Induction of apoptosis and suppression of angiogenesis of hepatocellular carcinoma by HS-159, a novel phosphatidylinositol 3-kinase inhibitor

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Abstract. The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in cell proliferation and survival in human cancer and is emerging as an attractive therapeutic target. In this study, we synthesized a novel PI3Kα inhibitor, HS-159 [N-(5-(3-(3-cyanophenyl)imidazo[1,2-a]pyridin-6-yl)pyridin-3-yl)benzenesulfonamide] and evaluated its anticancer effects on Huh-7 human hepatocellular carcinoma (HCC) cells. HS-159 effectively inhibited the phosphorylation of downstream PI3K effectors such as Akt, mTOR and P70S6 kinases in a dose-dependent manner. This compound also induced apoptosis and increased the fraction of apoptotic cells in the sub-G_1 phase as well as the levels of cleaved PARP, caspase-3 and -9. Furthermore, HS-159 decreased the expression of hypoxia inducible factor-1α and vascular endothelial growth factor which play important roles in angiogenesis. The anti-angiogenic effect of HS-159 was confirmed by the suppression of tube formation and migration of human umbilical vein endothelial cells *in vitro*. Collectively, our results demonstrate that HS-159 exhibited anticancer activities including the induction of apoptosis and inhibition of angiogenesis by blocking the PI3K/Akt pathway in Huh-7 cells. Therefore, we suggest that this drug may be potentially used for targeted HCC therapy.

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Introduction

Worldwide, hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most prevalent cause of cancer-related mortality. HCC is also responsible for over 600,000 deaths annually (1). Although this disease is most prevalent in Asia and Africa, the incidence of HCC is increasing in Europe and the United States (2-5). Due to its asymptomatic nature during the early stages, HCC is often diagnosed at a late stage with a very poor prognosis. Although tumor ablation surgery can be successfully performed in patients with early stages of HCC, the recurrence rate is ~50% within 3 years (6). Even with relatively aggressive treatments such as systemic chemotherapy, radiotherapy and trans-arterial chemoembolization, the 5-year relative survival rate for patients with HCC is only 7% (7). Moreover, systemic therapy with classical cytotoxic drugs has been reported to be poorly tolerated and ineffective (8). In particular, sorafenib, a compound globally approved for treating unresectable and advanced cases of HCC, is associated with low response rates and several unwanted side effects such as hypertension, diarrhea and skin reactions on the hands and feet (9,10). Therefore, an effective and well-tolerated pharmaceutical agent for treating advanced HCC is needed.

In recent years, studies of signaling pathways that regulate cancer cell proliferation, angiogenesis, invasion and metastasis have led to the identification of several possible therapeutic targets. In particular, many clinical investigations have indicated that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway plays an integral role in the regulation of many key cellular processes such as proliferation, growth, survival, differentiation and metabolism. Additionally, this pathway is one of the most commonly activated in various cancers (11-13). PI3K generates a lipid secondary messenger, phosphatidylinositol 3,4,5-triphosphate (PIP3), from phosphatidylinositol 4, 5-diphosphate (PIP2) and then leads to Akt activation. Akt subsequently phosphorylates mTOR and several other proteins. mTOR is a serine/threonine protein

kinase that exists as two functional complexes: mTORC1 and mTORC2 (14,15). This kinase promotes cell growth and cell cycle progression by phosphorylating several translational regulators such as P70S6 kinase (P70S6K).

Abnormal activation of the PI3K/Akt pathway is observed in ~50% of patients with HCC (16). Additionally, Akt phosphorylation is involved in early HCC recurrence and poor prognosis (17) and 23% of HCC patients have elevated levels of Akt phosphorylation on Ser^{473} (18). Concurrently, total mTOR expression has been found to be increased in 5% of HCC cases and elevated levels of phosphorylated mTOR have been reported in 15% of HCC patients. In addition, phosphorylated (p)-mTOR positively correlates with increased expression of S6K, which is found in 45% of HCC cases (19). For this reason, the PI3K/Akt/mTOR pathway has emerged as a key therapeutic target and seems to offer a promising strategy for treating patient with HCC.

Although several PI3K/Akt pathway inhibitors have been identified, clinical trials for these agents are only in the early stages. *In vivo* applications have also been limited due to problems with inhibitor stability, solubility and toxicity (20-23). To discover a new structural class of PI3K inhibitors, we used a pharmacophore-directed design. As a result, we recently identified a novel scaffold belonging to the imidazopyridine family that acts as a PI3K/mTOR inhibitor (24). Based on this finding, we designed, synthesized and screened a new series of imidazo[1,2-a]pyridine derivatives. Among these, a new compound, N-(5-(3-(3-cyanophenyl)imidazo[1,2-a]pyridin-6-yl)pyridin-3-yl)benzenesulfonamide (HS-159) was found to be the most potent PI3K inhibitor. In the present study, we determined whether HS-159 has anticancer effects against HCC and explored the molecular mechanism involved in this process.

