhibit CYP3A4 [6], CYP1A1 [8], and other CYPs [9]. In addition, bergamottin reduces the formation of DNA adducts in MCF-7 cells induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene [10] and skin tumors in mice [11], and the generation of NO [12], while it induces leukemia differentiation [13]. Furthermore, some case-control studies suggest that citrus fruit intake reduces the risk of gastric cancer [14]. Also, Sasaki *et al.* [15] have reported that bergamottin attenuates tumor necrosis factor-α (TNF-α)-induced endothelial molecule expression and leukocyte adhesion.

Tumor invasion and metastasis are major causes of cancer-related death and involve several biological processes. Cell–extracellular matrix (ECM) interactions, disconnection of intercellular adhesion, degradation of ECM and invasion of lymph and blood vessels are critical steps for cancer invasion and metastasis [16–18]. A number of proteolytic enzymes participate in the degradation of environmental barriers such as the ECM and basement membrane [19, 20]. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases, play an important role in the proteolysis of various ECM components and are involved in the metastasis and angiogenesis of cancer cells [21, 22]. MMPs are synthesized as preproenzymes and are secreted from cells as proenzymes. MMPs are divided into four subclasses based on substrate: collagenase, gelatinase, stromelysin and membrane-associated MMPs [23]. Among the human MMPs reported to date, MMP-2 and MMP-9 are the key enzymes involved in degrading type I and IV collagen and ECM [22, 24]. Tumor-secreted MMPs destroy ECM components in the tissues surrounding the tumor, and tumor cells subsequently invade through the basement membrane of blood vessels and facilitate the spread to distant organs, resulting in organ failure and patient mortality. Both MMP-2 and MMP-9, which are abundantly expressed in various malignant tumors [25], contribute to cancer invasion and metastasis [26]. Generally, MMP-2 is constitutive and overexpressed in highly metastatic tumors, whereas MMP-9 can be stimulated by the inflammatory cytokine, TNF-α, by the growth factor, epidermal growth factor, or by phorbol ester through activation of different intracellular-signaling pathways [27–29]. Its main activation occurs on the cell surface and is mediated by membrane-type MMPs such as MT1-MMP [30]. The concerted action of highly expressed MT1-MMP and adequate expression of tissue inhibitor of metalloproteinase (TIMP) leads to activation of MMPs [31, 32].

Mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase and protein kinase C (PKC) signaling pathways are the predominant cascades participating in MMP-9 expression [33–35]. In addition, transcriptional regulation by activating transcription factors, including activator protein-1 (AP-1), nuclear factor-κB (NF-κB) or stimulatory protein-1, was also reported to occur in the regulation of MMP-9 gene expression [36]. NF-κB regulates the expression of a number of genes, the products of which are involved in tumorigenesis [37, 38]. Indeed, NF-κB is a key transcription factor involved in the activation of genes that encode inflammatory cytokines such as TNF-α and IL-1β. NF-κB can also induce the activation of MMP-9 and cyclooxygenase-2 [37, 38]. Thus, agents able to suppress NF-κB activation have the potential to suppress tumorigenesis and metastasis, and several such agents show therapeutic potential.

Several studies have indicated that inhibition of MMP expression or enzyme activities can serve as an early target for treating cancer metastasis [39, 40]. Recent results demonstrate downregulation of MMP-2 by furanocoumarins, which suppress the invasiveness of glioblastoma cells [41, 42]. These results provide insight into the different mechanisms involved in the chemopreventive actions of bergamottin. However, the antimetastatic activity of bergamottin has not been demonstrated until now. In the present study, we demonstrate that bergamottin significantly suppresses MMP-9 gene expression by blocking the PKCδ/p38 MAPK and c-Jun N-terminal kinase (JNK)/NF-κB signaling pathways and consequently reduces migration and invasion of human HT-1080 cells.

2 Materials and methods

2.1 Materials

Bergamottin and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chemical (St. Louis, MO, USA). NF-κB activation inhibitor was purchased from Calbiochem (La Jolla, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-based colorimetric assay kit was purchased from Roche (Indianapolis, IN, USA). RPMI1640, fetal bovine serum (FBS), sodium pyruvate and Trizol were supplied by Gibco BRL (Grand Island, NY, USA). Antibodies against phospho-MAP kinase, phospho-PKCδ MMP-2, MMP-9 and NF-κB were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against c-jun, c-fos, IαBα lamin B and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The other chemicals and reagents were of analytical grade.

2.2 Cell culture and cell treatments

HT-1080 cells from ATCC (American Type Culture Collection, Manassas, VA, USA) were grown in RPMI1640 supplemented with 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a 5% CO_2 humidified incubator. Cells were treated with different concentrations of bergamottin in the absence or presence of PMA (30 nM) for 24 h. Bergamottin was dissolved in DMSO, the final concentration of which did not exceed 0.1%.

2.3 Measurement of cell viability

HT-1080 cells were plated at a density of 4×10^4 cells/500 μL in 48-well plates and the cell viability was determined by the conventional MTT reduction assay. After incubation, cells were treated with MTT solution (final concentration, 1 mg/mL) for 1 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO and the absorbance at 570 nm was measured with a microplate reader (Varioskan, Thermo Electron, Berthold, Germany).

2.4 *In vitro* wound-healing assay

HT-1080 cells were seeded in 6-well plates and grown overnight to confluence. The monolayer cells were scratched with a 200-μL pipette tip to create a wound; the cells were washed twice with serum-free RPMI1640 to remove floating cells and then incubated in medium without serum. The rate of wound closure was investigated through photography 24 h later. Each value is derived from three randomly selected fields.

2.5 Matrigel invasion assay

HT-1080 cells were incubated in RPMI1640 with 10% FBS and then collected by trypsinization. Cells (1×10^5 cells/mL) in serum-free medium were added to an inner cup of the 48-well Transwell chamber (Corning Life Sciences, Corning, NY, USA) that had been coated with 50 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA; 1:10 dilution in serum-free medium). Medium supplemented with 10% serum or the indicated agent was added to the outer cup. After 24 h, cells that had migrated through the Matrigel and the 8-μm pore size membrane were fixed, stained, and counted under a light microscope. Each experiment was performed in triplicate.

2.6 RT-PCR

Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK) and the concentration of total RNA was measured spectrophotometrically. RNA (2 μg) was converted to complementary DNA by a RT-PCR Bead kit (Amersham Pharmacia) according to the manufacturer's protocol. The amplification sequence protocol was 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

2.7 Western blotting analysis

After treatment, cells were collected and washed with PBS. The harvested cells were then lysed on ice for 30 min in 100 μL lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP40) and centrifuged at 13 000 × *g* for 15 min. Supernatants were collected from the lysates and protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed on a 10% SDS–polyacrylamide gel. The membranes were blocked with 1% BSA at room temperature for 1 h and then incubated with the indicated specific primary antibodies for 3 h followed by incubation with the respective alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 h. Finally, protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology).

