Breast cancer exhibits high lethality in women because it is frequently detected at an advanced stage and aggressive forms such as triple-negative breast cancer (TNBC), which are often characterized by metastasis through colonization of secondary tumors. Thus, developing therapeutic agents that target the metastatic process is crucial to successfully treat aggressive breast cancer. We evaluated SP-8356, an anti-inflammatory synthetic verbenone derivative, with respect to its regulation of breast cancer cell behavior and cancer progression. Treatment of SP-8356 arrested cell cycle and reduced growth in various types of breast cancer cells with mild cytotoxicity. Particularly, SP-8356 significantly reduced the motility and invasiveness of TNBC cells. Assays using an in vivo xenograft mouse model confirmed the cell-specific anti-proliferative and anti-metastatic activity of SP-8356. Functional studies revealed that SP-8356 suppressed serum response element-dependent reporter gene expression and NF-κB-related signaling, resulting in downregulation of many genes related to cancer invasion. We conclude that SP-8356 suppresses breast cancer progression through multimodal functions, including inhibition of NF-κB signaling and growth-related signaling pathways.

Breast cancer is one of the most fatal diseases in women and exhibits high incidence and mortality among various cancers1,2. In addition, global burden of breast cancer surpasses that of other cancers and incidence rate is increasing. Although early detection and appropriate treatment with surgical resection increase survival rate, detection in advanced stages and diagnosis of atypical triple-negative breast cancer (TNBC) usually result in poor therapeutic outcomes with high lethality. The most common causes of death related breast cancer are metastasis and subsequent colonization of secondary tumors in other organs or tissues. Extensive studies of cellular signaling networks have identified proteins necessary for metastasis and uncontrolled growth of cancers, which has led to the development of targeted therapies against cancer-driving molecules in the last decade3. However, because of its heterogeneous nature, inherent chromosomal instability, and the complex context of tumor environments, breast cancers may easily acquire resistance to targeted drugs. Thus, to overcome the limitations of current therapies, new effective drug identification and development are necessary4-7.

Among various signaling pathways which promote tumor growth and progression, an inflammatory transcription factor NF-κB plays pivotal roles on cell proliferation and survival. Interestingly, NF-κB has been reported to be highly activated in various types of cancers, including breast, bladder, prostate carcinoma and melanoma8-10. In breast cancer, aberrant constitutively active NF-κB exacerbates malignancy without hormonal dependency11. IKKβ is an essential kinase in the canonical NF-κB activation pathway that induces phosphorylation and degradation of IκB and subsequent nuclear translocation of NF-κB. According to previous reports, IKKβ

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Cell cycle analysis revealed an increase of SP-8356-treated MDA-MB231 cells in sub-G0 phase compared to the
and 29.65% in 4T1 cells, 25.53% and 31.07% in MDA-MB453 cells, and 24.73% and 30.93% MCF-7 cells (Fig. 1B).

Breast cancer cells also occurs lower tumor weights in the SP-8356-treated mice than in the vehicle group, confirming SP-8356 inhibition of
tumors that were injected with SP-8356 or a vehicle control. The tumor volumes of mice treated with SP-8356 were
taken together, our results suggest that SP-8356 downregulates metastasis and progression of breast cancer
pathological analysis revealed no apparent abnormalities (data not shown), implying that SP-8356 is potentially safe in
adversely affected the mice, we applied the reagents to naïve mice for the same time period. Blood and gross ana-
nodules were also decreased in SP-8356-treated mice (Fig. 3F). To investigate if either the vehicle or SP-8356 itself
graft to mammary fat pad is not applicable for MDA-MB231 cells, cells were injected to tail vein, which is cur -
in vivo. The ability of SP-8356 to potently block in vitro invasion of the breast cancer
cells led us to investigate its effectiveness in limiting in vivo metastasis. Since metastatic model using orthotopic
graft to mammary fat pad is not applicable for MDA-MB231 cells, cells were injected to tail vein, which is cur -
currently acceptable lung metastasis model. Lungs isolated from the xenograft mice treated with SP-8356 exhibited
significantly reduced tumor burdens compared to the vehicle-treated group (Fig. 3D,E). The numbers of tumor
nODULES were also decreased in SP-8356-treated mice (Fig. 3F). To investigate if either the vehicle or SP-8356 itself adversely affected the mice, we applied the reagents to naïve mice for the same time period. Blood and gross anatomical analysis revealed no apparent abnormalities (data not shown), implying that SP-8356 is potentially safe in
mice. Taken together, our results suggest that SP-8356 downregulates metastasis and progression of breast cancer
in a cell- and tissue-specific manner.

