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Suppression of PMA-induced tumor cell invasion by dihydroartemisinin via inhibition of PKCα/Raf/MAPKs and NF-κB/AP-1-dependent mechanisms

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ABSTRACT

Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, has recently been shown to possess antitumor activity in various cancer cells. However, the effects of DHA in preventing the invasion of cancer cells have not been studied. In the present study, we investigated the inhibitory effects of DHA on tumor invasion and migration and the possible mechanisms involved using human fibrosarcoma HT-1080 cells. DHA reduced PMA-induced activation of MMP-9 and MMP-2 and further inhibited cell invasion and migration. DHA suppressed PMA-enhanced expression of MMP-9 protein, mRNA, and transcriptional activity through suppressing NF-κB and AP-1 activation without changing the level of tissue inhibitor of metalloproteinase (TIMP)-1. DHA also reduced PMA-enhanced MMP-2 expression by suppressing membrane-type 1 MMP (MT1-MMP), but did not alter TIMP-2 levels. DHA-inhibited PMA-induced NF-κB and c-Jun nuclear translocation, which are upstream of PMA-induced MMP-9 expression and invasion. Furthermore, DHA strongly repressed the PMA-induced phosphorylation of Raf/ERK and JNK, which are dependent on the PKCα pathway. In conclusion, we demonstrated that the anti-invasive effects of DHA may occur through inhibition of PKCα/Raf/ERK and JNK phosphorylation and reduction of NF-κB and AP-1 activation, leading to down-regulation of MMP-9 expression. The data presented show that DHA is an effective anti-metastatic agent that functions by down-regulating MMP-9 gene expression.

1. Introduction

Artemisinin is a sesquiterpene lactone endoperoxide found in the traditional Chinese medicinal plant *Artemisia annua* [1,2], and its derivatives are very effective blood schistocidal antimalarials with fewer adverse side effects than other antimalarial drugs. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, is recommended as a first-line antimalarial drug, with low toxicity [3]. In recent years, certain artemisinin derivatives have been shown to inhibit the growth of a limited set of human cancer cell lines [4,5], such as fibrosarcoma cells [6], breast cancer cells [7,8], cervical cancer cells [9], leukemia cells, and ovarian cells [10,11]. Moreover, Jiao et al. also demonstrated that DHA was the most effective and potent agent in inhibiting cell growth of ovarian cancer cells, as compared with artemisinin, artesunate, arteether, artemether, and arteannuin [12]. More importantly, DHA has been shown to be selectively toxic to breast cancer cells, versus normal human breast cells [13], and exerted potent cytotoxic effects on ovarian carcinoma cells, but had minimal effects on non-tumorigenic human ovarian surface epithelial cells [11], suggesting that DHA is well-tolerated and may represent a promising potent therapeutic agent to treat cancers. Consequently, in recent years a hypothesis has been advanced that artemisinin and its derivatives may be useful as anticancer drugs [8]. Although various bioactivity studies of DHA have been carried out, the molecular mechanisms by which DHA acts on the expression of matrix metalloproteinase (MMP)-9 and the invasiveness of HT-1080 are still unclear.

Metastasis and invasion are fundamental properties of malignant cancer cells. An inability to control metastasis and invasion is the leading cause of death in patients with cancer. The control of metastasis and invasion, therefore, represents an important therapeutic target. Tumor invasion and metastasis require increased expression of matrix metalloproteinases (MMPs) [14]. Cell-extracellular matrix (ECM) interactions, disconnection of intercellular adhesion, degradation of the ECM, and the invasion of lymph and blood vessels are important steps in cancer invasion and metastasis [15–17]. Members of the MMP family are involved

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in the degradation of ECM, and MMPs have been implicated in malignancy [14,18]. MMPs are synthesized as preproenzymes and are secreted from cells as proenzymes. Among the human MMPs reported to date, MMP-2 and -9 are the key enzymes involved in degrading Type-I and -IV collagens and ECM [17–19]. Both MMP-2 and -9, which are abundantly expressed in various malignant tumors [17], contribute to cancer invasion and metastasis [20]. Generally, MMP-2 is constitutive and is over-expressed in highly metastatic tumors, whereas MMP-9 can be stimulated by the inflammatory cytokine tumor necrosis factor (TNF)-α, by the growth factor epidermal growth factor, and by phorbol esters, through activation of different intracellular signaling pathways [21–23]. Its primary activation occurs on the cell surface and is mediated by membrane-type matrix metalloproteinases (MT-MMPs), such as MT1-MMP [24]. The concerted action of highly expressed MT1-MMP and adequate expression of tissue inhibitor of metalloproteinase (TIMP) leads to activation of MMPs [25,26].

Mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and protein kinase C (PKC) signaling pathways are the predominant cascades participating in MMP-9 expression [27–29]. Furthermore, stimulators, such as cytokines and phorbol myristate acetate (PMA), control the expression of MMP-9 by modulating the activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) and through Ras/Raf/MAPKs and PI-3K/AKT signaling pathways [21,28,30], because the promoter region of MMP-9 has AP-1 and NF-κB binding sites [31]. NF-κB and AP-1 are well-known transcription factors that regulate the expression of a number of genes, the products of which are involved in metastasis, tumorigenesis, and inflammation [21,32,33]. Indeed, NF-κB and AP-1 are key transcription factors in the activation of genes encoding inflammatory cytokines, such as TNF-α and IL-1β [33–35]. NF-κB can also induce the activation of MMP-9 and COX-2 [32,36]. Several studies have indicated that inhibition of MMP expression or enzyme activity can be used as early targets for preventing cancer metastasis [17,20]. Thus, agents possessing the ability to suppress the expression of MMP-2 or -9 are worthy of investigation with regard to cancer cell invasion and metastasis.

In this research, we studied the effects of DHA on PMA-induced MMP expression and explored the underlying upstream signaling molecular mechanisms. We found that DHA significantly suppressed MMP-9 gene expression by blocking the conventional PKCα/Raf/MAPKs and NF-κB/AP-1 signaling pathways, consequently reducing invasion and metastasis of HT-1080 cells.

2. Materials and methods

2.1. Materials

Dihydroartemisinin (DHA), curcumin, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chemical (St. Louis, MO). NF-κB activation inhibitor (JSH-23), Gö6976, U0126, SB203580, and SP600125 were purchased from Calbiochem (La Jolla, CA). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide]-based colorimetric assay kit was purchased from Roche (Indianapolis, IN). RPMI1640, fetal bovine serum (FBS), sodium pyruvate, and Trizol were from Gibco BRL (Grand Island, NY). Antibodies against phospho-MAP kinase, phospho-PKCα, PKCα, MMP-2, MMP-9, and NF-κB were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against c-Jun, c-Fos, IκBα, lamin B, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The MMP-9 promoter vector was kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany) [37]. The MMP-2 promoter vector was kindly provided by Dr. E.N. Benvenist (Department of Cell Biology, University of Alabama, Birmingham, Alabama, USA) [38]. The pNF-κB-Luc and pAP-1-Luc reporter plasmid were obtained from Stratagene (La Jolla, CA). 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) 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 DHA in the absence or presence of PMA (30 nM) for 24 h. DHA was dissolved in dimethyl sulfoxide (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 cell viability was determined by a 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 absorbance at 570 nm was measured with a microplate reader (Varioskan, Thermo Electron Co.).

