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RESEARCH PAPER

6-Shogaol, an active constituent of ginger, inhibits breast cancer cell invasion by reducing matrix metalloproteinase-9 expression via blockade of nuclear factor-κB activation

H Ling, H Yang, S-H Tan, W-K Chui and E-H Chew

Department of Pharmacy, National University of Singapore, Singapore, Singapore

Correspondence
Eng-Hui Chew, Department of Pharmacy, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore. E-mail: phaceh@nus.edu.sg

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BACKGROUND AND PURPOSE
Shogaols are reported to possess anti-inflammatory and anticancer activities. However, the antimetastatic potential of shogaols remains unexplored. This study was performed to assess the effects of shogaols against breast cancer cell invasion and to investigate the underlying mechanisms.

EXPERIMENTAL APPROACH
The anti-invasive effect of a series of shogaols was initially evaluated on MDA-MB-231 breast cancer cells using the matrigel invasion assay. The suppressive effects of 6-shogaol on phorbol 12-myristate 13-acetate (PMA)-induced matrix metalloproteinase-9 (MMP-9) gelatinolytic activity and nuclear factor-κB (NF-κB) activation were further determined.

KEY RESULTS
Shogaols (6-, 8- and 10-shogaol) inhibited PMA-stimulated MDA-MB-231 cell invasion with an accompanying decrease in MMP-9 secretion. 6-Shogaol was identified to display the greatest anti-invasive effect in association with a dose-dependent reduction in MMP-9 gene activation, protein expression and secretion. The NF-κB transcriptional activity was decreased by 6-shogaol; an effect mediated by inhibition of IκB phosphorylation and degradation that subsequently led to suppression of NF-κB p65 phosphorylation and nuclear translocation. In addition, 6-shogaol was found to inhibit JNK activation with no resulting reduction in activator protein-1 transcriptional activity. By using specific inhibitors, it was demonstrated that ERK and NF-κB signalling, but not JNK and p38 signalling, were involved in PMA-stimulated MMP-9 activation.

CONCLUSIONS AND IMPLICATIONS
6-Shogaol is a potent inhibitor of MDA-MB-231 cell invasion, and the molecular mechanism involves at least in part the down-regulation of MMP-9 transcription by targeting the NF-κB activation cascade. This class of naturally occurring small molecules thus have potential for clinical use as antimetastatic treatments.

Abbreviations
AP-1, activator protein-1; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IKK, IκB kinase; IκBα, inhibitor of κBα; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PMA, phorbol 12-myristate 13-acetate

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Introduction

Breast cancer is the most frequently diagnosed type of cancer and the second leading cause of cancer-related mortality among females worldwide (Jemal *et al*., 2009). The high mortality rates associated with breast cancer are mainly caused by the metastatic spread of tumour cells from the site of their origin to other parts of the body (Weigelt *et al*., 2005). A critical early event in tumour cell invasion and metastasis is degradation of the extracellular matrix (ECM) by proteolytic enzymes (Bogenrieder and Herlyn, 2003). Matrix metalloproteinases (MMPs), a family of structurally related zinc-dependent endopeptidases, are prime candidates for the degradation of ECM and thus are implicated in tumour invasion and metastasis (Overall and Lopez-Otin, 2002). To date, more than 20 human MMPs have been identified (Overall and Lopez-Otin, 2002; Clark *et al*., 2008). Among them, MMP-2 (EC 3.4.24.24) and MMP-9 (EC 3.4.24.35) are believed to be the most important enzymes in tumour invasion due to their ability to degrade type IV collagen, the major structural protein component in ECM and basement membrane (Brinckerhoff and Matrisian, 2002). The function of MMPs is tightly controlled at multiple levels including transcription, pro-enzyme activation and inhibition by tissue inhibitors of MMPs (Overall and Lopez-Otin, 2002; Clark *et al*., 2008). Due to structural differences in the gene promoter regions, MMP-2 is usually constitutively expressed and MMP-9 is highly inducible by a large variety of stimuli including growth factors, cytokines, UV and phorbol ester (Egeblad and Werb, 2002). Nevertheless, agents reducing either MMP-2 or MMP-9 expression have been shown to effectively inhibit breast cancer cell invasion (Deshane *et al*., 2003; Woo *et al*., 2004; Hong *et al*., 2005; Huang *et al*., 2005).

Ginger (*Zingiber officinale Roscoe*) has been widely used as a condiment throughout the world for centuries. In Asian countries, ginger has also been used as a herbal medicine to treat a wide range of disorders such as inflammation, dyspepsia, nausea, vomiting, pain, the common cold and diarrhoea (Ali *et al*., 2008). The biologically active components contained in ginger are reported to be phenylpropanoid-derived compounds including gingerols and shogaols (Kundu *et al*., 2009). As dehydrated products of gingerols, shogaols exist in fresh ginger at low levels but are present in larger amounts in dried ginger (Jolad *et al*., 2005). Earlier studies have indicated that shogaols possess anti-cancer and anti-inflammatory effects. For example, 6-shogaol has been shown to induce apoptosis in human colorectal carcinoma cells via the production of reactive oxygen species and activation of caspase (Pan *et al*., 2008b). In another study, 6-shogaol was reported to reduce gastric cancer viability by impairing tubulin polymerization (Ishiguro *et al*., 2007). In addition, 6-shogaol has been shown to be effective at inhibiting the expression of inflammatory mediators, inducible nitric oxide synthase and cyclooxygenase-2 induced by either lipopolysaccharide or phorbol 12-myristate 13-acetate (PMA) (Pan *et al*., 2008a). Furthermore, a recent study showed that 6-shogaol suppressed the TRIF-dependent signalling pathway of Toll-like receptors by targeting TBK1 (Park *et al*., 2009). An anti-inflammatory effect of 6-shogaol has also been demonstrated *in vivo*; it reduced the chronic inflammation in the knees of rats induced by complete Freund's adjuvant (Levy *et al*., 2006).

Despite the various studies performed to investigate the biological activities of shogaols, to date, their effects on cancer cell invasion and metastasis have not been reported. In this study, we evaluated the anti-invasive potential of shogaols of different side-chain lengths, namely 6-, 8- and 10-shogaol using MDA-MB-231 cells, a known breast carcinoma cell line with highly invasive characteristics. All shogaols, with 6-shogaol being the most potent, were found to inhibit PMA-induced invasion of MDA-MB-231 cells by reducing MMP-9 activity. We focused on 6-shogaol to elucidate the mechanism(s) underlying this anti-invasive effect, and revealed the nuclear factor-κB (NF-κB) signalling pathway as a molecular target. Importantly, our findings indicate the potential of this class of naturally derived small molecules for clinical use as antimetastatic agents.

Methods

Materials and cell culture

BD BioCoat™ Matrigel™ Invasion Chambers were obtained from BD Biosciences (San Jose, CA, USA). Antibodies specific for ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-JNK (Thr183/Tyr185), p38, phospho-p38 MAPK (Thr180/Tyr182), NF-κB p65, phospho-NF-κB p65 (Ser536), IκBα, phospho-IκBα (Ser32), IKKα, IKKβ, phospho-IKKα/β (Ser176/180) were purchased from Cell Signaling Technology (Beverly, MA, USA), and antibodies against MMP-9, β-Actin and histone H1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). pAP-1-Luc and pNF-κB-Luc luciferase reporter plasmids were purchased from Stratagene (La Jolla, CA, USA).

Human-derived breast carcinoma MDA-MB-231 and MCF-7 cells were maintained in RPMI 1640

medium supplemented with 10% foetal bovine serum, 100 U·mL^{-1} penicillin G and streptomycin 100 μg·mL^{-1}, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Chemicals and inhibitors

The 6-, 8- and 10-shogaol were prepared from vanillin according to previously published procedures (Denniff *et al.*, 1981; Kim and Kim, 2004). Shogaol stocks (50 mM) were prepared in dimethyl sulphoxide (DMSO) and stored at –20°C. PMA was purchased from Sigma (St. Louis, MO, USA). MMP-2/MMP-9 inhibitor I, SB203580, a p38 inhibitor, and Bay 11-7082, a NF-κB inhibitor, were obtained from Calbiochem (San Diego, CA, USA). SB-3CT was obtained from Santa Cruz Biotechnology, SP600125 (JNK inhibitor) was from Biomol International LP (Plymouth Meeting, PA, USA), and U0126 (MEK1/2 inhibitor) was from Cell Signaling Technology.

