Liver cancer is a common tumor and currently the second leading cause of cancer-related mortality globally. Liver cancer is highly related to inflammation as more than 90% of liver cancer arises in the context of hepatic inflammation, such as hepatitis B virus and hepatitis C virus infection. Despite significant improvements in the therapeutic modalities for liver cancer, patient prognosis is not satisfactory due to the limited efficacy of current drug therapies in anti-metastatic activity. Therefore, developing new effective anti-cancer agents with anti-metastatic activity is important for the treatment of liver cancer. In this study, SP-8356, a verbenone derivative with anti-inflammatory activity, was investigated for its effect on the growth and migration of liver cancer cells. Our findings demonstrated that SP-8356 inhibits the proliferation of liver cancer cells by inducing apoptosis and suppressing the mobility and invasion ability of liver cancer cells. Functional studies revealed that SP-8356 inhibits the mitogen-activated protein kinase and nuclear factor-kappa B signaling pathways, which are related to cell proliferation and metastasis, resulting in the downregulation of metastasis-related genes. Moreover, using an orthotopic liver cancer model, tumor growth was significantly decreased following treatment with SP-8356. Thus, this study suggests that SP-8356 may be a potential agent for the treatment of liver cancer with multimodal regulation.

Key Words: SP-8356, Liver cancer, Proliferation, Motility, ERK, NF-κB

INTRODUCTION

Liver cancer is a frequently diagnosed tumor and currently the second leading cause of cancer-related mortality worldwide (Torre et al., 2015). Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, and the major risk factor of HCC development is viral hepatitis, such as that caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infection (El-Serag and Rudolph, 2007; Marrero and Marrero, 2007). Despite significant improvement in treatment options, including surgical resection, liver transplantation and other therapeutic modalities, the 5-year survival rate remains extremely poor due to the limited efficacy of the therapies (Bruix and Sherman, 2011; Zhu et al., 2017; Siegel et al., 2018).

As an advanced systemic therapeutic option, several multi-kinase inhibitors are available for unresectable HCC treatment. Sorafenib and lenvatinib are multi-kinase inhibitors approved by United States Food and Drug Administration as first-line treatment options for unresectable HCC. However, these systemic therapeutic options, particularly sorafenib, merely improve the survival time of HCC patients by 3-5 months and its use is occasionally restricted due to adverse side effects such as bleeding, diarrhea, and hand-foot skin reactions (Llovet et al., 2008; Je et al., 2009; Zhu et al., 2017).

Besides the limitations of the current therapies, a factor that exacerbates the prognosis of HCC patients is metastasis. Approximately one-third of HCC patients eventually develop extrahepatic metastases, with the most common sites being the lungs, lymph nodes, and bones (Poddar et al., 2017). The major pathophysiological modes for HCC metastasis are di-
rect extension of the tumor, hematogenous spread, and/or lymphatic invasion (Poddar et al., 2017). Because of its systemic nature and the resistance of dispersed tumor cells to existing therapeutic agents, metastasis accounts for 90% of cancer mortality (Okusaka et al., 1997; Chaffer and Weinberg, 2011; Valastyan and Weinberg, 2011). Moreover, metastatic liver cancer often leads to the recurrence of liver cancer after surgical resection (Tung-Ping Poon et al., 2000). Therefore, identifying novel and effective systemic agents with anti-metastasis activity is urgent for the treatment of HCC.

In cancer progression, inflammation has been considered an important component of the development of various cancers (Coussens and Werb, 2002). Among them, HCC is highly related to inflammation as more than 90% of HCCs arise in the context of chronic hepatic injury and inflammation (Nakagawa and Maeda, 2012). Nuclear factor-kappa B (NF-κB) is an important transcription factor that functions as a regulator of inflammation. Because inflammation predisposes cancer progression, it seems logical to speculate the link between NF-κB and cancer. NF-κB is also involved in cancer proliferation, apoptosis, metastasis, and angiogenesis (Naugler and Karin, 2008). In HCC, NF-κB is constitutively activated to promote tumor growth, indicating that NF-κB plays a pivotal role in HCC pathogenesis (Wang et al., 2003; Pikarsky et al., 2004; Li et al., 2009). Thus, the inhibition of NF-κB activation is a potential therapeutic target for liver cancer treatment.