Materials and methods

In vitro PI3K activity assay. Active PI3Kα (100 ng) was pre-incubated with various doses of HS-159 or LY294002 for 5 min in kinase reaction buffer (25 mM MOPS, pH 7.0; 5 mM $MgCl_2$ and 1 mM EGTA) and 10 μg L-α-phosphatidylinositol. The L-α-phosphatidylinositol was sonicated in water for 20 min before being added to the reaction to facilitate micelle formation. The reaction was initiated by adding 10 μM ATP and allowed to run for 180 min at room temperature. To terminate the kinase reaction, an equal volume of Kinase-Glo® buffer (Promega, Madison, WI) was added. After 10 min, luminescence of the plates was measured with a GloMax plate reader.

Cell lines. Human liver cancer (HepG2, Huh-7 and Hep3B) cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Huh-7 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HepG2 and Hep3B cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were grown in a gelatin-coated 75-cm^2 flask with M199 medium containing 20 ng/ml basic fibroblast growth factor (bFGF), 100 U/ml heparin and 20% FBS. All cell cultures were maintained at 37˚C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2.

Cell viability assay. Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded at a density of $1x10^4$ cells/well in a 96-well plate and incubated for 24 h. The medium was then removed and the cells were treated with either DMSO as a control or various concentrations (0.1-10 μM) of HS-159 for 48 h at 37˚C. Next, 10 μl of MTT solution (2 mg/ml) was added to each well and the plate was incubated for another 4 h at 37˚C. The medium was then removed and the formazan crystals that had formed were dissolved in DMSO (200 μl/well) with constant shaking for 5 min. Absorbance of the solution was then measured with a microplate reader at 540 nm. This assay was conducted in triplicate.

Western blotting. Total cellular proteins were extracted with lysis buffer containing 1% Triton X-100, 1% Nonidet P-40 and a 1% protease and phosphatase inhibitor cocktail containing aprotinin (10 mg/ml), leupeptin (10 mg/ml; ICN Biomedicals, Asse-Relegem, Belgium), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 mM), $NaVO_3$ (500 mM) and $Na_4P_2O_7$ (500 mg/ml; Sigma-Aldrich, St. Louis, MO). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The blots were incubated with the appropriate primary antibodies followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Antibody binding was detected with an enhanced chemiluminescence reagent (Amersham Biosciences). Antibodies against p-mTOR (Ser^{2448}), mTOR, p-Akt (Ser^{473}), Akt, p-P70S6K (Thr^{389}), P70S6K, PARP, cleaved caspase-3, hypoxia inducible factor-1α (HIF-1α), vascular endothelial growth factor (VEGF) and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA).

Immunofluorescence microscopy. Huh-7 cells were plated on 18-mm cover glasses in RPMI-1640 medium and incubated for 24 h at 37˚C so that ~70% confluence was reached. The cells were then incubated in the presence or absence of 10 μM HS-159, washed twice with PBS and fixed in an acetic acid: ethanol solution (1:2, v/v) for 10 min at -20˚C. The cells were blocked in 1.5% horse serum in PBS for 30 min at room temperature and then incubated overnight at 4˚C with primary antibodies against p-Akt, p-mTOR and p-P70S6K (Cell Signaling) in a humidified chamber. After washing twice with PBS, the cells were then incubated with rabbit TRITC secondary antibody (1:100, Dianova, Germany) for 1 h at room temperature. The cells were also stained with 4, 6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. The slides were then washed twice with PBS and covered with fluorescent mounting medium (Dako North America Inc., Carpinteria, CA) before viewing with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Cell cycle analysis. Huh-7 cells were plated in 100-mm culture dishes with RPMI-1640 medium at ~70% confluence and incubated for 24 h at 37˚C. The next day, the cells were treated with either DMSO as a control or various concentra-

tions (0.01-10 μM) of HS-159. Floating and adherent cells were collected and fixed overnight in cold 70% ethanol at 4˚C. After washing with PBS, the cells were subsequently stained with 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A for 1 h at room temperature in the dark. Flow cytometry was then performed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) to determine the percentage of cells in specific phases of the cell cycle (sub-G_1, G_0/G_1, S and G_2/M). The data were analyzed using FlowJo software (Tree Star, Inc.). All the experiments were performed in triplicate.

DAPI staining and terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL). Huh-7 cells were plated on an 18-mm cover glass with RPMI-1640 medium at ~70% confluence and incubated for 24 h at 37˚C. The cells were then incubated in the presence or absence of 10 μM HS-159 for 24 h. The cells were then fixed in cold 2% para-formaldehyde (PFA), washed with PBS and stained with 2 μg/ml of DAPI (Sigma-Aldrich) for 20 min at 37˚C. The stained cells were examined under a fluorescence microscope for evidence of nuclear fragmentation. A TUNEL assay was subsequently performed using a commercial kit (Chemicon, Temecula, CA) according to the manufacturer's instructions.

