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ARTICLE

α-Pinene inhibits tumor invasion through downregulation of nuclear factor (NF)-κB-regulated matrix metalloproteinase-9 gene expression in MDA-MB-231 human breast cancer cells

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Abstract 2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (α-Pinene) is an organic compound of the terpene class found in the essential oil of many plants. In this study, the inhibitory effect of α-pinene on tumor invasion in highly metastatic MDA-MB-231 human breast cancer cells was evaluated. α-Pinene inhibited tumor necrosis factor (TNF)-α-induced invasiveness of MDA-MB-231 cells as revealed by three-dimensional spheroid invasion assay. Further analysis showed that α-pinene reduced TNFα-induced matrix metalloproteinase-9 gene promoter activation and mRNA expression in a dose-dependent manner. In addition, α-pinene treatment attenuated TNFα-induced nuclear factor κB (NF-κB) activation and NF-κB-dependent transcriptional activity. These results suggest that α-pinene has a significant effect on the inhibition of tumor invasion and may potentially be developed into an anti-metastatic drug.

Keywords 2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene · Breast cancer · Matrix metalloproteinase-9 · Nuclear factor kappa B · Tumor invasion · Tumor necrosis factor alpha

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Introduction

Tumor necrosis factor alpha (TNFα) is one of the major cytokines involved in controlling systemic inflammation and is produced by various types of cells, including macrophages, lymphocytes, natural killer cells, neutrophils, mast cells, and fibroblasts. Tumor cells at a primary site interact with nearby stromal cells creating tumor microenvironment (Balkwill et al. 2012), which may influence tumor growth, invasion, and metastasis (Friedl and Alexander 2011). In tumor microenvironment, TNFα is produced by tumor and tumor-associated stromal cells, which in turn promote tumor invasion and metastasis through proteolysis of extracellular matrix proteins (Balkwill 2009). Matrix metalloproteinases are zinc-containing proteases involved in tissue remodeling by degrading extracellular matrix proteins. Among them, matrix metalloproteinase-9 (MMP-9) is a 92 kDa type IV collagenase (also known as gelatinase-B) that plays a critical role in the migration and invasion of tumor cells through the breakdown of basement membranes (Deryugina and Quigley 2006).

2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene (α-Pinene) (Fig. 1(A)) is a naturally occurring monoterpene commonly found in essential oils of rosemary and many species of pine trees, and may possess anti-inflammatory, bronchodilator, hypoglycemic, sedative, antioxidant, and broad-spectrum antibiotic activities (Mercier et al. 2009; da Silva et al. 2012). In a recent study, α-pinene isolated from pine needle oil showed anti-proliferative effects on hepatic carcinoma BEL-7402 cells through induction of cell cycle arrest at the G2/M phase (Chen et al. 2015). However, the effect of α-pinene on tumor invasion is currently unknown. In this study, we examined the effect of α-pinene on the expression of MMP-9 mRNA in highly metastatic MDA-MB-231 human breast cancer

