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Suppression of PMA-induced human fibrosarcoma HT-1080 invasion and metastasis by kahweol via inhibiting Akt/JNK1/2/p38 MAPK signal pathway and NF-κB dependent transcriptional activities

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ABSTRACT

Coffee is one of the widely sales beverage worldwide and contains numerous phytochemicals that are beneficial to health. Kahweol acetate (KA), a coffee-specific diterpene, exhibits anti-tumoric properties in human tumoric cells. However, the effect of KA on the metastasis and invasion of cancer cells and the underlying mechanisms remain unclear. The objectives of this study were to estimate the anti-tumor activity of KA and reveal the possible molecular mechanisms. KA markedly inhibited the cell proliferation enhanced by phorbol 12-myristate 13-acetate (PMA) in human fibrosarcoma cells. As well as, KA attenuated PMA-induced cell migration and invasion in a concentration-dependent manner. KA suppressed PMA-enhanced activation of matrix metalloproteinase-9 (MMP-9) through suppression of nuclear factor kappa B (NF-κB) activation. KA repressed the PMA-induced phosphorylation of Akt, c-Jun N-terminal kinase (JNK) 1/2, and p38 MAPK, which are signaling molecules upstream of MMP-9 expression. In summary, we demonstrated that the anti-tumor effects of KA might occur through the inhibition of Akt/JNK1/2/p38 MAPK phosphorylation and downregulation of NF-κB activation, leading to a decrease in MMP-9 expression. Thus, KA is a useful chemotherapeutic agent that may contribute to prevent to the metastatic tumor.

1. Introduction

Metastasis is a complex process caused by the movement and attachment of malignant cells from the primary tumor to target tissues. Unfortunately, most newly diagnosed patients display progressed cancer, with malignant tumor cells moving through the blood vessels or spreading through the lymphatic system to distant sites (Saranga-Perry et al., 2014). Therefore, preventing adhesion, migration, and invasion of tumor cells has become the key to cancer therapy. Extracellular matrix (ECM) remodeling is involved in many physiological conditions including metastasis and tumorigenesis (Nagase and Woessner, 1999; Pasco et al., 2005). During tumorigenesis, several proteolytic enzymes contribute to ECM remodeling and degradation of the basement membrane (Nagase and Woessner, 1999; Pasco et al., 2005). MMPs are a family of zinc-dependent proteases and more than 20 different MMPs have been identified, including gelatinases, collagenases, stromalysines, matrilysines, and membrane-bound MMPs. Among the proteolytic enzymes, MMP-2 and MMP-9 can degrade type IV collagen, which is abundant in the ECM. MMP-9 plays an important role in ECM remodeling and degradation of the basement membrane, and is involved in many biological processes such as angiogenesis, cancer, arthritis, inflammation, and cardiovascular disease. MMP-9 is highly expressed in various malignant tumors, and is thought to play a critical role in metastasis and tumorigenesis (John and Tuszynski, 2001).

The transcriptional regulation of MMP-9 is controlled by various inflammatory cytokines, growth factors, and 12-phorbol 13-myristate acetate (PMA) (Tauro et al., 2014). Furthermore, the regulation of MMP-9 activity is controlled by proenzyme activation and inhibition by tissue inhibitors of metalloproteinases-1 (Yamamoto et al., 2015). Recently, it was demonstrated that both sustainable activation of

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Abbreviations

AP-1	Activator protein-1
ECM	Extracellular matrix
ERK1/2	Extracellular signal-regulated kinase 1/2
JNK1/2	c-Jun N-terminal kinase 1/2
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinases
NF-κB	Nuclear factor-kappaB
PMA	Phorbol 12-myristate 13-acetate
STAT3	Signal transducer and activator of transcription 3
VEGF	Vascular endothelial growth factor

transcription factors, such as STAT3 and AP-1, and NF-κB pathways by proinflammatory cytokines, growth factors, and PMA, have provided a strong link to tumor progression (Gray et al., 2014). Furthermore, the nuclear translocation of NF-κB requires the activation of several signal transduction pathways, such as Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) 1/2 pathways, which are critical for increased MMP-9 activation (Cheng et al., 2006; Hong et al., 2005).

Coffee, a highly complex chemical mixture, is reported to contain different potentially bioactive compounds, such as kahweol, cafestol, caffeine, and chlorogenic acid (Spiller, 1984; Schmit et al., 2016). Natural constituents of coffee beans are released from roast and ground coffee beans by hot water. Content of kahweol is different in different types of coffee brews. The content of kahweol has been reported that 0.7–10 mg of Scandinavian boiled, 0.1–7.1 mg of Turkish/Greek, 1.7–5.3 mg of french press, 0.1–2.6 mg of espresso, and 0–0.1 mg of filtered per cup (100 mL) of coffee (Ranheim and Halvorsen, 2005). These compounds exert various medical and pharmacological effects, including anti-cancer (Wang et al., 2016a,b), anti-oxidant (Hwang and Jeong, 2008), and anti-inflammatory activities (Cardenas et al., 2011). Kahweol is a diterpene molecule that is found in high quantities in Arabica coffee beans. Recently, the anti-tumor (Cardenas et al., 2011) and pro-apoptotic effects (Um et al., 2011; Choi et al., 2015) of kahweol have been reported for several types of cancer cells. In addition, our previous studies have reported that kahweol attenuates hydrogen peroxide-induced oxidative damage in NIH3T3 cells (Lee and Jeong, 2007) and protects neurons against 6-OHDA-induced oxidative damage in SH-5Y5Y cells by heme-oxygenase 1 upregulation via phosphatidylinositol 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) pathways (Hwang and Jeong, 2008). We also previously reported that kahweol induces apoptosis and inhibits metastasis by modulating the expression levels of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) via signal transducer and activator of transcription 3 (STAT3) inactivation (Kim et al., 2009, 2012; Lee et al., 2012). Although kahweol has been shown to reduce cell metastasis in tumor cells, the underlying mechanism in human tumorigenic cells is not fully understood.

Nevertheless, the molecular mechanism associated with cancer metastasis by kahweol in cancer cells has not been fully explored. In this study, we examined whether kahweol acetate significantly inhibits MMP-9 expression by inhibiting the Akt/p38 MAPK/JNK1/2 and NF-κB signaling pathways to attenuate the metastasis of human fibrosarcoma cells.