Measurement of mitochondrial membrane potential (ψ_m). Mitochondrial membrane potential was assessed with a mitochondria staining kit (MitoPT, Immunohistochemistry Technologies, Bloomington, MN) using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1), which indicates potential-dependent accumulation in mitochondria. Huh-7 cells were plated on 18-mm cover glass in RPMI-1640 medium and incubated for 24 h at 3˚C. When confluence reached ~70%, the cells were incubated in the presence or absence of 10 μM HS-159 at 37˚C for 6 h. Next, 100 μl of a JC-1 solution (12.5 μg/ml final concentration) was added to each well and the plate was incubated for 30 min at 3˚C. After washing twice with PBS, the cells were stained with DAPI to visualize the nuclei. The slides were then washed twice with PBS and covered with DABCO before viewing with a confocal laser scanning microscope (Olympus). The results [green/red (G/R) fluorescence ratio] were expressed as a percentage of the control.

Capillary tube formation assay. Matrigel (10 mg/ml, 200 μl; BD Biosciences) was polymerized for 30 min at 37˚C. HUVECs were suspended at a density of $2.5x10^5$ cells/well in M199 medium supplemented with 5% FBS. Aliquots (0.2 ml) of the cell suspension were added to each well coated with Matrigel and incubated with or without different concentrations of HS-159 for 24 h at 37˚C. Morphological changes of the cells and tube formation were observed with a phase-contrast microscope. The cells were captured at magnification x200.

Wound migration assay. HUVECs were plated on 60-mm culture dishes at ~90% confluence. A razor blade was then used to create a wound 2 mm in width in the cell monolayer and the injury line was marked. After wounding, the detached cells were removed with serum-free medium and the remaining HUVECs were further incubated in M199 medium supplemented with 5% FBS, 1 mM thymidine (Sigma-Aldrich) and HS-159 (0.5 μM). The cells were allowed to migrate for 18 h before being rinsed with serum-free medium and fixed in absolute methanol. Cell migration was quantitated by counting the number of cells that had moved beyond the injury line.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using an ANOVA and unpaired Student's t-test. P-values ≤0.05 were considered statistically significant. All calculations were performed using SPSS software for the MS Windows operating system (version 10.0; SPSS, Chicago, IL).

Results

HS-159 inhibits PI3K activity. A new PI3Kα inhibitor, HS-159 (N-(5-(3-(3-cyanophenyl)imidazo[1,2-a]pyridin-6-yl)pyridin-3-yl)benzenesulfonamide) was synthesized as described in our previous study (Fig. 1A) (25). Three-dimensional (3D) coordinates in the X-ray crystal structure of PI3Kα in the resting form (PDB code: 2RD0) were selected as the receptor model for docking simulations. After removing the solvent molecules, hydrogen atoms were added to each protein atom. We used the Auto Dock program for studying docking between PI3Kα and HS-159 because the outperformance of its scoring function over those of the others had been shown in several target proteins. The predicted binding mode of HS-159 in the ATP-binding site of PI3Kα is shown in Fig. 1B. The imidazopyridine of HS-159 maintained stable hydrogen-bonding in the hinge region (Val851). The pyridyl group was anchored by hydrogen bonds to Asp933 and interacted with gatekeeper residue Tyr836. Two stable hydrogen bonds were established between the cyano group of C3 and Thr856. These hydrogen bonds were received from the enzyme and seemed to play a crucial role as an anchor for HS-159 binding. HS-159 could be further stabilized in the ATP-binding site by forming hydrophobic interactions with Met772, Pro778, Lys802, Val850, Ser854, Gln859 and Ile932. Judging from the overall structural features derived from our docking simulations, the inhibitory activity of HS-159 is likely to stem from multiple hydrogen bonds and hydrophobic interactions simultaneously established in the ATP-binding site of PI3Kα. We also found that HS-159 more strongly inhibited PI3Kα activity (EC_{50} 1.892 nM) compared to LY294002, a conventional specific PI3K inhibitor (EC_{50} 488.8 nM, Fig. 1C).

HS-159 inhibits the PI3K/Akt signaling pathway in Huh-7 cells. Since HS-159 inhibited PI3Kα activity, we next determined whether this drug also affected downstream signal transduction factors that promote PI3K-mediated cell survival. When Huh-7 cells were treated with various concentrations of HS-159 for 6 h, the phosphorylation of Akt and its downstream components such as mTOR and P70S6K was effectively suppressed in a dose-dependent manner (Fig. 2A). We further assessed the effect of HS-159 on Akt, mTOR and P70S6K using a fluorescent imaging system. Results from this assay again showed that HS-159 strongly suppressed phosphorylation of these proteins in Huh-7 cells, similar to the western blotting results (Fig. 2B).