Cell viability assay

The effect of 6-, 8- and 10-shogaol on the viability of MDA-MB-231 cells was evaluated using a cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's protocol. Following shogaol treatment for 24 h, cell viability was expressed as % cell viability as compared to that of DMSO vehicle control cells.

Cell invasion assay

The cell invasion assay was conducted using BD BioCoat™ Matrigel™ Invasion Chambers according to the manufacturer's instructions. Briefly, 5×10^4 MDA-MB-231 cells suspended in 500 μL of serum-free medium were seeded into the upper chamber of Matrigel-coated filter inserts. After treatment with different concentrations of shogaols for 1 h, 750 μL of serum-free medium containing 80 nM of PMA was added to the bottom wells as chemoattractant. The chambers were incubated at 37°C for 20 h. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the filter were removed using cotton swabs. Cells that had invaded on the underside of the filter were first fixed with methanol (15 min), then stained with 2% ethanol containing 0.2% crystal violet powder (15 min). After being dried, the stained cells were enumerated under light microscope at 10X objective.

Gelatin zymography

Gelatin zymography was performed as reported previously with slight modifications (Liotta and Stetler-Stevenson, 1990). MDA-MB-231 cells were seeded in 6-well plates and allowed to grow to 80% confluency. The cells were then maintained in serum-free medium for 12 h prior to designated treatments with shogaols and PMA for 20 h. Conditioned medium was then collected, cleared by centrifugation (400× *g*) and equal volumes were mixed with non-reducing sample buffer, and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. After electrophoresis, the gels were washed in renaturing buffer (10 mM Tris-HCl pH 8, 2.5% Triton X-100) for 30 min, equilibrated in developing buffer (50 mM Tris–HCl pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, and 1 mM $ZnCl_2$) for 30 min and finally incubated in fresh developing buffer at 37°C for 24 h to allow digestion of the gelatin. The gelatinolytic activity of MMPs was visualized by staining the gels with 0.5% Coomassie blue R-250 in 45% methanol, 10% acetic acid (v/v) and destained with 45% methanol, 10% acetic acid (v/v) until clear bands suggestive of gelatin digestion appeared.

Reverse transcription – polymerase chain reaction (RT-PCR)

MDA-MB-231 cells were seeded and cultured in 6-well plates until 80% confluent. The cells were then maintained in serum-free medium for 12 h before 6-shogaol and PMA treatment. Following the indicated treatment, total RNA from each sample was isolated using a RNeasy® Mini Kit (Qiagen, Hilden, Germany). Single-stranded cDNA was synthesized from 2 μg of RNA by reverse transcription using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA). Fragments specific to examined genes were amplified in 20 μL of reaction solution containing 2 μL generated cDNA, 2 μL deoxyribonucleotide triphosphate (2 mM), 0.2 μL Taq DNA Polymerase (New England Biolabs) and 1 μL of each specific sense and antisense primers (5 nM). PCR was run for 30 cycles of 94°C for 15 s (denaturation), 57°C for 15 s (annealing) and 72°C for 30 s (extension). The primer sequences used and their product sizes were as follows: MMP-1 (516 bp) forward, 5′-TGAGGGGAACCCTCGCTGGG-3′; MMP-1 reverse, 5′-GGGGTTTGTGGGCCGATGGG-3′; MMP-3 (644 bp) forward, 5′-ACGGGGAAGCTG GACTCCGA-3′; MMP-3 reverse, 5′-CAGTTGGCT GGCGTCCCAGG-3′; MMP-7 (363 bp) forward, 5′-AGGAGGCGGGAGGCATGAGT-3′; MMP-7 reverse, 5′-GCAGGGGGATCTCTTTGCCCC-3′; MMP-9 (620 bp) forward, 5′-CGATGACGAGTTGTGGTCCC TGGGC-3′; MMP-9 reverse, 5′-AATGATCTAAGCC CAGCGCGTGGC-3′ (Liang *et al.*, 2009); MMP-13 (663 bp) forward, 5′-CCCTGCCCCTTCCCAGTGGT-3′; MMP-13 reverse, 5′-TGAGTGCTCCAGGGTCC TTGG-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (325 bp) forward, 5′-TGAAGGTC GGAGTCAACGGATTTGGT-3′; GAPDH reverse, 5′-AAATGAGCCCCAGCCTTCTCCATG-3′ (Wang

et al., 2004). The PCR products were subjected to horizontal electrophoresis on 1.2% agarose gels and images were captured in a Bio-Rad ChemiDoc imaging system (Hercules, CA, USA).

AP-1 and NF-κB-dependent luciferase reporter assay

MDA-MB-231 cells were seeded in 24-well culture plates. At a confluency of 50%, cells were cotransfected with 0.7 μg pAP-1-Luc or pNF-κB-Luc plasmids (PathDetect luciferase *cis*-reporting system containing 7 x AP-1 and 5 x NF-κB enhancer elements respectively) and 14 ng pRL-CMV (Promega) per well. After transfection for 24 h, the cells were maintained in serum-free medium and pretreated with 6-shogaol for 1 h followed by PMA stimulation for another 20 h. Firefly and renilla luciferase activities were assayed using the Dual Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Results, expressed as firefly luciferase activity normalized to renilla luciferase activity were representative of three independent experiments.

Preparation of whole cell lysates and nuclear fractions

Whole cell lysates were prepared using SDS sample buffer. Briefly, at the end of designated treatments, MDA-MB-231 cells were lysed in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 0.01% (w/v) bromophenol blue, 42 mM DTT, and protease inhibitor cocktail), sonicated on ice, boiled, and centrifuged at 16 200× *g*. The supernatant was subjected to SDS-PAGE and Western blot analysis. For the preparation of nuclear fractions, cells were collected and re-suspended in ice-cold Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 1 $mg \cdot mL^{-1}$ leupeptin and 1 $mg \cdot mL^{-1}$ aprotinin). After incubation on ice for 15 min, NP-40 (final concentration 0.5%) was added to the cell suspension, followed by centrifugation at 16 200× *g* for 30 s to obtain the supernatant as cytosolic extracts. The remaining nuclear pellets were re-suspended in Buffer B (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 $mg \cdot mL^{-1}$ leupeptin, 1 $mg \cdot mL^{-1}$ aprotinin). Final nuclear extracts were obtained after preclearing by centrifugation and protein concentrations were quantified using the BCA colorimetric assay (Pierce, Rockford, IL, USA) as described in the manufacturer's manual.

Western blot

Proteins in whole cell lysates and nuclear fractions were resolved by SDS-PAGE and electroblotted onto nitrocellulose membrane. The membranes were probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. Protein bands on the membranes were detected by enhanced chemiluminescence [Western Lightning, Perkin-Elmer (Boston, MA, USA) or SuperSignal West Femto, Pierce (Rockford, IL, USA)].

For detection of MMP-9 protein secreted into the medium, conditioned medium was collected and centrifuged at 400× *g* to remove cells and debris. Equal volumes of conditioned medium were subjected to SDS-PAGE. After the resolved proteins had been transferred onto membranes, the levels of MMP-9 protein were determined using a specific antibody against MMP-9.

Statistical analysis

Numerical data were presented as means ± SD of different determinations. Statistical significance between treatment and control groups was analysed using Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Inhibitory effects of 6-, 8- and 10-shogaol on PMA-induced invasion of MDA-MB-231 cells

The 6-, 8- and 10-shogaol are the main shogaols with different alkyl carbon chain lengths contained in ginger (Figure 1A). We first evaluated the effect of these shogaols on the viability of MDA-MB-231 breast cancer cells using the CCK-8 assay. At the concentrations tested, between 5 and 30 μM, for a duration of 24 h, the shogaols demonstrated negligible antiproliferative effects on the cells (Figure 1B). To ascertain that any possible anti-invasive effects of the shogaols observed was not due to their antiproliferative activities, non-lethal concentrations (≤30 μM) were used for the following experiments.

The anti-invasive potential of 6-, 8- and 10-shogaol was first evaluated by studying their effects on PMA-induced invasion of MDA-MB-231 cells (a known cell line with highly invasive property) using matrigel-coated transwell plates. Compared to DMSO control, 80 nM PMA caused a 4.5-fold increase in cell invasion (Figure 1C). All three shogaols were observed to inhibit this PMA-induced invasion significantly (Figure 1C,D). In particular, 6-shogaol at a concentration of 10 μM caused almost complete inhibition of PMA-induced invasion. Collectively, these results demonstrate that shogaols have an inhibitory effect on cancer cell invasion.