In previous studies, essential oils containing (1S)-(−)-verbenone were identified to possess anti-inflammatory activity through the inhibition of NF-κB signaling (Choi et al., 2010; Kuo et al., 2011). Since then, a series of (1S)-(−)-verbenone derivatives has been synthesized by adding functional moieties to improve their cytotoxic effects with stronger anti-inflammatory and anti-oxidant activities (Ju et al., 2013). Given the roles of NF-κB signaling in liver cancer progression, synthesized (1S)-(−)-verbenone derivatives could be a new therapeutic agent for liver cancer.

In the present study, the effect of (1S)-(−)-verbenone derivatives on liver cancer cells was investigated. Among various (1S)-(−)-verbenone derivatives, SP-8356 demonstrated the most significant anti-proliferative effect on liver cancer cells by inducing apoptosis. In addition, SP-8356 inhibited liver cancer cell motility by regulating metastasis-related genes. Functional studies suggested that these anti-cancer activities of SP-8356 are mediated by its inhibitory effect on the mitogen-activated protein kinase (MAPK) and NF-κB pathways.

MATERIALS AND METHODS

Reagents, culture media, and antibodies

(1S)-(−)-verbenone derivatives were synthesized as previously reported (Ju et al., 2013). The structure of SP-8356 was already described in previous report (Pahk et al., 2019). Cell culture media were obtained from WELGENE Inc (Daegu, Korea). Human recombinant TNF-α was purchased from R&D systems (Minneapolis, MN, USA), human recombinant epidermal growth factor (EGF) was purchased from Peprotech Inc. (Hamburg, Germany), and protease inhibitor cocktail was purchased from Roche (Mannheim, Germany). Antibodies against PARP, caspase-3, p-ERK1/2, and Akt were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against survivin, ERK1/2, p-Akt, p-Erk-1, NF-κB p65, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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

Huh-7, Hepa1-6, Hep3B, and SK-Hep1 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The Huh-7, Hepa1-6, and Hep3B cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 IU/mL) streptomycin (100 μg/mL). The SK-Hep1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin (100 IU/mL) streptomycin (100 μg/mL). Cells were cultured at 37°C in a humidified chamber containing 5% CO2.

Cell growth assay

Huh-7, Hep3B, SK-Hep1 (3,000 cells/well), and Hepa1-6 (1,000 cells/well) cells were seeded into 96-well plates and treated with various concentrations of (1S)-(−)-verbenone derivatives for the indicated times in complete culture medium. Cell growth was measured using a Cell Counting Kit-8 (CCK-8) kit from Dojindo Molecular Technologies, Inc. (Rockville, MD, USA) following the manufacturer’s instructions. Cells were incubated with 10 μL of CCK-8 solution for 2 h, and the absorbance of each well was measured at 450 nm using a microplate reader, SpectraMax iD3 (Molecular Devices, LLC, San Jose, CA, USA).

Lactate dehydrogenase (LDH) assay

Cell cytotoxicity was quantitatively assessed by measuring LDH released from plasma membrane-damaged cells using a cytotoxicity detection kit according to the manufacturer’s instructions (Takara Bio Company, Shiga, Japan). Huh-7, Hepa1-6, and Hep3B cells were seeded in a 96-well plate in DMEM with 10% FBS and incubated for 24 h. Next, cells were incubated with 200 μL serum-free DMEM medium with SP-8356 for 24 h and 48 h. Cells treated with vehicle (DMSO) were used as a negative control. Some of the vehicle-treated cells were lysed with 1% Triton X-100 buffer and used as a positive or high control. Microtiter plates were centrifuged at 250×g for 10 min; 100 μL of supernatant was transferred to another 96-well plate with the addition of 100 μL of reaction mixtures. After 30 min of incubation at room temperature, absorbance was measured at 490 nm using a microplate reader (SpectraMax iD3, Molecular Devices, LLC). The relative activity of LDH (%) was calculated as ([A]sample−[A]negative control)/([A]high control−[A]negative control)×100.

