HS-116, a novel phosphatidylinositol 3-kinase inhibitor induces apoptosis and suppresses angiogenesis of hepatocellular carcinoma through inhibition of the PI3K/AKT/mTOR pathway

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Abstract
The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in cell proliferation and survival of human cancers. As PI3K is active in many cancer patients, resulting in cancer development and progression, we developed an azaindole derivative, HS-116 as a novel PI3K inhibitor. This study aimed to clarify the anticancer effect of HS-116 in human hepatocellular carcinoma (HCC). To identify the effect of HS-116 on HCC cells, a PI3K assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, and Western blotting were conducted. IC50 of HS-116 for PI3K was 31 nM, and it effectively suppressed the phosphorylation of PI3K downstream factors such as AKT, mTOR, p70S6K, and 4EBP1. Also, HS-116 induced apoptosis by increasing the proportion of sub-G1 apoptotic cells from 1.8% to 35% and increasing the expressions of Bax, cleaved-caspase-3, and cleaved-PARP as well as decreasing the expression of Bcl-2. In addition, chromatin condensation and apoptotic bodies were detected in HS-116-treated HCC cells. Furthermore, HS-116 decreased protein expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF), and inhibited the tube formation and migration of human umbilical vein endothelial cells (HUVECs). In vivo, the ability of mice to vascularize subcutaneously implanted Matrigel plugs was diminished when the mice were treated with HS-116. These results show that HS-116 inhibits the PI3K/AKT/mTOR pathway via apoptosis and anti-angiogenesis in HCC cells. We suggest that HS-116 may be an effective novel therapeutic candidate against HCC.

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1. Introduction
Hepatocellular carcinoma (HCC) is the sixth most common malignancy and the third most common cause of cancer-related mortality worldwide [1]. Being most prevalent in Asia and Africa, it is the second leading cause of cancer death in China [2]. One of the reasons for the high mortality rate in patients with HCC is the lack of effective treatment, especially for those with advanced disease. Although tumor ablation surgery can be achieved in patients with early HCC, recurrence rates are very high, approximately 50% within 3-year [3]. Also, even with treatment such as trans-arterial chemoembolization, intra-arterial or systemic chemotherapy, and radiotherapy, the 5 year relative survival rate for patients with HCC is only 7% [4]. Moreover, systemic therapy with classical cytotoxic drugs has been reported to be poorly tolerated and ineffective [5]. Especially, sorafenib, globally approved for the treatment of unsalvageable and advanced HCC has shown low response rate and side effect such as hypertension, diarrhoea, rash, fatigue, and hand and foot skin reactions [6–8]. For this reason, an effective and well-tolerated pharmaceutical development for advanced HCC highlights the need for new therapeutic approaches.

In recent years, studies of an oncogenic signalling pathway that regulates cancer cell proliferation, angiogenesis, invasion, and metastasis have led to the identification of several possible therapeutic targets. The phosphoinositide 3-kinase (PI3K)/AKT/mTOR signaling pathways is one of the most commonly activated signaling pathways in human cancer [9]. PI3K catalyzes the phosphorylation of the 3-hydroxyl position of PIP2 (phosphatidylinositol 4,5-diphosphate) to PIP3 (phosphatidylinositol 3,4,5-triphosphate). Deregulation of PI3K leads to elevated PI3P levels and downstream activation of AKT [10]. Indeed, overexpression of
AKT has been established in many human cancers including HCC [11], which inhibits apoptosis and promotes cell proliferation [12]. mTOR is a serine/threonine protein kinase that exists as two functional protein complexes, mTORC1 and mTORC2 [13,14]. This kinase also promotes cell growth and cell cycle progression by phosphorylating the translational regulators p70S6 kinase (p70S6K) and eukaryotic initiation factor (eIF) 4E binding protein 1 (4EBP1). To this end, PI3K/AKT/mTOR pathway has emerged as a key therapeutic target for cancer treatment. In fact, the levels of the phosphorylated form of mTOR have been shown to be elevated in 15% of cases of HCC patients, and the levels of p70S6K have been shown to be increased in 45% of the cases [15]. Therefore, the inhibition of PI3K signalling in HCC seems to be a promising strategy for the treatment.