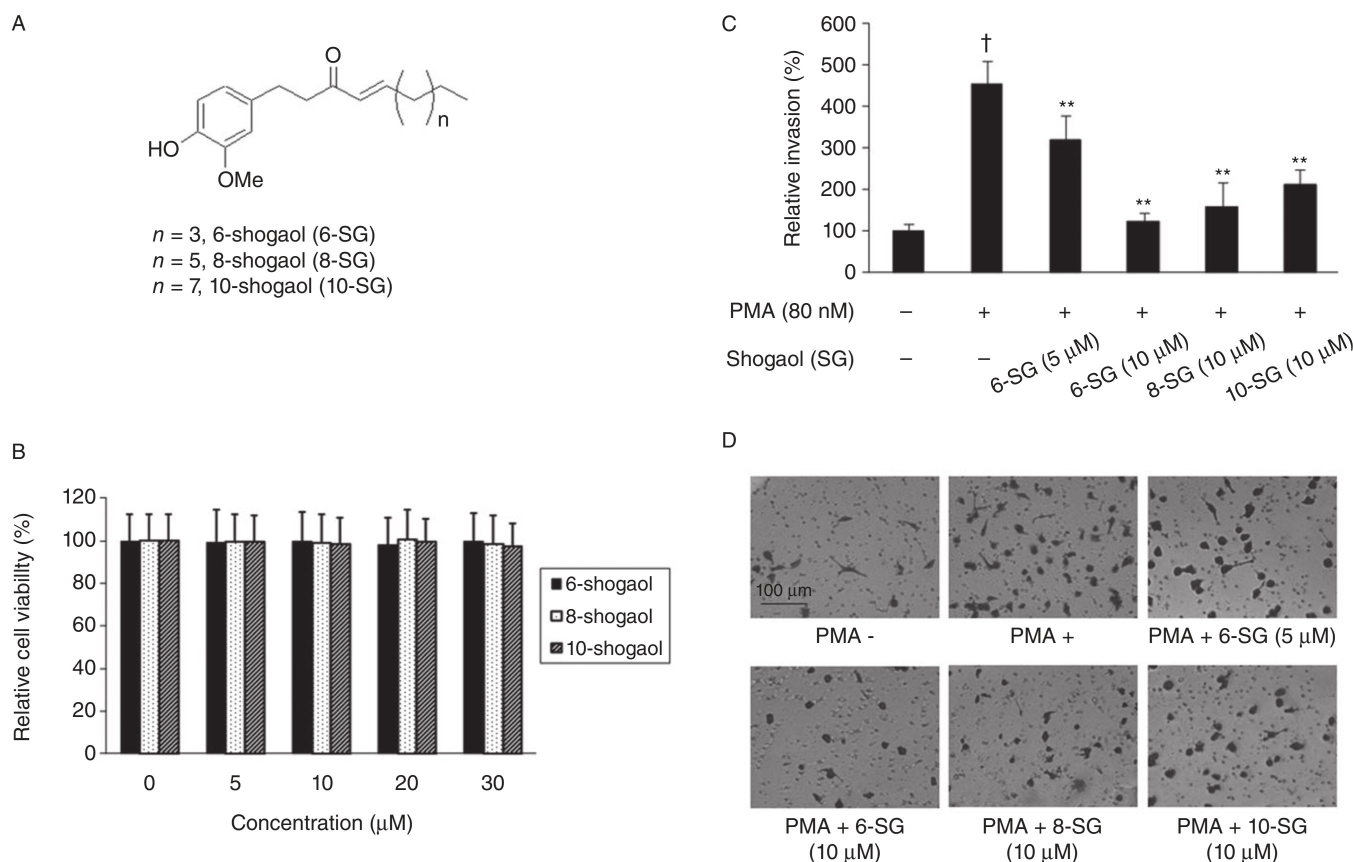
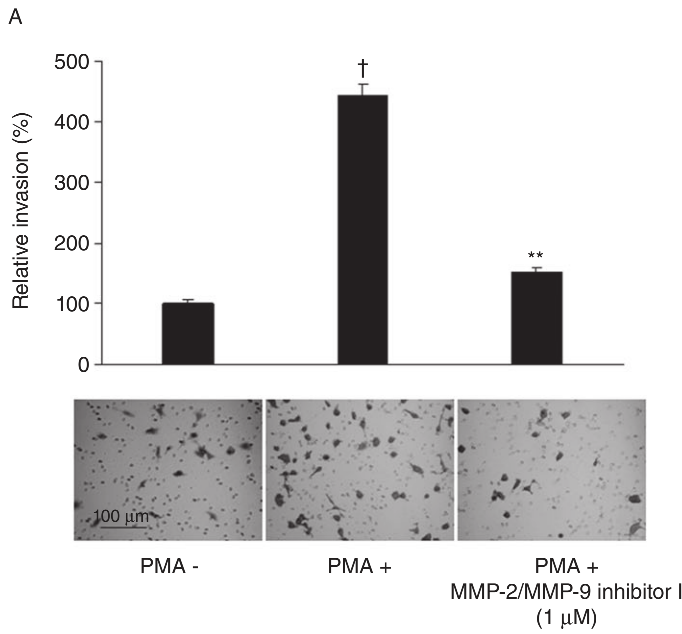


Figure 1

Shogaols inhibit PMA-induced invasion of MDA-MB-231 breast cancer cells at sublethal doses. (A) Chemical structures of 6-, 8- and 10-shogaol. (B) Effects of 6-, 8- and 10-shogaol on viability of MDA-MB-231 cells. Following 24 h treatment with dimethyl sulphoxide (DMSO) or different concentrations of shogaols, viability of MDA-MB-231 cells was determined using CCK-8 according to the manufacturer's instructions. Columns represent means of three independent experiments and bars show SD. (C) Inhibition of PMA-induced invasion by shogaols. MDA-MB-231 cells suspended in serum-free medium were seeded onto the upper chamber of matrigel-coated filter inserts. After treatment with various shogaols for 1 h, cells were stimulated with PMA (80 nM) for another 20 h. The cells invading into the underside of filter inserts were stained with crystal violet and counted under a microscope. Results are expressed as relative % of cell invasion to that of basal invasion of PMA-untreated cells. Columns show means of three independent experiments and bars SD; †$P < 0.05$ versus DMSO control (without PMA treatment); **$P < 0.01$ versus PMA-only group. (D) Representative microscopic images illustrating the *in vitro* inhibitory effects of shogaols on PMA-induced cell invasion.

The suppressive effect of shogaols on PMA-induced MMP-9 expression and secretion

The MMP-9 and MMP-2 have been reported to play an essential role in degradation and remodelling of the basement membrane, and thus contribute towards tumour invasion (Brinckerhoff and Matrisian, 2002). Indeed, using the *in vitro* matrigel cell invasion assay, we showed that a synthetic MMP-2/MMP-9 inhibitor (MMP-2/MMP-9 inhibitor I) clearly brought about a significant reduction in PMA-stimulated invasion of MDA-MB-231 cells (Figure 2A), thus affirming the essential role of MMP-9 and MMP-2 in cancer cell invasion. Similarly, SB-3CT, a more specific MMP-9 inhibitor (Brown *et al.*, 2000) also blocked PMA-induced cell invasion (Figure 2B). We therefore examined whether the inhibitory effect of shogaols against MDA-MB-231 cell invasion was associated with alterations in the extracellular secretion of MMP-9 and MMP-2. Cells were treated with 10 µM 6-, 8- or 10-shogaol 1 h prior to PMA (80 nM) addition for another 20 h. Conditioned medium was collected, and secreted forms of MMP-9 and MMP-2 were analysed using gelatin zymography. As shown in the upper panel of Figure 2C, PMA significantly enhanced MMP-9 proteolytic activity at 92 kDa (band corresponded to the MW of pro-MMP-9) and this enhancement was reduced after treatment with the shogaols. However, MMP-2 proteolytic activity at 62 kDa (band corresponded to the MW of MMP-2) was not significantly affected by PMA-only or combined treatment with shogaols (Figure 2C, upper panel). Consistent with the down-regulation