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% sodium dodecyl sulfate (SDS) (w/v)] containing protease inhibitor. The lysates were centrifuged at 15,000 rpm at 4°C for 15 min. Protein concentrations of the clarified lysates were determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Next, 20 μg of cell lysates de-natured with sodium dodecyl sulfate (SDS) sample buffer was separated by polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed with
and Hepa1-6 cells in serum-free media were placed into 4% paraformaldehyde solution and stained with Hemacolor. The membranes were fixed in Immunocytochemistry.

Invasion assay

Huh-7 cells were seeded into 24-well plates and then transfected with plasmids containing the serum response element (SRE)-luc and NF-κB-luc reporter genes. Cells cultured in serum-free DMEM media for 18 h were treated with different concentrations of SP-8356 for 30 min and then treated with 10% serum, 100 ng/mL of epidermal growth factor (EGF), 1 µM PMA (phorbol 12-myristate 13-acetate), and 10 ng/mL TNF-α as a stimulant. After 6 h, cells were washed with phosphate-buffered saline (PBS) and solubilized with lysis buffer. The luciferase activity of the cell extracts was determined using the standard luciferase assay system from BioTek Instruments, Inc (Winooski, VT, USA).

Wound healing assay

Huh-7 and Hepa1-6 cells (5×10⁴ cells/well) were seeded into 6-well plates. Confluent monolayers were manually scratched with a pipette tip and washed with PBS to remove the cell debris. Cells were incubated in DMEM media with different concentrations of SP-8356, and the scratch area was photographed at the indicated times. The area between two cell edges was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). The percentage of wound closure was calculated as follows: [(area of original wound−area of remaining wound)/area of original wound]×100.

Invasion assay

For the invasion assay, the upper chambers of Transwell inserts (8-µm pore size; Corning, NY, USA) were coated with 1:6 diluted Matrigel (Invitrogen, Carlsbad, CA, USA) and allowed to solidify in an incubator. Next, 2×10⁴ Huh-7 cells and 1×10⁴ Hepa1-6 cells in serum-free media were placed into the upper chambers of the inserts and then treated with SP-8356. The lower wells were filled with DMEM containing 10% FBS. Cells were incubated at 37°C in a humidified chamber containing 5% CO₂ for 18 h. The inserts were washed in PBS, and the cells in the upper chamber that had not invaded were removed with a cotton swab. The membranes were fixed in 4% paraformaldehyde solution and stained with Hemacolor Rapid staining of blood smear (Merck, Darmstadt, Germany); cells that had invaded were counted under a microscope.

Immunocytochemistry

Huh-7 cells were grown on poly-L-lysine-coated glass coverslips in 24-well plates. After 24 h, the cells were incubated in serum-free DMEM for 18 h, treated with 20 µL of 1:6 diluted Matrigel (Invitrogen, Carlsbad, CA, USA) and allowed to solidify in an incubator. Next, 2×10⁴ Huh-7 cells and 1×10⁴ Hepa1-6 cells in serum-free media were placed into the upper chambers of the inserts and then treated with SP-8356. The lower wells were filled with DMEM containing 10% FBS. Cells were incubated at 37°C in a humidified chamber containing 5% CO₂ for 18 h. The inserts were washed in PBS, and the cells in the upper chamber that had not invaded were removed with a cotton swab. The membranes were fixed in 4% paraformaldehyde solution and stained with Hemacolor. Rapid staining of blood smear (Merck, Darmstadt, Germany); cells that had invaded were counted under a microscope.