2.8 Gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded and allowed to grow to confluence for 24 h and then maintained in serum-free medium. The conditioned media were collected 24 h after stimulation, mixed with non-reducing sample buffer and subjected to electrophoresis in 10% polyacrylamide gel containing 0.1% w/v gelatin. The gel was washed with washing buffer containing 2.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5) and incubated at 37°C for 24 h in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 40 mM NaN_3. The gel was stained with 0.25% w/v Coomassie brilliant blue in 45% v/v methanol and 1% v/v acetic acid.

2.9 Transient transfection and luciferase assay

To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI, USA). The cells were plated in 48-well plates and incubated at 37°C. At 70–80% confluence, the cells were washed with RPMI1640 and incubated with RPMI1640 without serum or antibiotics for 6 h. The cells were then transfected with the MMP-9 promoter vector (gift from Dr. Wolfgang Eberhardt., Klinikum der johann wolfgang Goethe-universitat, Germany), AP-1 or NF-κB reporter vector and *Renilla* luciferase reporter

vector using LipofectAMINE 2000 reagent according to the manufacturer's protocol. After incubation, cells were lysed and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron).

2.10 Immunocytochemistry

HT-1080 cells grown on poly-L-lysine-coated coverslips was treated with bergamottin for 6 h, after which the cells were washed with PBS and fixed with 2% w/v paraformaldehyde. After permeabilization, coverslips were blocked with 1% BSA, followed by incubation with the rabbit anti-NF-κB (p65) polyclonal antibody (1:500 dilution) for 1 h. The secondary antibody was Alexa Fluor 488-conjugated donkey anti-rabbit antibody (1:500 dilution, Molecular Probes, Eugene, OR, USA) for 30 min. After two further washes in PBS, the sections were counterstained with the DNA dye 4′,6-dianidino-2-phenylindole at a concentration of 1 μg/mL for 5 min. The cells were finally mounted with mounting medium (Dako, Hamburg, Germany) and analyzed by fluorescence microscopy (Axiovert 200M; Carl Zeiss, Germany).

2.11 NF-κB activity assay

For analysis of basal NF-κB activity, cells were simultaneously infected with Lenti-NF-κB-luc or Lenti-NF-κB-GFP and packaging plasmids pFIV-34N/pVSV-G (System Biosciences) mixed with polybrene (4 μg/mL) to develop stable cell lines expressing the NF-κB reporter. Stable cells were cultured in 96-well plates until cells were 70% confluent, and functional validation of the NF-κB reporter activity was conducted *in vitro* using TNF-α (10 ng/mL; Sigma) as a positive control. Cell lysates were prepared, and luciferase activities were quantified according to the manufacturer's instructions (Luciferase Reporter Assay System; Promega). Fluorescence of GFP protein was analyzed by fluorescence microscopy (Axiovert 200M; Carl Zeiss).

2.12 Statistical analysis

All experiments were repeated at least three times. Means ± SD were calculated for each group and Dunnett's *t*-test was used to calculate statistical significance. Differences were considered statistically significant when $p < 0.01$.

3 Results

3.1 Bergamottin prevents invasion and metastasis of human fibrosarcoma cells

Prior to the experiments for invasion in medium containing 10% FBS and for MMP activity in serum-free medium, the cytotoxic effect of bergamottin on the human HT-1080 cell line was determined in the corresponding medium using the MTT assay. Treatment with 1 to 50 μM bergamottin caused a 1–10% decrease in cell viability in medium containing serum and a 3–13% decrease in cell viability in serum-free medium (Fig. 1B). Therefore, bergamottin had no significant cytotoxicity in tumor cells at these concentrations. *In vitro* invasion and migration assays, including transwell and wound-healing assays, were used to investigate the inhibitory effects of bergamottin on the invasive potency of fibrosarcoma HT-1080 cells. As illustrated in Fig. 1C, the data from the wound-healing assay indicated that migration of HT-1080 cells was inhibited by bergamottin (Fig. 1C). Similarly, the data obtained from the Matrigel invasion assay showed that PMA stimulated cell invasion, while 50 μM bergamottin inhibited the PMA-induced invasion of HT-1080 cells by 50% (Fig. 1D). These results suggest that bergamottin prevents invasion and migration of human fibrosarcoma at non-toxic concentrations.

3.2 Bergamottin suppresses MMP-9 activity

The fact that bergamottin had an inhibitory effect on invasion and migration prompted us to examine the effect of bergamottin on MMP-9 and MMP-2 activities using gelatin zymography. We used a gelatin zymography assay to investigate the inhibitory effect of bergamottin on PMA-induced MMP-9 and MMP-2 secretions. The secretion of MMP-9 into the conditioned medium of HT-1080 cells was dramatically induced by PMA in a dose-dependent manner, when cells were cultured in serum-free medium with various concentrations of PMA for 24 h (Fig. 2A). As shown in Fig. 2B, treatment of HT-1080 cells with bergamottin at doses above 5 μM suppressed PMA-induced MMP-9 and MMP-2 activities in a dose-dependent manner (Fig. 2B). Similar experiments were carried out with human breast carcinoma MCF-7 cells. TNF-α is one of the physiological inducers for MMP-9 [29]. Bergamottin significantly inhibited TNF-α-induced MMP-9 and-2 production in a dose-dependent manner (Fig. 2C). These results indicate that bergamottin inhibits MMP-9 and MMP-2 induction with physiological relevance.

3.3 Bergamottin suppresses PMA-induced MMP-9 and MMP-2 expression through inhibition of its transcriptional activity

The results obtained on zymography were further confirmed by Western blot analysis and semi-quantitative RT-PCR (Fig. 3). The secretion of MMP-9 and MMP-2 proteins into the medium was gradually decreased in a dose-dependent manner, indicating that the reduced MMP-9 enzyme activity is the result of decreased amounts of MMP-9 and MMP-2 proteins (Fig. 3A). In the semi-quantitative RT-PCR, the treatment of HT-1080 cells with bergamottin decreased the