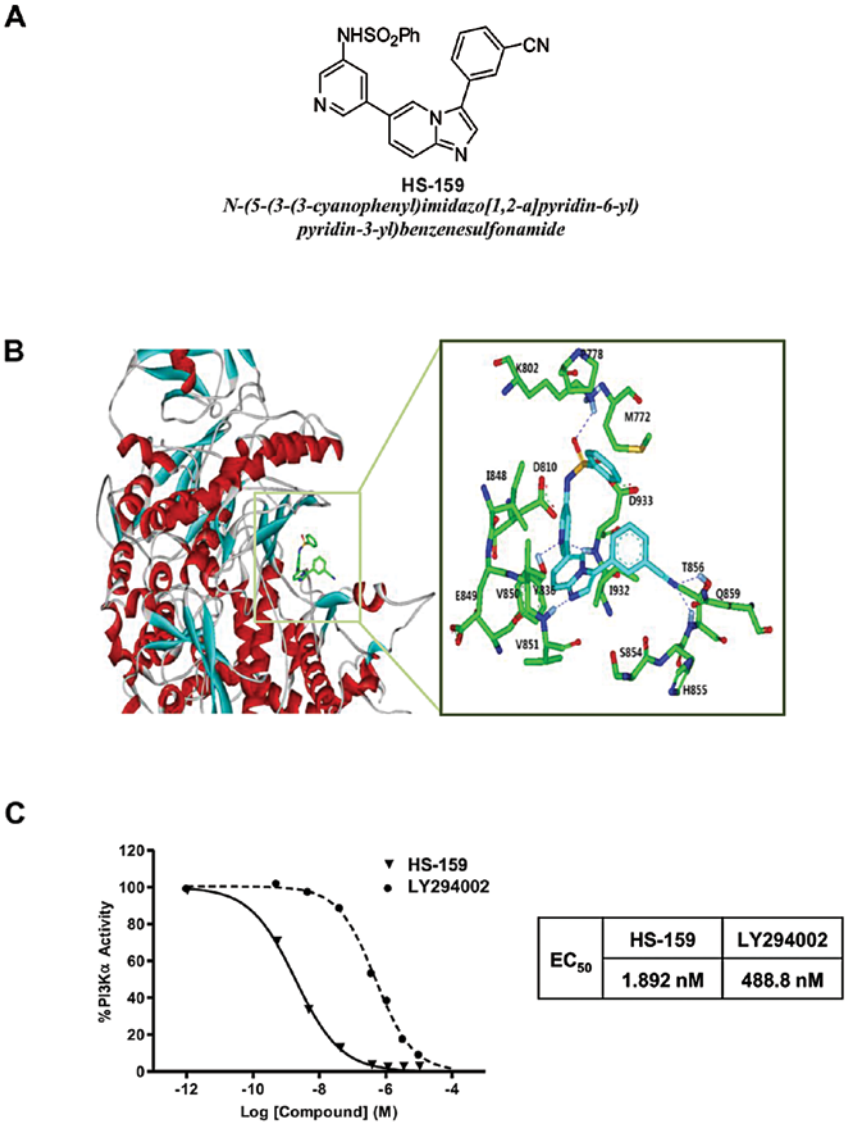

Figure 1. Chemical structure and characterization of HS-159. (A) Chemical structure of HS-159, [N-(5-(3-(3-cyanophenyl)imidazo[1,2-a]pyridin-6-yl)pyridin-3-yl)benzenesulfonamide]. (B) Docking mode of HS-159 according to the PI3Kα homology model based on PI3Kγ crystal structures. (C) Inhibition of PI3Kα by HS-159 or LY294002 *in silico*.

HS-159 inhibits the growth of HCC cells. Next, we further evaluated the anti-proliferative effects of HS-159 on HCC cells using an MTT assay. Huh-7, HepG2 and Hep3B cells were exposed to various concentrations (0.1-10 μM) of HS-159 for 48 h. HS-159 markedly reduced viability of the three HCC cell lines in a dose-dependent manner (Fig. 3). These results indicated that HS-159 exerts potent anti-proliferative effects on HCC cells.

HS-159 induces apoptosis in Huh-7 cells. Cell apoptosis and growth are correlated with cell cycle progression (26). In the present study, HS-159 (0.1-10 μM) inhibited the growth of HCC cells in a dose-dependent manner (Fig. 3). Thus, we performed flow cytometry to monitor changes of the cell cycle profile induced by HS-159. Data from this analysis revealed that treatment with 5 and 10 μM HS-159 increased the number of cells in a sub-G_1 stage, indicative of apoptosis and decreased the fraction of cells in the G_0/G_1 phase in Huh-7 cells (Fig. 4A).