To investigate the in vivo relevance of our cellular findings, we generated tumor-induced mice via MDA-MB231 cell implanta-
tion that were injected with SP-8356 or a vehicle control. The tumor volumes of mice treated with SP-8356 were
significantly lower than those of vehicle-treated mice after 42 days (Fig. 3A,B). Figure 3C shows substantially
lower tumor weights in the SP-8356-treated mice than in the vehicle group, confirming SP-8356 inhibition of
breast cancer cells also occurs in vivo. The ability of SP-8356 to potently block in vitro invasion of the breast cancer
cells led us to investigate its effectiveness in limiting in vivo metastasis. Since metastatic model using orthotopic
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nODULES were also decreased in SP-8356-treated mice (Fig. 3F). To investigate if either the vehicle or SP-8356 itself adversely affected the mice, we applied the reagents to naïve mice for the same time period. Blood and gross anatomical analysis revealed no apparent abnormalities (data not shown), implying that SP-8356 is potentially safe in
mice. Taken together, our results suggest that SP-8356 downregulates metastasis and progression of breast cancer
in a cell- and tissue-specific manner.
SP-8356 decreased NF-κB activity in a dose-dependent manner (Fig. 5A). We then treated the cells with PMA (protein kinase C activator), as some protein kinase C subgroups can activate IKK, an upstream kinase of NF-κB activation. As shown in Fig. 5B, SP-8356 downregulated PMA-dependent NF-κB activation. SP-8356 also significantly decreased TNF-α-stimulated NF-κB activity (Fig. 5C). Interestingly, all related experiments revealed that NF-κB activity in unstimulated cells was significantly decreased in the presence of more than 5 μM SP-8356.

To determine the effect of SP-8356 on other signaling pathways which may be involved in cancer progression, STAT3-derived reporter gene expression was examined in IL-6-treated MDA-MB231 cells. Unlike NF-kB, IL-6-dependent STAT3 activation was not inhibited by SP-8356, suggesting that this molecule may downregulate NF-kB signaling with specificity (Fig. 5D). In unstimulated cells, NF-κB predominantly localized to the cytosol, whereas in its active state, the RelA/p65 subunit of NF-κB was translocated to the nucleus. To establish if SP-8356 inhibits the nuclear translocation of p65, we determined protein location by immunostaining with anti-p65 antibodies. Prior to TNF-α treatment, p65 was found mainly in the cytosol but then localized to the nucleus after TNF-α exposure. However, p65 remained in the cytosol of TNF-α-stimulated cells pre-treated with 10 μM SP-8356 (Fig. 5E), and the percentage of cells undergoing p65 nuclear translocation was significantly lower in the presence of SP-8356 and TNF-α compared to cells exposed to TNF-α alone (Fig. 5F).

Because IKK-mediated phosphorylation and degradation of IκB precedes nuclear translocation of NF-κB, SP-8356 may block IκB degradation and indirectly inhibit NF-κB. However, SP-8356 neither changed basal levels of IκB nor blocked TNF-α-stimulated IκB degradation, which indicates SP-8356 may target events following NF-κB's release from IκB (Fig. 5G). According to previous reports, nuclear translocation of free NF-κB is mediated by a subset of importin molecules that directly bind subunits p50 and p6522,23. Immunoprecipitation with...
cells expressing hemagglutinin (HA)-importin α3 or α5 showed that TNF-α-dependent p65 interactions with importins was significantly inhibited by SP-8356 (Fig. 5H). These findings suggest that SP-8356 negatively regulates TNF-α-induced nuclear translocation of RelA/p65 by interfering with direct interaction between p65 and importin proteins.