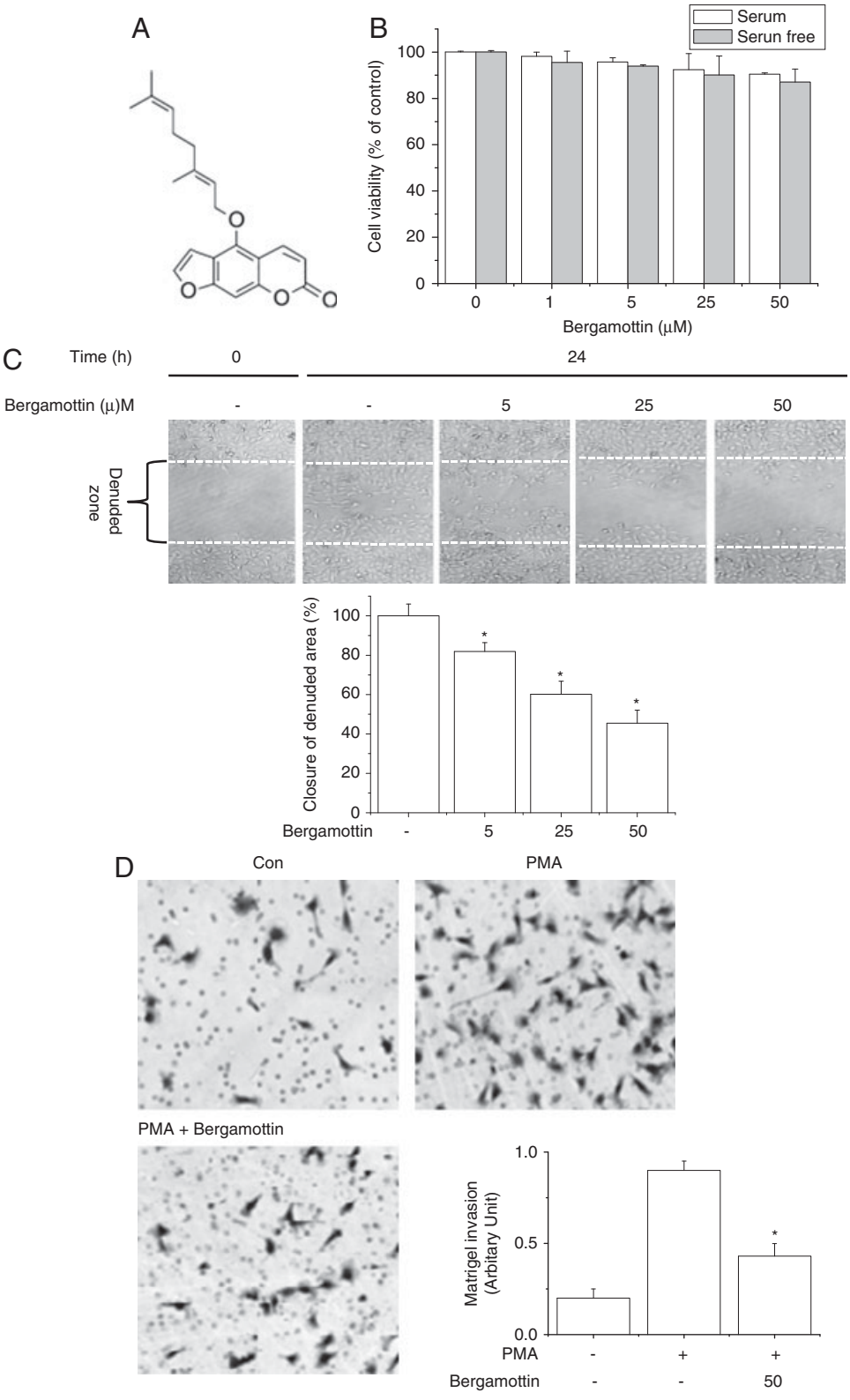

Figure 1. Inhibitory effects of bergamottin on the migration and invasion of human fibrosarcoma HT-1080 cells. (A) Structure of bergamottin. (B) HT-1080 cells were treated with bergamottin for 24 h in medium containing 10% serum (white bar) or in serum-free medium (gray bar), and viability was determined by an MTT assay. (C) Cells were scratched with a pipette tip and then treated with bergamottin (5–50 μM) for 24 h. Migrating cells were photographed under phase contrast microscopy. *Significantly different from control ($p<0.01$). (D) Cells were pretreated with bergamottin (50 μM) followed by PMA (30 nM) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($p<0.01$).

levels of PMA-stimulated MMP-9 mRNA expression (Fig. 3B), indicating that bergamottin prevents the transcription of MMP-9 in response to PMA. Significantly, MMP-9 and MMP-2 activation are mediated by high expression of MT1-MMP and adequate expression of TIMPs [31]. We further examined the transcription levels of TIMP-1 and TIMP-2 by

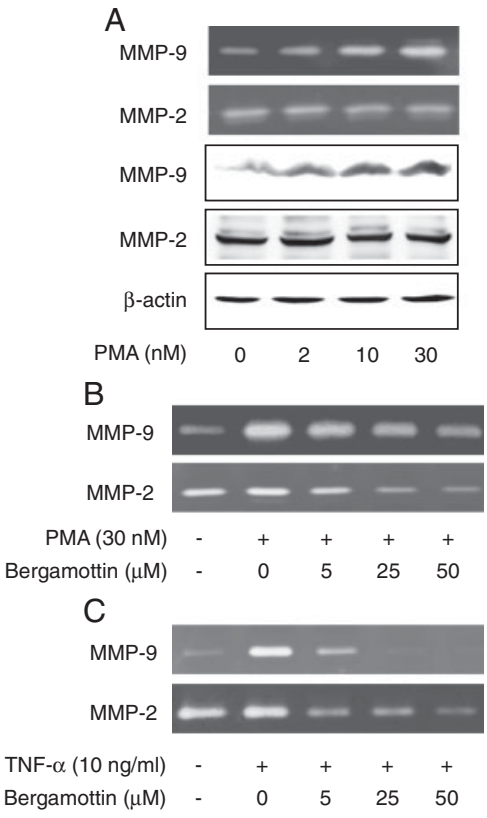

Figure 2. Inhibition of MMP-9 and MMP-2 activities by bergamottin. (A) HT-1080 cells (80% confluent) were treated with various concentrations of PMA in serum-free medium. Conditioned media were collected after 24 h and gelatin zymography and Western blot analysis were performed. (B) HT-1080 cells were incubated with varying concentrations of bergamottin in the presence of PMA (30 nM) for 24 h. MMP activity in the medium was analyzed by gelatin zymography. (C) MCF-7 cells were incubated with varying concentrations of bergamottin in the presence of TNF-α. MMP activity in the medium was analyzed by gelatin zymography.

semi-quantitative RT-PCR, but bergamottin had no effect on the levels of TIMP-1 and TIMP-2 (Fig. 3B). Western blot and semi-quantitative RT-PCR data revealed that bergamottin suppressed both MT1-MMP mRNA (Fig. 3C) and protein expression (Fig. 3D) induced by PMA. The effect of bergamottin on MMP-9 promoter activity was also investigated using HT-1080 cells that had been transiently transfected with a luciferase reporter gene linked to the MMP-9 promoter sequence. As shown in Fig. 3E, luciferase gene expression was activated up to fivefold in cells treated with PMA as compared with untreated cells. Treatment of cells with bergamottin (5–50 μM) decreased PMA-mediated luciferase activity in a dose-dependent manner, indicating that bergamottin inhibits MMP-9 expression at the transcriptional level and that the MMP-9 promoter contains bergamottin response elements. Also, bergamottin affected the luciferase reporter gene containing the MMP-2 promoter (Fig. 3F).

