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SKI-349, a Sphingosine Kinases 1/2 Inhibitor, Suppresses Cell Viability, Invasion, and AKT/mTOR Signaling Pathway, and Shows Synergistic Cytotoxic Effects with Sorafenib in Hepatocellular Carcinoma

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SKI-349 is a novel sphingosine kinases (SPHK) inhibitor with anti-tumor effects. This study aimed to assess the effect of SKI-349 on cell biological behaviors, downstream pathways, and its synergistic effect with sorafenib in hepatocellular carcinoma (HCC). HCC cell lines (Huh7 and Hep3B) were treated with SKI-349 at concentrations of 1, 2, 4, or 8 μM. Then, SPHK1/2 activity, cell viability, proliferation, apoptosis, invasion, and protein expressions of phosphorylated-protein kinase B (p-AKT), AKT, phosphorylated-mammalian target of rapamycin (p-mTOR) and mTOR were detected. Combination index values of SKI-349 (0, 1, 2, 4, or 8 μM) and sorafenib (0, 2.5, 5, 10, or 20 μM) were calculated. SKI-349 decreased the relative SPHK1 and SPHK2 activity compared with blank control in a dose-dependent manner in the Huh7 and Hep3B cell lines. Meanwhile, SKI-349 reduced cell viability, 5-ethynyl-2′-deoxyuridine (EdU) positive cells, and invasive cells, while it increased apoptotic cells compared to blank control in a dose-dependent manner in Huh7 and Hep3B cell lines. Based on the western blot assay, SKI-349 decreased the ratio of p-AKT to AKT and that of p-mTOR to mTOR compared with blank control in a dose-dependent manner in the Huh7 and Hep3B cell lines. Additionally, SKI-349 combined with sorafenib declined cell viability with concentration gradient effects compared to SKI-349 sole treatment, and they had synergistic cytotoxic effects in Huh7 and Hep3B cell lines. SKI-349 suppresses SPHK1 and SPHK2 activity, cell viability, invasion, and AKT/mTOR signaling pathway, as well as exhibits a synergistic cytotoxic effect with sorafenib in HCC.

Keywords: AKT/mTOR signaling pathway; hepatocellular carcinoma; SKI-349; sorafenib; sphingosine kinases 1/2
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Introduction

Hepatocellular carcinoma (HCC) accounts for 75.0%-85.0% of primary liver cancer, with over 700,000 new cases and more than 600,000 death cases worldwide each year (Sagnelli et al. 2020; Chidambaranathan-Reghupaty et al. 2021; McGlynn et al. 2021; Sung et al. 2021). The treatment strategies for early-stage HCC are surgical resection, ablation therapy, and liver transplantation, while for advanced-stage HCC, transarterial chemoembolization and systemic therapy (such as sorafenib and lenvatinib) are used (Galle et al. 2021; Renne et al. 2021). However, due to the inadequacy of current treatment options, the prognosis of HCC patients continues to be poor, with a global 5-year survival rate of 5.0%-30.0% (Sim and Knox 2018; Raees et al. 2021; Wang et al. 2021). Thus, it is important to search

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for potential new targets for the management of HCC.

Sphingosine kinases (SPHK) 1 and SPHK2 are oncogenic kinases that phosphorylate sphingosine to sphingosine-1-phosphate (S1P), and they play a key role in the progression of HCC by regulating cell proliferation, apoptosis and invasion (Bao et al. 2012; Diaz Escarcega et al. 2021; Gupta et al. 2021; Liu et al. 2022). For example, one study shows that SPHK1 facilitates cell migration and invasion through the S1P/endothelial differentiation sphingolipid G-protein-coupled receptor 1 axis in HCC (Bao et al. 2012). Another study illustrates that the ablation of SPHK2 downregulates ceramide transfer protein, which suppresses hepatocyte proliferation and the progression of non-alcoholic fatty liver disease-related HCC (Liu et al. 2022). One study also suggests that the combination of SPHK inhibitor with sorafenib leads to synergistic effects to suppress cell proliferation and induce cell apoptosis in HCC (Beljanski et al. 2011). Considering the above studies, SPHK1 and SPHK2 may be potential therapeutic targets of HCC.