A
Relative invasion (%)
500
400
300
200
100
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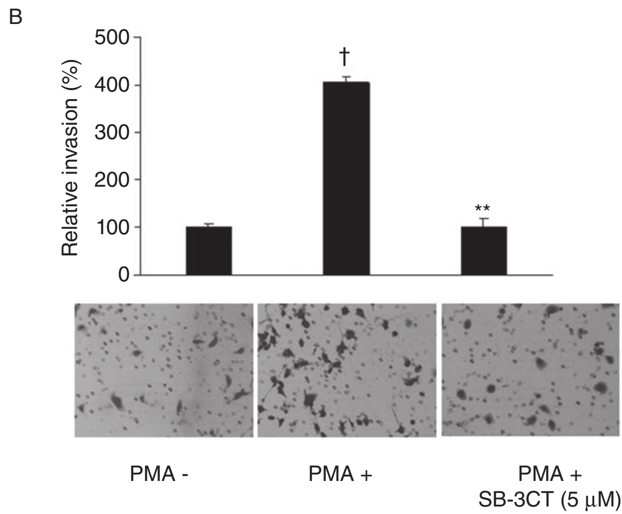
**
100 µm
PMA -
PMA +
PMA +
MMP-2/MMP-9 inhibitor I
(1 µM)


B
Relative invasion (%)
500
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300
200
100
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†
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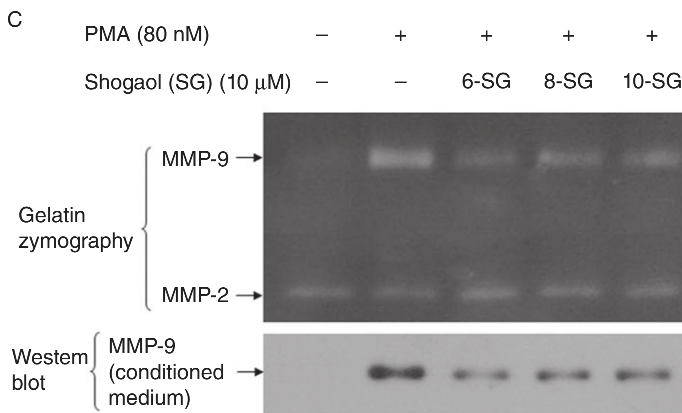
PMA -
PMA +
PMA +
SB-3CT (5 µM)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PMA (80 nM)
Shogaol (SG) (10 µM)
6-SG
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Gelatin zymography
MMP-9
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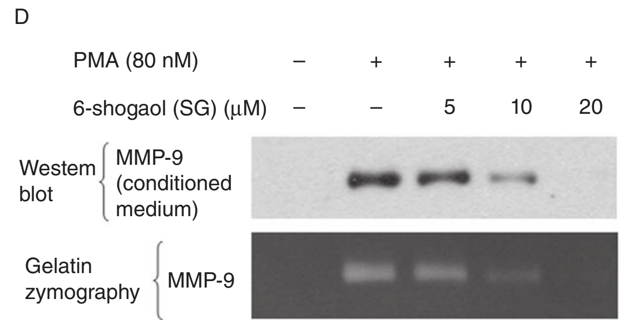
MMP-9 (conditioned medium)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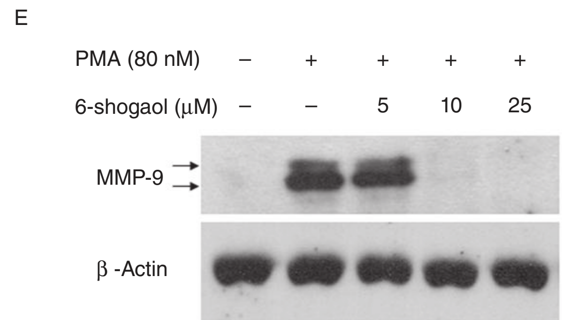
Westem blot
MMP-9 (conditioned medium)
Gelatin zymography
MMP-9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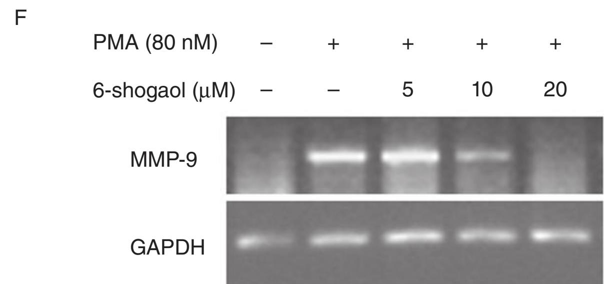
MMP-9
β -Actin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6-shogaol (µM)
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MMP-9
GAPDH

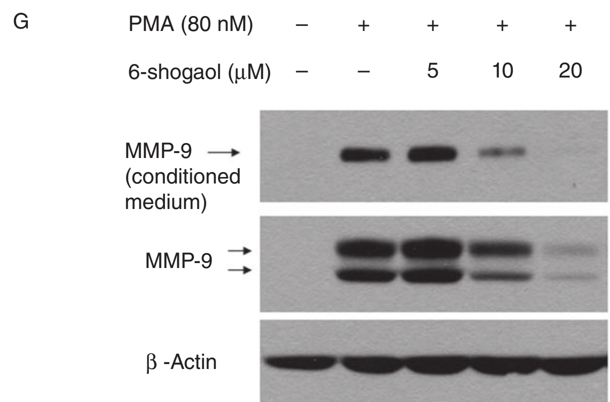
G
PMA (80 nM)
6-shogaol (µM)
5
10
20
MMP-9 (conditioned medium)
MMP-9
β -Actin

Figure 2

Shogaols suppress PMA-induced expression and secretion of MMP-9. (A) Inhibition of PMA-induced invasion by MMP-2/MMP-9 inhibitor I. MDA-MB-231 cells suspended in serum-free medium were seeded onto the upper chamber of Matrigel-coated filter inserts. After treatment with vehicle control or MMP-2/MMP-9 inhibitor I (1 μM) for 1 h, cells were stimulated with PMA (80 nM) for another 20 h. The cells invading into the underside of filter inserts were stained with crystal violet and counted under a microscope. (B) Inhibition of PMA-induced invasion by SB-3CT. MDA-MB-231 cells were treated and analysed as described in (A), except that SB-3CT (5 μM) was used instead of MMP-2/MMP-9 inhibitor I. Results are expressed as relative % of cell invasion to that of basal invasion of PMA-untreated cells. Columns show means of three independent experiments and bars SD; †$P < 0.05$ versus dimethyl sulphoxide control (without PMA treatment); **$P < 0.01$ versus PMA-only group. (C) Suppression by shogaols of PMA-induced extracellular secretion of MMP-9. MDA-MB-231 cells were pretreated with 10 μM of each shogaol (SG) for 1 h, followed by treatment with PMA for another 20 h. The conditioned medium was collected and subjected to gelatin zymography (top panel) and Western blot analysis (lower panel). (D) Dose-dependent reduction of PMA-induced MMP-9 protein secretion by 6-shogaol. MDA-MB-231 cells were pretreated with 6-shogaol at indicated concentrations for 1 h prior to PMA stimulation for another 20 h. Conditioned medium was analysed by Western blot (top panel) and gelatin zymography (lower panel) for MMP-9 level and gelatinolytic activity respectively. (E) Inhibition by 6-shogaol of PMA-induced MMP-9 protein expression in MDA-MB-231 cells. Cells were incubated with 6-shogaol for 1 h, followed by PMA treatment for another 20 h. Whole cell lysates were subjected to Western blot analysis for detection of MMP-9 levels. (F) Dose-dependent inhibition by 6-shogaol of PMA-induced *MMP-9* gene expression. MDA-MB-231 cells were incubated with 6-shogaol for 1 h, followed by PMA treatment for another 20 h. *MMP-9* gene expression was detected by RT-PCR analysis. *GAPDH* was used here as a housekeeping gene. (G) Inhibition of PMA-induced MMP-9 protein expression and secretion in MCF-7 cells by 6-shogaol. MCF-7 cells were pretreated with 6-shogaol for 1 h prior to PMA stimulation for another 20 h. Whole cell lysates or conditioned medium were subjected to Western blot analysis for detection of MMP-9 levels. All experiments were conducted three times with similar results and a representative image is shown.

by shogaols of PMA-induced MMP-9 gelatinolytic activity observed with gelatin zymography, Western blot analysis of the collected conditioned medium also revealed that shogaols caused a reduction in the level of MMP-9 secreted (Figure 2C, lower panel). These results, together with the inhibitory effects against cell invasion demonstrated in matrigel-coated transwell plates (Figure 1C,D), suggest that shogaols bring about inhibition of MDA-MB-231 breast cancer cell invasion by decreasing the extracellular secretion of MMP-9.