Reverse transcription–quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNA was generated using reverse transcriptase (Promega, Madison, WI, USA) for 2 µg of extracted total RNA, and qPCR was performed using iQTM SYBR Green Supermix and the iCycler PCR Thermocycler (Bio-Rad) with gene-specific primer sets designed using Beacon Designer version 2.1 (Premier Biosoft International, Palo Alto, CA, USA) as follows: urokinase-type plasminogen activator (uPA; 5′-tgctcaccacacagaacat-3′ and 5′-ggcaggcagatgtcat-3′), plasminogen activator inhibitor (PAI; 5′-actgaagcaacatgta-3′ and 5′-ctcagggctgcaggtg-3′), vascular endothelial growth factor (VEGF)-A (5′-ggaggtccacatcaccat-3′ and 5′-ctcttgctctatatctttc-3′), VEGF-C (5′-tcacagcaacagactat-3′ and 5′-tcctcaacattcaactatctt-3′), matrix metalloproteinase (MMP)-7 (5′-gctgtcatatgtcatc-3′ and 5′-tctcctcgagacatact-3′), MMP-9 (5′-atccgagcactatgc-3′ and 5′-ctgagggggtcagaggtg-3′), and β-actin (5′-ggcagcaatgccaggttac-3′ and 5′-atccgagtgggtcacaggg-3′). The mRNA level of each gene was normalized to that of β-actin.

Orthotopic xenograft model and in vivo imaging

All mice were housed in a temperature-controlled (22-23°C) facility with a specific pathogen-free barrier under a 12-h light/dark photoperiod (lights on at 8:00 am). Mice were allowed standard mouse chow and water ad libitum. All animal experiments and procedures were performed in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) at Korea University (KOREA-20160153-C4).

Four- to six-week-old male NOD/SCID mice were purchased from KOATECH (Pyeongteck, Korea). Huh-7 cells (2×10⁶) were injected into the left liver lobe of NOD/SCID mice. After 1 day, the mice were randomized into two groups comprised of five mice each. The experimental group was treated with SP-8356 (30 mg/kg) every day by intraperitoneal injections until the end of the experiment and the control group was similarly treated with vehicle (saline). Body weight was measured every other day. After 40 days, the mice were intraperitoneally injected with luciferin and subjected to in vivo live imaging using NightOWL II LB 983 (Berthold Technologies, Bad Wildbad, Germany). After imaging, the mice were sacrificed and their livers were post-fixed with 4% paraformaldehyde. The nodule number and size in the isolated livers were assessed visually.

Statistical analysis

All statistical analysis was performed using PRISM5 software (GraphPad, La Jolla, CA, USA). The group means were presented as means ± standard deviation (SD) and statistical significance was evaluated using Student’s t tests and/or one-way or two-way analysis of variance (ANOVA) with Bonferroni post hoc tests. A p-value less than 0.05 was considered statistically significant. All experiments were performed in triplicate unless otherwise indicated.
RESULTS

SP-8356 inhibits the growth of liver cancer cells

Based on the various effects of (1S)-(+) -verbenone, such as anti-inflammatory, anti-oxidant, and anti-proliferation, we designed and synthesized (1S)-(+) -verbenone derivatives to investigate their anti-cancer effects on liver cancer cells. Among these derivatives, the S form of SP-8356 significantly inhibited the growth of Huh-7 cells in a time- and dose-dependent manner, while the R form of the molecule had no effect (Fig. 1A). Therefore, in further experiments, the S form of SP-8356 was used. Because SP-8356 showed the most significant growth inhibitory effect, we focused only on SP-8356 in subsequent experiments. The effect of SP-8356 was further investigated in several liver cancer cell lines, and its growth inhibitory activity was observed in all tested cell lines, Hepa1-6, Hep3B and SK-Hep1, with different efficiencies (Fig. 1B).

SP-8356 shows mild cytotoxicity by inducing apoptosis in liver cancer cells

Because the growth inhibitory effect of SP-8356 may be related to the cytotoxic activity of the molecule to liver cancer cells, LDH assays were performed to measure cell death due to SP-8356. The LDH activities in the cells were slightly increased by SP-8356 in a dose-dependent manner. Maximum LDH activities were observed at 48 h to be 21.83%, 31.56%, and 8.77% with 20 µM SP-8356 in Huh-7, Hepa1-6, and Hep3B cells, respectively (Fig. 2A). In particular, the LDH activities in Huh-7 and Hep3B did not change over time as they were similar at both 24 and 48 h. These results may be ascribed to mild cytotoxicity of SP-8356 in these liver cancer cells.