2. Materials and methods

2.1. Chemicals and reagents

Kahweol acetate (KA) and PMA were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), lactate dehydrogenase (LDH), and WST-1-based colorimetric assay kits were purchased from Roche (Mannheim, Germany). JSH-23, LY294002, SB203580, and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). RPMI1640, fetal bovine serum (FBS), sodium pyruvate, penicillin-streptomycin solution, and LipofectAMINE 2000 were supplied by Gibco BRL (Grand Island, NY, USA). Antibodies against phospho-MAPK, total MAPKs, phospho-Akt, Akt, as well as the secondary antibodies (horseradish peroxidase [HRP]-linked anti-rabbit and anti-mouse IgG), were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against and NF-κB p65, β-actin, and Lamin B1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals were of the highest commercially available grade.

2.2. Cell culture and cell viability assay

Human fibrosarcoma HT-1080 cells were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in RPMI1640 containing 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml). KA was dissolved in dimethylsulfoxide (DMSO) for all experiments in this study. The final DMSO concentration never exceeded 0.1%, and the solvent had no noticeable effect on the assays. The effects of KA on the viability and proliferation of cells were assessed using the MTT reduction, LDH release, and WST-1 assay kits according to the manufacturer's instructions.

2.3. Migration assay

Cells were seeded in a 24-well plate and incubated to 90% confluence. The monolayers of cells were scratched with a 200-μl micropipette tip to create a wound. Next, the cells were washed twice with Hank's balanced salt solution to remove floating cells and then were changed to serum-free medium with KA. And then, cells were treated with PMA for 24 h. The rate of wound closure was determined under an inverted light microscope (50 ×; Axiovert-200M; Carl Zeiss, Jena, Germany).

2.4. Invasion assay

Invasion assays were performed using Transwell inserts pre-coated with Matrigel. The inner part of the Transwell chamber was precoated with Matrigel overnight. Cells (5×10^5 cells/ml) were seeded in serum-free medium with KA and added to the upper chamber of a Transwell chamber for 24 h. The lower chamber was filled with 10% FBS in RPMI1640 as a chemoattractant. Migrating cells were stained with crystal violet, imaged, eluted, and transferred to a 96-well plate for absorbance readings at 595 nm. The membrane inserts were dried and observed under a microscope (100 ×; Axiovert-200M; Carl Zeiss, Jena, Germany).

2.5. Gelatin zymography

Cells were pre-treated with different concentrations of KA in the presence of PMA (20 nM) for 24 h. The conditioned medium was collected, centrifuged to remove cell debris, and concentrated using Centricon YM-30 concentrator (Millipore, USA). After the protein concentrations were determined, gelatin zymography samples were separated by 8% SDS-PAGE containing 0.2% gelatin under non-reducing conditions. Following electrophoresis, the gel was washed twice with renaturing buffer (50 mM Tris-Cl at pH 7.5, 100 mM NaCl, and 2.5% Triton X-100), followed by brief rinsing in washing buffer without Triton X-100, and incubated overnight in developing buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 200 mM NaCl, 0.2% Brij-35) at overnight. The gel was stained with 0.5% coomassie brilliant blue G250

(Sigma-Aldrich Co.) in 40% methanol and 10% acetic acid. The gelatinolytic activity of MMPs was represented by a clear zone of gelatin digestion.

2.6. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from KA-treated cells using an RNAiso reagent (Takara Shuzo, Kyoto, Japan) according to the manufacturer's protocol. Accumulated PCR products were detected directly by monitoring the increase in the reporter dye (SYBR®) signal. The quantity of each transcript was calculated according to the manufacturer's instructions and normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene.

2.7. Luciferase and β-galactosidase assays

Cells were incubated in 24-well (5×10^5 cells/well) plates for overnight and transiently co-transfected with the MMP-9 promoter vector, AP-1 $(5'\text{-TGACTAA-}3')_7$ or NF-κB $(5'\text{-GGGGACTTTCC-}3')_5$ reporter vector (1 μg), and pCMV-β-gal (0.2 μg) using Lipofectamine™ 2000 reagent according to the manufacturer's instructions. After 4 h, the transfection medium was replaced with basal medium. The cells were then treated with KA and PMA for 24 h. The cells were washed with PBS, and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron Co., Germany). Luciferase activities were normalized to β-galactosidase and expressed relative to the luciferase activity of control cells. The MMP-9 promoter vector was kindly provided by Dr. W. Eberhardt (Klinikum der Johann Wolfgang Goethe-Universitat, Frankfurt am Main, Germany) (Eberhardt et al., 2002). The pNF-kB-Luc and pAP-1-Luc reporter plasmids were obtained from Stratagene (La Jolla, CA, USA).

2.8. Preparation of the nuclear fraction

KA-treated cells were washed with ice-cold PBS and 100 μL of lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% NP-40, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. Cell membranes were disrupted by vortexing, and the lysates were incubated for 5 min on ice and centrifuged (7200 × g, 5 min). Pellets containing crude nuclei were resuspended in 50 μL of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, and incubated for 30 min on ice. The samples were centrifuged (15,800 × g, 30 min) to obtain supernatants containing the nuclear extracts. The nuclear extracts were stored at − 80 °C until use.