SP-8356 attenuates cell migration-associated gene expression. Metastasis of a primary tumor relies on the tumor cells’ ability to degrade the extracellular matrix (ECM) so we examined SP-8356’s effect on the expression of genes influencing cell adhesion and invasion targeted by NF-κB. The mRNA levels of uPA, MMP-2, MMP-7, and MMP-9 in SP-8356-treated MDA-MB231 cells were significantly reduced, whereas PAI was elevated compared to control cells (Fig. 6A). Zymography assays revealed that levels of exogenous MMP-2 and MMP-9 were remarkably reduced in the presence of 10 μM SP-8356 (Fig. 6B), and Western blotting showed decreased MMP-9 and urokiase plasminogen activator (uPA) levels in cells treated with 10 μM SP-8356 (Fig. 6C). These results indicate that SP-8356 likely limits the migration and invasion activity of aggressive MDA-MB231 cells by reducing expression of MMPs and uPA and upregulating PAI.

Pharmacokinetics and concentration-response relationships of SP-8356. Catechol or polyphenol compounds are rapidly metabolized to glucuronides and/or sulfates by phase II reactions, and therefore non-conjugated aglycones are scarcely detected in human plasma. SP-8356 with a catechol moiety can be rapidly metabolized by phase II reactions. Thus, we further determined the concentrations of SP-8356 and its metabolites in plasma. After intraperitoneal injection, plasma levels of SP-8356 and its major metabolite SP-8356 monoglucuronide conjugate (Glu-8356) reached to micromolar ranges. The maximal concentration (Cmax) and area under the curve (AUC) of Glu-8356 in plasma were over 11.4 times higher than that of SP-8356 (Fig. S1A). In addition, both SP-8356 and its glucuronide conjugate reduced NF-κB activity to a similar extent (Fig. S1B).

Discussion
In this study, the verbenone derivative SP-8356 inhibited growth of all breast cancer cell lines tested, which was closely related to cell death or cell cycle arrest. SP-8356 was cytotoxic to these cells by inducing apoptotic molecule activity and halting cell cycle progression at S phase. SP-8356 also showed strong inhibitory effects on wound healing and invasion activity of highly motile TNBC cells. Many signaling pathways that regulate cellular proliferation involve ERK phosphorylation, which declined in cells treated with SP-8356 long-term, although short-term treatment of SP-8356 did not significantly alter basal or serum-stimulated ERK phosphorylation. We
also found that SP-8356 slightly reduced serum-dependent SRE activation, a downstream target of ERK whose indirect regulation may contribute SP-8356-mediated growth inhibition of breast cancer cells.

SP-8356 also inhibits transcriptional activity of NF-κB, which induces expression of genes responsible for cell proliferation, survival, and motility. Growth inhibitory effect of SP-8356 was slightly higher than that of JSH-23, another inhibitor of NF-κB transcriptional activity (data not shown). The study on underlying mechanism revealed that SP-8356 negatively regulates NF-κB by blocking its importin-mediated nuclear translocation of p65 rather than inhibiting upstream signaling events of the transcription factor. Thus, regulation of NF-κB and ERK are likely related to SP-8356’s inhibitory action in breast cancer cells, which was generally confirmed in in vivo tumor suppression xenograft model. Because NF-κB regulates genes involved in epithelial-mesenchymal transition and metastasis, its inhibition by SP-8356 is extremely relevant to limiting cancer progression. In regard to nuclear translocation of NF-κB, SP-8356 is not likely to act on importin since it has no effect on STAT3 of which nuclear translocation also requires importin28.