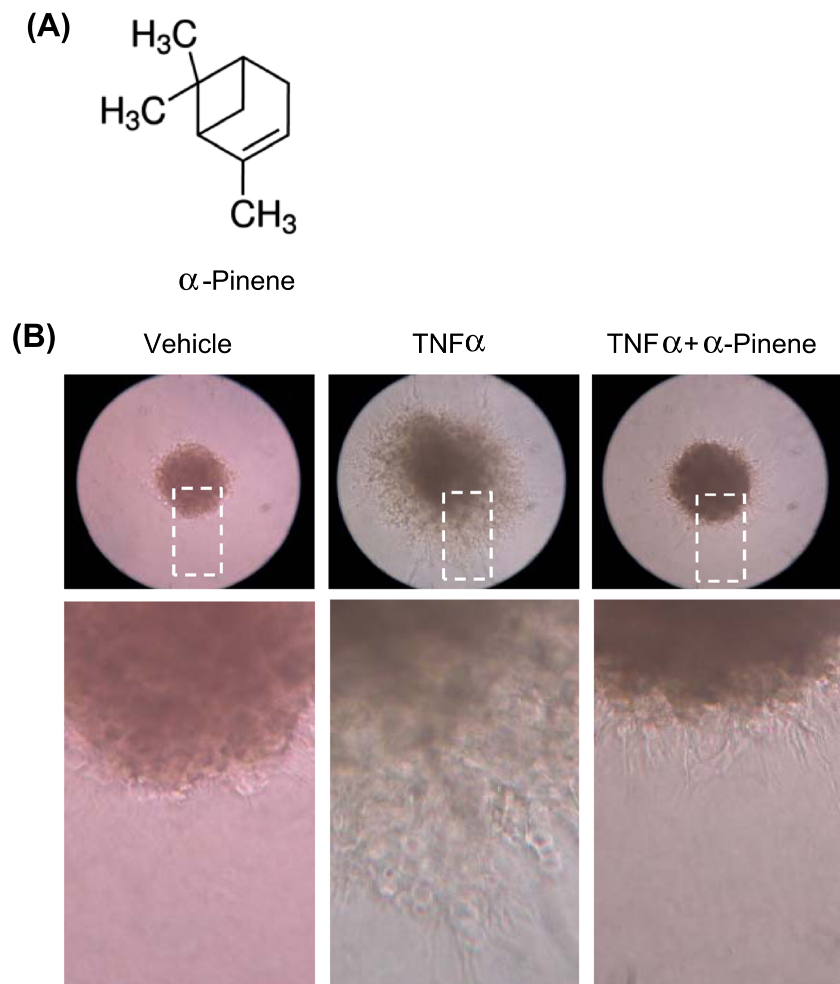


Fig. 1 Inhibitory effect of α-pinene on the invasion of MDA-MB-231 cells. (**A**) Chemical structure of α-pinene. (**B**) MDA-MB-231 cells growing in 3-D spheroids in the extracellular matrix were treated with vehicle (DMSO) or 10 ng/mL TNFα in the absence and presence of 50 µM α-pinene

cells. Our results reveal that α-pinene inhibits TNFα-induced MMP-9 gene expression and invasive capability of MDA-MB-231 cells through the inhibition of nuclear factor kappa B (NF-κB) activity.

Materials and methods

Cells and chemicals

MDA-MB-231 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium containing 10 % HyClone™ fetal bovine serum (Thermo Scientific, USA) at 37 °C in a 5 % CO_2 atmosphere. α-Pinene and TNFα were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Three-dimensional (3-D) spheroid cell invasion assay

Three-dimensional invasion assay was performed using Cultrex 3-D Spheroid Cell Invasion Assay kit (Trevigen, Inc., Gaithersburg, MD, USA) as described previously (Lee et al. 2015). Briefly, MDA-MB-231 cells were cultured for 7 days in a spheroid formation extracellular matrix to drive aggregation and spheroid formation of cells, followed by the addition of an invasion matrix composed of basement membrane proteins and medium, with and without 10 ng/mL TNFα in the absence and presence of 50 µM α-pinene. Cell invasion was visualized with a Nikon Eclipse TS100

microscope (Nikon Instruments Inc., Tokyo, Japan) equipped with a digital sight camera.

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time PCR

MDA-MB-231 cells were treated with different concentrations of α-pinene for 18 h, and total RNA was extracted using Isol-RNA lysis reagent (5 PRIME, Gaithersburg, MD, USA). The first-strand cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instruction. PCR was performed as described previously (Shin et al. 2013b). The amplified products were subjected to electrophoresis in a 1 % agarose gel. Relative expression levels of mRNAs were measured by quantitative real-time PCR with a TaqMan-iQ^{TM} supermix kit (Bio-Rad) using the Bio-Rad iCycler iQ^{TM} thermal cycler according to the manufacturer's instruction. The TaqMan^{TM} fluorogenic probes and gene-specific PCR primers for MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed as described elsewhere (Shin et al. 2013b). The relative fold changes were normalized to GAPDH mRNA in the same sample.