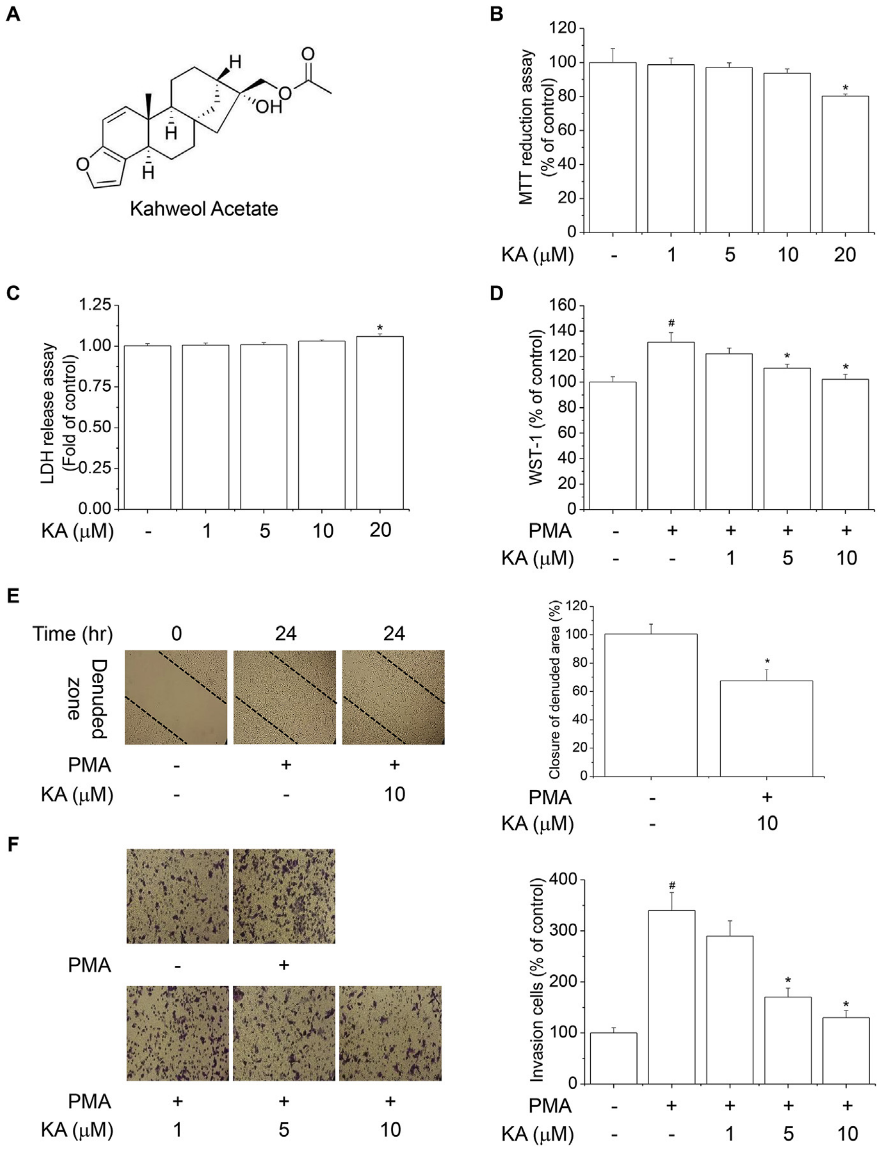

Fig. 1. Inhibitory effects of KA on the viability, proliferation, migration, and invasion in human fibrosarcoma cells. (A) Chemical structure of kahweol acetate. (B and C) Effect of kahweol acetate (KA) on cells viability in human fibrosarcoma cells. Cells were treated with various concentrations of KA at 37 °C for 24 h, and cell viability was determined by the MTT reduction and LDH release assays. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. *Significantly different from the control ($P < 0.01$). (D) Effect of kahweol acetate (KA) on cells proliferation in cells. Cells were pretreated with various KA (1, 5, and 10 μM) concentrations for 1 h, and then stimulated with PMA (20 nM) for 24 h. Cell proliferation was determined by the WST-1 kit. (E) Effects of KA on migration in cells. Cells were cultured to an 90% confluent monolayer in a 24-well plate, which was scratched with a 200-μl micropipette tip. After washing with serum free medium, the cells were pretreated with various KA (1, 5, and 10 μM) concentrations for 1 h, and then stimulated with PMA (20 nM) for 24 h to investigate the cell migration. The denuded zone was observed at 0 and 24 h, and images were photographed using a microscope (50 × magnification). (F) Effects of KA on invasion in cells. Cells were pretreated with different concentrations of KA (1, 5, and 10 μM) followed by PMA (20 nM) treatment for 24 h. After 24 h, cells on the bottom side of the filter were counted. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. #Significantly different from the control ($P < 0.01$). *Significantly different from the PMA-treated group ($P < 0.01$).

2.9. Western blotting

The cell lysates, nuclear fraction, or concentrated medium samples were separated by SDS-PAGE and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and incubated with the appropriate primary and HRP-conjugated secondary antibodies (Cell signaling technology, Beverly, MA, USA). Membranes were visualized using an enhanced chemiluminescence Western blotting detection kit (iNtRON Biotechnology Co., Ltd., Korea). Band intensity was quantified by densitometry using Image J software (Image Processing and Analysis in Java, NIH, USA). The relative expression levels of target protein were normalized using β-actin or Lamin B1 as an internal control.

2.10. Statistical analysis

All experiments were performed in triplicate. Results reported are means ± the standard deviation (SD). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test, with $p < 0.01$ indicating significance.

3. Results

3.1. KA reduces cells proliferation

To determine the anti-tumor effects of kahweol acetate (KA) (Fig. 1A), we first determined the cytotoxic effects in human fibrosarcoma cells following treatment with various KA concentrations. KA at concentrations lower than 10 μM exhibited no cytotoxic effect on the cells (Fig. 1B and C). To explore the effect of KA on the PMA-induced cells proliferation treated with KA (1, 5, and 10 μM), cell proliferation was measured using the WST-1 assay kit. As shown in Fig. 1D, KA suppressed PMA-induced cells proliferation in a concentration-dependent manner.

3.2. KA inhibits cells migration and invasion

To investigate the effect of KA on the invasion and migration of cancer cells, the Transwell invasion assay and wound healing assay were performed in cells. In the wound healing assay, KA treatment suppressed the migration of cells across the wounded space (Fig. 1E). In addition, the invasion ability of cells was inhibited depending on the treatment concentration of KA (Fig. 1F).