To explore the mechanisms underlying the cytotoxic effects of SP-8356, protein extracts of affected cells were subjected to western blotting with antibodies against death- or survival-related proteins. Caspase activation was examined because it is a hallmark of apoptosis (Ola et al., 2011). Cleaved caspase-3 was detected in Hepa1-6 cells treated with 20 µM SP-8356. Although cleaved caspase-3 was not detected in Huh-7 and Hep3B cells, pro-caspase-3 was decreased, indicating that pro-caspase-3 was activated in these cells. Additionally, the cleavage of PARP, another apoptotic marker, was notably detected in all cells, even with 15-µM SP-8356 treatment in Huh-7 cells. Expression of survivin, a member of the apoptosis-inhibitory family, was decreased by SP-8356 in all cell lines (Fig. 2B). Taken together, these results suggest that the anti-proliferative effect of SP-8356 is likely correlated with the cytotoxic activities of SP-8356.

SP-8356 suppresses the migration and invasion of liver cancer cells

Because metastasis accounts for most liver cancer-related deaths, the effect of SP-8356 on cell motility was determined using wound healing migration assays (Okusaka et al., 1997; Chaffer and Weinberg, 2011; Uchino et al., 2011; Valastyan and Weinberg, 2011). SP-8356 significantly reduced cell migration at 15 µM and 20 µM in Huh-7 cells and 20 µM in Hepa1-6 cells compared with control-treated cells (Fig. 3A).

The effect of SP-8356 on cell invasiveness was investigated using a Matrigel invasion assay. Both Huh-7 and Hepa1-6 cell lines penetrated the matrix toward serum stimulation. The number of invading cells decreased in a dose-dependent manner in response to SP-8356 treatment; in particular, invading Huh-7 cells were not detected following 20-µM SP-8356 treatment (Fig. 3B). Thus, SP-8356 likely exerts a suppressive effect on the migration and invasion of liver cancer cells. Hep3B cells were excluded in the cell motility assay because they are not invasive.

Fig. 1. SP-8356 inhibits proliferation of liver cancer cells. (A) The effect of enantiomers of synthesized (1S)-(+) -verbenone derivative SP-8356 on Huh-7 cell proliferation was investigated using the CCK-8 assay. The cells were treated with various doses of verbenone derivatives for 24, 48, 72, and 96 h. For SP-8356 panel; F(4, 30)=434.78, p<0.001 across SP-8356 concentration. (B) The effect of SP-8356 on different liver cancer cells. For Huh-7 panel; F(4, 30)=434.78, p<0.001 across SP-8356 concentration; F(3, 30)=1508.20, p<0.001, Hepa1-6 panel; F(3, 30)=1508.20, p<0.001, Hep3B panel; F(3, 30)=394.64, p<0.001 and SK-Hep1; F(3, 30)=464.87, p<0.0001 across SP-8356 concentration. All values are shown as means ± SD. **p<0.01, ***p<0.001 versus Veh by two-way analysis of variance (ANOVA) with Bonferroni post hoc test.
SP-8356 inhibits the MAPK pathway via blocking the nuclear translocation of p-ERK1/2

Following analysis of the anti-cancer activities of SP-8356, the effects of the reagent on signaling molecules were examined. Dysregulation of the MAPK and PI3K/Akt signaling pathways commonly occur in liver cancer; the sustained activation of these pathways facilitates liver cancer proliferation and survival (Liu et al., 2009; Min et al., 2011). SRE-dependent luciferase activity by serum, EGF, and PMA (a protein kinase C activator) was significantly reduced by SP-8356 treatment in Huh-7 cells (Fig. 4A).

To understand which molecular mechanism is involved when SP-8356 suppresses SRE activity in response to the tested stimulants, the phosphorylation of ERK1/2 and Akt was investigated. Although the levels of phosphorylated ERK1/2 and Akt remained unchanged in the presence of SP-8356, it inhibited the phosphorylation of Elk-1, a downstream target of p-ERK1/2, which later forms a temporal complex with SRE (Fig. 4B). Because the nuclear translocation of Elk-1 is needed to phosphorylate nuclear Elk-1 (Flores and Seger, 2013), it was hypothesized that SP-8356 may inhibit the nuclear translocation of phosphorylated ERK1/2. To test this hypothesis, we compared the presence of phosphorylated ERK1/2 in the cytoplasm and nucleus. In the cytoplasm, the degree of phosphorylated ERK1/2 was increased when the cells were treated with SP-8356 and EGF compared with that in the cells treated with EGF only (Fig. 4C). Phosphorylated ERK1/2 was notably increased in the nucleus of EGF-treated cells. However, nuclear phosphorylated ERK1/2 was unchanged in the presence of SP-8356, suggesting an inhibitory effect of SP-8356 on p-ERK1/2 nuclear translocation. Taken together, these data suggest that SP-8356 regulates the MAPK pathway by inhibiting the nuclear translocation of p-ERK1/2.