For the goal of discovery of a new structural class of PI3K inhibitors, we initiated a pharmacophore-directed design. Our previous study reported that azaindole substructures enhance the cytotoxicity against cancer cells through stronger hydrogen bonding with the target enzymes [16]. Based on this result, we synthesized and screened a chemical library of azaindole derivatives [17]. Among them, HS-116 (N-(5-(3-(3-cyanophenyl)-1H-pyrrolo[2,3-b]pyridin-3-yl)pyridin-3-yl)benzenesulfonamide) was selected as the most potent PI3K inhibitor. In this study, we investigated whether HS-116 has anti-cancer activity against HCC, and the molecular mechanism involved in this process. Our results show that HS-116 induces apoptosis and inhibits proliferation and angiogenesis by inhibiting the PI3K/AKT/mTOR pathway in human HCC cells.

2. Material and methods

2.1. Cells and materials

The human HCC cell lines Huh-7, Hep3B, and HepG2 were purchased from ATCC (Manassas, VA), and normal liver cell line HL-7702 was purchased from Shanghai Institute of Cell Biology (Shanghai, China). Huh-7 and HL-7702 cells were cultured in Roswell Park Memorial Institute Media 1640 (RPMI-1640), and Hep3B and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. FBS, cell culture media, penicillin-streptomycin, and all other agents used in cell culture studies were purchased from Gibco (Grand Island, NY). Cultures were maintained at 37 °C in a CO2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO2. Human umbilical vein endothelial cells (HUVECs) were grown in a gelatin coated 75 cm2 flask in M199 medium containing 20 ng/mL basic fibroblast growth factor (bFGF), 100 U/mL heparin, and 20% FBS at 37 °C. Propridum iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO). RNase A was purchased from Qagen (Valencia, CA).

2.2. Synthesis of a new compound, HS-116

HS-116 was synthesized as described in our previous report [14]. Briefly, a solution of 2-chloroaniline in 20% hydrochloric acid was slowly added to a solution of sodium nitrite in water. Acreolin was slowly added to the reaction mixture, and then extracted with dichloromethane. The organic layers were washed with sodium hydroxide and extracted with dichloromethane and water containing 4 M sodium hydroxide. The organic layer was extracted with 4 M hydrochloric acid to give the desired product in a 25% yield. 5-(2-chlorobenzyl) thiazol-2-amine was extracted with dichloromethane and water containing sodium bicarbonate. Next, the residue was purified using flash column chromatography (DCM:Hexane = 2:1 to only DCM) to give a desired product. The final product was more than 98% pure and was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 50 mM, and was stored at −20 °C.

2.3. Measurement of cell proliferation

Cell viability was performed by the MTT assay. Briefly, Huh-7, Hep3B, and HepG2 cells were plated at a density of 3 × 104 cells/well in a 96-well plate for 24 or 48 h. The medium was removed, and cells were treated with either DMSO as a control or various concentrations of HS-116. The final concentration of DMSO in the medium was <0.1% (v/v). After the cells were incubated for 24 or 48 h, 20 μL of the MTT solutions (2 mg/mL) was added to each well for another 4 h at 37 °C. The formazan crystals that formed were dissolved in DMSO (200 μL/well) by constant shaking for 5 min. The plate was then read on a microplate reader at 540 nm. Three replicate wells were used for each analysis. The median inhibitory concentration (IC50, defined as the drug concentration at which cell growth was inhibited by 50%) was assessed from the dose-response curves.

2.4. PI3K activity assay

An active PI3K (100 ng) was preincubated with HS-116 or LY294002 for 5 min in kinase reaction buffer (3-(N-morpholino)propan sulfonic acid [MOPS, pH 7.0], 5 mM MgCl2, and 1 mM ethylene glycol tetraacetic acid [EGTA]) and 10 μg λ-γ-phosphatidylinositol. Before addition of γ-γ-phosphatidylinositol, it was sonicated in water for 20 min to allow micelle formation. The reaction was started by the addition of 10 μM ATP and was run for 180 min. To terminate the kinase reaction, the same volume of Kinase-Glo Max buffer (Promega; Madison, WI) was added. After 10 min, the plates were then read on a GloMax plate reader for luminescence detection.