The experimental results presented so far indicated that 6-shogaol exhibited greater inhibitory activity against cell invasion than either 8- or 10-shogaol. Indeed, 6-shogaol inhibited PMA-induced MMP-9 secretion in a dose-dependent manner, with a complete inhibition achieved at 20 μM (Figure 2D, upper panel). In corroboration, a similar 6-shogaol-mediated dose-dependent decrease in PMA-induced MMP-9 secretion was detected by gelatin zymography (Figure 2D, lower panel). We therefore focused on 6-shogaol in further studies.

We were interested in investigating whether the observed reduction in MMP-9 secretion brought about by 6-shogaol was the result of a reduction in the intracellular expression of MMP-9. To do this, we analysed whole cell lysates of MDA-MB-231 cells exposed to PMA and 6-shogaol; a PMA-induced elevation of MMP-9 expression that was dose-dependently attenuated by 6-shogaol was detected (Figure 2E). It should be pointed out that the two bands observed were all latent MMP-9 enzymes, the upper band (92 kDa) corresponding to a mature form and the lower band (85 kDa) representing a glycosylated form (Toth *et al.*, 1997). Using RT-PCR, we also demonstrated that 6-shogaol treatment led to a decrease in PMA-induced *MMP-9* gene expression in a dose-dependent fashion (Figure 2F). Taken together, these results indicate that shogaol-mediated regulation of MMP-9 expression occurs at the transcriptional level.

Using a different breast cancer cell line, MCF-7 expressing wild-type p53, we found that 6-shogaol also brought about a dose-dependent decrease in PMA-induced MMP-9 expression and extracellular secretion (Figure 2G).

Effect of 6-shogaol on PMA-activated AP-1 signalling pathway

Activator protein-1 (AP-1), known to be regulated by MAPKs including ERK, JNK and p38 kinase, is one of the important transcription factors involved in PMA-elicited activation of *MMP-9* gene transcription (Overall and Lopez-Otin, 2002; Woo *et al.*, 2004; Lin *et al.*, 2008). Therefore, 6-shogaol may act through AP-1 signalling to exert its inhibitory effect on *MMP-9* gene expression. To test this possibility, we first examined the effect of 6-shogaol on the phosphorylation status (presumably the activation) of the MAPK family members. As shown in Figure 3A,C, phosphorylated forms of ERK, JNK and p38 MAPK were increased by PMA, whereas their respective total protein levels remained unchanged. Exposure to 6-shogaol markedly suppressed PMA-induced phosphorylation of JNK, but had no effect on that of ERK and p38 MAPK (Figure 3A). Parallel to this observation, we also detected a decrease in p-c-Jun, the downstream signal of JNK, upon treatment with 6-shogaol (Figure 3B), thus suggesting that JNK signalling activated by PMA was selectively blocked by 6-shogaol. Notably, specific MAPK

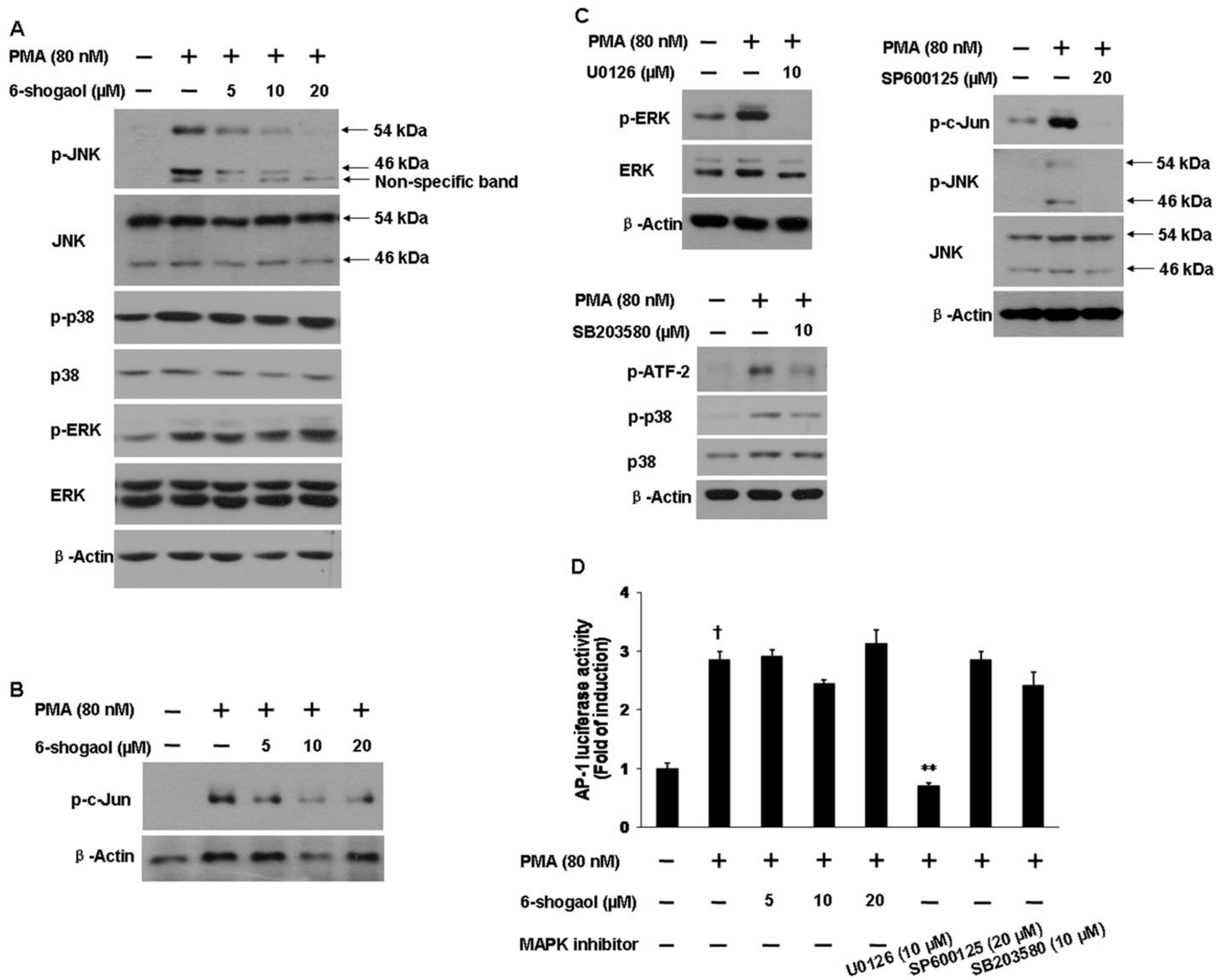

Figure 3

6-Shogaol lacks an inhibitory effect on PMA-elicited AP-1 activation. (A) Inhibitory effect of 6-shogaol on PMA-induced JNK phosphorylation, but not on ERK and p38 MAPK phosphorylation. MDA-MB-231 cells were incubated with 6-shogaol for 1 h before addition of PMA for another 1 h. Cell lysates were analysed by Western blot using the antibodies indicated. (B) Inhibitory effect of 6-shogaol on c-Jun phosphorylation. Lysates collected from cells exposed to 6-shogaol for 1 h and subsequently to PMA for 1 h were analysed by Western blot for p-c-Jun protein expression. (C) Effects of specific MAPK inhibitors on PMA-induced MAPK activation. MDA-MB-231 cells were preincubated with U0126 (10 μM), SP600125 (20 μM) or SB203580 (10 μM) for 1 h, and then exposed to PMA for 1 h. The lysates collected were analysed using indicated antibodies. (D) Effect of 6-shogaol and various MAPK inhibitors on PMA-induced AP-1 transcriptional activity. MDA-MB-231 cells co-transfected with pAP-1-Luc and pRL-CMV reporter plasmids for 24 h were pretreated with 6-shogaol for 1 h and then exposed to PMA for another 20 h. Luciferase activity was determined using a dual-luciferase reporter assay system with AP-1 firefly luciferase activity normalized to *Renilla* luciferase activity. Columns show means of three independent experiments and bars, SD; †$P < 0.05$ versus dimethyl sulphoxide control (without PMA treatment); **$P < 0.01$ versus PMA-only group. The analyses in (A) to (C) were performed three times with similar results and a representative image is shown.

inhibitors were demonstrated to down-regulate their respective target proteins (Figure 3C). Phosphorylation of ERK was completely blocked by a specific MEK1/2 inhibitor, U0126 (Figure 3C, top panel). Similarly, treatment of cells with SP600125, a JNK inhibitor, not only resulted in a decrease in JNK phosphorylation, but also abolished the phosphorylation of downstream signal c-Jun (Figure 3C, middle panel). On the other hand, SB203580, a p38 MAPK inhibitor, while having no significant inhibitory effect on p38 phosphorylation, inhibited p38 MAPK enzyme activity as evident from the observed reduction in p-ATF-2 expression (a downstream signal of p38 MAPK) (Figure 3C, bottom panel). Our results therefore are in agreement with previous reports revealing SB203580's inhibitory effect on

p38 MAPK kinase activity but not its phosphorylation (Ravanti *et al.*, 1999; Xu *et al.*, 2006).