SKI-349 is a novel SPHK1 and SPHK2 dual inhibitor, whose effects on inhibiting SPHK and inducing cytotoxicity exhibit log-fold enhancements compared with previously-discovered and widely-studied SPHK inhibitor SKI-178 (Hengst et al. 2020). Notably, SKI-349 is considered an effective therapeutic strategy for malignant tumors by targeting SPHK (Hengst et al. 2020). One previous research observes that SKI-349 suppresses non-small cell lung cancer cell proliferation, migration, and viability, as well as promotes apoptosis, with non-cytotoxicity to lung epithelial cells (Xue et al. 2022). Nevertheless, there is no study to investigate the role of SKI-349 on the progression of HCC up to now.

Therefore, this study aimed to explore the effect of SKI-349 on cell biological behaviors and potential pathways, as well as its synergistic anti-tumor effect with sorafenib in HCC.

Methods

Cell lines

The immortalized HCC cell lines, including p53 mutation (Huh7) and p53 deletion (Hep3B), were obtained from the iCell Bioscience (Shanghai, China). Huh7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (iCell), and Hep3B cells were maintained in modified Eagle's medium (MEM) (iCell). All cells were grown with medium plus 10% fetal bovine serum (FBS) (iCell) and 100 U/mL penicillin/streptomycin (iCell).

SKI-349 treatment

Huh7 and Hep3B cells were seeded and treated with gradient concentration (1, 2, 4, or 8 μM) of SKI-349 (MCE, Beijing, China), and the blank group (BLK) was cultured without SKI-349. The doses of SKI-349 were determined referring to one previous literature (Xue et al. 2022). Following 24 h of treatment, cells were collected for SPHK1 and SPHK2 enzyme activity, cell viability, 5-ethynyl-2′-deoxyuridine (EdU) staining, annexin V/propidium-iodide (AV/PI) staining, transwell, and western blot assays.

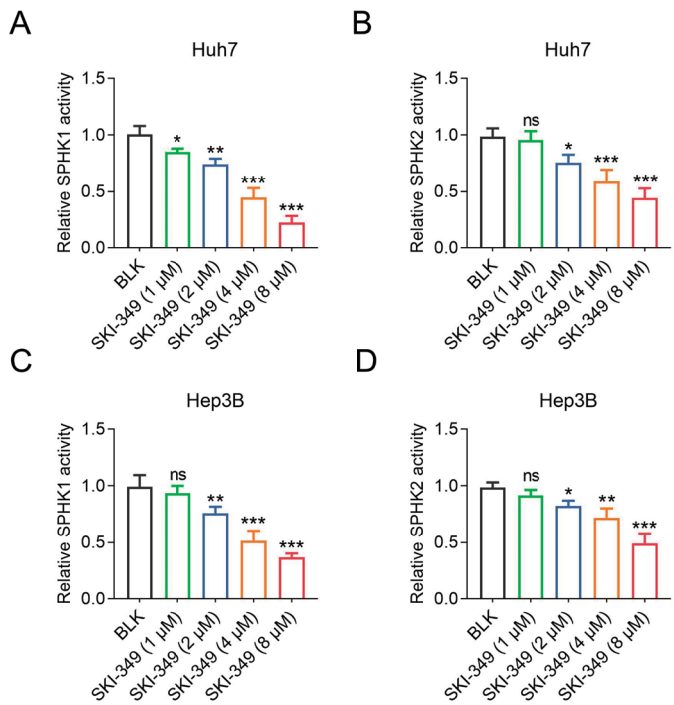

Fig. 1. Effect of SKI-349 on SPHK1 and SPHK2 activity in HCC cell lines.
SKI-349 reduced relative SPHK1 (A) and SPHK2 (B) activity in the Huh7 cell line. SKI-349 declined relative SPHK1 (C) and SPHK2 (D) activity in the Hep3B cell line. ns, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. blank control (BLK); n = 3.

SPHK1 and SPHK2 enzyme activity assay

The enzyme activity of SPHK1 and SPHK2 was detected using Sphingosine Kinase Activity Assay (Echelon Biosciences, Salt Lake City, UT, USA) referring to the kit's protocol. In brief, Huh7 and Hep3B cells were lysed by reaction buffer. Then, 10 μL of cell lysis buffer, 20 μL of adenosine 5′-triphosphate (ATP) and 10 μL of sphingosine solution was added into 96-well plate, and cells were treated for 1 h at room temperature (RT). The luminescence (550 nm) was assessed after being treated with 40 μL of ATP Detector (Echelon Biosciences) for 10 min at RT.