2.5. Western blotting

Cells were washed three times with ice-cold phosphate buffered saline (PBS) before lysis. Cells were lysed with buffer containing 1% Triton X-100, 1% Nonidet P-40 (NP-40), and the following protease and phosphatase inhibitors: apotinin (10 mg/mL), leupeptin (10 mg/mL) [ICN Biomedicals, Assse-Releagen, Belgium], phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 mM), Na3VO4 (500 mM), and Na2P2O7 (500 mg/mL) (Sigma–Aldrich, St. Louis, MO). Equal amounts of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes and the protein transfer was checked by staining with Ponceau S solution (Sigma–Aldrich). Immunostaining of the blots was performed using the primary antibodies, followed by the secondary antibody conjugated to horseradish peroxidase (HRP) and detection by enhanced chemiluminescence reagent (ECL, Seoul, Korea). Primary antibodies were mouse monoclonal antibodies: anti-caspase-3, Bax, and Bcl-2 (Santa Cruz Biotechnology, SantaCruz, CA), anti-HIF-1α (BD Biosciences, SanJose, CA), and cleaved caspase-3, PARP, p-AKT, p-mTOR, and p-p70S6K (Cell Signaling Technologies, Danvers, MA). The secondary antibodies were purchased from Amersham Biosciences (Piscatway, NJ).

2.6. Cell cycle analysis

Huh-7 cells were plated in 100 mm-diameter culture dishes. The next day, cells were treated with various concentrations of HS-116 or 0.1% DMSO for 24 h. Floating and adherent cells were collected and fixed in cold 70% ethanol at 4 °C overnight. After washing, the cells were subsequently stained with 50 μg/mL propidium iodide (PI) and 100 μg/mL RNase A for 1 h in the dark and subjected to flow cytometric analysis to determine the percentage of cells at specific phases of the cell cycle. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, SanJose, CA), and cleaved caspase-3, PARP, p-AKT, p-mTOR, and p-p70S6K (Cell Signaling Technologies, Danvers, MA). The secondary antibodies were purchased from Amersham Biosciences (Piscatway, NJ).

2.7. DAPI staining and TUNEL assay

Huh-7 cells were plated onto a 18-mm cover glass in RPMI-1640 medium at approximately 70% confluence for 24 h. The cells were then treated with HS-116 at 10 μM for 24 h. They were fixed in ice-cold 2% para-formaldehyde (PFA), washed with PBS and then stained with 2 μg/mL 4,6-diamidino-2-phenylindole (DAPI) for 20 min at 37 °C. The stained cells were examined under a fluorescence of nuclear fragmentation. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed following the manufacturer’s protocol for TUNEL kit (Chemicon, Temecula, CA).

2.8. Tube formation assay

A 10 mg/mL (200 μL) of Matrigel (BD Biosciences) was polymerized by constant shaking for 4 min at 37 °C. HUVECs were suspended in M199 (2% FBS) medium at a density of 2.5 × 104 cells/mL, and 0.2 mL of cell suspension was added to each well coated with Matrigel, together with or without the indicated concentrations of HS-116 and VEGF (50 ng/mL) for 20 h. The morphological changes of the cells and tubes formed were observed under a phase-contrast microscope and photo-graphed at 200× and 400× magnification.
2.9. Wound migration assay

HUVECs plated on 60 mm-diameter culture dishes at 90% confluence, were wounded with a razor blade score 2 mm in width and marked at the injury line. After wounding, the peeled off cells were removed with a serum-free medium and further incubated in M199 with 2% FBS, 1 mM thymidine (Sigma-Aldrich), HS-116 (0.1–10 μM) and/or VEGF (50 ng/mL). HUVECs were allowed to migrate for 18 h and were rinsed with a serum-free medium, followed by fixing with absolute. Migration was quantitated with counting the number of cells that moved beyond the reference line.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The amount of VEGF secreted into media was measured by sandwich ELISA. ELISA plates (Nunc, Roskilde, Denmark) were coated with 100 μL of 2 ng/ml anti-VEGF (R&D Systems, Minneapolis, MN) antibody in PBS for 24 h at 25°C. The plates were washed with PBS containing 0.1% Tween-20 and incubated for 1 h at 25°C with 200 μL/well of 1% bovine serum albumin (BSA, Sigma–Aldrich) in PBS. The conditioned medium or various concentrations of recombinant human VEGF were incubated for 2 h at 25°C with 100 μL of 75 ng/mL biotinylated anti-VEGF antibody, the plates were washed and further incubated for 30 min with 100 μL of HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA). After washing, the reaction was stopped by adding 50 μL of 2 N H₂SO₄. The absorbance at 450 nm was measured with a 96-well plate reader.