2.4. In vitro wound-healing assay

HT-1080 cells were seeded in six-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 by photography 24 h later. Each value was 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) that had been coated with 50 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ; 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. RNA preparation, semi-quantitative RT-PCT and real-time 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 using the RT-PCR Bead kit (Amersham Pharmacia, Buckinghamshire, UK), 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. PCR product formation was monitored continuously during the reaction using Sequence Detection System software, version 1.7 (Applied Biosystems, Foster City, CA, USA). Accumulated PCR products were detected directly by monitoring the increase of the reporter dye (SYBR®). The mRNA expression levels of MMP-2 and MMP-9 in the treated cells were compared to

the expression levels in control cells at each time point using the comparative cycle threshold (Ct)-method [53]. The following primers were used in this study: MMP-2 forward: 5′-AGT CTG AAG AGC GTG AAG-3′, MMP-2 reverse: 5′-CCA GGT AGG AGT GAG AAT G-3′, MMP-9 forward: 5′-TGA CAG CGA CAA GAA GTG-3′, MMP-9 reverse: 5′-CAG TGA AGC GGT ACA TAG G-3′, GAPDH forward: 5′-CCA CCC ATG GCA AAT TCC-3′, GAPDH reverse: 5′-TGG GAT TTC CAT TGA TGA CAA-3′. The quantity of each transcript was calculated as described in the instrument manual and normalized to the amount of GAPDH, a housekeeping gene.

2.7. Western blotting analysis

After treatment, cells were collected and washed with phosphate-buffered saline (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 (13,000 × *g*, 15 min). Supernatants were collected from the lysates and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). 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% bovine serum albumin 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, Santa Cruz, CA) for 1 h. Finally, protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Pierce Biotechnology, Rockford, IL).

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 were then maintained in serum-free medium. Conditioned media were collected 24 h after stimulation, mixed with non-reducing sample buffer, and subjected to electrophoresis in a 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 then 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). The cells were plated in 48-well plates and incubated at 37 °C. At 70–80% confluency, the cells were washed with PBS and incubated with RPMI1640 without serum or antibiotics for 6 h. The MMP-9 and -2 promoter vector, AP-1 or NF-κB reporter vector (Stratagene, Grand Island, NY) (1 μg) and pCMV-β-gal (0.5 μg) plasmids were transfected using LipofectAMINE 2000 (Invitrogen, San Diego, CA, USA) reagent according to the manufacturer's protocol. After incubation, cells were lysed, and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron Co., Berthold, Germany). Luciferase activity was normalized by β-galactosidase activity in cell lysates and expressed as an average of three independent experiments. For analysis of basal NF-κB and AP-1 activity, cells were transiently transfected with Lenti-NF-κB-GFP or Lenti-AP-1-GFP (System Biosciences, CA) mixed with the Lipofectamine reagent. Fluorescence of GFP protein was analyzed by fluorescence microscopy (Axiovert 200 M; Carl Zeiss, Germany).

2.10. Statistical analyses

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 deemed to be statistically significant when $P < 0.01$.

3. Results

3.1. DHA suppresses PMA-induced invasion and migration of HT-1080 cells through inhibition of MMP-9 expression

Prior to investigating the pharmacological potential of DHA on PMA-induced MMP activity, we first determined the dose dependence of the cytotoxic effects of DHA in the absence or presence of PMA (30 nM) for 24 h in HT-1080 cells using the MTT assay. DHA at concentrations lower than 50 μM had no cytotoxic effect on the cells and DHA at 100 μM showed about a 20–25% decreases in cell viability in the absence or presence of PMA (Fig. 1B). We next used a gelatin zymography assay to investigate the inhibitory effect of DHA on PMA-induced MMP-9 and -2 secretion. Treatment with PMA for 24 h dramatically induced MMP-9 secretion, in a dose-dependent manner, in HT-1080 cells, while the level of MMP-2 secretion was not affected by PMA (data not shown). As shown in Fig. 1C, treatment of HT-1080 cells with DHA at doses above 5 μM suppressed PMA-induced MMP-9 and -2 activities dose-dependently (Fig. 1C). The results obtained by zymography were further confirmed by Western blot analysis (Fig. 1C). The secretion of MMP-9 and -2 proteins into the medium gradually decreased in a dose-dependent manner, indicating that the reduced MMP-9 enzyme activity was the result of decreased amounts of MMP-9 and -2 proteins (Fig. 1C). It has been reported that up-regulation of MMP expression contributes to invasion and metastasis of cancer cells [17,20]. *In vitro* invasion and migration assays, including Transwell and wound-healing assays, were used to investigate the inhibitory effects of DHA on the invasive potency of HT-1080 fibrosarcoma cells. The data obtained from the Matrigel invasion assay showed that PMA caused the stimulation of cell invasion; however, DHA at 50 μM inhibited the PMA-induced invasion of HT-1080 cells, by 55% (Fig. 1D). Similarly, the data from the wound-healing assay indicated that migration of HT-1080 cells was inhibited by DHA (Fig. 1E). These results, suggesting the inhibition of cell invasion and migration by DHA, are consistent with the inhibition of MMP-9 expression.

3.2. DHA suppresses PMA-induced MMP-9 and -2 expression through inhibition of transcription

To determine whether the inhibition of MMP-9 secretion by DHA was due to a decreased level of transcription, we performed RT-PCR and promoter assays using transiently transfected cells with a luciferase reporter gene linked to the MMP-9 promoter sequence. In the semi-quantitative RT-PCR or real-time PCR, treatment of HT-1080 cells with DHA decreased the levels of PMA-stimulated MMP-9 and -2 mRNA expression (Fig. 2A and E), indicating that DHA decreased transcription of MMP-9 in response to PMA. Because activities of MMPs are tightly regulated by endogenous inhibitors, the TIMPs [39,40], we further examined the expression level of TIMP-1 and -2 by semi-quantitative RT-PCR, but their expression was essentially unchanged by treatment with DHA (Fig. 2A). Because MT1-MMP plays an important role in regulating MMP-9 and -2 activation, we determined whether DHA suppressed its expression in HT-1080 cells stimulated with PMA. Western blot and semi-quantitative RT-PCR data revealed that DHA suppressed both MT1-MMP mRNA (Fig. 2B) and protein expression (Fig. 2C) induced by PMA. The effect of DHA on MMP-9