3.3. KA inhibits MMP-9 expression and activity

Previous studies have reported that the invasion and migration of tumor cells are typical features of cancer metastasis, and these features are related to increased expression of MMPs. Therefore, we examined the effects of KA on expression and enzymatic activity of MMP-9 associated with cancer cell metastasis in cells. KA inhibited PMA-induced MMP-9 mRNA expression in a concentration-dependent manner (Fig. 2A). Because the expression and activity of MMP-9 are regulated by endogenous inhibitors, tissue inhibitor of metalloproteinases (TIMPs), we assessed the level of TIMP-1 mRNA expression (Groblewska et al., 2012). KA had no effect on TIMP-1 mRNA expression at the same concentrations tested (Fig. 2B). We then analyzed the protein activity and expression of MMP-9 secreted in culture media. KA treatment inhibited the enzyme activity and protein expression of MMP-9 by PMA-stimulated in a concentration-dependent manner as shown by gelatin zymography and western blot (Fig. 2C). Next, we studied the effect of KA on the levels of MMP-9 luciferase activity stimulated by PMA. As shown in Fig. 2D, pretreatment of KA decreased PMA-mediated luciferase activity in a concentration-dependent manner. These results indicate that KA exerts an inhibitory effect on the metastasis and tumorigenic cancer cells by inhibiting MMP-9 activity.

3.4. KA inhibits MMP-9 expression via the suppression of PMA-induced NF-κB activity

Transcriptional factors such as NF-κB and AP-1 are centrally involved in the regulation of MMP-9 gene expression in various cell types

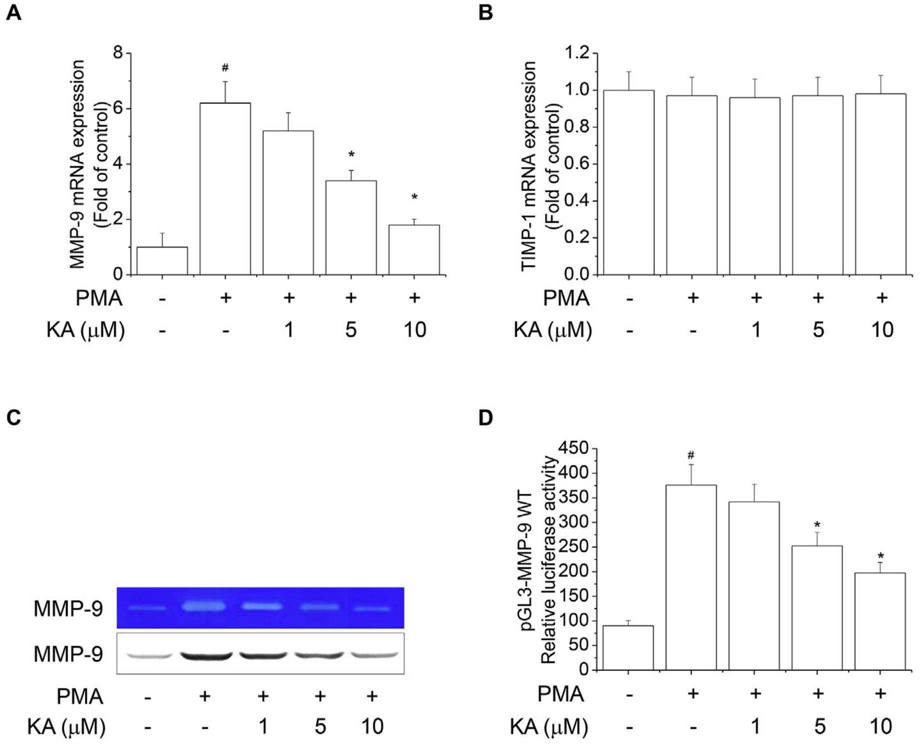

Fig. 2. Inhibitory effects of KA on PMA-induced matrix metalloproteinase (MMP)-9 expression and activity in human fibrosarcoma cells. (A and B) Effect of KA on PMA-induced MMP-9 and TIMP-1 expression in cells. Cells were pretreated with the indicated concentrations of KA (1, 5, and 10 μM) in serum-free medium for 1 h, and then stimulated with PMA (20 nM) for 24 h. MMP-9 and TIMP-1 mRNA expression was assessed by real-time polymerase chain reaction analysis in cells. (C) Effect of KA on PMA-induced MMP-9 expression and activity in cells. Cells were pretreated with the indicated concentrations of KA (1, 5, and 10 μM) in serum-free medium for 1 h, and then stimulated with PMA (20 nM) for 24 h. The conditioned media were collected and concentrated, and gelatin zymography and western blot was performed. (D) Effect of KA on PMA-induced MMP-9 luciferase activity in cells. Cells were transiently transfected with a wild-type (WT)-MMP-9 promoter-containing reporter vector and incubated with various concentrations of KA in the presence of PMA (20 nM) as indicated. Luciferase activity was measured 24 h after transfection. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. $^{\#}$Significantly different from the control ($P < 0.01$). *Significantly different from the PMA-treated group ($P < 0.01$).

(Lee et al., 2015; Woo et al., 2004). To evaluate which transcription factors may regulate MMP-9 gene expression in cells, cells were transiently transfected with reporter genes that included the wild-type MMP-9 promoter or a promoter with mutations in the AP-1 or NF-κB site (Fig. 3). As shown in Fig. 3A, pretreatment of cells with KA decreased PMA-induced wild-type MMP-9 luciferase activity in a concentration-dependent manner. To further investigate the promoter structure used by KA, we transfected cells with pNF-κB-Luc or pAP-1-Luc plasmid DNAs. Following treatment with KA in the presence of PMA, the transcriptional activity of the reporter with the AP-1 mutations was inhibited, suggesting that the targets of KA were the NF-κB transcription factor in MMP-9 reporter gene (Fig. 3B and D). Continuously, we assessed the inhibitory effect of KA on reporter gene used by NF-κB- or AP-1-responsive elements, respectively. As shown in Fig. 3E and F, KA suppressed NF-κB-responsive promoters in a concentration-dependent manner. These results showed KA inhibits PMA-enhanced MMP-9 expression via regulation of NF-κB transcription.