3.4 Bergamottin suppresses NF-κB activity

The expression of the MMP-9 gene is regulated through the transcriptional level interaction of AP-1 and NF-κB with their binding sequences in the MMP-9 gene promoter [43]. To test which of these transcription factors may regulate the MMP-9 gene in HT-1080 cells, the cells were transiently transfected with reporter genes that included the wild-type MMP-9 promoter or a promoter with mutations in the NF-κB site or one or both AP-1 sites (Fig. 4A). Treatment with bergamottin in the presence of PMA decreased the transcription activity of the reporter with the AP-1 mutation, but had no effect for the reporter with NF-κB mutations, suggesting that the target of bergamottin is the NF-κB transcription factor. The luciferase activity in cells transfected with the NF-κB reporter was significantly and dose-dependently reduced by treatment with bergamottin (Fig. 4B), whereas bergamottin had no statistically significant effect on the luciferase activity of cells transfected with the AP-1 reporters (Fig. 4C). This suggests that bergamottin inhibits MMP-9 gene expression by suppressing the MMP-9 promoter activity. HT-1080 cells were incubated with different concentrations of bergamottin in the presence of PMA for 3 h, and nuclear extracts were prepared and tested by Western blot analysis. As shown in Fig. 5A, bergamottin dramatically reduced the PMA-induced nuclear translocation of NF-κB in a dose-dependent manner, whereas it did not affect the PMA-induced protein levels of c-Jun and c-Fos (Fig. 5A). Next, we measured the levels of IκBα in whole-cell extracts. Application of PMA resulted in IκBα degradation, which was significantly repressed upon bergamottin pretreatment at 3 h (Fig. 5B). Immunocytochemical analysis further elucidated this effect. In untreated cells, cytoplasmic immunostaining was seen with anti-p65 antibody whereas cells treated with PMA showed an intense nuclear fluorescence (Fig. 5C). Predictably, cells pretreated with bergamottin and then exposed to PMA showed a significant diminution in nuclear p65 immunostaining (Fig. 5C). To confirm the specificity of bergamottin-mediated inhibitory effects on NF-κB in human breast carcinoma MCF-7 cells, cells were stably transfected with reporter vectors that included the tandem repeat of the NF-κB binding sites. As shown in Figs. 5D and E, treatment of cells with bergamottin decreased the TNF-α-mediated luciferase activity and GFP fluorescence. The above findings collectively suggest that bergamottin inhibits PMA or TNF-α-induced activation of MMP-9 by suppressing NF-κB activation in cancer cells.

3.5 Bergamottin inhibits MMP-9 activation through suppression of PMA-stimulated NF-κB activity

The NF-κB inhibitor, which blocks the nuclear translocation of NF-κB, was used to examine the involvement of NF-κB in MMP-9 activation. HT-1080 cells were pretreated with NF-κB inhibitor (20 nM) or bergamottin for 1 h and then

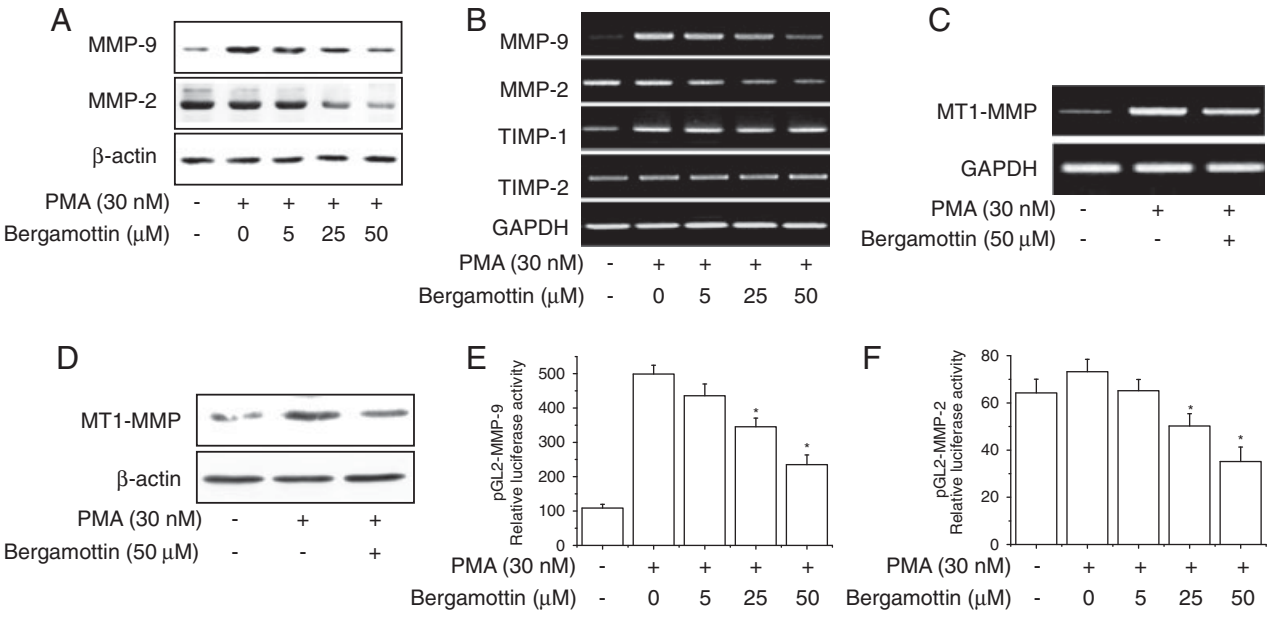

Figure 3. Inhibition of PMA-induced MMP and MT1-MMP expression by bergamottin. (A) Effects of bergamottin on MMP-9 and MMP-2 expression. HT-1080 cells were incubated with bergamottin and/or PMA (30 nM) for 24 h. MMP-9 and MMP-2 expressions in the medium were analyzed by Western blotting. β-actin in the cell lysate is shown as a control. (B) HT-1080 cells were incubated with bergamottin and/or PMA (30 nM) for 24 h. mRNA expression of MMP-9 and MMP-2 in the cells was analyzed by semi-quantitative RT-PCR. GAPDH expression was included as an internal control. (C and D) Effects of bergamottin on MT1-MMP expression. HT-1080 cells were incubated with bergamottin and/or PMA (30 nM) for 24 h. The expression of MT1-MMP in the cells was analyzed by Western blotting and semi-quantitative RT-PCR. GAPDH expression or β-actin expression was included as an internal control. (E) Cells were transfected with WT-MMP-9 promoter-containing reporter vector and incubated with various concentrations of bergamottin in the absence or presence of PMA (30 nM) as indicated. Luciferase activity was measured 24 h after transfection. *Significantly different from PMA treatment only ($p<0.01$). (F) Cells were transfected with WT-MMP-2 reporter vector, and luciferase activity was measured 24 h after transfection. *Significantly different from PMA treatment only ($p<0.01$).

stimulated with 30 nM PMA for 24 h. In the semi-quantitative RT-PCR, the treatment of HT-1080 cells with NF-κB inhibitor decreased the levels of PMA-stimulated MMP-9 mRNA expression (Fig. 6A), indicating that NF-κB inhibitor prevents the transcription of MMP-9 in response to PMA. The culture media were subjected to gelatin zymography and Western blot analysis. As shown in Fig. 6B, NF-κB inhibitor inhibited the PMA-induced MMP-9 expression and activation. The effect of NF-κB inhibitor on the activity of the MMP-9 promoter was investigated using HT-1080 cells that had been transiently transfected with a luciferase reporter gene linked to the MMP-9 promoter sequence. As shown in Fig. 6C, treatment of cells with NF-κB inhibitor decreased the PMA-mediated luciferase activity.