SP-8356 suppresses NF-κB activation by inhibiting the nuclear translocation of p65

Activation of NF-κB is important for cancer progression and metastasis, and its inhibition contributes to cancer suppression (Pikarsky et al., 2004; Naugler and Karin, 2008; Wu et al., 2009). Because (1S)-(-)-verbenone regulates NF-κB activation under certain conditions, including inflammation (Choi et al., 2010; Kuo et al., 2011), the effect of SP-8356 on NF-κB signaling was examined in Huh-7 cells. TNF-α-induced NF-κB-dependent luciferase expression was significantly decreased by SP-8356 (Fig. 5A). Huh-7 cells were treated with PMA because some protein kinase C subgroups can activate IKK, an upstream kinase of NF-κB activation. Interestingly, SP-8356 also suppressed PMA-induced NF-κB transcriptional activation (Fig. 5B).

SP-8356 reduced the basal transcriptional activity of NF-κB without stimulation. In unstimulated cells, NF-κB is sequestered in the cytoplasm bound to inhibitor of kappa B (IκB). After exposure to stimuli such as TNF-α, PMA, and lipopolysaccharide, IκB is phosphorylated by IκB kinase (IKK), followed by ubiquitination and degradation that lead to the nuclear translocation of the RelA/p65 subunit of NF-κB (Hoesel and Schmid, 2013).
To investigate whether SP-8356 inhibits the nuclear translocation of p65, immunocytochemistry was performed. Before TNF-α and PMA treatment, p65 was mainly located in the cytoplasm; after TNF-α and PMA treatment, p65 was translocated to the nucleus. However, 20-µM SP-8356 pretreatment suppressed the nuclear translocation of p65 by TNF-α and PMA (Fig. 5C). The quantitative data of the nuclear translocation showed that the percentage of nuclear p65 induced by TNF-α or PMA was significantly decreased by SP-8356 (Fig. 5D). These results indicate that SP-8356 inhibits NF-κB activation by blocking the nuclear translocation of p65.

Because the nuclear translocation of p65 was carried out by importins, blockade of the interaction between free p65 and importin may be the inhibitory mechanism of SP-8356. This idea was confirmed by immunoprecipitation and subsequent western blotting with cells expressing HA-importin α5; p65

**Fig. 3.** SP-8356 downregulates migration and invasion of liver cancer cells. (A) Wound healing assay. Huh-7 and Hepa1-6 cells were grown to confluence and scratched with a pipette tip. The monolayer was captured under a microscope. After incubation with the indicated doses of SP-8356, the images were captured at 24 h in Huh-7 cells and 9 h in Hepa1-6 cells. Representative microscopic images are shown; the right panels show the percentages of wound closure in the various treatment groups. For Huh-7 panel, F(4, 15)=16.82, p<0.0001 and Hepa1-6 panel, F(4, 15)=5.520, p<0.0062 across SP-8356 concentration. (B) Invasion assay. Huh-7 and Hepa1-6 cells treated with SP-8356 were seeded into Matrigel-coated Transwell chambers and incubated for 18 h. The lower membrane surface to which cells migrated were fixed and stained. Representative images are shown; the number of invading cells was calculated as a percentage of invasion. For Huh-7 panel, F(4, 15)=14.75, p<0.0001 and Hepa1-6 panel, F(4, 15)=13.63, p<0.0001 across SP-8356 concentration. All values are shown as means ± SD. *p<0.05, **p<0.01, ***p<0.001 versus Vehicle by one-way analysis of variance (ANOVA) with Bonferroni post hoc test.
was not detected in the anti-HA antibody-mediated immuno-precipitation of SP-8356-treated cells (Fig. 5E).