To better understand the signaling pathways affected by KA treatment, NF-κB and AP-1, the two major molecular pathways involved in PMA-induced MMP-9 expression, were investigated. As shown in Fig. 4A, cells were incubated with various concentrations of KA (1, 5, and 10 μM) in the presence of PMA (20 nM) for 3 h, and nuclear lysates were prepared and analyzed by Western blot. KA significantly reduced the PMA-induced nuclear translocation of NF-κB p65 in a concentration-dependent manner without affecting c-Jun and c-Fos (Fig. 4A). Also, we showed that KA pretreatment was inhibited the IκBα, NF-κB p65 inhibitor, proteasomal degradation in cells (Fig. 4B). To further confirm the role of NF-κB in the reduction of MMP-9 expression caused by KA, we used JSH-23 (20 nM), a selective NF-κB activation inhibitor (Shin et al., 2004). As shown in Fig. 4C and D, pretreating cells with JSH-23 effectively inhibited PMA-induced MMP-9 expression and activity. Furthermore, combination treatment with JSH-23 and KA synergistically reduced PMA-induced MMP-9 expression and activity. These results indicate that NF-κB signaling pathway accounted for the anti-tumor effect of KA.

3.5. KA inhibits MMP-9 activation via the Akt, p38 MAPK, and JNK1/2 signaling pathways

To understand the upstream modulator of NF-κB, we examined whether Akt and MAPKs are involved in KA-reduced MMP-9 expression and activity. Western blot analysis showed that the phosphorylation of Akt, p38 MAPK, and JNK1/2, but not that of ERK1/2, was inhibited by KA in cells (Fig. 5A). To clarify whether Akt, p38 MAPK, and JNK1/2 were involved in KA-reduced MMP-9 activity, cells were treated with PMA in the presence of the Akt inhibitor LY294002, the p38 MAPK

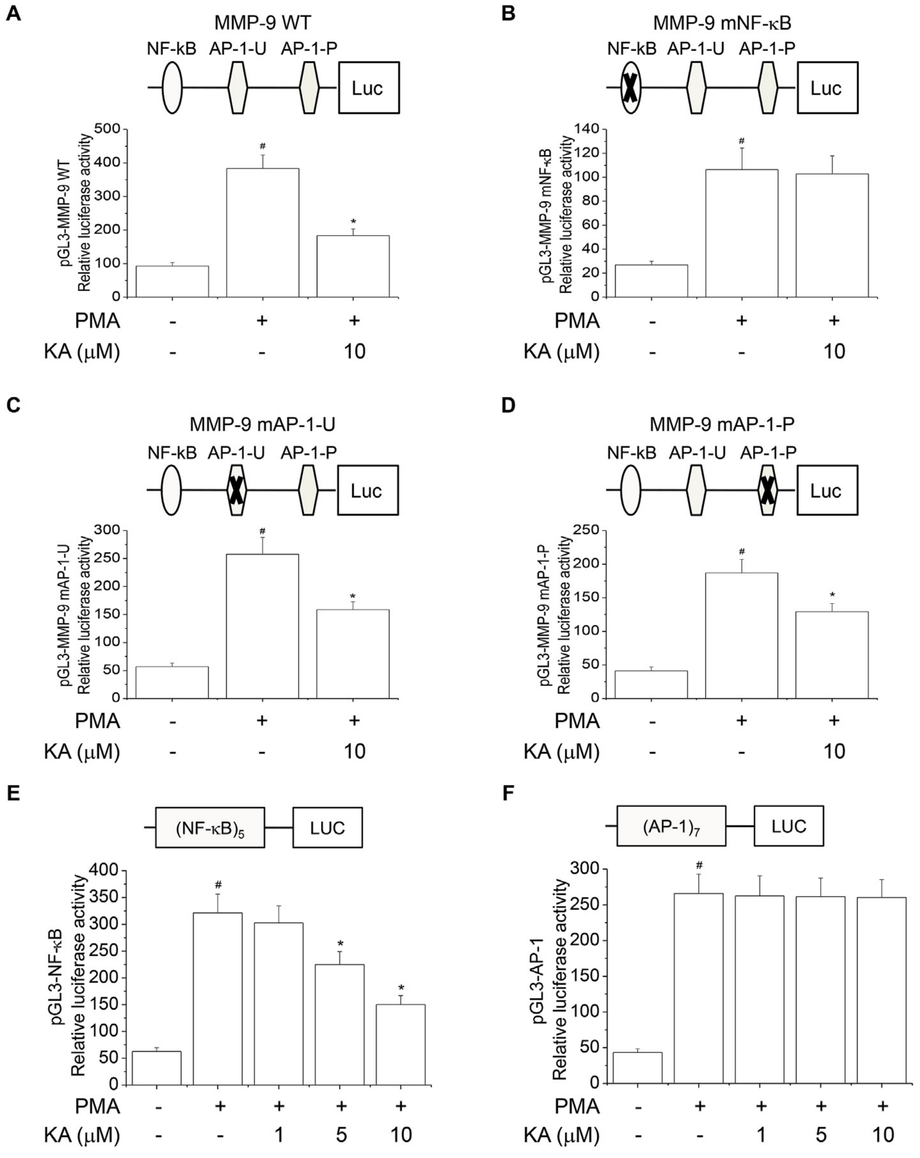

Fig. 3. Inhibitory effects of KA on PMA-induced MMP-9 activity via suppression of nuclear factor kappa B (NF-κB) in human fibrosarcoma cells. (A–F) Effect of KA on PMA-induced MMP-9 luciferase activity in cells. Cells were transiently transfected with pGL3-MMP-9 WT, pGL3-MMP-9 mNF-κB, pGL3-MMP-9 mAP-1-U, or pGL3-MMP-9 mAP-1-P reporter plasmids or with reporter plasmids containing tandem NF-κB or AP-1 binding sites. Cells were cultured with KA (10 μM) and/or PMA (20 nM) for 24 h, and the relative luciferase activity in the cell extract was determined. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. $^{\#}$Significantly different from the control ($P < 0.01$). *Significantly different from the PMA-treated group ($P < 0.01$).