NF-κB-dependent transcriptional activity assay

MDA-MB-231 cells cultured in 12-well plates were transfected with 0.1 μg of the 5 × NF-κB-Luc plasmid. At 24-h post-transfection, cells were treated with 10 ng/mL TNFα in the absence and presence of α-pinene along with 50 ng of the pRL-null plasmid encoding *Renilla* luciferase, as described previously (Lee et al. 2015). Firefly and *Renilla* luciferase activities were measured using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA) and normalized to *Renilla* activity. The luminescence was measured with a Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

MMP-9 promoter reporter assay

Construction of the MMP-9 promoter, pMMP9(–925/+13)_Luc was described elsewhere (Shin et al. 2010). For luciferase promoter reporter assay, MDA-MB-231 cells were seeded onto 12-well plates and transfected with 0.5 μg of the pMMP9(–925/+13)_Luc using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, USA) as described previously (Shin et al. 2010). To monitor transfection efficiency, 50 ng of the pRL-null plasmid encoding *Renilla* luciferase was included in all the samples. At 48-h post-transfection, cells were treated with 10 ng/mL TNFα in the absence and presence of α-pinene. After 8 h, cells were collected and the firefly luciferase activities were measured and normalized to *Renilla* activities using the Dual-Glo luciferase assay system. Luminescence was measured using a Centro LB960 luminometer.

Immunoblot analysis

MDA-MB-231 cells were lysed, and immunoblotting was performed as described previously (Shin et al. 2013a). Antibodies specific to phosph-IKKα/β (Ser176/180), phospho-RelA (Ser536), and phospho-IκB (Ser32) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Signals were detected using an enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ, USA).

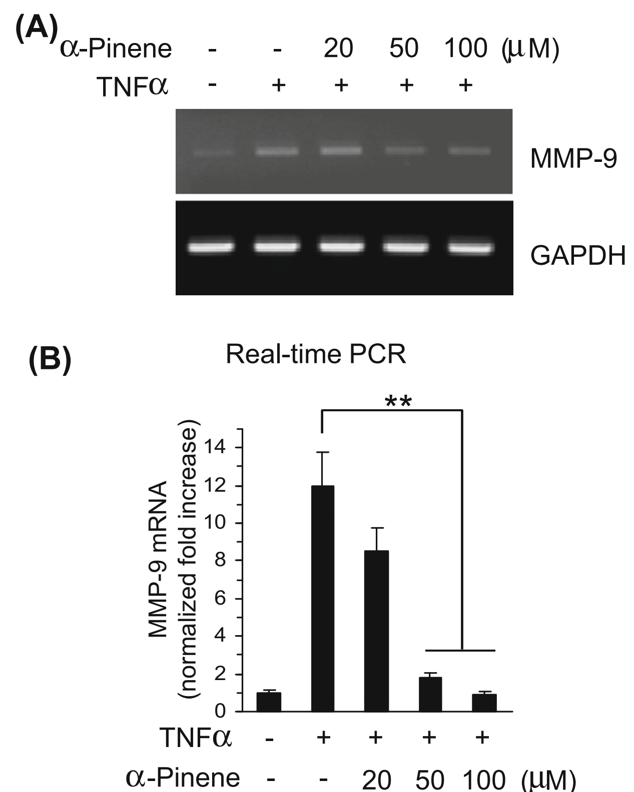


Fig. 2 Effect of α-pinene on the inhibition of TNFα-induced MMP-9 mRNA expression. (**A**) RT-PCR analysis. MDA-MB-231 cells were treated with 10 ng/mL TNFα in the absence or presence of α-pinene for 18 h. GAPDH mRNA was used as an internal control. (**B**) MDA-MB-231 cells were treated with 10 ng/mL TNFα in the absence or presence of α-pinene for 18 h. Relative fold changes in the mRNA levels between untreated control and TNFα- or TNFα plus α-pinene-treated cells were measured by quantitative real-time PCR. The relative fold changes were normalized to GAPDH mRNA in the same sample. The data shown represent the mean ± SD of three independent experiments performed in triplicate (**B**). ** $P < 0.01$ versus TNFα-only treatment ($n = 9$)

Immunofluorescence microscopy

MDA-MB-231 cells plated on coverslips were either untreated or treated with 10 ng/mL TNFα in the absence and presence of 50 μM α-pinene for 30 min. They were then fixed with 4 % paraformaldehyde and permeabilized using 0.1 % Triton X-100, as described previously (Shin et al. 2013a). Briefly, primary antibodies specific to α-tubulin and phospho-p65/RelA (Ser536) were preincubated for 2 h, followed by incubation with Alexa Fluor 488-conjugated (Invitrogen; green signal for α-tubulin) and Alexa Fluor 555-conjugated secondary antibodies (Invitrogen; red signal for phospho-RelA) for 30 min. Nuclear DNA (blue signal) was stained with 1 μg/mL Hoechst 33258 (Sigma-Aldrich) for 10 min. Stained cells were examined under an EVOS f1 fluorescence microscope (Advanced Microscopy Group, Bothell, WA, USA).