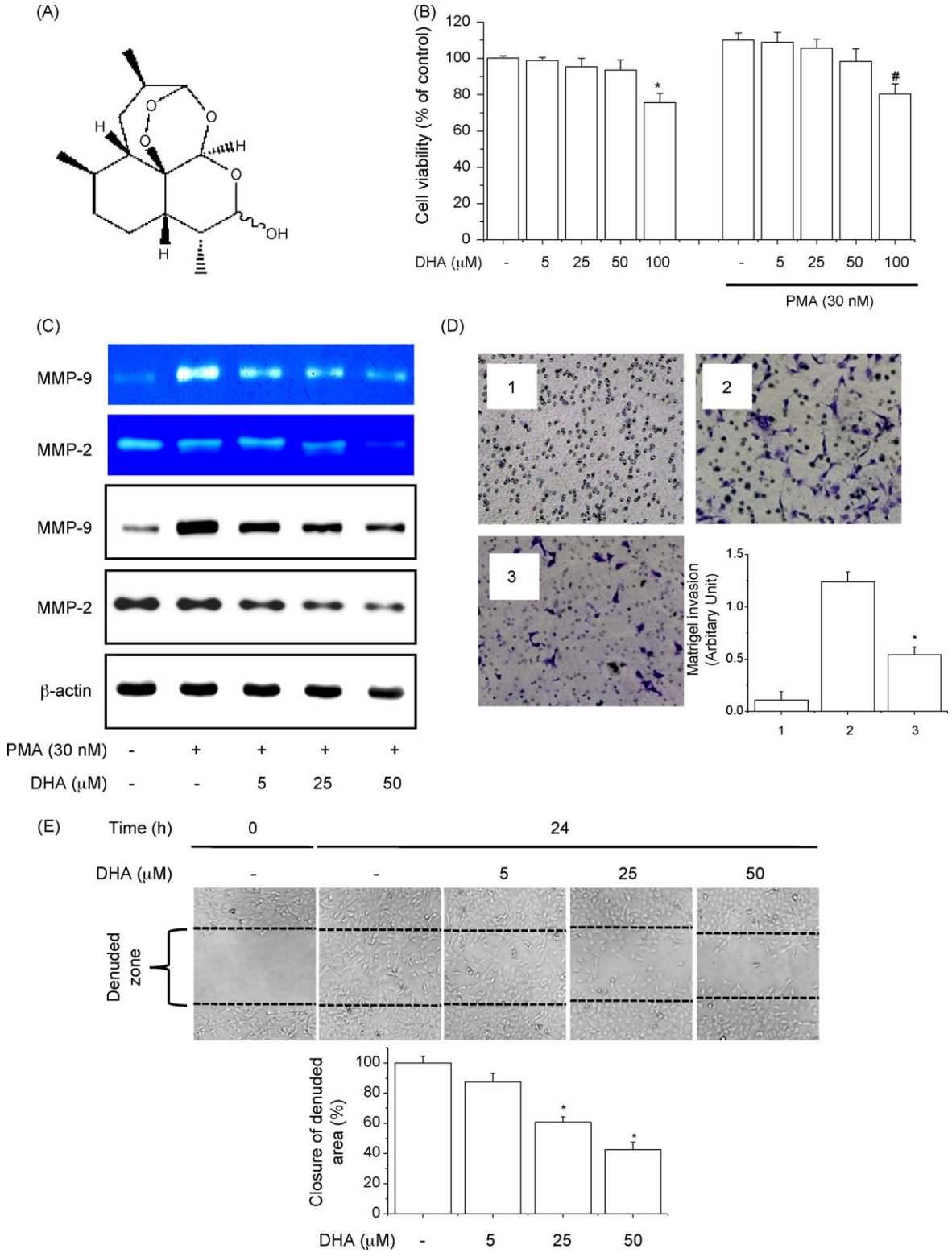

Fig. 1. Inhibitory effects of DHA on migration, invasion, and MMP-9 activity in human fibrosarcoma HT-1080 cells. (A) Structure of DHA. (B) HT-1080 cells were treated with DHA in the absence or presence of 30 nM PMA for 24 h in serum-free medium, and viability was determined by the MTT assay. *Significantly different from control ($P < 0.01$). #Significantly different from treatment only ($P < 0.01$). (C) Effects of DHA on the MMP-9 activity in HT-1080 cells. Cells were treated with various concentrations of DHA (5, 25, 50 μM) for 24 h in the presence of 30 nM PMA. MMP-9 and -2 activities in HT-1080 cells were determined by gelatin zymography and Western blotting. (D) Effects of DHA on the invasion in HT-1080 cells. Cells were pretreated with DHA (50 μM) followed by PMA (30 nM) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. (1) Control; (2) PMA alone; (3) PMA with DHA (50 μM). *Significantly different from PMA treatment only ($P < 0.01$). (E) Effects of DHA on the migration in HT-1080 cells. Cells were scratched with a pipette tip and then treated with DHA (5–50 μM) for 24 h. Migrating cells were photographed under phase contrast microscopy. *Significantly different from control ($P < 0.01$).

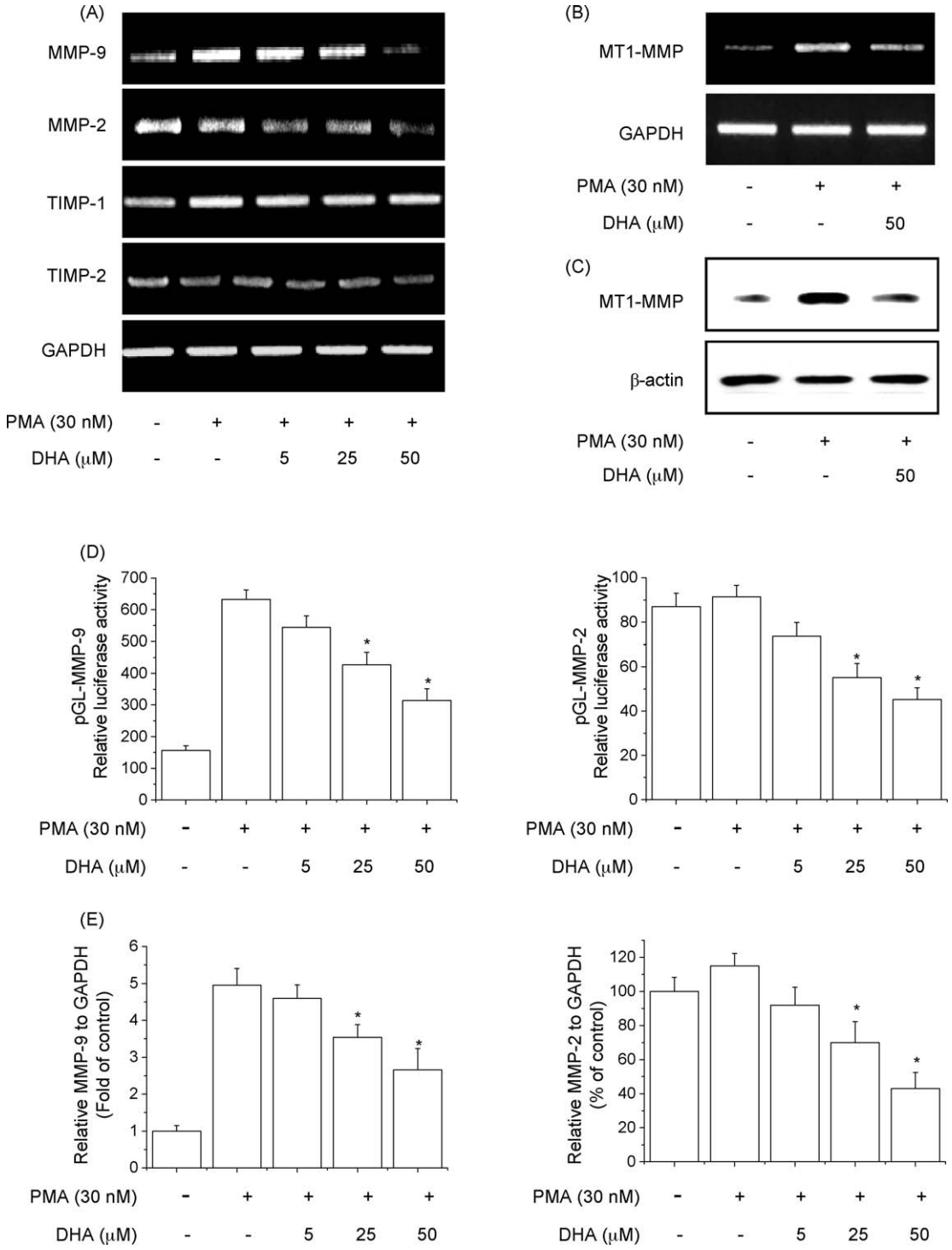

Fig. 2. Inhibition of PMA-induced MMP-9, -2, and MT1-MMP expression by DHA. (A and E) Effects of DHA on MMP-9 and -2 mRNA expression. HT-1080 cells were incubated with DHA and/or PMA (30 nM) for 24 h. MMP-9 and -2 mRNA expression was analyzed by semi-quantitative RT-PCR or real-time PCR. GAPDH expression was included as an internal control. (B and C) Effects of DHA on MT1-MMP expression. HT-1080 cells were incubated with DHA 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 or β-actin expression was included as an internal control. (D) Cells were transfected with the WT-MMP-9 or WT-MMP-2 promoter-containing reporter vector and incubated with various concentrations of DHA 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 ($P < 0.01$).