HS-159-induced apoptosis was also evaluated by DAPI and TUNEL staining to characterize nuclear morphology. As shown in Fig. 4B, cells treated with 10 μM HS-159 had morphological features characteristic of apoptotic cells such as bright nuclear condensation and perinuclear apoptotic bodies observed with DAPI staining. HS-159-induced apoptosis was also confirmed by detecting DNA fragmentation using a TUNEL assay. In addition, we performed JC-1 staining to measure the effect of HS-159 on changes in mitochondrial membrane potential that correlate with the intrinsic pathway of apoptosis. As shown in Fig. 4C, the cytoplasm of control cells showed heterogeneous staining with both red and green fluorescence coexisting in the same cells. Consistent with mitochondrial localization, red fluorescence (corresponding to high mitochondrial membrane potential, ψ_m) was mostly found in granular structures distributed throughout the cytoplasm. Treatment with 10 μM HS-159 decreased the red fluorescence while frequent clusters of mitochondria were still observed. HS-159 induced marked changes

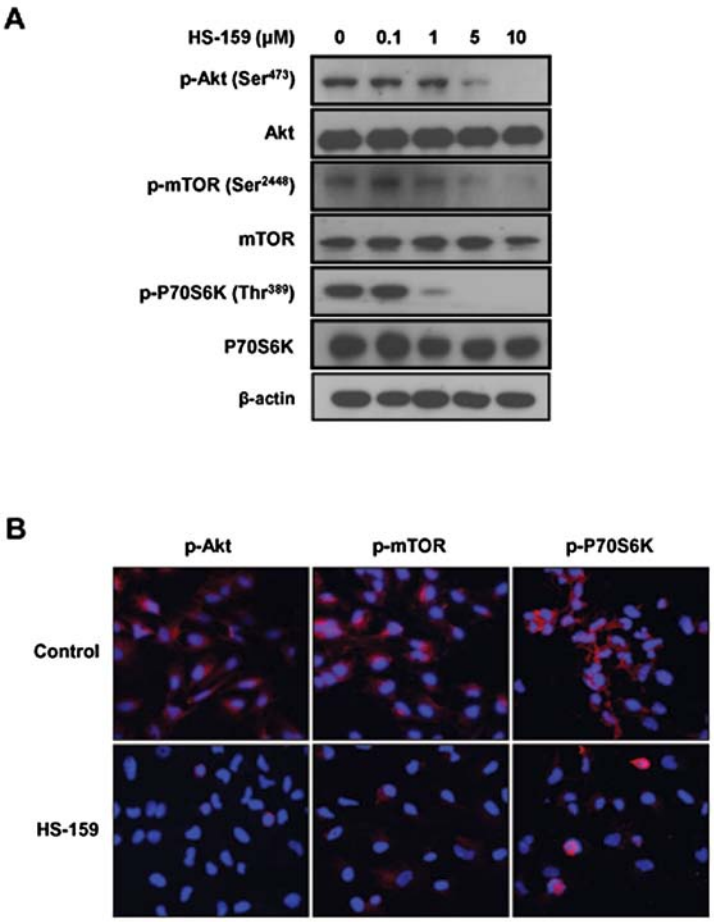

Figure 2. Effect of HS-159 on PI3K/Akt signaling in Huh-7 cells. (A) Cells were treated with various concentrations (0.1-10 μM) of HS-159 for 6 h. The protein expression and phosphorylation of Akt, mTOR and P70S6K were analyzed by western blotting. (B) Huh-7 cells were treated with or without 10 μM HS-159. p-Akt, p-mTOR and p-P70S6K were detected by immunostaining and confocal microscopy. DAPI was used to counterstain the nucleus. Images were taken at magnification x400.

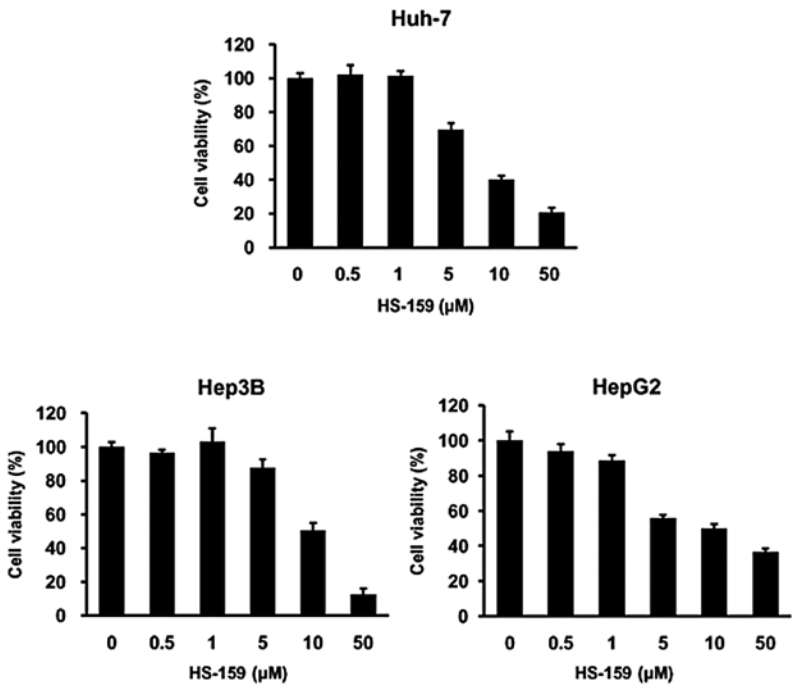

Figure 3. Effect of HS-159 on the proliferation of human HCC cells. Inhibitory effect of HS-159 on the proliferation of Huh-7, HepG2 and Hep3B cells was assessed with an MTT assay. Results are expressed as the percent cell proliferation relative to the proliferation of the control. Each value represents the mean ± SD of triplicate wells.