Statistical analysis

Statistical analysis was performed by the Student's *t* test or two-factor ANOVA using the InStat version 3.0 software (GraphPad Software Inc., La Jolla, CA, USA). A *P* value of <0.05 was considered statistically significant. Each experiment was repeated at least three times. Data are presented as the mean ± SD.

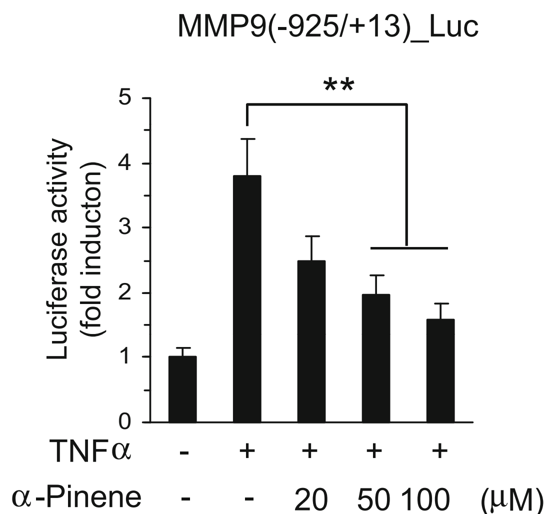
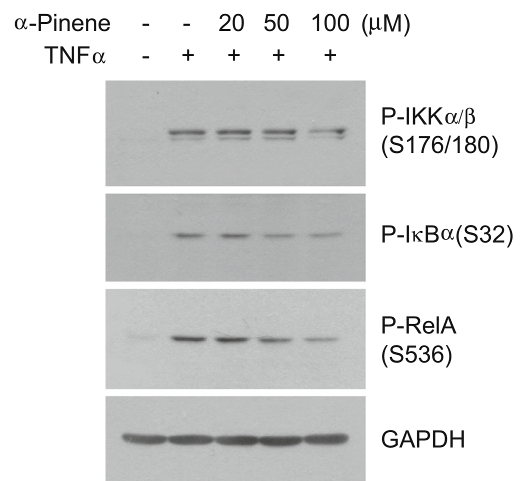


Fig. 3 Effect of α-pinene on the inhibition of TNFα-induced MMP-9 promoter activity. MDA-MB-231 cells were transfected with 50 ng pRL-null vector and 0.2 μg of pMMP9(–925/+13)_Luc. Then, 48-h post-transfection, cells were treated with 50 μM α-pinene for 8 h, and their luciferase activities were determined. Values for firefly luciferase were normalized to those for Renilla luciferase. Data represent the mean ± SD of three independent experiments, performed in triplicate. The data shown represent the mean ± SD of three independent experiments performed in triplicate. ** $P < 0.01$ versus TNFα-only treatment ($n = 9$)

Results and discussion

TNFα promotes tumor invasion and metastasis by stimulating the expression of MMPs in many cancer cells (Rao et al. 1999; Van den Steen et al. 2002; Lin et al. 2008; Joyce and Pollard 2009). The effect of α-pinene on the invasion of MDA-MB-231 cells was examined using a 3-D spheroid culture system (Fig. 1(B)) where control cells (vehicle) were grown into non-invasive aggregates. However, treatment of cells with 10 ng/mL TNFα resulted in cell invasion out of the spheroid into the extracellular