3.6 Bergamottin suppresses PMA-mediated MMP-9 gene activation through the PKC, p38 MAPK and JNK signaling pathways

Several studies have identified signal transduction pathways that are involved in the regulation of MMP-9 expression in tumor cells [33–35]. The role of MAP kinases as upstream modulators of NF-κB or AP-1 is well understood in the regulation of MMP-9 expression in malignant cells [44]. Several studies have shown that PMA-induced MMP-9 activation was decreased by ERK1/2, p38 MAPK or JNK inhibitors or by PKC inhibitor [45]. The subsequent experiments were designed to elucidate which of these signal transduction pathways was involved in PMA-stimulated MMP-9 expression and bergamottin inhibition of MMP-9 expression in HT-1080 cells. First, the effects of specific kinase inhibitors on the expression of MMP-9 in PMA-induced HT-1080 cells were analyzed by gelatin zymography. PMA-induced MMP-9 secretion was completely inhibited by inhibitor of MEK (U0126), p38 MAPK (SB203580), JNK (SP600125) or PKCs (Rottlerin) (Fig. 7A). Next, we investigated whether bergamottin inhibited the activation of these three signaling pathways. Bergamottin inhibited the phosphorylation of p38 MAPK and JNK, but not ERK1/2, in a dose-dependent manner at 30 min after PMA treatment (Fig. 7B). These results suggest that the specific inhibitions of p38 MAPK and JNK signaling pathways by bergamottin are directly involved in its regulation of PMA-induced MMP-9 expression. Activation of PKCs has been correlated with the potential for tumor metastasis [46]. PMA has been reported as a PKC activator, and activation of the PKC isoforms by PMA has been identified [35, 47]. To

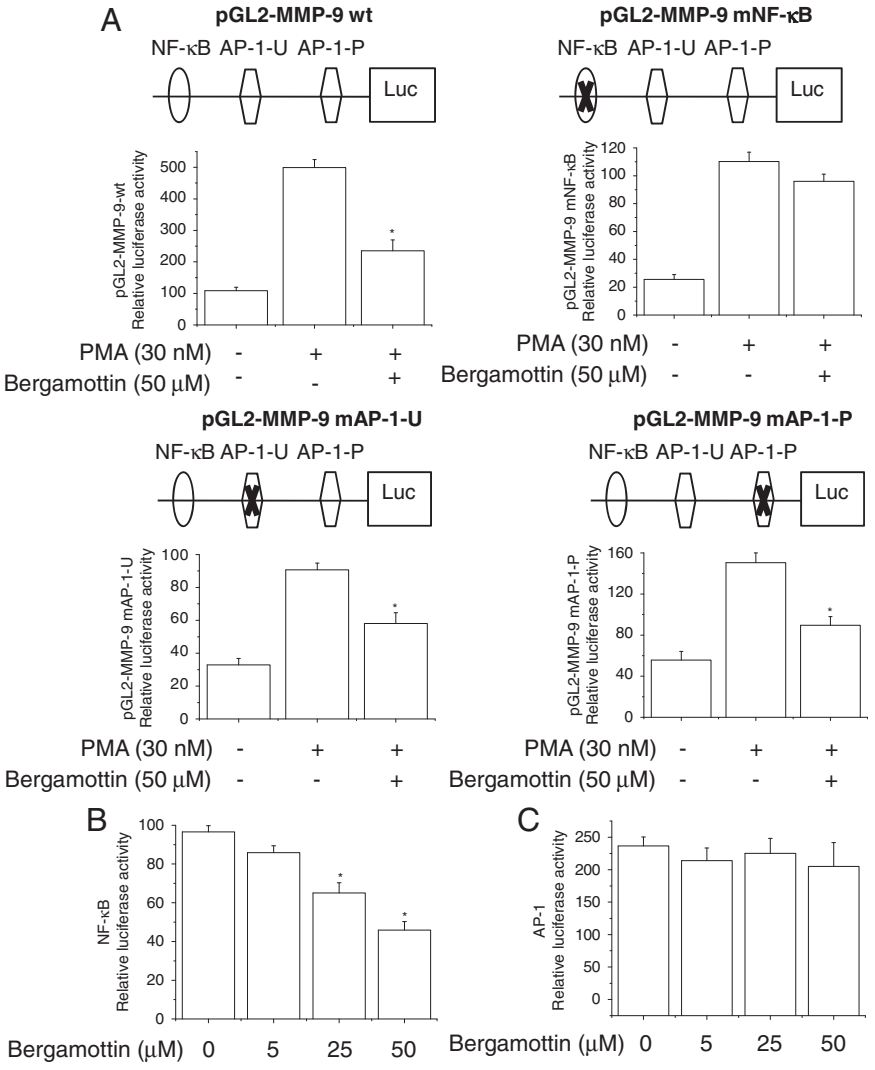

Figure 4. Inhibition of NF-κB activity in the MMP-9 promoter by bergamottin. Mutations were introduced in the NF-κB or AP-1 binding sites of pGL2-MMP-9WT. HT-1080 cells were transfected with pGL2-MMP-9WT, pGL2-MMP-9mNF-κB, pGL2-MMP-9 mAP-U or pGL2-MMP-9 mAP-P reporter plasmids (A) or with reporter plasmids containing tandem elements for NF-κB (B) or AP-1 binding sites (C). Cells were cultured with bergamottin and/or PMA for 24 h, and the relative luciferase activity in the cell extract was determined. Data are expressed as the means ± SD of triplicate experiments. *Significantly different from PMA treatment only ($p < 0.01$).

further evaluate the effect of bergamottin on the PKC signaling pathway, we examined whether bergamottin enhanced PMA-mediated modulation of the translocation of PKCä isoenzymes using Western blot analysis. Remarkably, 50 μM bergamottin had a dramatic effect on the cytosol-to-membrane translocation of PKCä after 1-h incubation (Fig. 7C). Interestingly, rottlerin, a PKCs inhibitor, inhibited PMA-induced phosphorylation of p38 MAPK and JNK (Fig. 7D) and the nuclear translocation of NF-κB p65 (Fig. 7E). PMA mainly stimulates MMP-9-mediated cell invasion through PKCδ-triggered p38 MAPK and JNK/NF-κB activation in HT-1080 cells, and these results indicated that bergamottin suppresses PMA-induced PKCδ activation.