Cell viability assay

Cell Counting Kit-8 (CCK-8; Selleck, Shanghai, China) was utilized to assess cell viability. Shortly, Huh7 and Hep3B cells were washed and treated with 10 μL of CCK-8 solution for 2 h at 37°C, followed by absorbance measurement at 450 nm using BioTek microplate reader (Agilent, Santa Clara, CA, USA).

EdU staining assay

The EdU staining kit (Beyotime, Shanghai, China) was adopted for detection of cell proliferation. In brief, Huh7 and Hep3B cells were washed and fixed with 4% paraformaldehyde (Sangon, Shanghai, China). Then, cells were treated with 10 μM of EdU reagent for 2 h at 37°C. The cell nuclei were stained by Hoechst 33342 (Beyotime).

AV/PI staining assay

The AV-fluorescein 5-isothiocyanate (FITC)/PI Apoptosis Kit (Vazyme, Nanjing, China) was utilized to evaluate cell apoptosis. Concisely, Huh7 and Hep3B cells were resuspended with AV solution. Subsequently, cells were treated by AV-FITC and PI solution for 30 min at 37°C. The flow cytometry was carried out with FACSCanto II (BD, Tempe, AZ, USA).

Transwell assay

The transwell assay was adopted to estimate the invasive potential of Huh7 and Hep3B cells. In brief, cells were resuspended with serum-free medium, and then added to top chamber of matrigel-coated insert (BD). Meanwhile, the complete medium was added into the bottom chamber. The crystal violet staining (Solarbio, Beijing, China) was carried out after 24 h of cultivation, and the invasive cells were counted under a microscope (Leica, Wetzlar, Germany).

Western blot assay

Huh7 and Hep3B cells were lysed in radio immunoprecipitation assay (RIPA) buffer plus protease inhibitors (Solarbio). The concentration of protein was measured with bicinchoninic acid (BCA) kit (Yeason, Shanghai, China). The protein was separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then electro-transferred from the gels to polyvinylidene flu-