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. 2D, luciferase gene expression was activated, up to 5-fold, in cells treated with PMA, compared with untreated cells. Treatment of cells with DHA (5–50 μM) decreased PMA-mediated luciferase activity in a dose-dependent manner, indicating that DHA-inhibited MMP-9 expression at the transcriptional level and that the MMP-9 promoter contains DHA-responsive elements. DHA also affected the luciferase reporter gene containing the MMP-2 promoter (Fig. 2D). No cytotoxicity was observed when cells were exposed to DHA (data not shown).

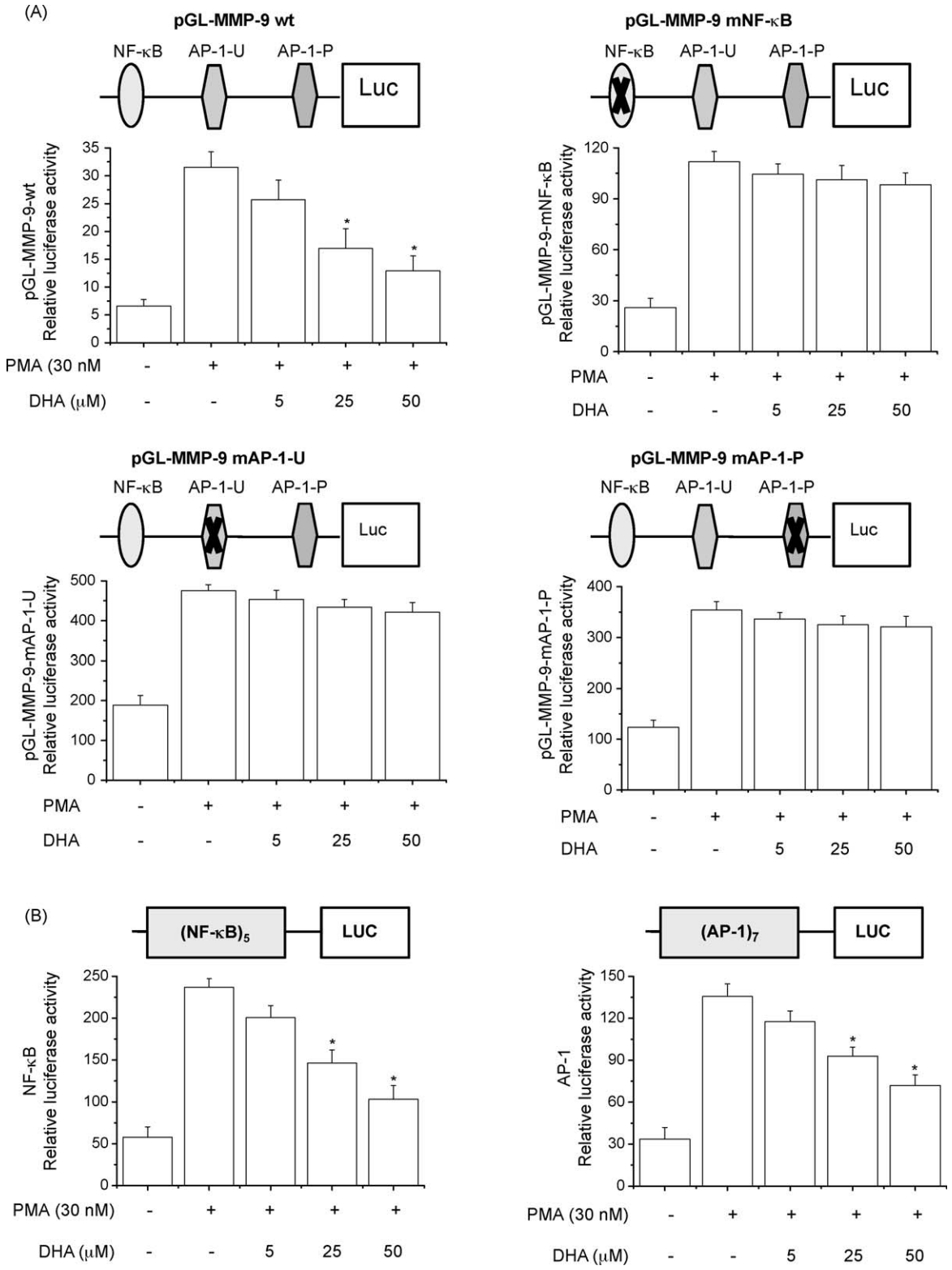

Fig. 3. Inhibitory effects of DHA on PMA-induced activation of NF-κB and AP-1. Mutations were introduced in the NF-κB or AP-1 binding sites of pGL-MMP-9WT. HT-1080 cells were transfected with pGL-MMP-9WT, pGL-MMP-9mNF-κB, pGL-MMP-9mAP-U, or pGL-MMP-9mAP-P reporter plasmids (A) or with reporter plasmids containing tandem NF-κB or AP-1 binding sites (B). Cells were cultured with DHA and/or PMA for 24 h, and the relative luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($P < 0.01$). (C) Effects of DHA on PMA-induced NF-κB and c-Jun nuclear translocation. HT-1080 cells were pretreated with DHA (5–50 μM) for 1 h and treated with 30 nM PMA for 3 h. The 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. (D) Effect of DHA on PMA-induced IκBα degradation and phosphorylation of p65 and c-Jun. HT-1080 cells were pretreated with DHA (5–50 μM) for 1 h and treated with 30 nM PMA for 3 h. Whole-cell extracts were subjected to SDS-PAGE, followed by Western blotting with anti-IκBα, anti-phospho-c-Jun, anti-phospho-p65, and anti-β-actin antibodies. (E) Effects of DHA on PMA-induced NF-κB and AP-1 activation. Cells were transiently transfected with Lenti-NF-κB-GFP or Lenti-AP-1-GFP. GFP protein fluorescence was analyzed by fluorescence microscopy.

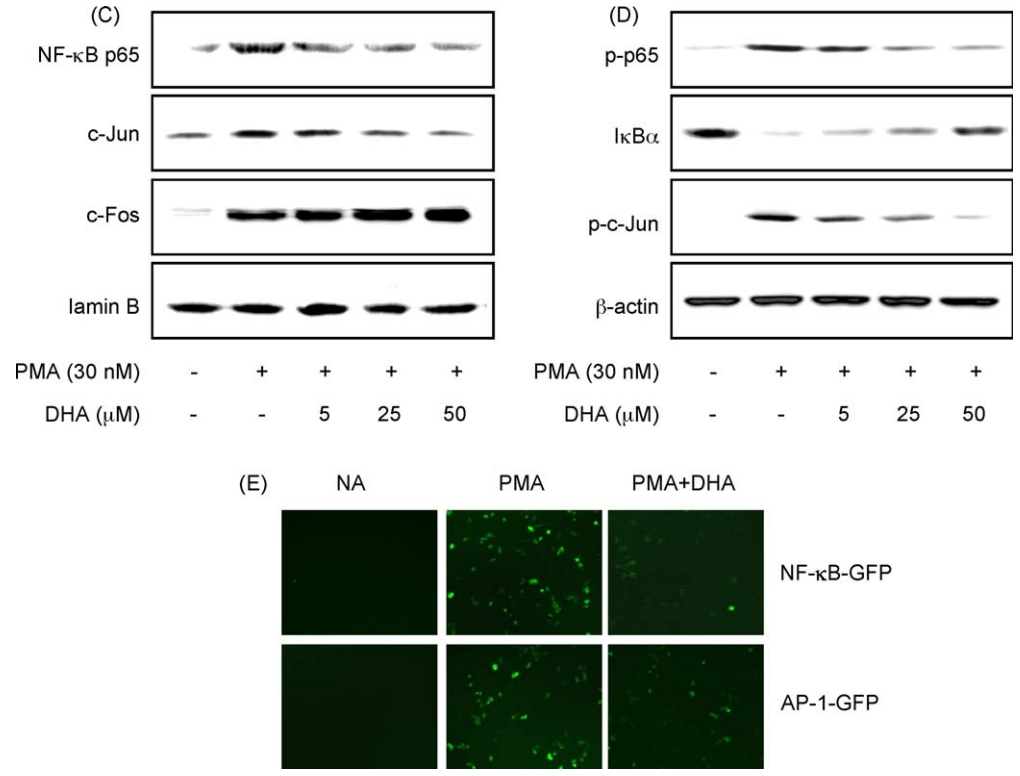

Fig. 3. (*Continued*).