2.11. Matrigel plug assay

Animal care and experimental procedures were conducted in accordance with the Guide for Animal Experiments by the Korean Academy of Medical Sciences. Male BALB/c 6-week-old mice were obtained from Orient-Bio Laboratory Animal Research Center Co., Ltd. (Gyeonggi-do, Kapyeong, Korea). Animals were fed with standard rat chow with free access to tap water in a temperature- and humidity-controlled animal house alternating 12 h light–dark cycles. The mice were subcutaneously injected with 500 μL of Matrigel containing concentrated VEGF (50 ng/mL), and either HS-116 (10 μM) or PBS (10 μL). After 7 days, mice were killed and the Matrigel plugs were removed.

2.12. Histopathological examination

Matrigel plugs were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned. The 8 μm-thick sections were stained with hematoxylin and eosin (H&E) for routine histology. For H&E staining, sections were stained with hematoxylin for 3 min, washed, and stained with 0.5% eosin for an additional 3 min. After a washing step with water, the slides were dehydrated in 70%, 96%, and 100% ethanol, and then in xylene.

2.13. Fluorescent immunohistochemistry

Ten micrometer thick frozen sections were incubated overnight at 4°C with 1:100 dilutions of rabbit anti-p-AKT, p-p70S6K, and p-4EBP antibodies (Cell Signaling Technologies). After washing three times with PBS, detection of primary antibodies were performed using a 1:200 dilution of rabbit cy5- and fluorescein isothiocyanate (FITC)-labeled secondary antibodies raised in a mouse and rabbit, respectively (Vector Laboratories). After washing with PBS three times, each slide was occluded with 50% glycerin buffer and was observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.14. Statistical analysis

Data are expressed as mean ± S.D. Statistical analysis was performed using ANOVA and an unpaired Student’s t-test. A P-value of <0.05 was considered statistically significant. Statistical calculations were performed using SPSS for Windows Version 10.0 (SPSS, Chicago, IL).

Fig. 1. Chemical structure and its docking mode of HS-116 and inhibition effects of HS-116 on phosphatidylinositol 3-kinase (PI3K) activity in vitro. (A) N-(5-(3-(3-cyanophenyl)-1H-pyrrolo [2,3-b] pyridin-5-yl) pyridin-3-yl)benzensulfonamide. [B] PI3K activity was estimated using PI3-kinase ELISA kit. Data represents as mean ± S.D. from the triplicate wells.
3. Results

3.1. HS-116 inhibits PI3K activity in vitro

We synthesized a novel PI3K inhibitor HS-116, N-(5-(3-(3-cyanophenyl)-1H-pyrrolo[2,3-b]pyridine-5-pyridine-3yl) benzenesulfonamide, which inhibited comparatively ATP binding site against PI3K. We observed that HS-116 had high binding affinity in the ATP-binding site of PI3K. As shown in Fig. 1A, HS-116 appeared to be in close contact with residues Val851, Tyr836, Asp810, and Lys802, which belong to the hinge region, the gatekeeper site, DFG pocket and catalytic lysine region, respectively. Among them, these hydrogen bonds seem to play a critical role of anchor for binding of HS-116. Owing to binding of hydrogen bond at PI3K, HS-116 may be further stabilized in the ATP-binding site. We first examined the ability of HS-116 to inhibit PI3K activity in vitro using a PI3K assay. For these experiments, we preincubated HS-116 or LY294002, the latter a well-characterized PI3K inhibitor, for 5 min in kinase reaction buffer. The reaction was started by the addition of 10 μM ATP and L-α-phosphatidylinositol and run for 180 min. As shown in Fig. 1B, HS-116 inhibited PI3K activity dramatically. Interestingly, at concentration of 0.1 μM, HS-116 reduced the PI3K activity similar to the effect of 10 μM LY294002. This result showed that HS-116 could act as a strong PI3K inhibitor.