To determine whether 6-shogaol-mediated inhibition of the JNK pathway resulted in a downstream effect on AP-1 signalling, we used a luciferase reporter assay to measure AP-1 transcriptional activity. Surprisingly, we did not observe a significant decrease in AP-1-dependent luciferase activity in cells treated with 6-shogaol at concentrations effective at inhibiting JNK signalling (refer to Figure 3D). Consistent with this finding, SP600125, the JNK inhibitor shown to block c-Jun activation (refer to Figure 3C, middle panel), failed to exert an inhibitory effect on AP-1 transcriptional activation (Figure 3D). Similarly, SB203580, a p38 MAPK inhibitor, also failed to inhibit AP-1 activation. However, the MEK1/2 inhibitor, U0126, produced a marked blockade of AP-1 transcriptional activation (Figure 3D) at the concentration effective at inhibiting ERK phosphorylation. Taken together, these results demonstrate that, in MDA-MB-231 cells, the ERK signalling pathway plays an essential role in PMA-elicited AP-1 activation, whereas JNK and p38 MAPK pathways do not appear to have an activating effect on AP-1 signalling. These results also strongly argue against the possibility that inhibition of JNK signalling mediates the inhibitory effect of 6-shogaol on MMP-9.

Inhibitory effect of 6-shogaol on PMA-activated NF-κB signalling pathway

The NF-κB is another important transcription factor involved in PMA-induced *MMP-9* gene transcription (Overall and Lopez-Otin, 2002; Shin *et al.*, 2007). We therefore investigated whether 6-shogaol had a possible effect on the NF-κB signalling pathway. To do this, we first carried out a NF-κB-responsive luciferase reporter assay, where MDA-MB-231 cells transfected with NF-κB reporter construct were treated with PMA and 6-shogaol. As shown in Figure 4A, PMA induced a 3.6-fold increase in NF-κB-dependent luciferase activity, which was suppressed dose-dependently by 6-shogaol. The results imply that 6-shogaol brings about suppression of *MMP-9* gene activation by an effect on the NF-κB signalling pathway.

NF-κB p65 subunit nuclear translocation is a critical step for its binding to NF-κB binding sites located in the promoter region of the *MMP-9* gene (Ghosh and Hayden, 2008). As shown in Figure 4B, 6-shogaol decreased the PMA-induced p65 protein expression in the nucleus, suggesting that p65 nuclear translocation was prevented by 6-shogaol. In addition, we found that while the total level of p65 in the whole cell lysates remained unchanged, PA-induced phosphorylation of p65 at serine 536 was inhibited by 6-shogaol in a dose-dependent fashion (Figure 4C). It has been reported that phosphorylation of p65 at serine 536 is involved in p65 nuclear translocation, which leads to NF-κB-dependent transcriptional activation (Ghosh and Karin, 2002; Viatour *et al.*, 2005). Therefore, the results obtained in this study further suggest that 6-shogaol exerts an inhibitory effect on p65 transcriptional activity.

We further studied whether 6-shogaol-mediated attenuation of p65 translocation was due to a reduction in the NF-κB-sequestering effect of the inhibitor of κBα (IκBα). PMA-induced reduction in IκBα level was reversed by 6-shogaol and this was accompanied by a corresponding decrease in phosphorylated IκBα (p-IκBα) (Figure 4D). These results accord with those obtained with the specific NF-κB inhibitor Bay 11-7082, which similarly inhibited IκBα phosphorylation (Figure 4E). We also investigated a possible effect of 6-shogaol on the IKK complex, a family of serine/threonine IκBα kinase involved in the phosphorylation of IκBα, and observed that while total expression of IKKα and IKKβ remained unchanged, phosphorylation of these two catalytic subunits in the IKK complex was reduced drastically by 6-shogaol at dose ≥10 μM (Figure 4D). Taken together, these results suggest that 6-shogaol targets PMA-induced *MMP-9* gene activation through suppression of NF-κB transcriptional activity by exerting an inhibitory effect on IKK phosphorylation.

Effect of specific MAPK inhibitors and NF-κB inhibitor on MMP-9 activity, secretion and protein synthesis

We had earlier investigated the effects of specific MAPKs inhibitors on AP-1 luciferase activity and the effect of the NF-κB inhibitor Bay 11-7082 on NF-κB transcriptional activity (results depicted in Figures 3D and 4A respectively). To further understand the role of MAPKs and NF-κB in PMA-induced MMP-9 expression, we studied the downstream effects of these specific inhibitors on MMP-9. Analysis by Western blot and gelatin zymography revealed that U0126 [demonstrated to inhibit ERK phosphorylation (Figure 3C) and AP-1 transcriptional activity (Figure 3D)], was able to markedly block PMA-elicited MMP-9 extracellular secretion (Figure 5). On the contrary, SP600125 (JNK inhibitor) and SB203580 (p38 MAPK inhibitor) did not attenuate MMP-9 secretion (Figure 5); this result agreed with the lack of effect of SP600125 and SB203580 on AP-1 transcriptional activity. On the other hand, Bay 11-7082, at a concentration that inhibited NF-κB, significantly reduced MMP-9 extracellular secretion (Figure 5). These results correlate well with those obtained using Western blot analysis