In the present study, plasma levels of SP-8356 monoglucuronide conjugate were much higher in comparison to SP-8356. In addition to SP-8356 monoglucuronide, sulfated and methylated metabolites were also found in plasma levels higher than the parent drug SP-8356 (Data not shown). Like SP-8356 with a catechol moiety, quercetin, a plant flavonol from the flavonoid group of polyphenols, and its water-soluble metabolites, quercetin-3′-glucuronide and quercetin-3′-sulfate possess strong anti-proliferative effects26,29. Resveratrol, a polyphenolic phytoalexin, and its metabolites, resveratrol-3-O-glucuronide and resveratrol-3-O-sulfate has cell proliferation-inhibiting activities30.

Matrix metalloproteases are a family of enzymes capable of degrading various ECM components and facilitating tumor migration34,31, and expression of various MMPs is upregulated in many cancers associated with a poor prognosis32,33. In addition, uPA binding to its receptor uPAR converts proenzyme plasminogen into active serine protease plasmin34, which cleaves ECM proteins and growth factor precursors to their active forms. Ultimately, these growth factors bind their cognate receptors, resulting in cell proliferation and migration35,36. Binding of uPA to uPAR also activates JAK-STAT signaling and induces integrin-mediated activation of MAPK and Akt, which leads to cell proliferation, survival, and motility37–39. The mRNA expression of these genes (except uPAR)
was prominently inhibited by SP-8356 in a dose-dependent manner, some of which were confirmed by Western blotting or protease activity assays. Interestingly, plasminogen activator inhibitor (PAI), a serine protease inhibitor, was dramatically enhanced by SP-8356. This molecule normally binds and inhibits the uPA-uPAR complex, induces endocytosis followed by complex degradation\(^3\), and blocks activity of MMPs\(^4\). Therefore, positive or negative regulation of genes involved in cell migration and invasion is a possible mechanism of SP-8356′s inhibitory effects against breast cancer cells.

Our recent experiments revealed that SP-8356 binds to CD147 and inhibits dimerization of the protein (unpublished data). CD147 was recently reported to be overexpressed in many human cancers and implicated in tumor progression, especially during proliferation, invasion, and metastasis\(^5\). As a member of the immunoglobulin superfamily and a type I transmembrane glycoprotein, CD147 is expressed in the plasma membrane as homodimer and responsible for activation of some matrix metalloproteases, which may enhance cell motility\(^6\). So, targeting to CD147 may contribute to the anti-metastatic activity of SP-8356 of which the mechanism should be verified in the future.

We confirmed the \textit{in vitro} effects of SP-8356 using a xenograft mouse model. Although aggressive TNBC cells initially migrated to the lung and formed large tumors, SP-8356 treatment blocked colonization of the cells and reduced tumor burdens without causing any adverse effects in the mice, further highlighting the efficacy and potential safety of SP-8356 as an anti-cancer agent. Its biological functions related to cell proliferation, survival, and migration may result from its regulation of NF-κB signaling and subsequent expression metastasis-associated genes. Moreover, blocking homophilic interactions of CD147 may be another mechanism for anti-metastatic

![Figure 4. SP-8356 decreases basal ERK activity.](A,B) SP-8356 suppresses serum- or PMA-stimulated SRE activity. MDA-MB231 cells were transfected with plasmids containing a SRE-luciferase reporter gene construct. After 24 h of serum starvation, cells were treated with different doses of SP-8356 prior to stimulation with 10% FBS or 1μM PMA, lysed, and analyzed in luciferase activity assays. Values are shown as means ± SEM. \(*p < 0.05, \#p < 0.001\) (compared to serum stimulation without SP-8356), \(*p < 0.001\) (compared to no serum or SP-8356 treatment) (C) To examine the effect of short time treatment with SP-3856, after 24 h starvation, MDA-MB231 cells were exposed to 10 μM SP-8356 for the indicated time points, and phosphorylation of ERK and Akt was determined by Western blotting. Veh: vehicle treatment (D) After 24 h serum starvation, cells were treated with 10 μM SP-8356 and stimulated with serum for the indicated times, after which lysates were subjected to Western blotting. (E) MDA-MB231 cells were treated with SP-8356 under normal culture conditions (no serum stimulation) for three days, and lysates were analyzed with Western blotting. (C,D,E) 20μg of cell lysates was used for SDS-PAGE. After transfer, nitrocellulose membranes were divided to two parts for Western blotting against pERK and pAkt around 55 kDa region. After first blotting, antibodies were removed from the nitrocellulose membrane by detachment solution, then the membranes were used for re-blotting for ERK or Akt. Exposure time for each blotting was around 15 sec.
activity of SP-8356. In summary, SP-8356 is a multi-target agent possessing cancer-specific anti-metastatic activity both in vitro and in vivo, making it a promising therapy for aggressive breast cancer.