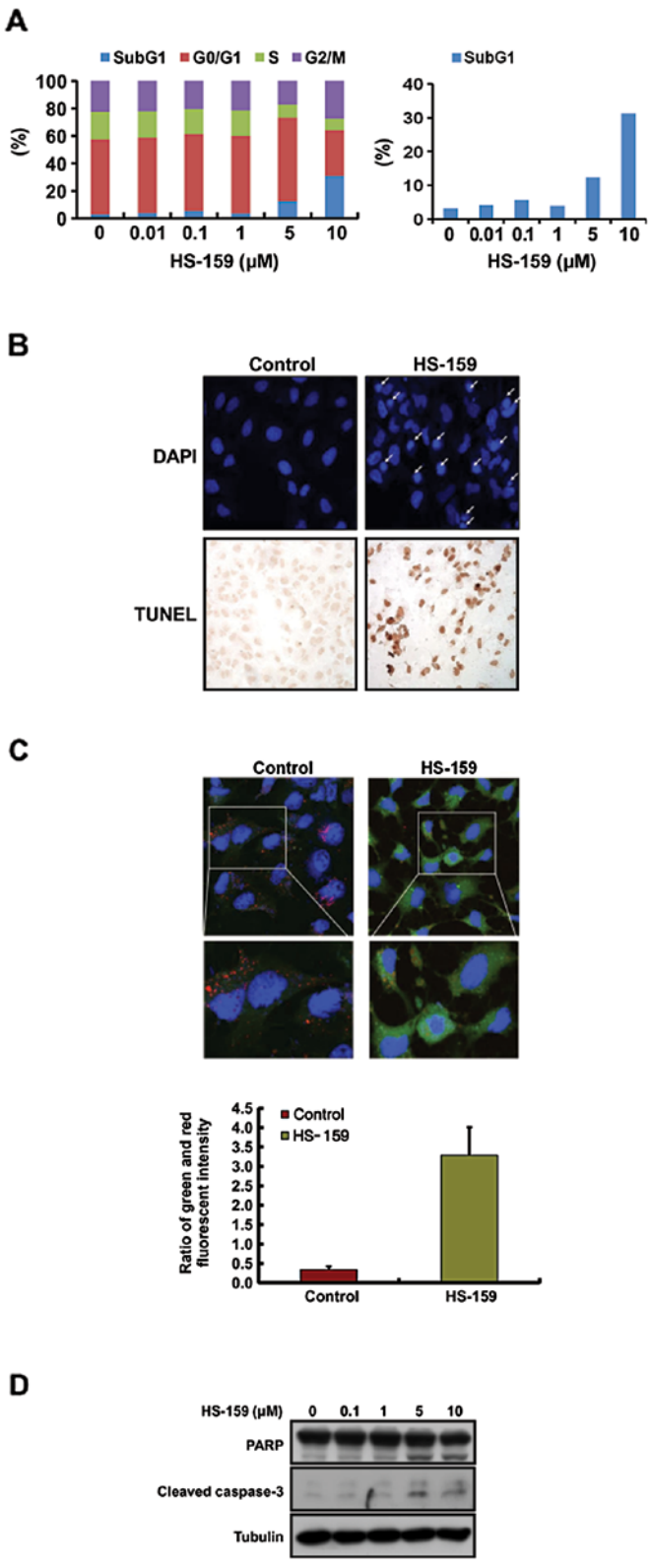

Figure 4. Effect of HS-159 on apoptosis of Huh-7 cells. (A) Huh-7 cells were treated with HS-159 (0.01-10 μM) for 24 h, stained with PI and analyzed with a FACSCalibur flow cytometer. Quantification of PI staining is presented as percentages corresponding to cell cycle distribution. (B) Huh-7 cells treated with or without HS-159 (10 μM) were evaluated by DAPI staining and a TUNEL assay. (C) Huh-7 cells were treated with HS-159 (10 μM) for 6 h. Mitochondrial membrane potential was measured by JC-1 staining and analyzed with a fluorescence microplate reader. The results (G/R ratio) are expressed as the percentage of cells treated with HS-159. Data are expressed as the mean ± SD of triplicate wells. (D) The levels of cleaved caspase-3 and PARP in cells treated with the indicated doses of HS-159 for 24 h were measured by western blotting.

in ψ_m as evident from the disappearance of red fluorescence or increased green fluorescence in most cells. We next performed western blotting to evaluate the activation of caspase-3 and PARP after treatment with HS-159. As expected, HS-159 increased the levels of cleaved caspase-3 and PARP in Huh-7 cells in a dose-dependent manner (Fig. 4D). Taken together, these results showed that HS-159 induces apoptotic cell death in Huh-7 cells.