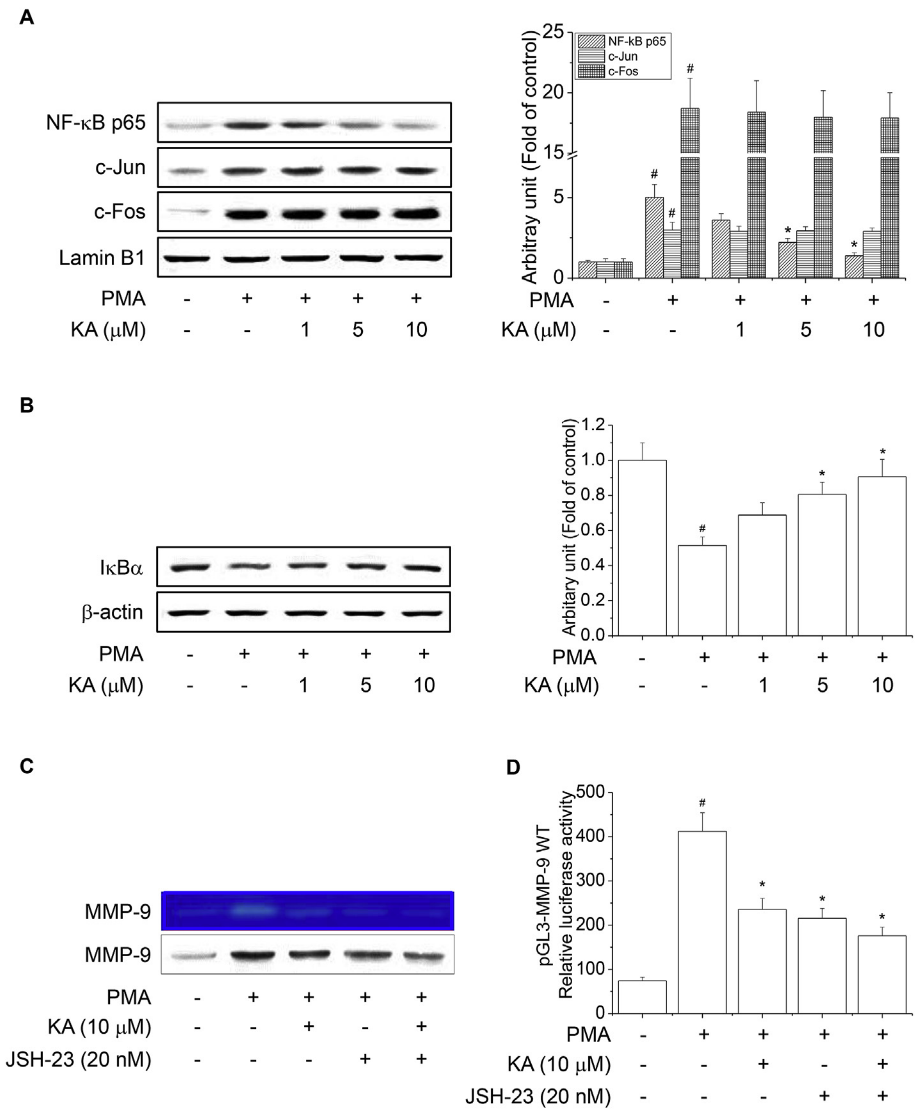

Fig. 4. Inhibitory effects of KA on PMA-induced MMP-9 expression through inhibition of NF-κB in human fibrosarcoma cells. (A) Effects of KA on PMA-induced NF-κB nuclear translocation in cells. Cells were pretreated with KA (1, 5, and 10 μM) for 1 h and treated with 20 nM PMA for 3 h. The nuclear extracts were subjected to SDS-PAGE, followed by Western blotting with NF-κB p65, c-Jun, c-Fos, and Lamin B1 antibodies. The intensity of western blot bands was measured using the NIH Image J program. (B) Effects of KA on PMA-induced IκBα proteasomal degradation in cells. Cells were pretreated with KA (1, 5, and 10 μM) for 1 h and treated with 20 nM PMA for 0.5 h. The total cellular protein were subjected to SDS-PAGE, followed by Western blotting with IκBα and β-actin antibodies. (C) Effects of KA on PMA-induced MMP-9 expression and activity via inhibition of NF-κB in cells. Cells were treated with 20 nM PMA for 24 h in the presence of KA and/or JSH-23. Conditioned media were collected after 24 h. The conditioned media were collected and concentrated, and gelatin zymography and western blot was performed. (D) Effect of KA on PMA-induced MMP-9 luciferase activity in cells. Cells were transiently transfected with pGL3-MMP-9 WT. Cells were treated with 20 nM PMA for 24 h in the presence of KA and/or JSH-23, and the relative luciferase activity in the cell extract was determined. The level of protein expression was analyzed by image j software. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. $^{\#}$Significantly different from the control ($P < 0.01$). *Significantly different from the PMA-treated group ($P < 0.01$).

inhibitor SB203580, and the JNK1/2 inhibitor SP600125. As shown in Fig. 5B and C, PMA-induced MMP-9 expression and activity were dramatically inhibited following treatment with the Akt, p38 MAPK, and JNK1/2 inhibitors. And, combination treatment with LY294002, SB203580, SP600125, and KA synergistically reduced PMA-induced MMP-9 expression and activity. These results demonstrated that the Akt, p38 MAPK, and JNK1/2 signaling pathways are involved in the regulation of PMA-induced MMP-9 expression and activity by KA.

4. Discussion

Metastasis and invasion of tumor cells are hallmarks of malignant tumors. Many anti-tumor drugs are believed to prevent the progression of tumor cells by modulating cell proliferation or metastasis (Wang et al., 2016a,b). However, these agents also have severe side effects, which limit their clinical treatment of cancer. Recent accumulating evidence has highlighted the coffee diterpene kahweol as a therapeutic agent in the treatment of malignant tumors. In particular, kahweol suppresses cell proliferation by inducing proteasomal degradation of cyclin D1 via regulation of ERK1/2, JNK, and GKS3β-dependent threonine-286 phosphorylation in human colorectal cancer cells (Park et al., 2016). We also previously reported that KA induces apoptosis and inhibits metastasis by modulating VEGF expression via STAT3 inactivation (Kim et al., 2009, 2012). These previous studies focused on proliferation, invasion, and metastasis in cancer cell lines. While recent attention for KA has focused on an anticancer effect, the mechanism responsible for its anticancer effects is not fully understood. In this paper, we determined the effect of KA on the metastasis or invasion of cancer cells and elucidated the molecular mechanism for KA-mediated MMP-9 downregulation via AP-1 or NF-κB signaling pathways in cancer cells.