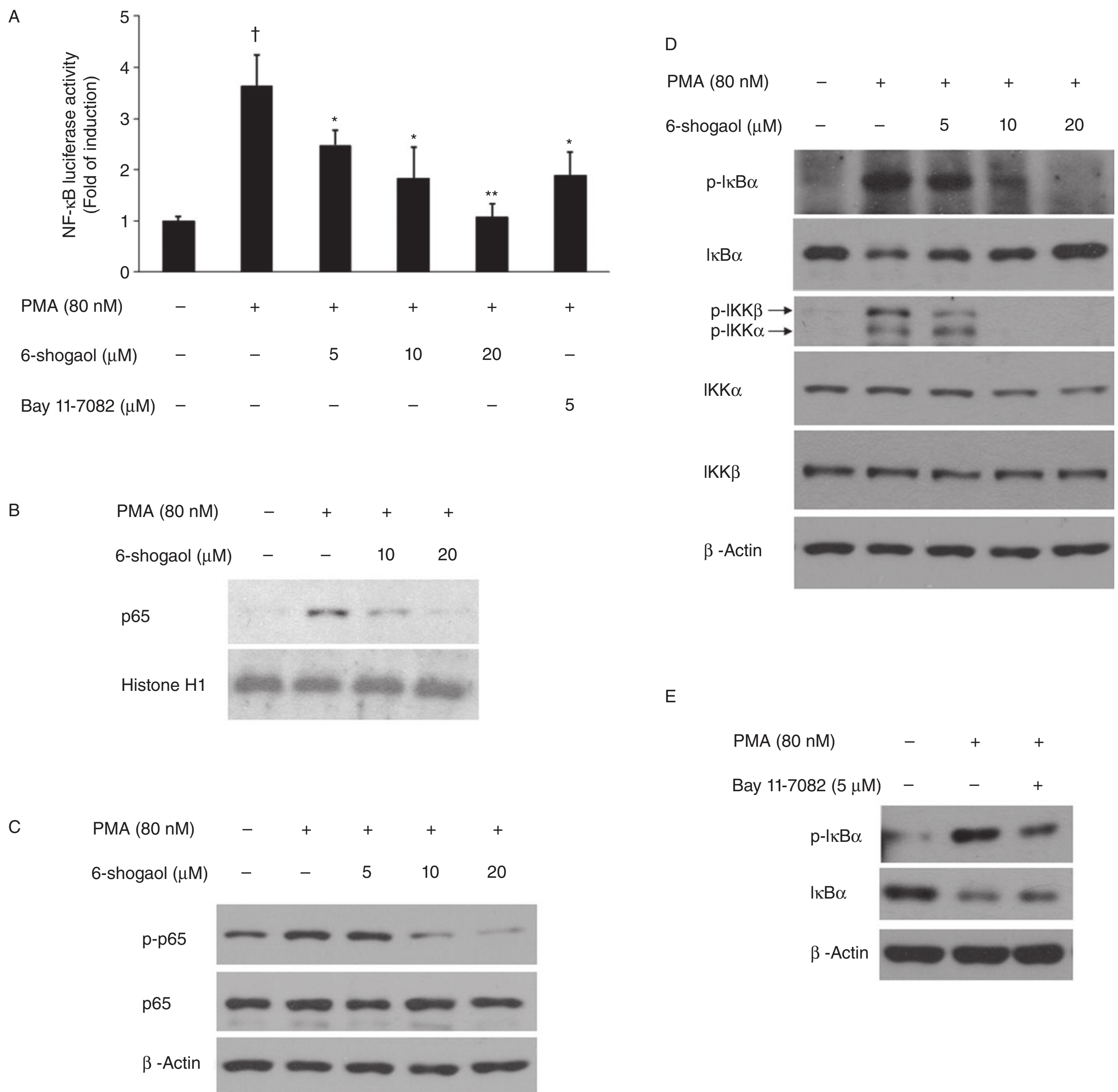

Figure 4

6-Shogaol suppresses PMA-induced NF-κB activation. (A) Inhibition of 6-shogaol on PMA-stimulated NF-κB transcriptional activity. MDA-MB-231 cells co-transfected with pNF-κB-luc and pRL-CMV reporter plasmids for 24 h were pretreated with 6-shogaol for 1 h and then exposed to PMA for another 20 h. NF-κB firefly luciferase activity was normalized to *Renilla* luciferase activity. Columns show means of three independent experiments and bars, SD; †$P < 0.05$ versus dimethyl sulphoxide control (without PMA treatment); *$P < 0.05$, **$P < 0.01$ versus PMA-only group. (B) Inhibition of NF-κB p65 nuclear translocation by 6-shogaol. MDA-MB-231 cells were incubated with 6-shogaol for 1 h before addition of PMA for another 1 h. The nuclear fractions collected were analysed by Western blot for detection of nuclear p65 protein. Histone H1 expression was used to verify equal loading. (C) Inhibitory effect of 6-shogaol on p65 phosphorylation. Lysates collected from cells exposed to 6-shogaol for 1 h and subsequently to PMA for another 1 h were immunodetected for total p65 or phosphorylated p65 proteins. (D) Prevention by 6-shogaol of the reduction of IκBα steady state level mediated by preventing IKK phosphorylation and resulting in a decrease in IκBα phosphorylation. Lysates collected from cells treated similarly as those described in (C) were analysed by Western blot using indicated antibodies. (E) Inhibitory effect of Bay 11-7082 on PMA-induced phosphorylation and reduction of IκBα protein. MDA-MB-231 cells were preincubated with Bay 11-7082 for 1 h, and then stimulated with PMA for 1 h. Lysates collected were immunodetected for total or phosphorylated IκBα. Images presented in (B) to (E) are representative of three independent experiments with similar results.

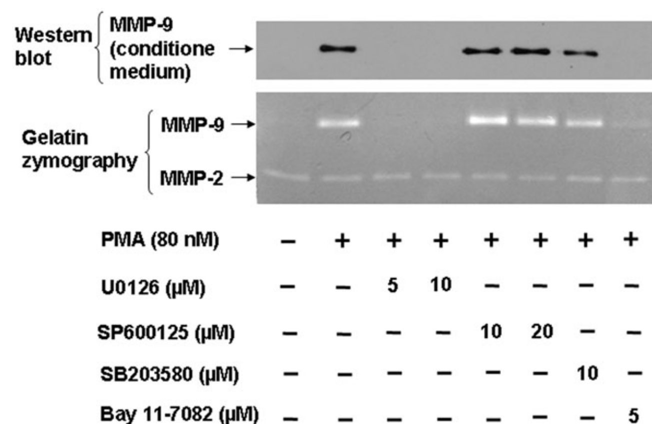

Figure 5

Effect of MAPK inhibitors, U0126, SP600125, SB203580, or NF-κB inhibitor, Bay 11-7082, on PMA-stimulated MMP-9 secretion. Cells were pretreated with designated treatments for 1 h, followed by treatment with PMA for another 20 h. Conditioned medium was collected and analysed using Western blotting (upper panel) and gelatin zymography (lower panel). The analyses were performed three times with similar results and a representative image is shown.

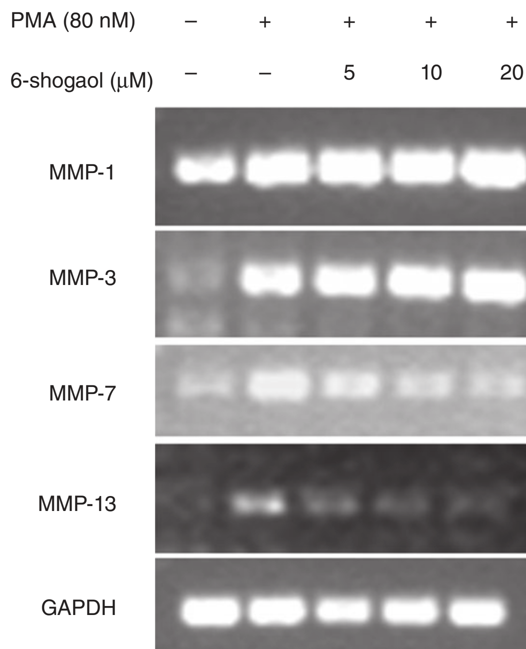

Figure 6

Effect of 6-shogaol on *MMP-1, -3, -7* and *-13* gene expression. MDA-MB-231 cells were incubated with 6-shogaol for 1 h, followed by PMA treatment for another 20 h. *MMP-1, -3, -7* and *-13* gene expression was detected by RT-PCR analysis. *GAPDH* was used here as a housekeeping gene. The experiments were conducted three times with similar results and a representative image is shown.

of cell lysates, which showed that the respective MAPK inhibitor brought about a similar change in intracellular MMP-9 expression (results not shown). Taken together, these results confirm that ERK and NF-κB pathways, but not JNK and p38, are involved in PMA-elicited MMP-9 expression through their regulation of the transcriptional activities of AP-1 and NF-κB respectively.

Effect of 6-shogaol on the expression of MMP-1, -3, -7 and -13 mRNA

To explore the possible effects of 6-shogaol on other types of MMP enzymes, we examined the mRNA expression of MMP-1, 3, -7 and -13. As shown in Figure 6, PMA treatment increased mRNA expression levels of all the examined MMP enzymes. While 6-shogaol had no effect on MMP-1 and MMP-3 mRNA expression, it caused a dose-dependent decrease in PMA-induced transcriptional activation of MMP-7 and MMP-13. Since MMP-7 and MMP-13 play roles in cancer cell invasion (Shi *et al*., 2009; Adams *et al*., 2010), these results suggest that inhibition of MMP-7 and MMP-13 expression by 6-shogaol may be an additional mechanism for its anti-invasive effect.

Discussion and conclusions

For centuries, people have been harnessing the power of nature to provide medicinal solutions to various diseases. Indeed, in the context of cancer, plants, in particular, comprise rich sources of natural compounds that not only possess potential anticancer activity, but also serve as novel lead compounds and chemical entities on which synthesis can be performed to derive superior new compounds. Known naturally occurring agents such as resveratrol (Woo *et al*., 2004), quercetin (Lin *et al*., 2008), curcumin (Woo *et al*., 2005), as well as gingerol (a major pungent constituent in ginger) (Lee *et al*., 2008; Yagihashi *et al*., 2008) have been demonstrated to exert effects against cancer cell invasion. In this study, we investigated whether shogaols, another class of biologically active component found in rhizomes of *Zingiber officinale Roscoe* (ginger), could inhibit cell migration and invasion. We demonstrated that shogaols of differing side chain lengths have an inhibitory effect on PMA-induced invasion of MDA-MB-231 breast carcinoma cells, which was associated with a decrease in the extracellular secretion of MMP-9. Among the shogaols tested, 6-shogaol with the shortest alkyl side chain displayed the greatest inhibition at sublethal concentrations (between 5 to 20 μM), suggesting that increasing the length of the alkyl carbon chain led to a hampering of the anti-invasive effect of shogaols.