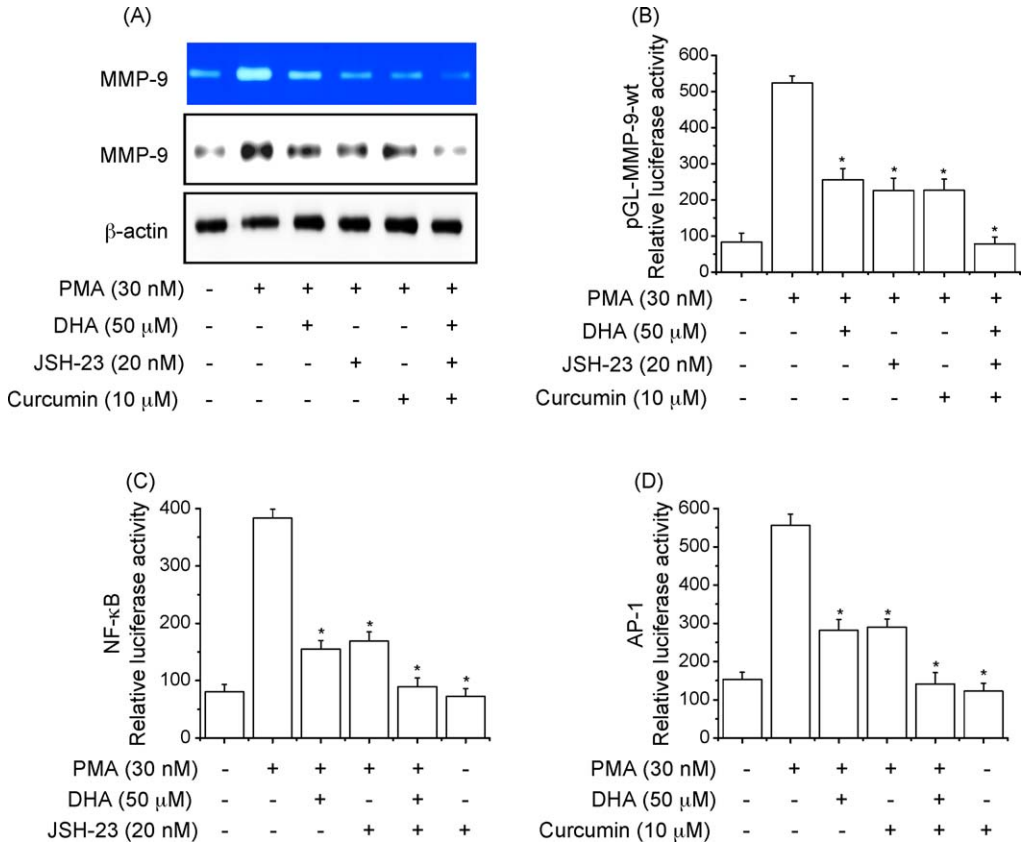

Fig. 4. NF-κB and AP-1 are involved in DHA-mediated down-regulation of MMP-9. Cells were treated with 30 nM PMA for 24 h in the absence or presence of DHA, JSH-23, or curcumin. (A) Conditioned media were collected after 24 h and then gelatin zymography or Western blotting was performed. (B) HT-1080 cells were transfected with pGL-MMP-9WT reporter plasmids and then cultured with DHA, JSH-23 or curcumin and/or PMA for 24 h. Luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($P < 0.01$). (C and D) Cells were transfected with pNF-κB or pAP-1 reporter plasmids and then cultured with DHA, JSH-23, or curcumin and/or PMA for 24 h. Luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($P < 0.01$).

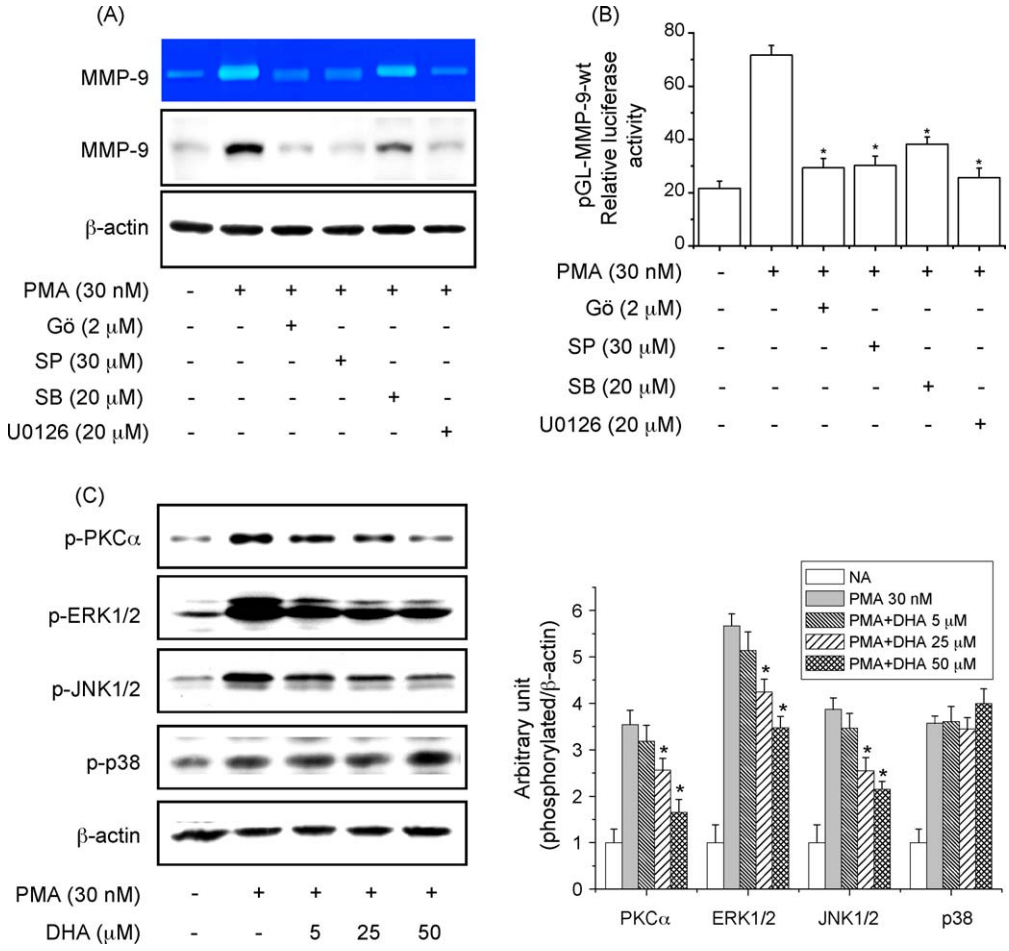

Fig. 5. Effects of DHA on PMA-induced activation of PKCα and MAPKs signaling pathways. (A and B) Effects of inhibitor on PMA-induced MMP-9 activation. Cells were pretreated for 1 h with Gö6976 (Gö, 2 μM), SB203580 (SB, 20 μM), SP600125 (SP, 30 μM) or U0126 (20 μM), followed by PMA stimulation for 24 h. The MMP-9 activity in the conditioned media was analyzed by gelatin zymography and Western blotting. The relative luciferase activity in the cell extract was determined. *Significantly different from PMA treatment only ($P < 0.01$). (C) Effects of DHA on PMA-induced phosphorylation of PKCα and three MAPKs signaling pathways. Cells were treated with PMA (30 nM) for 30 min in the presence or absence of DHA, and the phosphorylation levels of PKCα, ERK1/2, JNK, and p38 MAPK were measured by Western blotting. Densitometry ratios of phospho-PKCα and phospho-MAPKs were normalized to β-actin. *Significantly different from PMA treatment only ($P < 0.01$).