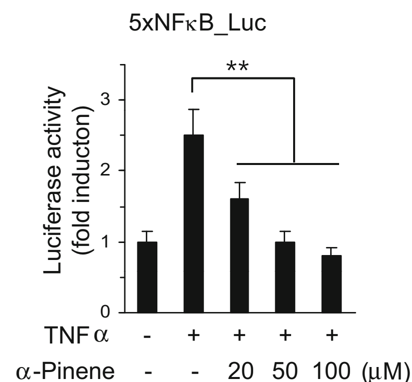


Fig. 4 Effect of α-pinene on the inhibition of TNFα-induced NF-κB activity. (**A**) Immunoblot analysis. MDA-MB-231 cells were treated with 10 ng/mL TNFα in the absence or presence of 50 μM α-pinene. Whole-cell lysates were prepared, and immunoblotting was performed using the phospho-specific antibody against IKK (Ser176/180), IκBα (Ser32) or RelA/p65 (Ser536). The anti-GAPDH antibody was used as an internal control. (**B**) NF-κB-dependent transcriptional activity assay. MDA-MB-231 cells were transfected with 5 × NFκB_Luc plasmid, along with 50 ng pRL-null. At 48-h post-transfection, the cells were either untreated or treated with 10 ng/mL TNFα in the absence or presence of 50 μM α-pinene. The data shown represent the mean ± SD of three independent experiments performed in triplicate. **$P < 0.01$ versus TNFα-only treatment ($n = 9$)

matrix as spindle-like protrusions. However, the presence of α-pinene drastically reduced the TNFα-induced invasiveness of spheroidal cells, suggesting that α-pinene inhibits TNFα-induced invasion of MDA-MB-231 cells.

As MMP-9 plays an important role in tumor invasion and metastasis (Ura et al. 1989), the effect of α-pinene on the expression of MMP-9 mRNA in MDA-MB-231 cells was investigated. RT-PCR analysis showed that α-pinene dose dependently inhibited TNFα-induced MMP-9 mRNA expression (Fig. 2(A)). To precisely measure the change in MMP-9 transcript expression, quantitative real-time PCR analysis was performed. Treatment with TNFα alone resulted in a 12.0-fold increase in MMP-9 mRNA level; however, this decreased to 8.5-, 1.8-, and 0.9-fold upon pretreatment with 20, 50, and 100 μM α-pinene, respectively (Fig. 2(B)). To determine whether α-pinene affects MMP-9 promoter activity, MDA-MB-231 cells were transiently transfected with the MMP-9 promoter reporter construct, pMMP9(-925/+13)_Luc, and luciferase activity measured. The result showed that treatment with α-pinene dose dependently reduced TNFα-induced MMP-9 promoter reporter activity (Fig. 3). These data suggest that α-pinene inhibits TNFα-induced MMP-9 mRNA expression at the transcriptional level.

The molecular mechanism underlying the α-pinene-induced downregulation of MMP-9 gene expression was subsequently investigated. The transcription factor NF-κB controls the production of multiple inflammatory cytokines and triggers pathological conditions in chronic inflammatory diseases (Barnes and Karin 1997). In tumor microenvironment, NF-κB plays an essential role in the regulation of MMP-9 gene expression (Han et al. 2001). TNFα stimulates the inhibitor of κB kinase (IκB kinase, or IKK), which subsequently phosphorylates IκB on serine-32, leading to the degradation of IκB and eventual activation of p65/RelA NF-κB. Since TNFα stimulates NF-κB in diverse cell types (Pikarsky et al. 2004), the effect of α-pinene on TNFα-induced NF-κB activation was tested. MDA-MB-231 cells were treated with TNFα in the absence and presence of α-pinene, and the activation status of IKK, IκB, and p65/RelA was examined by immunoblot analysis. As shown in Fig. 4(A), α-pinene dose dependently reduced TNFα-induced phosphorylation of IKK (serine-176/180), IκB (serine-32), and p65/RelA NF-κB (serine-536). Moreover, α-pinene attenuated TNFα-induced NF-κB-dependent transcriptional activity in a dose-dependent manner (Fig. 4(B)). Immunofluorescent microscopic analysis showed that phosphorylation of p65/RelA (serine-536) in the nucleus was evident upon TNFα stimulation, whereas phosphorylation was barely detectable in the presence of α-pinene (Fig. 5). These results suggest that α-pinene reduces TNFα-induced MMP-9 expression by inhibiting the NF-κB signaling pathway.