The role of MMP-2 and MMP-9 in tumour invasion has been intensively studied but has not been clearly defined. Consistent with findings of other studies (Woo *et al.*, 2004; Lee *et al.*, 2007; Lin *et al.*, 2008), our data revealed that at least under the condition of PMA stimulation, MMP-9 activity was increased while that of MMP-2 was not affected, thus implicating the involvement of MMP-9 in tumour invasion. The correlation of MMP-9 with cancer malignancy is evident with levels of the proteolytic enzyme detected to be higher in the serum of breast cancer patients than that in the patient group with benign tumours (Somiari *et al.*, 2006). It should be noted that latent MMP-9 (pro-MMP-9) can be activated during gelatin zymography analysis, which is not indicative of the activity of the gelatinase in its active form (Hibbs *et al.*, 1985; Toth *et al.*, 1997; Fridman *et al.*, 2003). Interestingly, in this study, we only detected the extracellular secretion of the 92 kDa pro-MMP-9, but not the presumably active MMP-9 (molecular weight of 82 kDa). Our observations are not uncommon; similar findings have been reported in numerous studies (Toth *et al.*, 1997; Woo *et al.*, 2004; Liang *et al.*, 2009). Indeed, the inability to detect the 82 kDa active form of MMP-9 in cultured cells is predominant (Fridman *et al.*, 2003). To date, the cellular and molecular mechanism(s) involved in the activation of pro-MMP-9 remain elusive. An alternative mechanism of pro-MMP-9 activation has been proposed, in which the propeptide domain becomes disengaged from the enzyme's active site without physical cleavage and this may partly explain MMP-9's activity in the absence of a change in molecular mass (Bannikov *et al.*, 2002; Fridman *et al.*, 2003). Nonetheless, our study clearly demonstrated that 6-shogaol caused a down-regulation of the activity of extracellular MMP-9.

In parallel with the reduction in MMP-9 secretion, we further revealed that 6-shogaol inhibited MMP-9 synthesis at the transcriptional level. Previous studies have suggested that AP-1 and NF-κB have a critical role in PMA-induced MMP-9 transactivation (Shin *et al.*, 2007; Lin *et al.*, 2008); in agreement, we found that PMA dramatically increased the transcriptional activity of both AP-1 and NF-κB. In addition, when this increased transactivation of AP-1 and NF-κB was effectively blocked by specific inhibitors, a reduction in MMP-9 expression and secretion was observed, thus affirming that MMP-9 is a downstream target of AP1 and NF-κB signalling. With respect to PMA-induced AP-1 and NF-κB transcriptional activity, a sublethal dose of 6-shogaol (20 μM) was found to selectively down-regulate the latter almost completely, but produce no effect on the former. Interestingly, 6-shogaol reduced PMA-activated *MMP-7* and *MMP-13* gene expression. Since the promoter regions of these two genes do not contain NF-κB binding site(s) (Overall and Lopez-Otin, 2002; Clark *et al.*, 2008), these findings suggest that 6-shogaol exerts its anti-invasive effect through inhibition of other MMPs via NF-κB-independent pathways.

NF-κB has been suggested to be a target for many biologically active natural products derived from plant foods such as curcumin, resveratrol, epigallocatechin gallate and sulforaphane (Surh *et al.*, 2001; Woo *et al.*, 2004). Moreover, studies have reported that 6-shogaol exerts an anti-inflammatory effect through down-regulation of NF-κB signalling (Pan *et al.*, 2008a). However, to date, the molecular mechanism by which 6-shogaol inhibits the NF-κB activation cascade is not fully understood. Our present study had demonstrated that 6-shogaol exerted anti-NF-κB effect through inhibition of phosphorylation of IKKα and IKKβ subunits of the IKK complex. This led to blockade of IκBα phosphorylation and prevention of IκBα proteosomal degradation with a resulting decrease in p65 nuclear translocation and NF-κB transcriptional activation. Furthermore, 6-shogaol inhibited p65 phosphorylation at serine 536 without affecting total p65 expression. The phosphorylation of p65 at serine 536 has been suggested to play an important role in p65 nuclear localization and transcriptional activity (Ghosh and Karin, 2002; Viatour *et al.*, 2005). Taken together, our findings demonstrate that the *in vitro* anti-invasive effect of 6-shogaol is mediated through its interference with NF-κB signalling.

Our experiments also explored the role of ERK, JNK and p38 kinase in PMA-enhanced AP-1 transcriptional activity and the subsequent activation of MMP-9. We found that the ERK pathway was involved in PMA-mediated AP-1 and MMP-9 activation, whereas the JNK and p38 pathways had no effect. Interestingly, although we observed inhibition of c-Jun phosphorylation by SP600125, a specific JNK inhibitor, this inhibition failed to lead to suppression of both AP-1 and MMP-9 activation. Because AP-1 is a heterodimer composed of c-Jun and c-Fos, the respective downstream signal of JNK and ERK, our results suggest that at least in the present experimental system, c-Fos is highly involved in PMA-induced increased induction of MMP-9, while c-Jun does not play a critical role. Conflicting findings have been obtained with regards the involvement of JNK signalling in MMP-9 activation, with a good number suggesting a positive relationship (Woo *et al.*, 2004; Lee *et al.*, 2007) while some others concluding otherwise (Hong *et al.*, 2005; Lin *et al.*, 2008). It is plausible that the role of individual MAPK in MMP-9 activation is

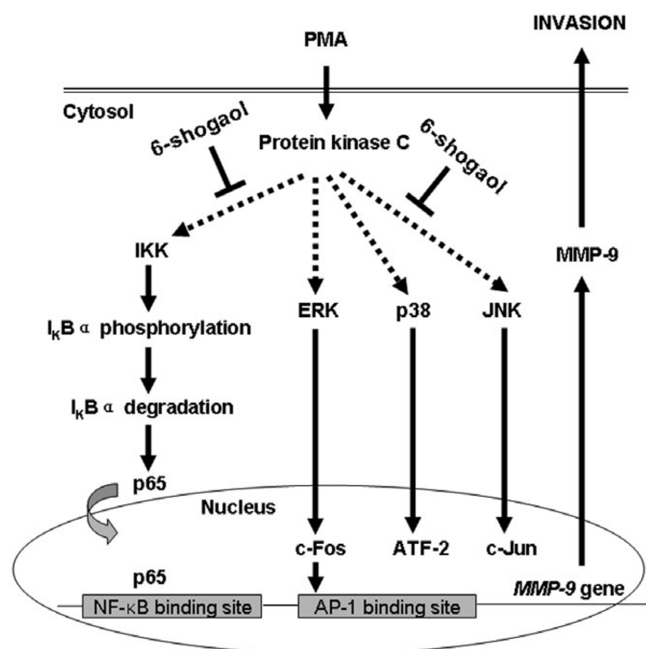

Figure 7

A summary of the proposed molecular mechanism through which 6-shogaol inhibits PMA-induced breast cancer cell invasion. PMA induces MMP-9 expression and subsequent cancer cell invasion through NF-κB transcriptional activation and ERK-dependent AP-1 transcriptional activation. 6-Shogaol targets the upstream signal IKK by suppressing IKK-dependent IκBα phosphorylation and degradation, leading to a retardation of p65 nuclear translocation and subsequent inhibition of NF-κB transcriptional activation. Although 6-shogaol has an inhibitory effect on the JNK signalling pathway, this effect is unlikely to contribute to its inhibition of MMP-9 activation.

cell-specific and stimulus-specific. Therefore, in our experiments, although 6-shogaol was found to inhibit phosphorylation of c-Jun, we deduce at this point that this event is unlikely to contribute towards 6-shogaol's inhibition of MMP-9 activation.

In summary, our study has focused on the naturally occurring shogaols found in ginger. We have demonstrated that sublethal doses of 6-, 8- and 10-shogaol, by reducing MMP-9 expression and secretion, have an inhibitory effect on PMA-induced breast cancer cell invasion. Furthermore, we provide evidence that 6-shogaol impairs breast cancer cell invasion, at least in part, through targeting the NF-κB activation cascade (summarized in Figure 7). In recent years, an increasing number of natural products possessing anticancer properties have been unveiled. Coupled with the elucidation of their antitumour mechanism(s), the exploitation of their use in clinical chemotherapeutic strategies is promisingly realizing. In view of the impressive *in vitro* potency of 6-shogaol in reversing PMA-induced cancer cell invasion at non-cytotoxic concentrations, and as specific antimetastatic agents with minimal or no complications from their cytotoxicities are preferred, this series of naturally occurring phytochemicals are indeed worthy of further development as antimetastatic agents for clinical use.

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Statement of conflicts of interest

None.

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