Materials and Methods

Reagents, culture media, and antibodies. (1S)-(–)-Verbenone derivatives were synthesized as previously reported. Cell culture media were obtained from WELGENE, Inc. (Daegu, Korea). Human recombinant TNFα was purchased from R&D Systems (Minneapolis, MN, USA), and protease inhibitor cocktail was purchased from Roche (Mannheim, Germany). Antibodies against actin, ERK, NF-κB p65, p-Akt, and p-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt, PARP, caspase-3 and MMP-9 were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against uPA and CD147 were purchased from Abcam (Cambridge, UK). Primers for gene cloning and materials for expression vector construction were
obtained from Cosmogenetech (Seoul, Korea), and DNA sequencing was conducted by the same company. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

**Cell culture.** MDA-MB231, MDA-MB453, MCF-7, and 4T1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were maintained in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). All cells were cultured at 37 °C in a humidified chamber containing 5% CO2.

**Cell growth assay.** MDA-MB231, MDA-MB453, MCF-7 (4,000 cells/well) and 4T1 (3,000 cells/well) were seeded into 96-well plates and treated with various concentrations of SP-8356 for indicated times in complete culture media (Fig. 1A). Cell growth was measured using the Cell Counting Kit-8 (CCK-8) from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA) following the manufacturer’s instructions. Cells were incubated with 10 μL CCK-8 solution for 2 h, and the absorbance of each well was then measured at 450 nm using a microplate reader.

**Cell cycle analysis.** Cell cycle distribution and apoptosis were determined by FACS analysis using propidium iodide (PI) staining to measure DNA content. Although all cells tested in cell growth were applied for this assay, only MDA-MB231 showed discrete fractions regarding to DNA contents. MDA-MB231 cells were plated at a density of 5 × 10^5 cells/well in a 6-well-plate. 24 h later, cells were treated with 10 μM SP-8356 every 24 h for three consecutive days. Both adherent and floating cells were harvested, washed with cold PBS, and processed for cell cycle analysis. Briefly, cells were fixed in absolute ethanol and stored in ice for 1 h. The fixed cells were then centrifuged at 500 × g for 5 min and washed with PBS. Cells were resuspended with 1 mg/mL PI and 100 μg/mL RNase A and incubated for 30 min at 37 °C. The mixture was then analyzed using the FACSCalibur platform (BD Biosciences; Franklin Lakes, NJ, USA), equipped with CellQuest Pro software.

**Lactate dehydrogenase (LDH) assay.** Cell cytotoxicity was quantified by measuring LDH released from plasma membrane-damaged MDA-MB231 cells using a cytotoxicity detection kit according to the manufacturer’s instructions (Takara Bio Company, Shiga, Japan). Briefly, cells were seeded in 96-well plates in RPMI with 10% FBS and incubated for 24 h. Cells were then incubated in 200 μL serum-free RPMI with various concentrations of SP-8356 for 24 h or 48 h (Fig. 1B). Cells treated with DMSO (vehicle) were used as a negative control. Some vehicle-treated cells were lysed with 1% Triton X-100 and used as a positive control. Microtitre plates were centrifuged at 250 × g for 10 min, and 100 μL the supernatant was transferred to another 96-well plate followed by the addition of 100 μL reaction mixtures (provided by company). After 30 min incubation at room temperature, the