Fig. 2. Effect of HS-116 on PI3K/AKT/mTOR pathway signalling in Huh-7 HCC cells. (A) Cells were treated with HS-116 at various doses (0.1–20 μM). Western blotting experiments for p-AKT, p-mTOR, and p-p70S6K were performed with the lysates of cells. (B) Immunofluorescent imaging of PI3K/AKT/mTOR target proteins after treatment of HS-116 is shown. For labelling, anti-rabbit antibodies against p-AKT, p-70S6K, and p-4EBP1 were used. DAPI was used to counter stain the nucleus. 400× magnification.

3.2. HS-116 inhibits PI3K/AKT/mTOR signaling pathway in Huh-7 HCC cells

As activation of PI3K/AKT/mTOR plays a crucial role in carcinogenesis by maintaining cancer cell proliferation and preventing apoptosis [9], recent studies have focused on developing novel anti-cancer agents targeting this pathway [18,19]. Therefore, we next investigated the effects of HS-116 on the PI3K/AKT/mTOR pathway in Huh-7 HCC cells. When HCC cells were treated with various concentrations of HS-116, the phosphorylation level of AKT and its downstream factor mTOR, were effectively suppressed in a dose-dependent manner (Fig. 2A). mTOR activation results in phosphorylation of effectors such as p70S6K and 4EBP1, which subsequently leads to mTOR-dependent gene transcription that regulates cell proliferation, protein synthesis, and metabolism [20]. Therefore, we further determined the effect of HS-116 on the p70S6K and 4EBP1 using a fluorescent imaging system. The phosphorylation of p70S6K and 4EBP1 was down-regulated by HS-116 as shown by decreased fluorescence in these proteins when compared with control (Fig. 2B).

3.3. HS-116 inhibits cell growth in Huh-7 HCC cells

To determine if HS-116 could function as a new therapeutic compound, we tested the cell growth inhibition on three HCC cell
lines (HepG2, Hep3B, and Huh-7 cells) using MTT assay. HCC cells were exposed to various concentrations (0.1–50 μM) of HS-116 for 24 h or 48 h. HS-116 treatment clearly reduced cell viability in all HCC cell lines in a time- and dose-dependent manner (Fig. 3A–C). Especially, 50 μM of HS-116 induced strong reduction in the growth rate of the three HCC cell lines which ranged between 10% and 40% at 48 h. To predict side effects of HS-116, it was exposed to HL-7702 normal liver cell line (Fig. 3D). Change of cell viability by exposure to HS-116 was less in HL-7702 than in HCC cells. Next, we compared the effects of HS-116 with those of sorafenib, a commercial drug, in Huh-7 cells. As shown in Fig. 3E, HS-116 inhibited cell growth over 20% compared with sorafenib at more than 10 μM concentration of each drug. In addition, HL-7702 cell toxicity of HS-116 (50 μM) was low (about 22%) compared with sorafenib (Fig. 3F). In summary, the effect of HS-116 was higher than sorafenib in HCC cells and cytotoxicity of HS-116 was lower than sorafenib in the normal liver cell line.

3.4. HS-116 induces apoptotic cell death in Huh-7 HCC cells

Cell apoptosis and growth correlated with cell cycle progression. Indeed, HS-116 induced a dose-dependent inhibition of cell growth at doses from 0.1–50 μM in Huh-7 HCC cells (Fig. 3). Thus, we performed flow cytometric analysis to determine changes of the cell cycle profile induced by HS-116. Flow cytometric data revealed that treatment with 10 and 20 μM of HS-116 increased the number of cells in subG1 phase (34–35%), indicating apoptosis and decreased G0/G1 phase in Huh-7 cells (Fig. 4A). Also, induction of apoptosis by HS-116 was evaluated by DAPI and TUNEL staining, to characterize nuclear morphology. As shown in Fig. 4B, cells treated with 10 μM HS-116 presented the characteristic morphological features of apoptotic cells, such as bright nuclear condensation and perinuclear apoptotic bodies by DAPI staining. Apoptosis by HS-116 was confirmed by detection of DNA fragmentation using TUNEL staining. We next examined the activation of caspase-3, cleaved PARP, Bax, and Bcl-2 by Western blotting after HS-116 treatment. As expected, HS-116 increased the expression of the cleaved caspase-3, cleavage of PARP, and Bax, as well decreasing the cleavage of Bcl-2 in Huh-7 HCC cells in a dose-dependent manner, starting at a concentration of 5 μM (Fig. 4C). These results showed that HS-116 could induce cell apoptosis in Huh-7 HCC cells.