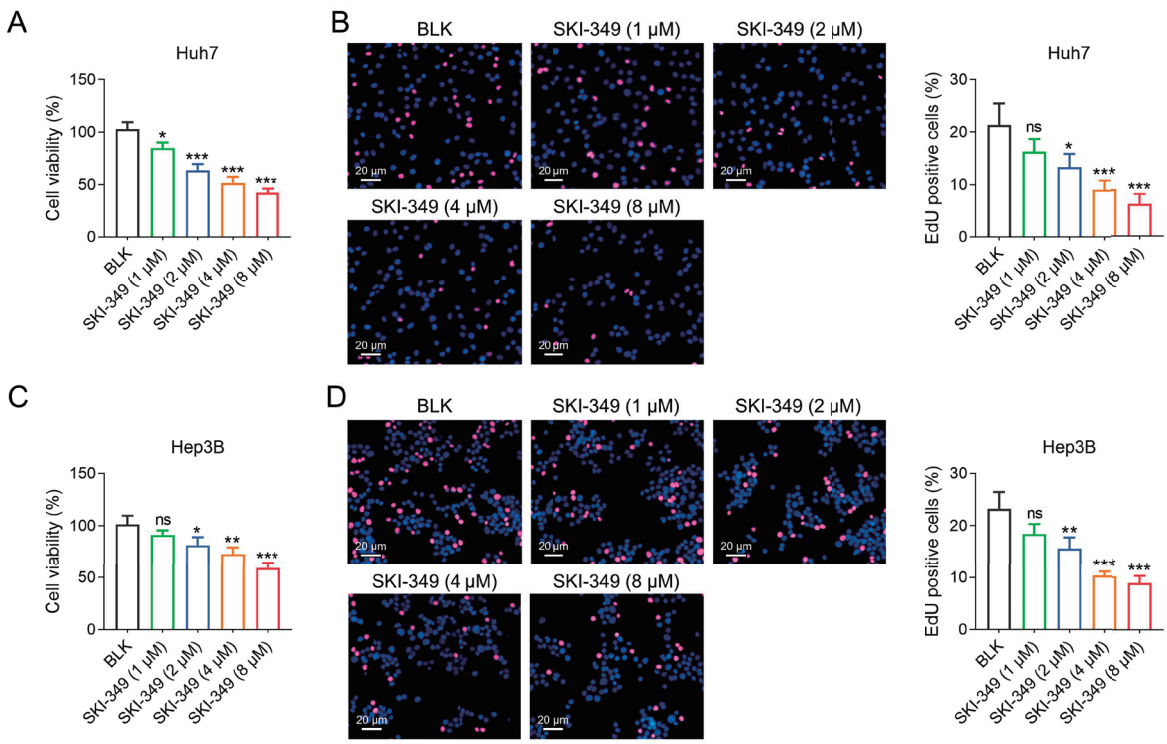

Fig. 2. Effect of SKI-349 on viability and proliferation in HCC cell lines.
SKI-349 reduced cell viability (A) and EdU positive cells (B) in the Huh7 cell line. SKI-349 decreased cell viability (C) and EdU positive cells (D) in the Hep3B cell line. ns, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. blank control (BLK); n = 3.

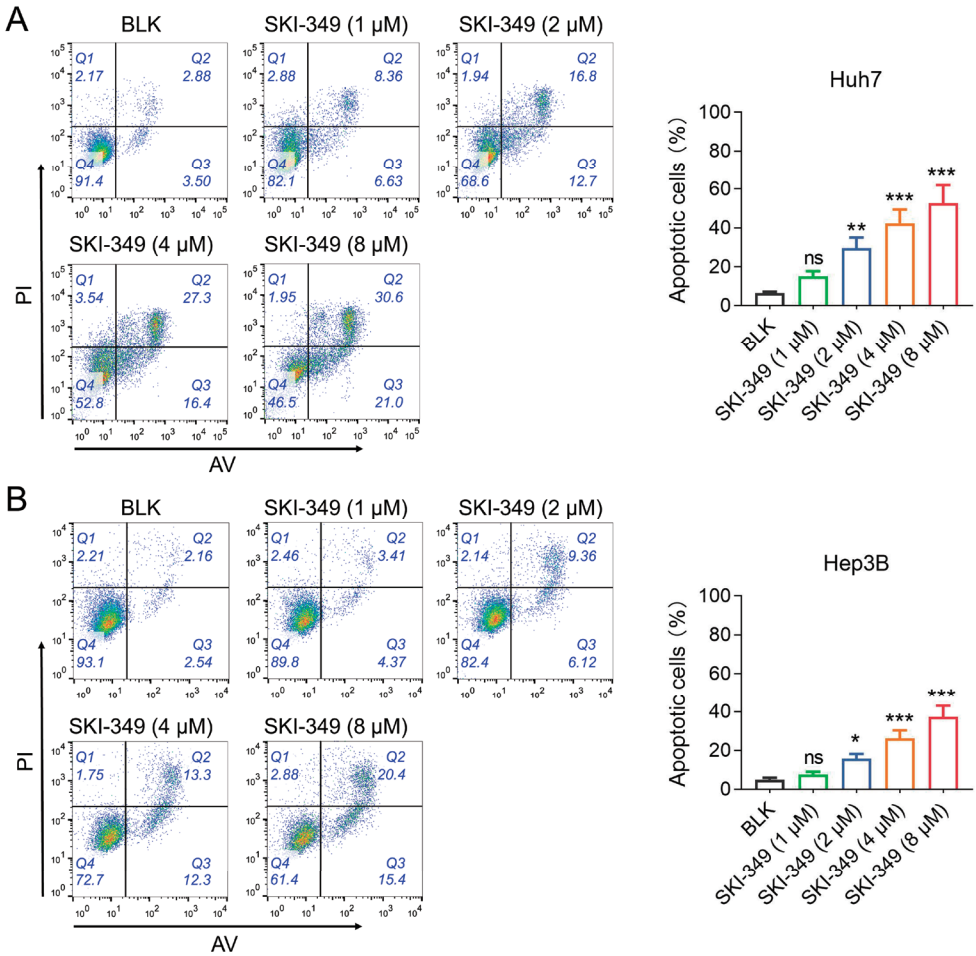

Fig. 3. Effect of SKI-349 on apoptosis in HCC cell lines.
SKI-349 increased apoptotic cells in the Huh7 (A) and Hep3B (B) cell lines; ns: no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. blank control (BLK); n = 3.