**SP-8356 regulates the expression of metastasis-related genes**

The invasive and metastatic properties of cancer cells are acquired by gene expression related to extracellular matrix degradation and new blood vessel formation around the tumor (Valastyan and Weinberg, 2011). Thus, the effect of SP-8356 on the expression of genes influencing cell adhesion, local invasion, and angiogenesis, most of which are targets of NF-κB, was examined. The mRNA levels of uPA, VEGF-A, and VEGF-C were decreased in Huh-7 cells treated with SP-8356 in a dose-dependent manner, whereas that of PAI was significantly increased compared with that in control cells (Fig. 6A). The mRNA levels of MMP-7 and MMP-9 were also reduced by SP-8356 treatment in cells stimulated with TNF-α (Fig. 6B). These results suggest that SP-8356 inhibits the migration and invasion of liver cancer cells by regulating the metastasis-related genes induced by NF-κB.

**Anti-proliferative effect of SP-8356 in a xenograft model**

To investigate the in vivo correlation of the cellular effects of SP-8356, we established a xenograft model by implanting Huh-7 cells expressing luciferase in the liver of SCID mice and then treated the mice with SP-8356 or saline every day. Forty days later, the mice were injected intraperitoneally with luciferin and subjected to in vivo live imaging. Average body weight was similar in both groups (Fig. 7A). The luminescence signals in saline-treated mice were much stronger than those in the SP-8356-treated group (Fig. 7B). After perfusion with PBS and fixing with 4% paraformaldehyde, nodule numbers and size in the isolated livers were higher in the control group than in the SP-8356-treated group, implying that SP-8356 inhibited Huh-7 growth in the liver (Fig. 7C). Unfortunately, we did not observe micrometastasis of the cells in the liver or metastasis into other organs, such as the lung, brain, and other peritoneal regions, in histological analysis. The cause may be the low motility of Huh-7 cells in the in vivo model.

**DISCUSSION**

Most HCC cases are closely associated with NF-κB-related chronic inflammation, and a significant portion of liver cancer patients eventually develop extrahepatic metastasis (Uka et al., 2007; Singh et al., 2018). Treatment of unresectable HCC largely relies on systemic therapeutics, such as multi-kinase inhibitors; however, current systemic treatment options do not produce satisfactory results. (Wang et al., 2003; Pikarsky et al., 2004; Li et al., 2009; Nakagawa and Maeda, 2012; Zhu et al., 2017). Thus, a new anti-cancer agent is needed for effective anti-inflammatory and anti-metastasis activities. In this study, some of the synthesized (1S)-(-)-verbenone derivatives with anti-inflammatory activity (Ju et al., 2013) were screened for growth inhibition of Huh-7 liver cancer cells. One of the de-
Fig. 5. SP-8356 inhibits the NF-κB signaling pathway in Huh-7 cells. (A, B) Treatment with SP-8356 reduced TNF-α- and PMA-stimulated NF-κB activity. Cells were transiently transfected with an NF-κB-luciferase reporter gene construct. After 18 h of serum starvation, the cells were treated with SP-8356 for 30 min and stimulated with 10 ng/mL TNF-α and 1 µM PMA. Cell lysates were prepared and subjected to the luciferase assay. For panel A, non-treated group; F(5, 6)=18.47, p<0.0014 and TNF-α treated group; F(5, 6)=25.81, p<0.0006 across SP-8356 concentration. For panel B, non-treated group; F(5, 6)=25.37, p<0.0006 and PMA treated group; F(5, 6)=41.12, p<0.0001 across SP-8356 concentration. (C) SP-8356 inhibits the nuclear translocation of the NF-κB p65 subunit. Huh-7 cells were serum starved for 18 h, pre-treated with 20 µM SP-8356 for 30 min and stimulated with 10 ng/mL TNF-α and 1 µM PMA for 30 min, and then stained with 4′,6-diamidino-2-phenylindole or FITC-conjugated antibodies for the subcellular localization of p65 (green). White arrows indicate cytosolic p65 and red arrows indicate nuclear p65. Scale bar=25 µm. (D) Quantitation of NF-κB p65 nuclear translocation in the indicated treatment groups. Data are expressed as the percentage of cells with nuclear NF-κB p65 subunit. (E) Immunoprecipitation. HEK293 cells expressing HA-importin α5 were treated with TNF-α and subjected to immunoprecipitation with anti-HA antibody. Western blotting was performed with anti-p65 and anti-HA antibodies. All values are shown as means ± SD. *p<0.05, **p<0.01 within non-treated groups and *p<0.05, **p<0.01, ***p<0.001 within treated groups versus Vehicle by one-way analysis of variance (ANOVA) with Bonferroni post hoc test and Students t-test.
Cells were pretreated with the indicated concentrations of SP-8356 for 1 h and then treated with 10 ng/mL TNF-α. The suppression of NF-κB by SP-8356 is strongly associated with inhibiting cancer progression. Cancer metastasis starts with the entry of cancer cells from a well-confined primary tumor into the surrounding tumor-associated stroma and then into the adjacent normal tissue parenchyma (Valastyan and Weinberg, 2011). To invade the surrounding tissue, it is necessary to degrade the extracellular matrix (ECM). Matrix metalloproteases (MMPs) are important enzymes that can degrade the ECM and promote cancer cell mobility; in particular, MMP7 and MMP9 are overexpressed in liver cancer and play a central role in liver cancer metastasis (Arii et al., 1996; Yeh et al., 2012; Chen et al., 2013; Lin et al., 2017). uPA is a serine protease that converts plasminogen into plasmin, the active proteinase, cleaving ECM proteins and upregulating the expression of uPA in liver cancer (Chan et al., 2004).