HS-159 inhibits the expression of HIF-1α and VEGF in Huh-7 cells. A low oxygen level is characteristic of solid tumors and a negative prognostic factor for cancer patient survival. The response of cancer cells to hypoxia not only drives neo-angiogenesis, but also enhances cell survival and malignant phenotype development. HIF-1α is a major regulator of cellular adaptive responses to hypoxia as well as a major transcriptional modulator of angiogenic factors such as VEGF (27). We therefore evaluated the effect of HS-159 on the expression of HIF-1α and VEGF in Huh-7 cells under hypoxia conditions. The cells were treated with various concentrations of HS-159 cultured for 16 h with 100 μM $CoCl_2$ for mimic hypoxic conditions. As shown in Fig. 5A, the expression of HIF-1α and VEGF was increased after exposure to hypoxia and subsequently suppressed by HS-159 treatment, respectively.

HS-159 suppresses angiogenesis. In endothelial cells, PI3K/Akt signaling actively mediates processes associated with angiogenesis including cell migration and capillary tube formation (28). Therefore, we performed capillary tube formation and migration assays to determine whether HS-159 suppresses the angiogenic activity of HUVECs. HS-159 inhibited the formation of vessel-like structures resulting from HUVEC elongation and alignment (Fig. 5B). HS-159 also markedly inhibited cell migration (Fig. 5C). These results indicate that HS-159 is a potential anti-angiogenic reagent that inhibits the tube formation and migration of HUVECs.

Discussion

Many clinical studies have indicated that hyper-activation of the PI3K/Akt pathway plays a critical role in the pathogenesis of various human cancers. Consequently, the PI3K/Akt pathway has emerged as an important therapeutic target for anticancer drug development. Several inhibitors targeting this pathway have been recently developed and some are being evaluated in clinical trials (29,30). These compounds have a wide variety of targets and, include pan-PI3K, isoform-specific PI3K, dual PI3K-mTOR, mTOR catalytic site and Akt inhibitors (23,31-33). Nevertheless, an optimal therapeutic strategy for targeting the PI3K/Akt pathways in cases of HCC has not been identified. In an effort to develop potent PI3K inhibitors, we screened a large number of novel imidazopyridine derivatives. Among them HS-159 acted as a specific PI3K inhibitor, which more strongly inhibited PI3Kα activity (EC_{50} 1.892 nM) than LY294002, a conventional PI3K-specific inhibitor (EC_{50} 488.8 nM). In addition it significantly suppressed proliferation and angiogenesis as well as induced apoptosis by blocking the PI3K/Akt signaling pathway in HCC cells.