oride (PVDF) membrane (Bio-rad, Shanghai, China). The membranes were incubated with Fast Blocking Western (Yeason), primary antibodies and secondary antibodies, successively. The primary antibodies (Affinity, Changzhou, China) were used, including phosphorylated-protein kinase B (p-AKT) (#AF0016, 1:200), protein kinase B (AKT) (#AF6261, 1:500), phosphorylated-mammalian target of rapamycin (p-mTOR) (#AF3308, 1:200), mammalian target of rapamycin (mTOR) (#AF6308, 1:500), and GAPDH (#AF7021, 1:4,000). The High Sensitivity ECL Substrate Kit (Abcam, Cambridge, UK) were utilized for visualizing the blots.

Combination index (CI) of SKI-349 and sorafenib

Huh7 and Hep3B cells were cultured and incubated with gradient concentration of SKI-349 (0, 1, 2, 4, or 8 μM) and sorafenib (0, 2.5, 5, 10, or 20 μM) (Selleck) in combination. The doses of sorafenib were determined referring to the previous literatures (Martin del Campo et al. 2015; Michel et al. 2019). The cell viability assay was performed after 24 h of treatment as indicated above. The CI values of SKI-349 and sorafenib were calculated using CalcuSyn2.0 software (Reachsoft, Beijing, China). The CI values > 1 were antagonistic, and CI values < 1 were considered synergistic (Li et al. 2023).

Statistical analysis

The statistical analysis was accomplished with SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The results were analyzed by one-way ANOVA with Dunnett's test. *P*-value < 0.05 was considered statistically significant.

Results

SKI-349 inhibited relative SPHK1 and SPHK2 activity in HCC cell lines

SKI-349 at concentrations of 1-8 μM decreased the relative SPHK1 activity in a concentration-dependent manner compared with blank control in the Huh7 cell line (all $P < 0.05$) (Fig. 1A). Meanwhile, SKI-349 at concentrations of 2-8 μM dose-dependently reduced the relative SPHK2 activity vs. blank control in the Huh7 cell line (all $P < 0.05$) (Fig. 1B). The SPHK1 and SPHK2 enzyme activity assay results exhibited that SKI-349 at concentrations of 2-8 μM dose-dependently declined the relative SPHK1 and SPHK2