Cancer cells are characterized by uncontrolled growth, metastatic spread to distant target tissues, and invasion to surrounding tissues (Cheng et al., 2006). In this study, we used KA at various concentrations to investigate whether it inhibits tumor cell proliferation in cells. Our data show that KA reduced cells proliferation in a concentration-dependent manner. KA also inhibited the cells migration and invasion in a concentration-dependent manner. To clarify the molecular mechanism underlying the anti-metastatic effects of KA, the key questions of this study asked whether KA downregulates MMP-9 expression in fibrosarcoma cells, because MMP-9 overexpression has been shown to be associated with the migration and invasion of tumor cells (John and Tuszynski, 2001). To further determine the molecular pathway of KA-induced inhibition of cell invasion and migration, we tested its effect on the expression and activity of MMP-9 using RT-PCR, western blot, and gelatin zymography. Our results demonstrated that KA inhibits PMA-induced MMP-9 enzymatic activity through suppression of the transcriptional and translational activity of MMP-9 in cells. These results

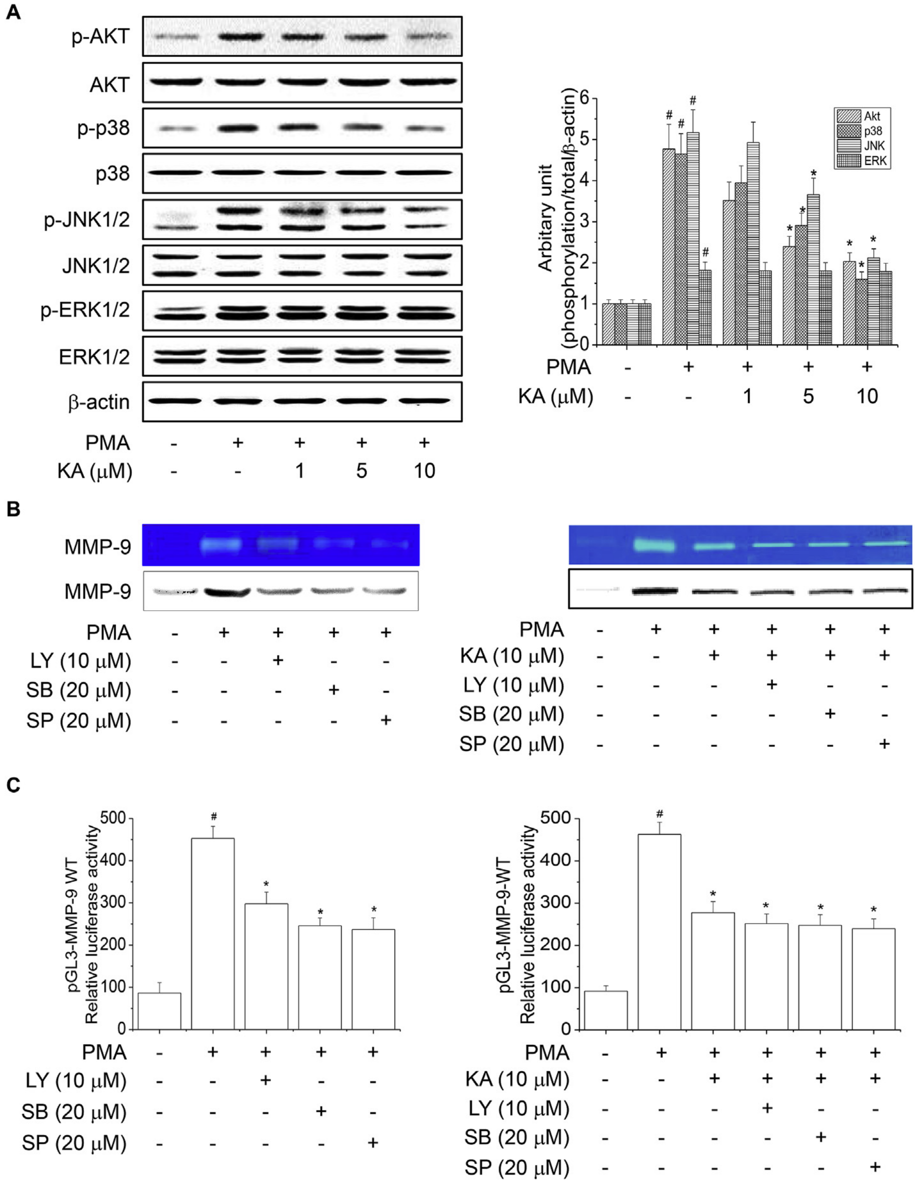

Fig. 5. Inhibitory effects of KA on the PMA-induced MMP-9 expression through inhibition of Akt and MAPK signaling pathways in human fibrosarcoma cells. (A) Effects of KA on PMA-induced Akt, p38 MAPK, and JNK 1/2 phosphorylation in cells. Cells were pretreated with KA (1, 5, and 10 μM) for 1 h and treated with 20 nM PMA for 30 min. The total cellular lysates were subjected to SDS-PAGE, followed by Western blotting with phospho- and total- Akt, ERK1/2, JNK1/2, p38 MAPK, and β-actin antibodies. The intensity of western blot bands was measured using the NIH Image J program. (B) Effects of KA on PMA-induced MMP-9 expression and activity via inhibition of Akt, p38 MAPK, and JNK 1/2 in cells. Cells were treated with 20 nM PMA for 24 h in the presence of KA and/or LY294002 (LY; 10 μM), SP600125 (SP; 20 μM), or SB203580 (SB; 20 μM). The conditioned media were collected and concentrated, and gelatin zymography and western blot was performed. (C) Effect of KA on PMA-induced MMP-9 luciferase activity in cells. Cells were transiently transfected with pGL3-MMP-9 WT. Cells were treated with 20 nM PMA for 24 h in the presence of KA and/or LY294002 (LY; 10 μM), SP600125 (SP; 20 μM), or SB203580 (SB; 20 μM), and the relative luciferase activity in the cell extract was determined. The level of protein expression was analyzed by image j software. Results are presented as mean ± standard deviation (SD) of data from three independent experiments. $^{\#}$Significantly different from the control ($P < 0.01$). *Significantly different from the PMA-treated group ($P < 0.01$).

suggest that KA is a strong candidate for the treatment of tumor cell metastasis via the inhibition of MMP-9 activity.