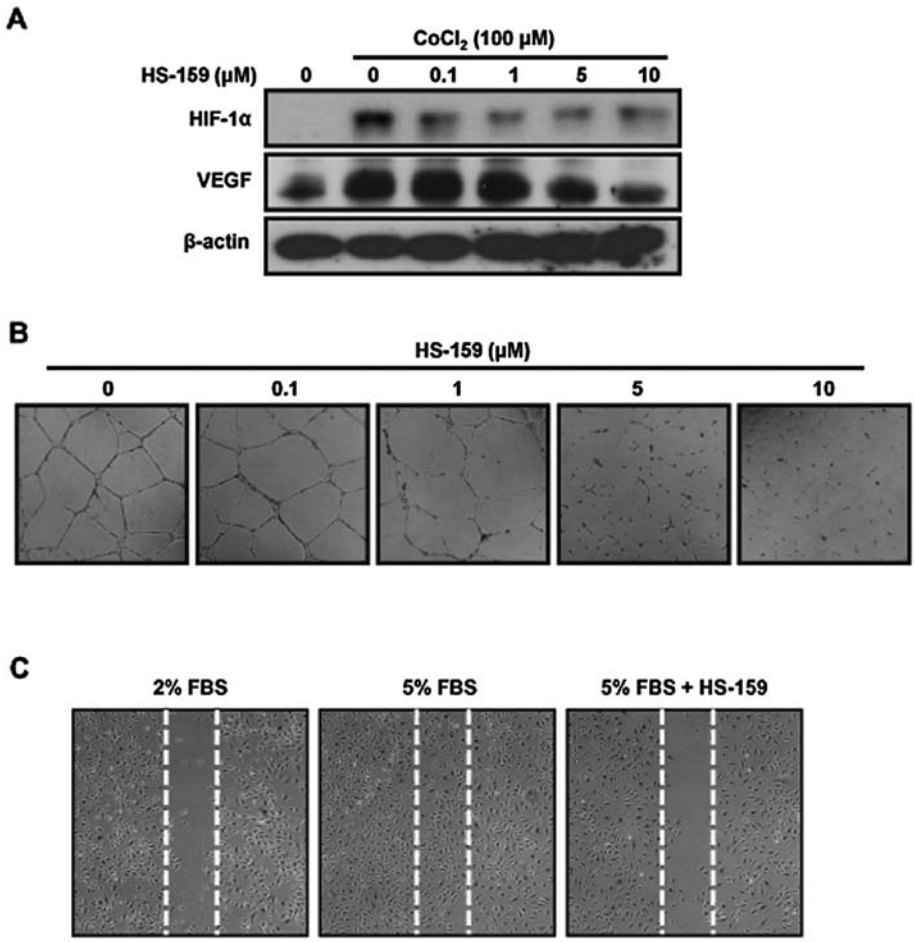

Figure 5. Effect of HS-159 on angiogenesis. (A) Expression of HIF-1α in hypoxic Huh-7 cells treated with HS-159. (B) Effects of HS-159 on HUVEC tube formation *in vitro*. HUVECs were plated on Matrigel (200 μl/well) and treated with different concentrations of HS-159. Capillary tube formation was assessed after 24 h. Morphological changes of the cells and tube formation were observed under a phase-contrast microscope and caprutred at magnification x200. (C) Effects of HS-159 (0.5 μM) on cell migration *in vitro*. HUVECs were plated at ~90% confluence and the cell monolayer was scratched with a razor blade.

Akt is considered the most important signaling factor in the PI3K pathway as it regulates various substrates affecting processes that control cell growth and survival (34). It was also reported that Akt phosphorylation on Ser^{473} is associated with poor prognosis in patients with different types of solid tumors including those in the pancreas, liver, prostate and breast (35). In our western blotting and immunofluorescent studies, HS-159 was found to effectively suppress the phosphorylation of downstream PI3K effectors including Akt, mTOR and P70S6K dose-dependently in HCC cells. Considering that the PI3K/Akt pathway is important for cell growth and progression, we also tested whether HS-159 inhibited the survival of three HCC cell lines. HS-159 inhibited the growth of all three cell lines in a dose-dependent manner. Thus, we suggest that HS-159 prevents cell growth by blocking the PI3K/Akt pathway in HCC cells.

Cell cycle arrest in tumor cells is a major indicator of anticancer activity. Additionally, cell cycle dysregulation has been implicated in tumor development and proliferation characteristic of different cancers, including HCC (30). Thus, we analyzed cell cycle changes following treatment with HS-159. We found that HS-159 induced cell cycle arrest during the G_0/G_1 phase. We also observed that the anti-proliferative activity of HS-159 was associated with the induction of apoptosis in Huh-7 cells. This was indicated by elevated levels of cleaved caspase-3 and PARP as well as increased number of TUNEL-positive cells. The alteration of mitochondrial membrane potential induces the release of cytochrome c from mitochondria into the cytosol and is associated with caspase-3 activation (36). We therefore monitored changes of mitochondrial membrane potential following treatment with HS-159. Our results showed that treatment with HS-159 disrupted the mitochondrial membrane potential in Huh-7 cells. Overall, these data suggest that HS-159 induced apoptotic cell death through activation of the intrinsic apoptotic pathway.

Rapid tumor cell proliferation in a stable vascular system leads to the development of hypoxia in almost all solid tumors (26). Because the liver is a highly vascular organ that depends on angiogenesis for cellular regeneration, angiogenesis may be specifically targeted as a novel therapeutic approach for treating HCC (37). Hypoxia in many solid tumors increases the expression of major angiogenic factors such as HIF-1α and VEGF to promote neo-angiogenesis (38,39). Additionally, it has been reported that PI3K signaling regulates HIF-1α and VEGF expression (40,41). We thus assessed the effect of HS-159 on expression of these two angiogenic factors.

As expected, HS-159 significantly reduced the expression of both HIF-1α and VEGF under hypoxic conditions induced by $CoCl_2$ in Huh-7 cells. The activation of endothelial cells by angiogenic processes results in cell proliferation and migration as well as cord tube formation for the creation of new microvessels (42). We performed tube formation and migration assays to study the effect of HS-159 on HUVECs. HS-159 significantly inhibited HUVEC tube formation as well as migration. Overall, our data suggest that HS-159 may be a potent anti-angiogenic agent.

In conclusion, our study demonstrated that HS-159 is a specific PI3K inhibitor and exerts potent anticancer effects against HCC cells. Additionally, blocking the PI3K/Akt pathway with HS-159 inhibited cell proliferation and angiogenesis and induced apoptosis of HCC cells. These finding suggest that HS-159 is a novel anticancer agent with the potential for treating patients with HCC and other diseases associated with dyregulated PI3K signaling.

Acknowledgements

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