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**Figure 6.** SP-8356 regulates expression of metastasis-related genes. (A) The relative mRNA expression levels of MMP-2, MMP-7, MMP-9, uPA, uPAR, PAI, VEGF-A, and VEGF-C in MDA-MB231 cells treated with varying doses of SP-8356 were evaluated by qRT-PCR. Values are shown as means ± SEM. *p < 0.05, **p < 0.001. (B) Effect of SP-8356 on the activity of MMPs. MDA-MB231 cells were treated with SP-8356 for 24 h, and MMP-2 and MMP-9 levels in conditioned culture media were assessed in zymography assays. (C) Western blot analysis shows decreased expression of MMP-9 and uPA in MDA-MB21 cells treated with SP-8356. Exposure time for MMP9 and uPA was 1 min. 15 sec. for β-actin.
absorbance of each was measured at 490 nm using a microplate reader. The relative activity LDH (%) was calculated as \((A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{high control}} - A_{\text{negative control}})\) \times 100%.

**Migration and invasion assays.** For migration assays, 2 \times 10^4 MDA-MB231 and 4T1 cells in serum-free medium were placed into the upper chamber of transwell insert (8-µm pore size; Corning, Inc.; Corning, NY, USA). For the invasion assays, the upper chamber of an insert was coated with 20 µL of diluted Matrigel (Invitrogen), which was allowed to solidify, after which 2 \times 10^4 cells in serum-free medium were added and treated with SP-8356. Medium with 10% FBS was added to the lower chamber. Transwell plates were incubated at 37 °C in a humidified chamber containing 5% CO₂ for 24 h. Cells remaining in the upper membrane were removed with a cotton swab, while cells that migrated or invaded through the membrane were fixed in 4% paraformaldehyde, stained with Hemacolor Rapid staining of blood smear (Merck; Darmstadt, Germany), and quantified microscopically.

**Wound healing assay.** 5 \times 10^5 MDA-MB231, 4T1, and MDA-MB453 cells were seeded into 6-well plates. Confluent monolayers of cells were treated with a pipette tip and washed with media to remove floating cells. Cells were then incubated with SP-8356 (1 µM, 2.5 µM, or 5 µM) and visualized after 18 h. The percentage of wound closure was calculated as follows: 100 \times (area of original wound − area of remaining wound)/area of original wound.

**Xenograft mouse model.** All animal experiments and procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee and the Institutional Animal Care & Use Committee in Korea University College of Medicine (Approval No. KUIA-CUC-20150406-2). Five-week-old female NOD/SCID mice were purchased from KOATECH (Pyeongtek, Korea) and housed under specific pathogen-free conditions in an individually ventilated caging system. MDA-MB231 cells (1 \times 10^6 cells) were injected subcutaneously into the right flank of the mice. After 1 day, the mice were randomized into two groups consisting of six mice each. One group was treated with SP-8356 (10 mg/kg) every day by intraperitoneal injection until the end of the experiment, and the other was treated with vehicle (5% DMSO and 10% cremophor in normal saline). Tumor diameter measurement began at \(>3\) mm, and tumor volume was calculated as \(0.5 \times \text{length} \times \text{width}^2\). When tumor volume reached approximately 2 cm³, tumors were surgically resected and weighed.

A lung metastasis model was established by lateral tail vein injection of MDA-MB231 cells (1 \times 10^6 cells) into NOD/SCID mice, which were treated with SP-8356 or vehicle as described above. On day 45, mice were sacrificed, and their lungs were fixed with 4% paraformaldehyde and processed for histological analysis. Sections (5 µm) were mounted on glass slides and subjected to hematoxylin and eosin staining. Representative images for each group were captured, metastatic tumor nodules were counted, and tumor burden was calculated as the percentage of lung area with tumors compared to total lung area.

**Western blot analysis and immunoprecipitation.** Cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate (w/v), and 0.05% SDS (w/v)] containing protease inhibitors. Protein concentrations of clarified lysates were determined using the Bradford protein assay kit (Bio-Rad; Hercules, CA, USA), and 20 µg cell lysates denatured with SDS sample buffer were separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and probed with appropriate antibodies. Signals were then detected using the ECL assay kit (GE Healthcare; Chicago, IL, USA).