3.5. HS-116 inhibits the expression of HIF-1α and VEGF in Huh-7 HCC cells

Considering the importance of HIF-1α in hypoxia and its potential correlation with HS-116, we attempted to examine the effect of HS-116 on expression pattern of HIF-1α in Huh-7 cells. Cells were treated with various concentrations of HS-116 under hypoxia-mimicking condition induced by 100 μM CoCl2 for 6 h. As shown
in Fig. 5A, HIF-1α expression was increased under the hypoxic condition. However, HS-116 in excess of 0.1 µM inhibited the hypoxia-induced HIF-1α expression. To further confirm the effect of HS-116 on hypoxia-induced VEGF, an immediate downstream target gene of HIF-1α, the protein and production of VEGF were determined by Western blotting and ELISA in Huh-7 cells. A notable increase of VEGF was observed after exposure to hypoxia, and the treatment of HS-116 suppressed hypoxia-induced VEGF expression and production in a dose-dependent manner under hypoxia (Fig. 5A and B).

3.6. HS-116 suppressed VEGF-induced tube formation and migration of HUVECs

To examine the effect of HS-116 on the angiogenesis of HCC, a capillary tube formation assay using HUVECs was done to mimic in vivo HCC associated angiogenesis. HS-116 inhibited VEGF-induced formation of vessel-like structures, consisting of the elongation and alignment of the cells at the indicated concentrations (Fig. 5C). Cell migration is critical for endothelial cells to form blood vessels in angiogenesis and is necessary for tumor growth and metastasis. Thus, we conducted a wound migration assay to identify the effect of HS-116 on cell migration. When the endothelial cells were wounded and incubated in a medium with VEGF in the presence of 10 µM HS-116 for 20 h, HS-116 markedly inhibited remarkably VEGF-induced cell migration (Fig. 5D). Considering that endothelial migration and tube formation are all highly relevant properties in the process of angiogenesis, our results show that HS-116 has the ability to block VEGF-induced in vitro angiogenesis.

3.7. HS-116 suppresses angiogenesis in the Matrigel plug model

To further confirm whether HS-116 had an anti-angiogenic activity, we performed Matrigel plug assay, which is an established in vivo angiogenesis model. Matrigel containing either VEGF or HS-116 was subcutaneously injected into male BALB/c mice and removed from the mice at 7 days after the implantation. As shown in Fig. 6A and B, blood vessels were rarely observed in Matrigel plugs without VEGF. VEGF strongly induced neovessels containing intact red blood cells inside the Matrigel, which were obviously inhibited by 10 µM HS-116 treatment. For histological analysis, each section of the Matrigel plug was stained with H&E and an endothelial marker CD34. The stained sections showed that the
plug with HS-116 treatment had fewer vessels within the gels than the VEGF-induced Matrigel plug. Expression of CD34 was also decreased by HS-116 treatment in VEGF-induced Matrigel plug. These results confirmed that HS-116 possessed a potent anti-angiogenic activity in vivo.

3.8. HS-116 inhibited the activation of VEGF-induced PI3K/AKT/mTOR signaling pathway in HUVECs

The activation of the PI3K/AKT/mTOR pathway is required for the proliferative and migratory effect of VEGF on endothelial cells [21]. Thus, we investigated the possibility that the inhibitory effect of HS-116 might be mediated through its ability to interfere with VEGF-induced activation of the PI3K/AKT/mTOR signaling pathway. To determine whether HS-116 could modulate the active signaling pathways that are involved in cell functions, HUVECs were incubated with increasing doses of HS-116 in vitro. When examined for the key pathway components that regulated the endothelial cell function in angiogenesis, we found that HS-116 effectively suppressed VEGF-induced activation of AKT (Fig. 6C). As a result of AKT inhibition, the activation of mTOR and p70S6K were blocked by HS-116, suggesting that HS-116 inhibits tumor angiogenesis by blocking the PI3K/AKT/mTOR signaling pathways.