4 Discussion

Natural compounds with anti-carcinogenic activities interfere with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis. Grapefruit and grapefruit-based products are a rich source of bioactive compounds [3] that have been inversely correlated to the occurrence of certain chronic diseases, such as several types of cancer and cardiovascular disease, in several cohort and case-control studies [1, 4]. Bergamottin is the predominant component of grapefruit juice and is a mechanism-based inactivator of CYP3A and major human liver microsomal CYPs [6, 7]. It also has anti-inflammatory [12], anticarcinogenic [10], antibacterial [14] and antitumor activities [41]. However, the effect of bergamottin against PMA-induced MMP-9 expression and invasiveness of fibrosarcoma HT-1080 cells is still unclear. Here we studied the inhibitory effects of bergamottin on MMP-9 activity and investigated its inhibitory mechanisms against human fibrosarcoma HT-1080 and breast carcinoma MCF-7 cells.

Tumor metastasis is a complex multistep process that includes cell proliferation, proteolytic digestion of ECM, cell

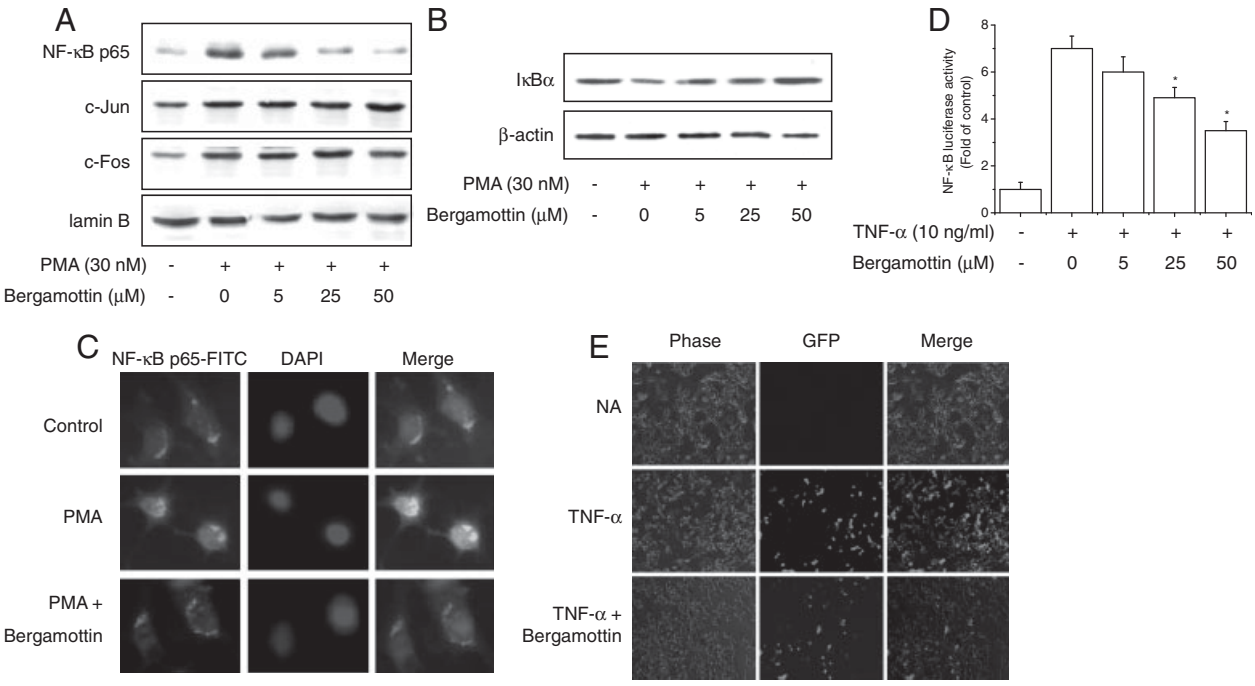

Figure 5. Inhibitory effects of bergamottin on PMA-induced activation of NF-κB. (A) Effects of bergamottin on PMA-induced NF-κB translocation. HT-1080 cells were pretreated with bergamottin (5–50 μM) for 1 h and treated with 30 nM PMA for 3 h. Nuclear extracts were subjected to SDS–PAGE followed by Western blotting with anti-NF-κB, anti-c-Jun, anti-c-Fos and anti-lamin B antibodies. (B) Effect of bergamottin on PMA-induced IκBα degradation. HT-1080 cells were pretreated with bergamottin (5–50 μM) for 1 h and treated with 30 nM PMA for 3 h. Whole-cell extract was subjected to SDS–PAGE followed by Western blotting with anti-IκBα and anti-β-actin antibodies. (C) Effects of bergamottin on PMA-induced nuclear translocation of NF-κB. Cells were incubated with 50 μM bergamottin for 1 h, treated with 30 nM PMA for 6 h, and then subjected to immunocytochemical analysis as described in Section 2. (D) Effects of bergamottin on PMA-induced NF-κB activation in MCF-7 cells. Cells were infected with Lenti-NF-κB-luc (D) or Lenti-NF-κB-GFP (E) and packaging plasmids pFIV-34N/pVSV-G mixed with polybrene (4 μg/mL) to develop stable cell lines expressing the NF-κB reporter. Stable cells were cultured in 96-well plates until cells were 70% confluent, and functional validation of the NF-κB reporter activity was conducted *in vitro* using TNF-α (10 ng/m) as a positive control. Cell lysates were prepared, and luciferase activities were quantified according to the manufacturer's instructions. Fluorescence of GFP protein was analyzed by fluorescence microscopy.

migration to the circulatory system and tumor growth at metastatic sites [48]. MMPs play a major role in promoting tumor metastasis, and overexpression of MMP-9 has been associated with the progression and invasion of tumors including mammary tumors [49, 50]. Thus, the inhibition of its expression can be studied in a therapeutic experimental model of tumor metastasis. Our data show that bergamottin inhibited PMA-induced cell migration and invasion (Figs. 1C and D). As described in previous studies [27], treatment with 2–30 nM PMA stimulated MMP-9 secretion in a dose-dependent manner (Fig. 2A). Our results also demonstrate that bergamottin inhibited the PMA-induced secretion of MMP-9 and MMP-2 in a dose-dependent manner through suppression of the transcriptional activity of the MMP-9 and MMP-2 genes in HT-1080 cells (Fig. 3). Bergamottin also inhibited the enzymatic activity of MMP-9 and MMP-2 secreted from PMA-induced HT-1080 cells in a dose-dependent manner (Fig. 2B), suggesting that bergamottin is a strong candidate for treatment of tumor metastasis and invasion *via* dual inhibition of MMP-9 enzyme activity and gene transcription. Bergamottin also significantly inhibited MMP-9 secretion in MCF-7 cells induced by TNF-α, which is an important physiological inducer for MMP-9 (Fig. 2C), indicating the possible physiological relevance of bergamottin. Protein levels of neither TIMP-1 nor TIMP-2 were altered by bergamottin in our system, possibly due to the different systems and concentrations. A recent study showed that enhanced production of MT1-MMP correlates with MMP-2 activation [51]. Here, we demonstrate that bergamottin markedly decreased the PMA-induced MT1-MMP mRNA and protein levels (Figs. 3C and D). The inhibitory effect of bergamottin on MT1-MMP expression provides a reasonable explanation for the observed decrease in MMP-2 activation.