For immunoprecipitation, MDA-MB231 cells transfected with HA-importin genes were lysed with the buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitors. The clarified cell extracts were incubated with anti-HA agarose for 2 h at 4°C. After washing, precipitated proteins were applied for SDSPAGE and Western blotting.

** Luciferase assays.** MDA-MB231 cells seeded into 24-well plates were transfected with plasmids containing a NF-κB-luc, STAT3-luc, or serum responsive element (SRE)-luc reporter gene. Cells cultured in serum-free medium for 18 h were treated with various assay-specific concentrations of SP-8356 prior to adding serum or TNFα as a stimulant. After 6 h, cells were harvested, and luciferase activity was measured using a standard assay program from BioTek Instruments, Inc. (Winooski, VT).

**Immunocytochemistry.** MDA-MB231 cells were fixed with 4% paraformaldehyde and blocked with 3% bovine serum albumin in PBS containing 0.1% Triton X-100 for 30 min. The preparation was then incubated for 2 h at room temperature with RelA/p65 antibody, washed with PBS, and probed with secondary antibody (fluorescein isothiocyanate-conjugated anti-rabbit IgG). Images were captured with a Leica TCS SP5 laser scanning microscope (Wetzlar, Germany).

**Quantitative real time RT-PCR (qRT-PCR).** MDA-MB231 cells were treated with SP-8356 for 24 h in the presence of complete medium. Total RNA was extracted with TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was generated using reverse transcriptase from Promega (Madison, WI, USA), and qRT-PCR was performed with iQTM SYBR Green Supermix and an iCycler PCR thermocycler (Bio-Rad) with gene-specific primer sets designed with Beacon Designer version 2.1 (Biosoft International; Palo Alto, CA, USA) as follows: uPA (5′-ttttgcacaaaccagctat-3′ and 5′-ggcaggcagatggtctgtat-3′), uPAR (5′-ctctgctgccacatctcg-3′ and 5′-ttgcc ttctcaggtgtgta-3′), PAI (5′-actgagaagccagacgaa-3′ and 5′-ctctggagctctcgctg-3′), MMP-2 (5′-cggaaagatgattcctg agta-3′ and 5′-ttgctgctgtagatcgg-3′), MMP-7 (5′-ggctgatctgatgtccttg-3′ and 5′-ttccctgagacatc-3′), MMP-9 (5′-atccgagacatttat-3′ and 5′-ctggacaatgacatc-3′), VEGF-A (5′-gagagcaccacactctac-3′ and 5′-ccttgcttgctctctct-3′), VEGF-C (5′-tcaggccagagagacact-3′ and 5′-ctccactatctaatcttctt-3′), GAPDH (5′-ctctgctc
tcgctgttgac-3′ and 5′-aatccgttgactccgacct-3′). The mRNA level of each gene was normalized to that of glyceraldehyde-3-phosphate dehydrogenase.

**Zymography.** The in vitro activities of matrix metalloproteases MMP-2 and MMP-9 from MDA-MB231 cells were analyzed by gelatin zymography using Novex® 10% Zymogram gels (Invitrogen; Carlsbad, CA, USA). After electrophoresis, gels were washed for 1 h at room temperature to remove SDS using 2.5% Triton X-100 (Sigma-Aldrich). The gels were then incubated overnight at 37°C in zymography reaction buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃ and stained with Coomassie Brilliant Blue R-250 (Bio-Rad) followed by destaining with 40% methanol and 10% glacial acetic acid to obtain contrast bands. Images were captured and analyzed by densitometry using ImageJ software (https://imagej.net).

**Statistical analysis.** Unpaired Student’s t-tests or ANOVA using PRISM5 software (GraphPad; La Jolla, CA, USA) were used. Group means were further analyzed using Bonferroni’s multiple comparison tests. Data were presented as means ± SEM, and all experiments were performed in triplicate unless otherwise indicated.

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