4. Discussion

The PI3K/AKT/mTOR pathway has a critical role in the pathogenesis of HCC. Indeed, the PI3K/AKT/mTOR pathway can be over-activated by enhanced stimulation of various receptor tyrosine kinase such as insulin growth like factor (IGF) and epithelial growth factor receptor (EGFR) in HCC [22]. Thus, the PI3K/AKT/mTOR pathway in cancer has been the subject of widespread and intense drug discovery for a long time [23]. Nevertheless, the optimal therapeutic strategy for targeting this pathway has not yet been identified in HCC. In this study, we developed HS-116, a novel PI3K inhibitor and explored its anticancer effects on HCC cells. For the first time, we report that HS-116 has a prominent effect on the proliferation, apoptosis, and angiogenesis through blocking the PI3K/AKT/mTOR signaling pathway in HCC.

The growth inhibitory effect is mainly mediated by inhibition of cell proliferation, which was observed in our three HCC cell lines to a similar extent at 20 μM HS-116 after 48 h. Among the three tested cell lines, Huh-7 cells were the most sensitive to HS-116. Since the PI3K/AKT/mTOR pathway regulates many different events involved in promoting cell survival and proliferation, this reduction of HCC cell proliferation by HS-116 seems to be associated with regulation of the PI3K/AKT/mTOR pathway.

Fig. 5. Effect of HS-116 on angiogenesis of Huh-7 HCC cells. (A) Expression of HIF-1α and VEGF by HS-116 in hypoxia-induced Huh-7 cells. (B) Production of VEGF by HS-116 in hypoxia-induced Huh-7 cells for 24 h. Data represents as mean ± S.D. from the triplicate wells. *P < .01, compared to control; †P < .05 and ‡P < .01, compared to hypoxia control (C) Effects of HS-116 on VEGF-induced tube formation in vitro. HUVECs were plated on Matrigel (200 μL/well) and treated with various concentrations of HS-116. Capillary tube formation was assessed after 18 h. The morphological changes of the cells and tube formation were observed under a phase-contrast microscope and photographed at 200× magnification. (D) Effects of HS-116 on VEGF-induced migration in vitro. HUVECs were plated at 90% confluence, scratched with a razor blade.
Bax is a proapoptotic member and is translocated to mitochondria overexpression is associated with many types of cancers, whereas Bcl-2 is a critical regulator of apoptotic pathways and its apoptotic role in Huh-7 cells. However, the inhibition of survival signalling [28,29]. So, we investigated the inhibition of various apoptotic signalling or mechanisms by which anticancer drugs induce apoptosis have been reported to involve the activation of various apoptotic signalling pathways [40,41]. In this regard, we wondered whether HS-116 may also inhibit angiogenesis by modulating the PI3K/AKT/mTOR pathway in endothelial cells. Our result indicates that HS-116 suppresses the VEGF-induced PI3K/AKT/mTOR signalling pathway in endothelial cells, which is consistent with the report that the PI3K inhibitor NVP-BEZ235 significantly inhibits VEGF-induced AKT and p70S6K phosphorylation in endothelial cells [42]. The results indicate that HS-116 inhibits not only the expression of HIF-1α and VEGF in HCC cells but also exerts an anti-angiogenic effect on endothelial cell by inhibiting the PI3K/AKT/mTOR pathway.

In conclusion, HS-116 inhibits the PI3K/AKT/mTOR pathway and produces potent anti-HCC activity by inhibiting cell growth/proliferation and angiogenesis in parallel with increasing apoptosis. The mechanism by which HS-116 inhibits cell proliferation along with inducing apoptosis seems to be associated with inhibition of the PI3K/AKT/mTOR pathway. Thus, HS-116 may be a potential anticancer agent to inhibit the tumor progression by the targeting of the PI3K/AKT/mTOR pathway in HCC.
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None declared.

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