It is well known that the initial response of the AP-1 and NF-κB signaling pathways triggered by PMA or cytokines such as tumor necrosis factor-α and interleukin-6 provide a mechanistic link to cancer and relevant chemotherapeutic approaches. Thus, we further investigated the mechanism of the anti-metastatic effects exerted by KA in the presence of PMA stimuli. Our results show that KA could significantly prevent the NF-κB signal activation cascades. To better understand the role of NF-κB in the decreased expression of MMP-9 caused by KA, this study used JSH-23, a selective NF-κB activation inhibitor. Treatment with the NF-κB activation inhibitor reduced PMA-induced enzymatic activity, protein expression and transcriptional activity of MMP-9. These results suggest that KA inhibits PMA-induced activation of MMP-9 by suppressing NF-κB activation in cells.

Akt and MAPKs, including ERK1/2, p38 MAPK, and JNK1/2, are important modulators of signaling pathways and are associated with a series of physiological processes such as cell proliferation, metastasis, and MMP-9 activation (Hong et al., 2005; Cheng et al., 2006). The expression of MMP-9 can be regulated by various stimulators, such as inflammatory cytokine, growth factor, and PMA. These stimulators can upregulate the expression of MMP-9 by modulating the activation of transcription factors such as AP-1 and NF-κB through the Akt, ERK1/2, JNK1/2, and p38 MAPK signaling pathways. In previous studies, KA was found to suppress cell proliferation and metastasis via the downregulation of MAPK and STAT3 signaling pathways in human colorectal cancer cells (Kim et al., 2012; Park et al., 2016). PMA is a well-known stimulator that increases the invasiveness of various types of cancer cells by activating MMP-9. Several studies have reported that agents suppressed PMA-induced MMP-9 expression by inhibition of NF-κB transcriptional activity via blocking of JNK1/2 and p38 MAPK signal pathways, not ERK1/2 (Cho et al., 2010; Kim et al., 2016; Ko et al., 2018). Our data shows that KA reduced the phosphorylation of Akt, p38 MAPK, and JNK1/2. We also confirmed that PMA-induced MMP-9 activation was decreased by Akt, p38 MAPK, and JNK1/2 inhibitors, which are major regulators of MMP-9 expression. These results are like to our provided evidences that kahweol suppresses PMA-induced MMP-9 expression and activity by inhibiting of NF-κB transcriptional activity via blocking of Akt, p38 MAPK, and JNK1/2 signaling pathways.

In conclusion, the present study provides evidences for the anti-metastatic effects of kahweol acetate (KA) in human fibrosarcoma cells. KA suppresses PMA-induced MMP-9 expression and activity by

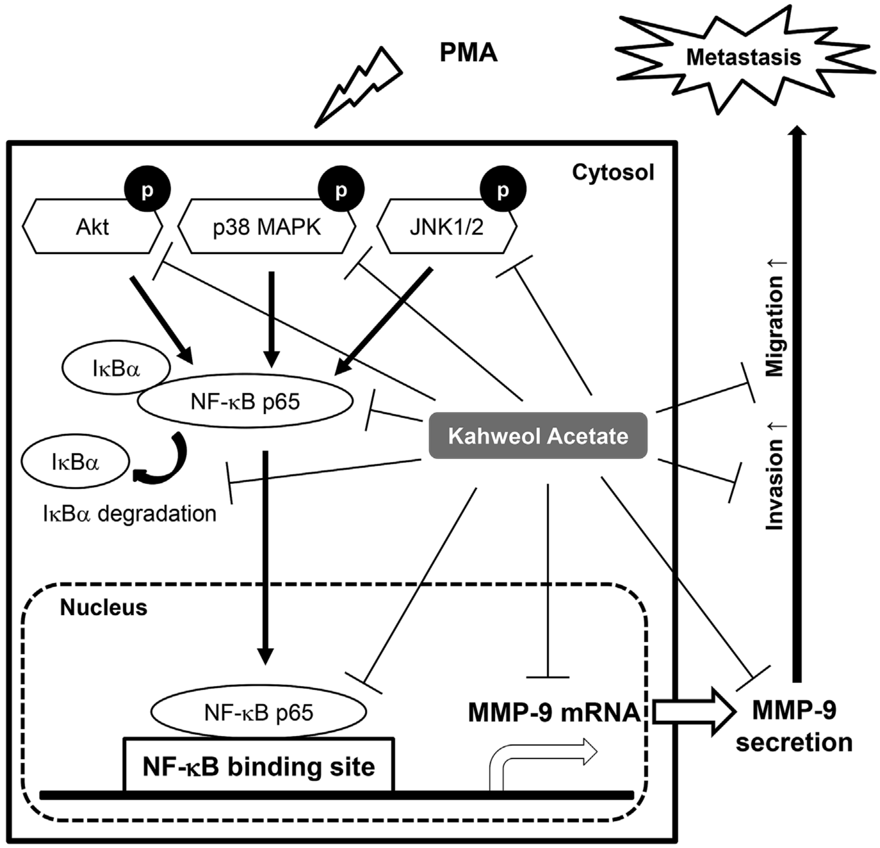

Fig. 6. Schematic highlights of the inhibitory effects of kahweol acetate (KA) on PMA-induced cells migration and invasion in human fibrosarcoma cells. KA significantly suppressed MMP-9 expression of human fibrosarcoma cells via blocking the Akt, p38 MAPK, and JNK 1/2 signaling pathways and NF-κB activity, with consequent suppression of tumorigenic cell invasion and migration. Therefore, KA has potential as a potent anticancer agent in the treatment of metastasis.

inhibiting the activation of NF-κB transcriptional activity via the Akt, p38 MAPK, and JNK1/2 signaling pathways (Fig. 6). We suggest that KA, diterpene found in coffee beans, could be a useful candidate for the discovery of new chemotherapeutic agents that may contribute to block the metastatic tumor.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.038.

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