Phosphatidylinositol-3-kinase (PI3K) is a ubiquitous intracellular lipid kinase capable of phosphorylating the position 3 hydroxyl group of the inositol ring of phosphatidylinositol. PI3K and its downstream protein kinase B (PKB, also known as AKT) participate in multiple cellular functions, including cell proliferation, differentiation, motility, and survival (Downes and Carter 1991). It has been reported that PKB plays an important role in the activation of NF-κB by phosphorylating IKKα (Ozes et al.

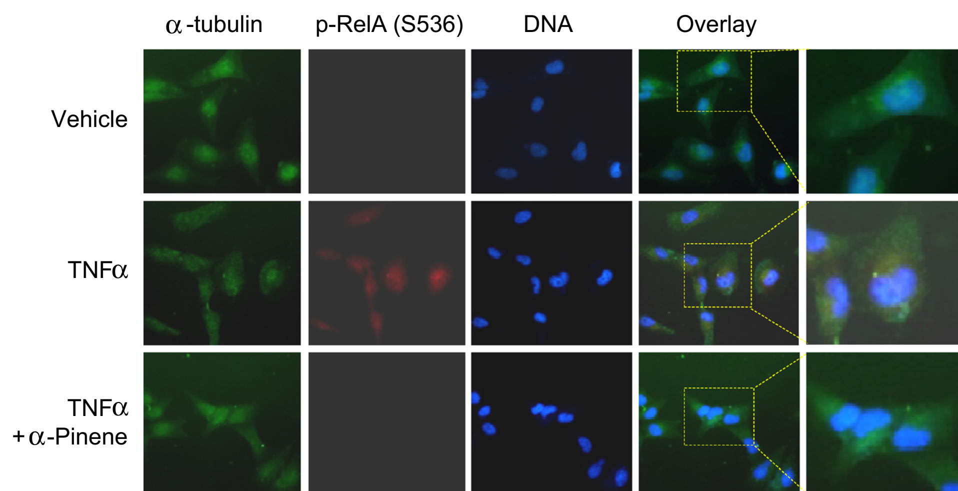


Fig. 5 Effect of α-pinene on the inhibition of TNFα-induced NF-κB phosphorylation. MDA-MB-231 cells were either treated with DMSO (vehicle) or treated with 10 ng/mL TNFα in the absence and presence of 50 μM α-pinene for 30 min. Primary antibodies specific to α-tubulin and phospho-p65/RelA (Ser536) were incubated for 2 h, followed by incubation with Alexa Fluor 488-conjugated (*green*) and Alexa Fluor 555-conjugated (*red*) secondary antibodies for 30 min. Nuclear DNA (*blue*) was stained with 1 μg/mL Hoechst for 10 min. Stained cells were analyzed by EVOSf1fluorescence microscope. Immunofluorescence microscopic analysis using Alexa Fluor 488-conjugated (*green signal*) or Alexa Fluor 555-conjugated (*red signal*) secondary antibodies. Nuclear DNA was stained with 1 μg/mL Hoechst 33258 (*blue signal*)

1999; Madrid et al. 2000, 2001). 1-2,4-Dihydroxy-3-(3-methyl-but-2-enyl)-phenyl]-3-(4-hydroxyphenyl)-propenone (Isobavachalcone), a natural chalcone derivative, inhibits PKB through binding to the ATP-binding site (Jing et al. 2010). We previously demonstrated that DK-139 (2-hydroxy-3′,5,5′-trimethoxychalcone) inhibited lipopolysaccharide-induced NF-κB activity via direct binding to PKB (Lee et al. 2012). Thus, it is possible to hypothesize that α-pinene inhibits IKK through the inhibition of PKB similar to DK-139. α-Pinene could also inhibit PKB and its upstream activators, PI3K and mTORC2 (Sarbassov et al. 2005). To clarify this point, future studies will be aimed at identifying the molecular target of α-pinene.

In summary, the present study demonstrates that α-pinene inhibits the invasiveness of highly metastatic MDA-MB-231 human breast cancer cells. Our experiments show that α-pinene inhibited TNFα-induced MMP-9 mRNA expression and TNFα-mediated NF-κB activity by suppressing IKK. These findings suggest that α-pinene has the potential to be developed into an anti-metastatic agent against highly metastatic malignancy.

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