3.3. DHA inhibits transcriptional activity of the MMP-9 gene through suppression of PMA-stimulated NF-κB and AP-1 activity

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 [21,30]. 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. 3A). As shown in Fig. 3A, in treatment with DHA in the presence of PMA, the transcriptional activity of the reporter with the AP-1 mutation or NF-κB mutations was not affected, suggesting that the targets of bergamottin were the NF-κB and AP-1 transcription factors themselves. To further determine the simplicity of promoter structure used by DHA, we transfected HT-1080 cells with pNF-κBLuc or pAP-1-Luc plasmid DNAs, which contain the luciferase gene driven by NF-κB (5′-GGGGACTTTCC-3′)$_n$ ($n = 5$) or AP-1 (5′-TGACTAA-3′)$_n$ ($n = 7$) responsive elements, respectively. Transfected HT-1080 cells were treated with DHA for 24 h, and luciferase activity was determined. As shown in Fig. 3B, DHA suppressed both NF-κB- and AP-1-containing promoters in a dose-dependent manner. HT-1080 cells were incubated with different concentrations of DHA in the presence of PMA for 3 h, and nuclear extracts were prepared and tested by Western blot analysis. As shown in Fig. 3C, PMA induced the nuclear translocation of p65, c-Jun, and c-Fos, and DHA inhibited the nuclear translocation of NF-κB and c-Jun, dose-dependently. However, DHA did not inhibit the PMA-induced nuclear translocation of c-Fos. Furthermore, the phosphorylation of NF-κB and c-Jun in whole-cell lysates was analyzed by Western blotting. As shown in Fig. 3D, PMA induced the phosphorylation of p65, c-Jun, a major subunit of NF-κB, and AP-1 [41], and DHA inhibited the phosphorylation of p65 and c-Jun in a dose-dependent manner. Because IκB phosphorylation and degradation is a predominant pathway for NF-κB activation [42], we next determined the levels of IκBα proteins in whole-cell extracts. Degradation of IκBα was stimulated by PMA, whereas DHA suppressed PMA-induced degradation of IκBα. To confirm the specificity of DHA-mediated inhibitory effects on NF-κB and AP-1 in HT-1080 cells, cells were transiently transfected with reporter vectors that included tandem repeats of the NF-κB and AP-1 binding sites. As shown in Fig. 3E, treatment of cells with DHA decreased PMA-mediated GFP fluorescence. No cytotoxicity was observed when cells were exposed to DHA (data not shown). Collectively, these findings suggest that DHA-inhibited PMA-induced activation of MMP-9 by suppressing NF-κB and AP-1 activation in HT-1080 cells.

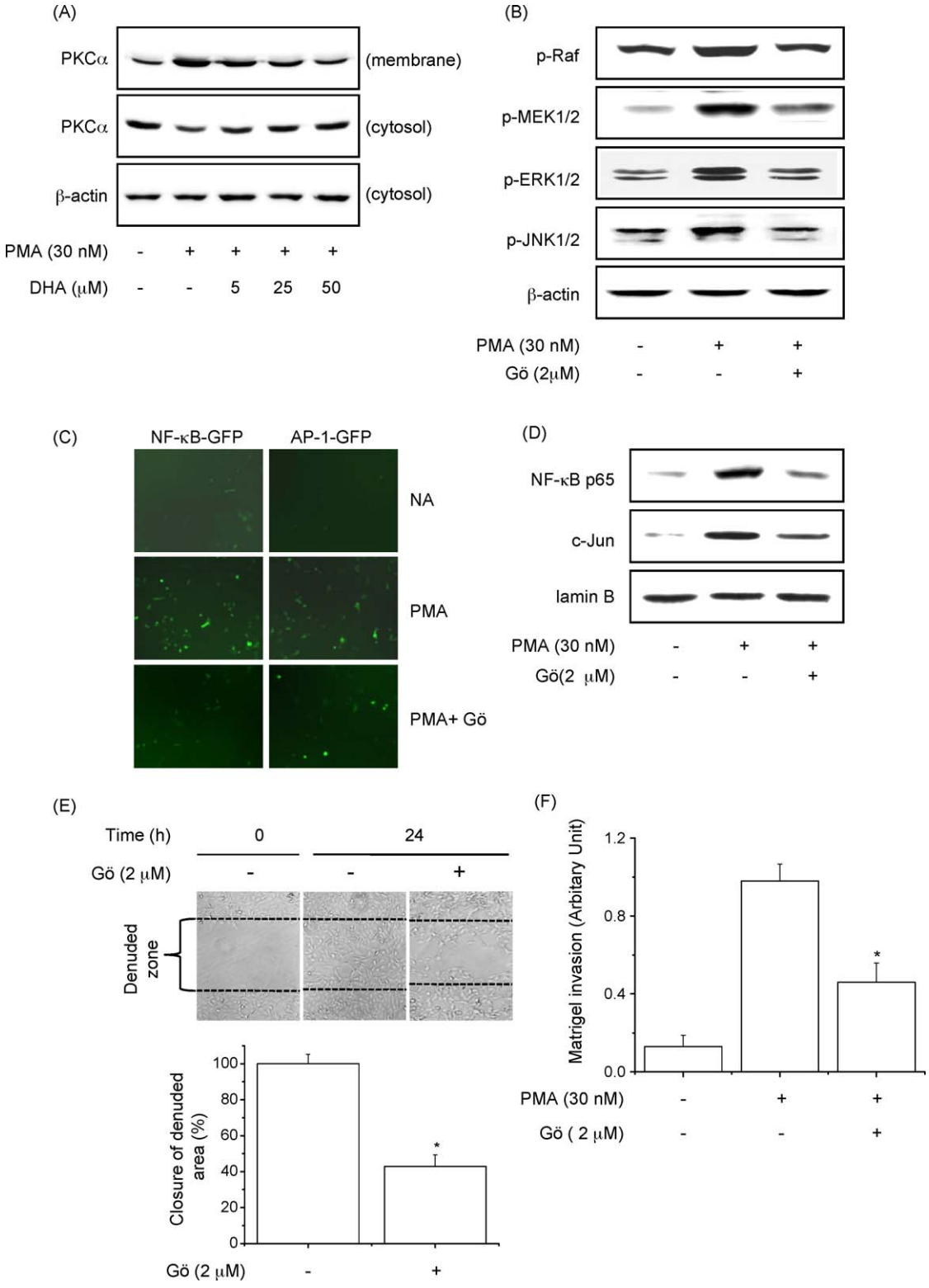

Fig. 6. Effects of DHA on PMA-induced PKCα activation. Cells were treated with DHA (5–50 μM) followed by PMA (30 nM) treatment for 1 h, and the PKCα levels in cytosol and membrane fractions were determined by Western blotting. (B) Cells were stimulated with PMA for 30 min after pretreatment with Gö6976 (Gö, 2 μM) for 1 h, and the levels of phospho-Raf, phospho-MEK, phospho-ERK, and phospho-JNK were determined by Western blotting. (C) Cells were transiently transfected with Lenti-NF-κB-GFP or Lenti-AP-1-GFP. Fluorescence of GFP protein was analyzed by fluorescence microscopy. (D) Cells were pretreated with Gö6976 (Gö, 2 μM) for 1 h followed by PMA stimulation for 3 h. Western blotting was performed to determine the nuclear levels of NF-κB p65 and c-Jun. (E) Cells were scratched with a pipette tip and then treated with Gö6976 (Gö, 2 μM) for 24 h. Migrating cells were photographed under phase contrast microscopy. *Significantly different from control ($P < 0.01$). (F) Cells were pretreated with Gö6976 (Gö, 2 μM), followed by PMA (30 nM) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. *Significantly different from PMA treatment only ($P < 0.01$).