The transcription of the MMP-9 gene is regulated by the upstream promoter sequence, which includes AP-1, NF-κB and stimulatory protein-1 sites [36]. Bergamottin suppressed MMP-9-induction by repressing the transcription activation in the MMP-9 promoter (Fig. 4). Mutational analysis of the promoter revealed that the major target of bergamottin was NF-κB, a finding that was further confirmed by the use of reporter plasmids containing synthetic elements that are

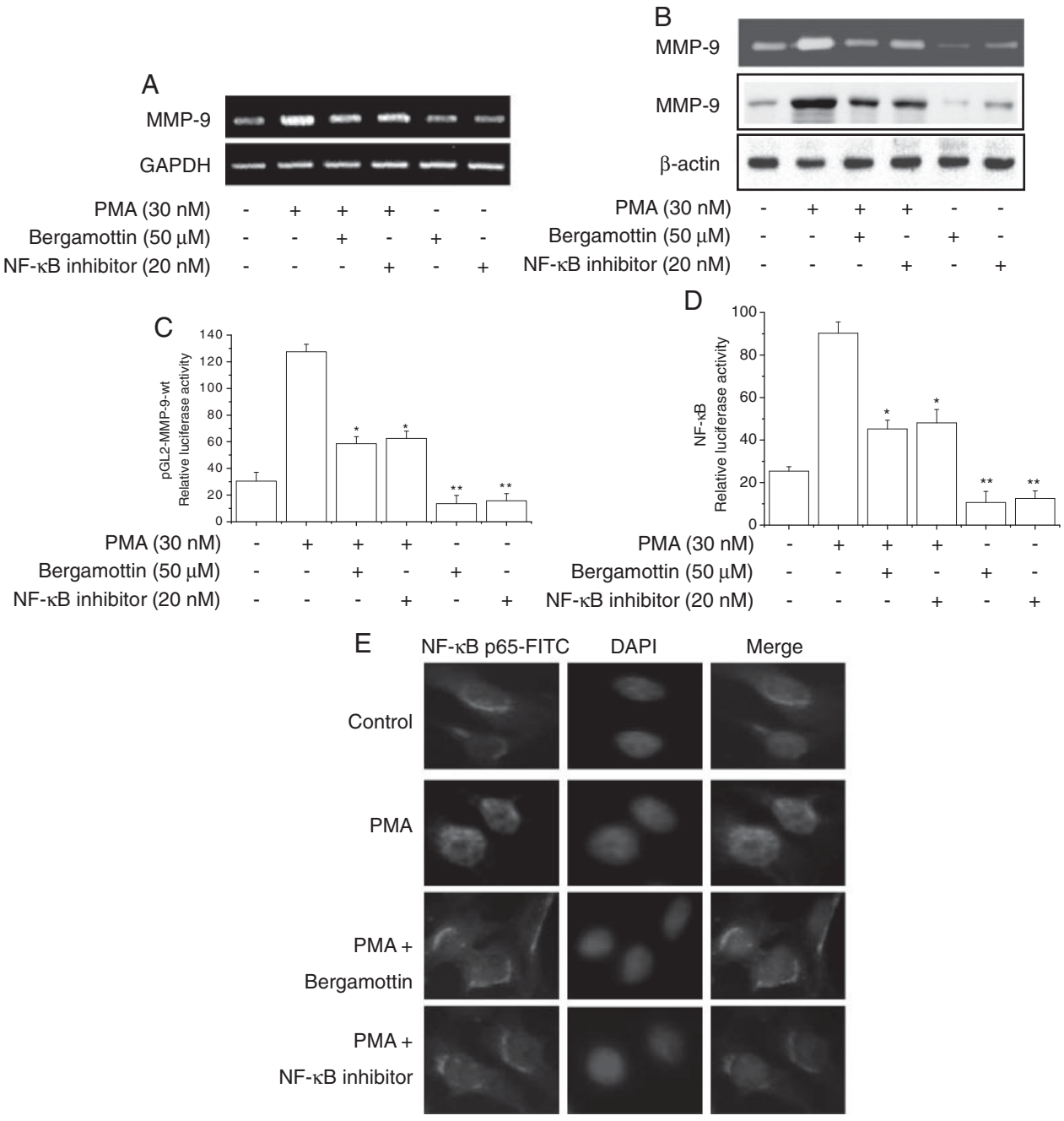

Figure 6. NF-κB is involved in bergamottin-mediated downregulation of MMP-9. (A) Cells were treated with 30 nM PMA for 24 h in the absence or presence of bergamottin or NF-κB inhibitor. The mRNA expression of MMP-9 in the cells was analyzed by semi-quantitative RT-PCR. GAPDH expression was included as an internal control. (B) Conditioned media were collected after 24 h and then gelatin zymography or Western blotting was performed. (C) HT-1080 cells were transfected with pGL2-MMP-9WT reporter plasmids and then cultured with bergamottin or NF-κB inhibitor and/or PMA for 24 h. The luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($p<0.01$). **Significantly different from control ($p<0.01$). (D) Cells were transfected with pGL2-NF-κB reporter plasmids and then cultured with bergamottin or NF-κB inhibitor and/or PMA for 24 h. The luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($p<0.01$). **Significantly different from control ($p<0.01$). (E) Nuclear translocation of NF-κB was determined by immunofluorescence staining. Cells were fixed and labeled with anti-NF-κB (p65) antibody and Alexa Fluor 488-conjugated secondary antibody.