Fig. 6. SP-8356 modulates the expression of metastasis-related genes. (A) The effect of SP-8356 on relative mRNA expression levels of uPA, PAI, VEGF-A, and VEGF-C in Huh-7 cells was determined by RT-qPCR. Cells were incubated with different doses of SP-8356 for 24 h. For uPA panel; F(2, 6)=14.76, p<0.0048, PAI panel; F(2, 6)=45.27, p<0.0002, VEGF-A panel; F(2, 6)=95.49, p<0.0001 and VEGF-C panel; F(2, 6)=37.61, p<0.0004 across SP-8356 concentration. (B) Effect of SP-8356 on relative mRNA expression levels of MMP-7 and MMP-9. Cells were pretreated with the indicated concentrations of SP-8356 for 1 h and then treated with 10 ng/mL TNF-α for 24 h. For TNF-α treated groups in MMP-7 panel; F(2, 6)=125.4, p<0.0001 and MMP-9 panel; F(2, 6)=205.3, p<0.0001 across SP-8356 concentration. All values are shown as means ± SD. *p<0.05, **p<0.01, ***p<0.001 within treated groups versus Vehicle by one-way analysis of variance (ANOVA) with Bonferroni post hoc test.
suppressed (Ulisse et al., 2009). Therefore, the regulation of genes implicated in cancer metastasis by SP-8356 is a possible mechanism to prevent liver cancer metastasis. Unfortunately, we could not determine the anti-metastatic activity in the SP-8356 orthotopic xenograft model with Huh-7 cells; however, its anti-proliferation activity was confirmed by tumor mass. Because Huh-7 is a relatively low metastatic cell line (Tong et al., 2017), the cells did not spread, even in the intrahepatic area. An animal model using highly metastatic liver cancer cells may help to elucidate the anti-metastatic activity of SP-8356 as demonstrated in breast cancer cells (Mander et al., 2019).

In summary, this study demonstrated that SP-8356 exerts an inhibitory effect on liver cancer cell growth and motility by regulating apoptosis- and metastasis-associated gene expression. The mechanisms of SP-8356 related to cell growth and migration may result from its regulation of signaling pathways involving NF-κB and MAPK by inhibiting their nuclear translocation. In conclusion, SP-8356 may inhibit liver cancer progression by modulating multiple target molecules in cancer cell activation mechanisms.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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