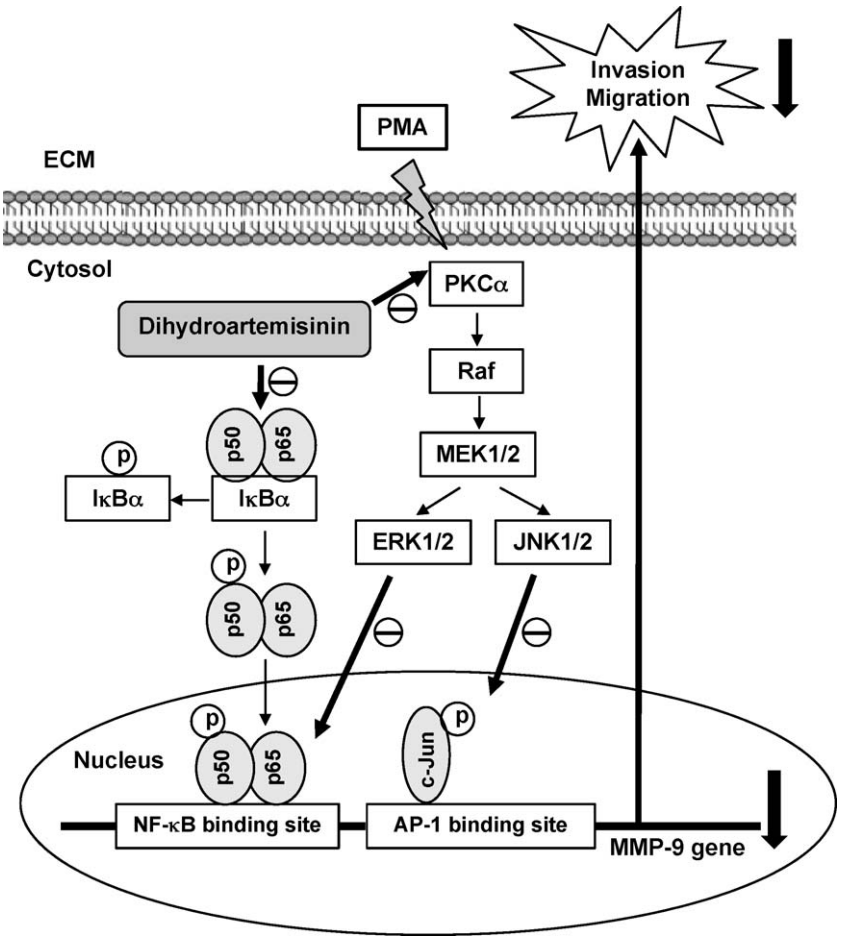

Fig. 7. Proposed signal transduction pathways by which PMA induced and DHA-inhibited invasion and migration of HT-1080 cells. 'O minus' means an inhibitory effect.

3.4. DHA inhibits MMP-9 activation through suppression of PMA-stimulated NF-κB and AP-1 activity

We found that the DHA could inhibit the PMA-induced invasion of HT-1080 cells by suppressing MMP-9 expression (Fig. 1), through regulating the activity of NF-κB and AP-1 (Fig. 3). Next, we further examined whether activation of NF-κB and AP-1 was involved in PMA-induced MMP-9 expression in HT-1080 cells, using a selective NF-κB activation inhibitor and an AP-1 inhibitor, JSH-23 and curcumin [43,44], respectively. HT-1080 cells were pretreated with JSH-23 (20 nM) or curcumin (10 μM) for 1 h and then stimulated with 30 nM PMA in the presence or absence of DHA (50 μM) for 24 h. The culture media were subjected to gelatin zymography and Western blotting. The results (Fig. 4A) indicated that JSH-23 and curcumin inhibited PMA-induced MMP-9 expression and combination treatment with JSH-23 or curcumin and DHA synergistically reduced PMA-induced MMP-9 expression. To test which of these transcription factors regulate the MMP-9 gene in HT-1080 cells, 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. 4B–D). As shown in Fig. 3B, JSH-23 or curcumin inhibited the PMA-induced transcriptional activation of MMP-9 gene and combination treatment with JSH-23 and DHA synergistically reduced PMA-induced transcriptional activation of the MMP-9 gene. Furthermore, as shown in Fig. 4C and D, JSH-23 or curcumin inhibited the PMA-induced NF-κB or AP-1 activities and combination treatment with JSH-23 and DHA synergistically reduced PMA-induced NF-κB or AP-1 activities.

3.5. DHA suppresses PMA-mediated invasion and migration activation through the PKC, ERK, and JNK signaling pathways

MMP-9 gene expression can be activated by a number of signal transduction pathways, including those involving PKCs, ERK1/2, p38 MAPK, and JNK, which are upstream modulators of AP-1 or NF-κB [28,29,44]. Subsequent experiments were designed to determine which of these signal transduction pathways were involved in PMA-stimulated MMP-9 expression and DHA 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 and Western blotting. The effects of specific kinase inhibitors on the promoter activity of MMP-9 in PMA-induced HT-1080 cells were analyzed using luciferase assays. PMA-induced MMP-9 secretion (Fig. 5A) and activity (Fig. 5B) were completely inhibited by inhibitors of PKCs (Gö6976), MEK (U0126), p38 MAPK (SB203580), and JNK (SP600125). Next, we investigated whether DHA inhibited the activation of these signaling pathways. DHA inhibited the phosphorylation of PKCα, ERK, and JNK, but not p38 MAPK, in a dose-dependent manner at 30 min after PMA treatment (Fig. 5C). These results suggest that specific inhibition of PKCα, ERK, and JNK signaling pathways by DHA is directly involved in its effects on PMA-induced MMP-9 expression.

3.6. DHA suppresses invasion and migration of HT-1080 cells by blocking the PKCα/Raf/MAPKs and NF-κB/AP-1 signaling pathways

Activation of PKCs has been correlated with the potential for tumor metastasis [45]. PMA has been reported to be a PKC activator, and activation of the PKC isoforms by PMA has been identified [29,46]. To further evaluate the effect of DHA on the PKC signaling pathway, we examined whether DHA enhanced PMA-mediated modulation of the translocation of PKCα isoenzymes by Western blot analysis. Remarkably, 50 μM DHA had a dramatic effect on the cytosol-to-membrane translocation of PKCα after a 1-h incubation (Fig. 6A). Interestingly, Gö6976, a PKCs inhibitor, inhibited PMA-induced phosphorylation of Raf, MEK, ERK, and JNK (Fig. 6B) and the nuclear translocation of NF-κB p65 and c-Jun (Fig. 6D). Further, treatment of cells with a PKC inhibitor decreased the PMA-mediated GFP fluorescence (Fig. 6C). Collectively, PMA primarily stimulates MMP-9-mediated cell invasion through PKCα-triggered Raf, MEK, ERK, and JNK/NF-κB and AP-1 activation in HT-1080 cells, and these results indicate that DHA suppressed PMA-induced PKCα activation (Fig. 7).