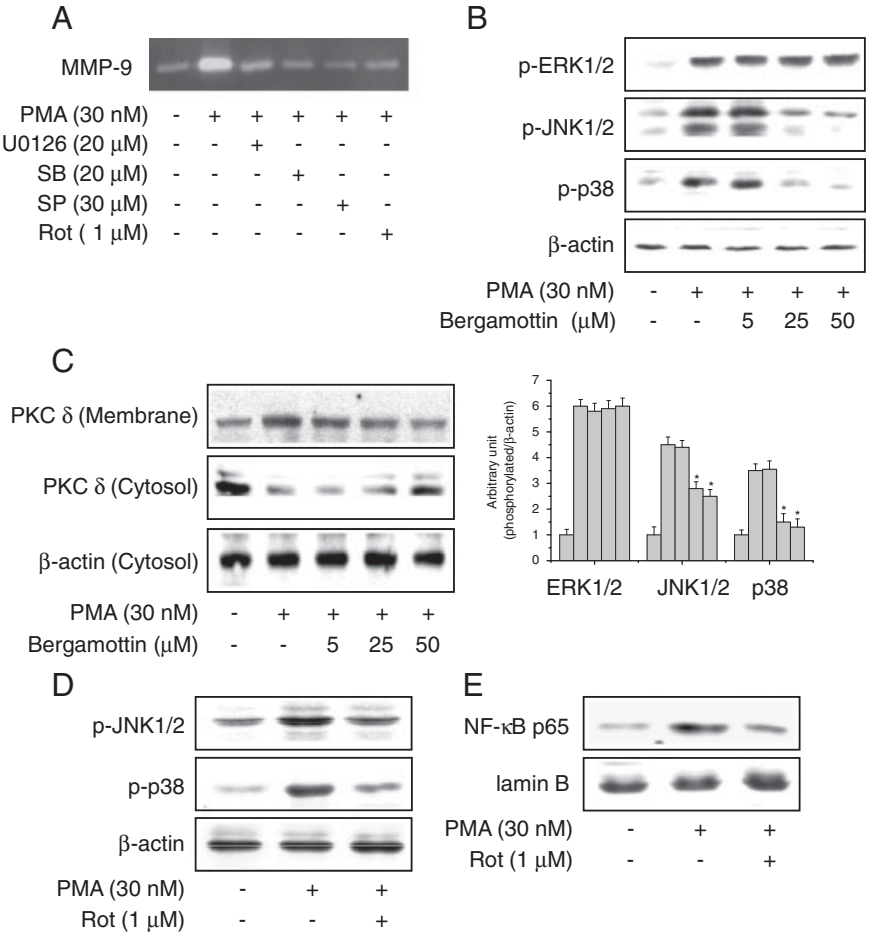

Figure 7. Effects of bergamottin on PMA-induced activation of MAPK and PKCδ signaling pathways. (A) Effects of inhibitor on PMA-induced MMP-9 activation. Cells were pretreated for 1 h with U0126 (20 μM), SB203580 (SB, 20 μM), SP600125 (SP, 30 μM) or rottlerin (Rot, 1 μM) followed by PMA stimulation for 24 h. The MMP-9 activity in the conditioned media was analyzed by gelatin zymography. (B) Effects of bergamottin on PMA-induced activation of MAPK signaling pathway. Cells were treated with PMA (30 nM) for 30 min in the presence or absence of bergamottin, and the phosphorylation levels of ERK1/2, p38 MAPK, and JNK were measured by Western blotting. Densitometry ratios of phospho-MAPKs were normalized to β-actin. *Significantly different from PMA treatment only ($p<0.01$). (C) Effects of bergamottin on PMA-induced PKCδ activation. Cells were treated with bergamottin (5–50 μM) followed by PMA (30 nM) treatment for 1 h, and the PKCδ levels in cytosol and membrane fractions were determined by Western blotting. (D) Cells were stimulated with PMA for 30 min after pretreatment with PKCs inhibitors (Rot, 1 μM) for 1 h, and the levels of phospho-p38 and JNK were determined by Western blotting. (E) Cells were pretreated with PKCs inhibitors (Rot, 1 μM) for 1 h followed by PMA stimulation for 3 h. Western blotting was performed to determine the nuclear levels of NF-κB p65.

specific for the transcription factors (Fig. 4). Bergamottin attenuated the degradation of IκBα protein in the cytoplasm (Fig. 5B) and the translocation of NF-κB subunits p65 to the nucleus in PMA-treated HT-1080 cells. But bergamottin did not suppress PMA-induced translocation of c-Jun and c-Fos, both of which are members of AP-1 (Fig. 5A). Our results indicate that bergamottin inhibited PMA-induced translocation of p65 to the nucleus through blockade of IκBα and p65 activation. To assess NF-κB transcriptional activity, a NF-κB reporter vector was transfected into HT-1080 cells. Treatment with bergamottin (5–50 μM) reduced NF-κB activity to the basal level (Fig. 4B). Next, we confirmed the NF-κB transcriptional activity in MCF-7 cells, which is a stable cell line expressing the NF-κB reporter. As shown in Figs. 5D and E, bergamottin decreased the TNF-α-mediated NF-κB luciferase activity and NF-κB GFP fluorescence. Our results suggest that bergamottin inhibits PMA or TNF-α-induced activation of MMP-9 by suppressing NF-κB activation in cancer cells. Next, we investigated the functional significance of NF-κB transactivation in MMP-9 activation in HT-1080 cells. Treatment with NF-κB activation inhibitor, which is a potent inhibitor of NF-κB transcriptional activation, reduced the PMA-induced enzyme activity and protein expression of MMP-9. Also, NF-κB activation inhibitor reduced the PMA-induced transcriptional activity and nuclear translocation of NF-κB (Fig. 6). The above findings

collectively suggest that bergamottin inhibits PMA-induced activation of MMP-9 by suppressing NF-κB activation in HT-1080 cells.

MAPKs are involved in MMP-9 expression in tumor cells, endothelial cells and keratinocytes; however, the signaling pathway related to MMP-9 expression evoked by PMA in HT-1080 cells is still unclear. In this study, we identified the signaling pathway-mediated regulation of the MMP-9 gene in PMA-induced HT-1080 cells in response to bergamottin treatment. The data here show that bergamottin suppressed PMA-induced phosphorylation of p38 MAPK and JNK, key pathways in PMA-induced cell invasion *via* MMP-9 expression (Fig. 7B). These results demonstrate that bergamottin reduced MMP-9 expression by blocking NF-κB activation *via* p38 MAPK and JNK and consequently inhibited MMP-9-mediated cell invasion in HT-1080 cells. Activation of PKC by PMA involves the translocation of PKC isoforms to the plasma membrane, causing proliferation, differentiation and malignant transformation in cancer cells. Recent studies also demonstrated that PKCδ plays a critical role in MMP-9 induction in cancer cells [52, 53]. In this study, PMA stimulation resulted in the translocation of PKCδ from the cytosol to the cell membrane. Treatment with a noncytotoxic dose of a PKCs inhibitor (Rottlerin) caused marked inhibition of PMA-induced activation of p38 MAPK, JNK (Fig. 7D) and NF-κB (Fig. 7E), as well as of PMA-induced MMP-9 secretion and cell invasion. These data indicate that PMA-activated PKCδ mediates MMP-9 expression and cell invasion *via* p38 MAPK, JNK and NF-κB. As expected, bergamottin reduced PMA-induced membrane localization of PKCδ.

In conclusion, bergamottin inhibited PMA-induced invasion and metastasis by reducing MMP-9 activation mainly through the PKCδ/p38 MAPK and JNK/NF-κB pathways in HT-1080 human fibroblast cancer cells. Bergamottin therefore has the potential to be a potent chemopreventive drug in therapeutic strategies for fibrosarcoma metastasis.

The authors have declared no conflict of interest.

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