4. Discussion

Artemisinin is a sesquiterpene lactone peroxide containing an endoperoxide moiety. DHA is the main active metabolite of artemisinin derivatives and is more water-soluble and a more effective antimalarial than artemisinin. After the discovery of artemisinin and its derivatives, especially DHA, as a novel and promising treatment for cancer, many studies have investigated the use of DHA in the treatment of cancers [12,13,47,48]. It is well established that tumor cell migration and invasion depend on gelatinase activity. MMP-9 and -2 are important enzymes in the process of tumor metastasis, resulting from ECM degradation [18]. MMPs are highly expressed in human cancer, and a direct relationship between cancer progression and MMPs expression and activity has been well established in many studies. As tumors manifest high levels of MMPs activity, inhibitors specific for the MMPs are highly sought. There are limited reports on the role of artemisinin in MMP-2 inhibition and migration [49]. However, the effect of DHA against PMA-induced MMP-9 expression and invasiveness of fibrosarcoma HT-1080 cells remains unclear. Here, we studied the inhibitory effects of DHA on MMP-9 activity and investigated its inhibitory mechanism in human fibrosarcoma HT-1080 cells. We first evaluated the inhibitory effect of DHA on PMA-induced invasion and migration in HT-1080 cells. PMA is a well-known inflammatory stimulus and tumor promoter that activates almost all PKC isozymes by direct binding. It causes dramatic PKC-mediated induction of invasiveness in cancer cells [29,45,50]. Our data show that DHA-inhibited PMA-induced cell invasion and migration (Fig. 1D and E). As described in previous studies [21,36], treatment with PMA, at concentrations ranging from 10 nM to 1 μM, stimulated MMP-9 secretion in a dose-related manner (data not shown). We also determined the role of MMPs in PMA-induced invasion of HT-1080 cells. Cells were pretreated with GM6001, a broad-spectrum MMPs inhibitor. PMA-induced invasion of HT-1080 cells was inhibited by GM6001 (Supplementary data). These results suggest that MMPs are involved in PMA-induced invasion of HT-1080 cells. Next, we determined the effect of DHA in PMA-induced iMMP-9 and -2 expressions. In PMA-treated HT-1080 cells, DHA suppressed the increased expression (Fig. 2A and E) and secretion of MMP-9 and -2 (Fig. 1C). Protein levels of neither TIMP-1 nor -2 were altered by bergamottin in our system, possibly due to the different systems and concentrations. MT1-MMP has been shown to be a key enzyme in tumor metastasis. The role of MT1-MMP in pericellular proteolysis can play a direct role in ECM turnover [24]. Moreover, it was identified as the first physiological activator of pro-MMP-2 [51]. Therefore, the inhibition of ECM degradation enzymes and cell adhesion to ECM molecules could be considered as a preventive approach for cancer metastasis. In the present study, we found that DHA significantly reduced MT1-MMP (Fig. 2B and C). This result suggests that DHA decreases pro-MMP2 activation through a reduced MT1-MMP level.

The effect of DHA on the activity of the MMP-9 and -2 promoters was investigated using HT-1080 cells that had been transiently transfected with a luciferase reporter gene linked to the MMP-9 and -2 promoter sequences. As shown in Fig. 2D, activity of the MMP-9 promoter was activated, up to ~5-fold, in cells that had been treated with PMA, versus untreated cells. Treatment of cells with DHA decreased the PMA-mediated luciferase activity, dose-dependently.

The MMP-9 promoter contains *cis*-acting regulatory elements for transcription factors, including two AP-1 sites (located at −79 and −533 bp) and an NF-κB site (located at −600 bp) [21]. DHA suppressed MMP-9-induction by repressing transcriptional activation of the MMP-9 promoter (Fig. 3A). Mutational analysis of the promoter revealed that the major target of DHA were both the NF-κB and AP-1 sites, a finding that was further confirmed by the use of reporter plasmids containing synthetic elements specific for these transcription factors (Fig. 3A and B). DHA inhibited the degradation of IκBα protein and the phosphorylation of NF-κB p65 and c-Jun in whole-cell lysates (Fig. 3D). DHA also blocked the translocation of NF-κB subunits, p65 and c-Jun, to the nucleus in PMA-treated HT-1080 cells. However, DHA did not suppress PMA-induced translocation of c-Fos, a component of AP-1 (Fig. 3C). Next, we confirmed NF-κB and AP-1 transcriptional activity in HT-1080 cells, by transiently expressing the NF-κB reporter. As shown in Fig. 3E, DHA decreased the PMA-mediated NF-κB-GFP and AP-1-GFP fluorescence. Next, we investigated the functional significance of NF-κB and AP-1 transactivation in MMP-9 activation in HT-1080 cells. Treatment with JSH-23 and curcumin, potent inhibitors of NF-κB and AP-1 transcriptional activation, respectively, reduced the PMA-induced enzyme activity and protein expression of MMP-9 (Fig. 4C and D). JSH-23 and curcumin also reduced the PMA-induced transcriptional activity of NF-κB and AP-1 (Fig. 4C and D). These findings collectively suggest that DHA inhibits PMA-induced activation of MMP-9 by suppressing both NF-κB and AP-1 activation in HT-1080 cells.

PMA increases the invasiveness of various types of cancer cells by activating MMP-9, via transcription factors, and the PKC, MAPKs, and PI3K/Akt pathways [27–29,52]. However, the distinct mechanisms regulating PMA-induced MMP-9 expression in different cell types remain unclear. To gain a better understanding of the PMA-induced signaling cascade underlying MMP-9 expression in human fibrosarcoma HT-1080 cells, we assessed the effects of specific inhibitors of three MAPKs and PKCs on PMA-induced MMP-9 activity in the conditioned medium and transcriptional activity. PMA-induced MMP-9 activity was more significantly inhibited by treatment with a PKCs inhibitor (Gö6976), a MEK inhibitor (U0126), and a JNK inhibitor (SP600125) than it was by a p38 MAPK inhibitor (SB203580; Fig. 5A and B). These results indicate that MMP-9 expression is primarily regulated by PKCs, MEK, ERK, and JNK, although p38 MAPK may also partially contribute to PMA-induced cell invasion and MMP-9 expression. In this study, we identified the signaling pathway-mediated regulation of the MMP-9 gene in PMA-induced HT-1080 cells in response to DHA treatment. The data presented here show that DHA suppressed PMA-induced phosphorylation of PKCα, ERK and JNK, key pathways in PMA-induced cell invasion via MMP-9 expression (Fig. 5C). It is generally accepted that PKC is the major cellular receptor for PMA. 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 PKCα plays an important role in MMP-9-induction in cancer cells [29]. In this study, PMA stimulation resulted in the translocation of PKCa from the cytosol to the cell membrane (Fig. 6A). Treatment with a non-cytotoxic dose of a PKCs inhibitor (Gö6976) caused marked inhibition of PMA-induced activation of Raf, MEK, ERK, and JNK (Fig. 6B). The PKCs inhibitor also inhibited activation of NF-κB and AP-1 (Fig. 6C and D), as well as of PMA-induced MMP-9 secretion, cell invasion, and migration (Fig. 6E and F). These data indicate that PMA-activated PKCα mediates MMP-9 expression and cell invasion, via Raf/MEK/ERK/NF-κB and JNK/AP-1.

In conclusion, we demonstrated that DHA could effectively inhibit the PMA-induced invasion and migration of HT-1080 cells; thus, DHA may be a useful anti-invasive drug in therapeutic strategies for fibrosarcoma metastasis. Fig. 7 shows the proposed mechanisms for the PMA-induced and DHA-inhibited invasion and migration of HT-1080 cells. The anti-invasive effects of DHA in PMA-induced HT-1080 cells may be through inhibiting the PKCα/Raf/MEK/ERK/NF-κB and JNK/AP-1 cascades, with consequent suppression of MMP-9 expression. With the clarification of signal transduction mediators and transcriptional factors involved in the DHA anti-invasive process of a human fibrosarcoma cell line, it might be possible to develop specific mediators to inhibit undesired cell invasion. DHA should be further tested in *in vivo* models to examine its effectiveness in the prevention of tumor invasion or metastasis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.02.003.

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