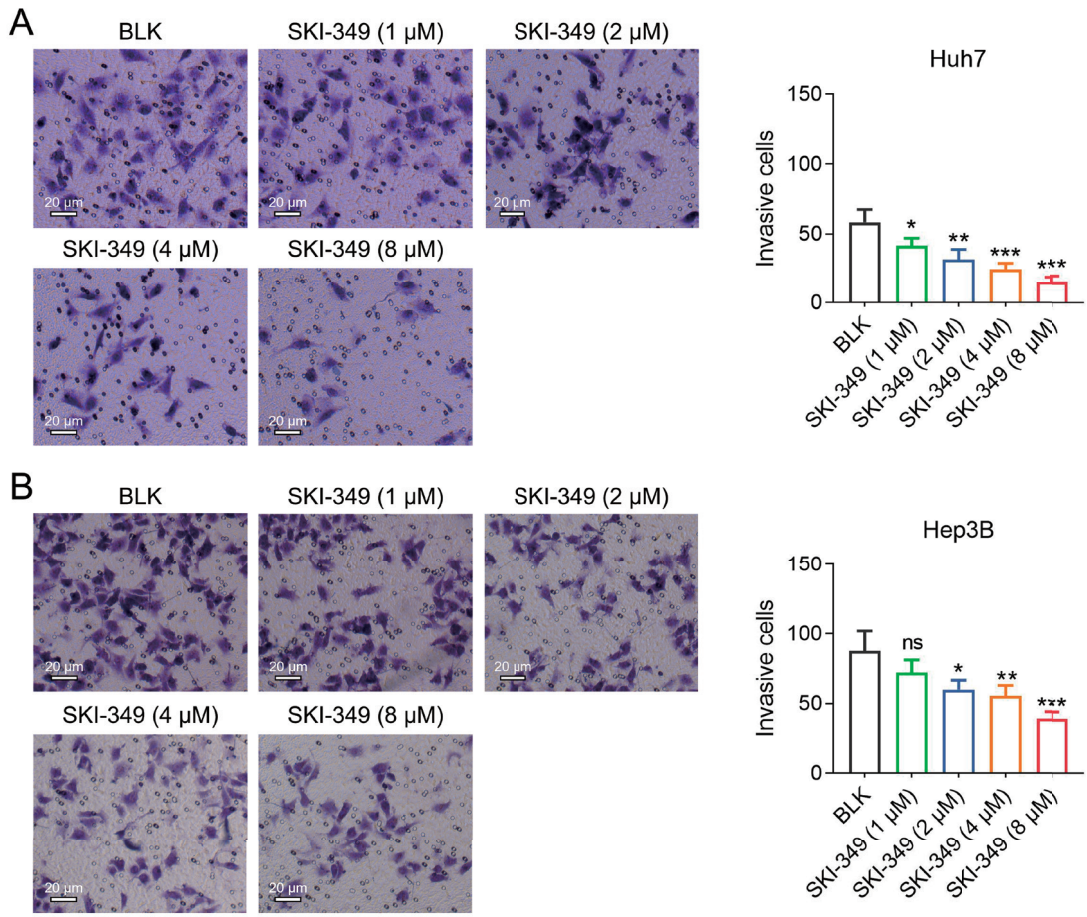

Fig. 4. Effect of SKI-349 on invasion in HCC cell lines.
SKI-349 reduced invasive cells in the Huh7 (A) and Hep3B (B) cell lines. ns, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. blank control (BLK); n = 3.

activity in comparison with blank control in the Hep3B cell line (all $P < 0.05$) (Fig. 1C, D).

SKI-349 inhibited viability and proliferation in HCC cell lines

SKI-349 at concentrations of 1-8 μM dose-dependently decreased cell viability compared to blank control in the Huh7 cell line (all $P < 0.05$) (Fig. 2A). Based on the EdU staining assays, SKI-349 at concentrations of 2-8 μM descended EdU positive cells in a dose-dependent manner vs. blank control in the Huh7 cell line (all $P < 0.05$) (Fig. 2B). Additionally, SKI-349 at concentrations of 2-8 μM dose-dependently reduced cell viability and EdU positive cells compared with blank control in the Hep3B cell line (all $P < 0.05$) (Fig. 2C, D).

SKI-349 promoted apoptosis and suppressed invasion in HCC cell lines

AV/PI staining assays were conducted to test cell apoptosis, which showed that apoptotic cells were increased with concentration gradient effects by treatment with SKI-349 at concentrations of 2-8 μM compared to blank treatment in the Huh7 and Hep3B cell lines (all $P < 0.05$) (Fig. 3A, B).

In addition, Transwell assay revealed that SKI-349 at concentrations of 1-8 μM dose-dependently reduced invasive cells compared to blank control in the Huh7 cell line (all $P < 0.05$) (Fig. 4A). SKI-349 at concentrations of 2-8 μM reduced invasive cells in a dose-dependent manner vs. blank control in the Hep3B cell lines (all $P < 0.05$) (Fig. 4B).

SKI-349 inhibited AKT/mTOR signaling pathway in HCC cell lines

The downstream signaling pathway of SKI-349 was assessed by the western blot assay. The results showed that SKI-349 at concentrations of 1-8 μM reduced the ratio of p-AKT to AKT, and SKI-349 at concentrations of 2-8 μM decreased the ratio of p-mTOR to mTOR vs. blank control in a concentration-dependent manner in the Huh7 cell line (all $P < 0.05$) (Fig. 5A). Moreover, SKI-349 at concentrations of 2-8 μM also revealed the same results in the Hep3B cell line (all $P < 0.05$) (Fig. 5B).

SKI-349 synergizes with sorafenib to kill HCC cells

Sorafenib at concentrations of 2.5-20 μM dose-dependently reduced cell viability compared to blank control in the Huh7 cell line (all $P < 0.05$). Meanwhile, SKI-349 at concentrations of 1-8 μM combined with sorafenib at concentra-

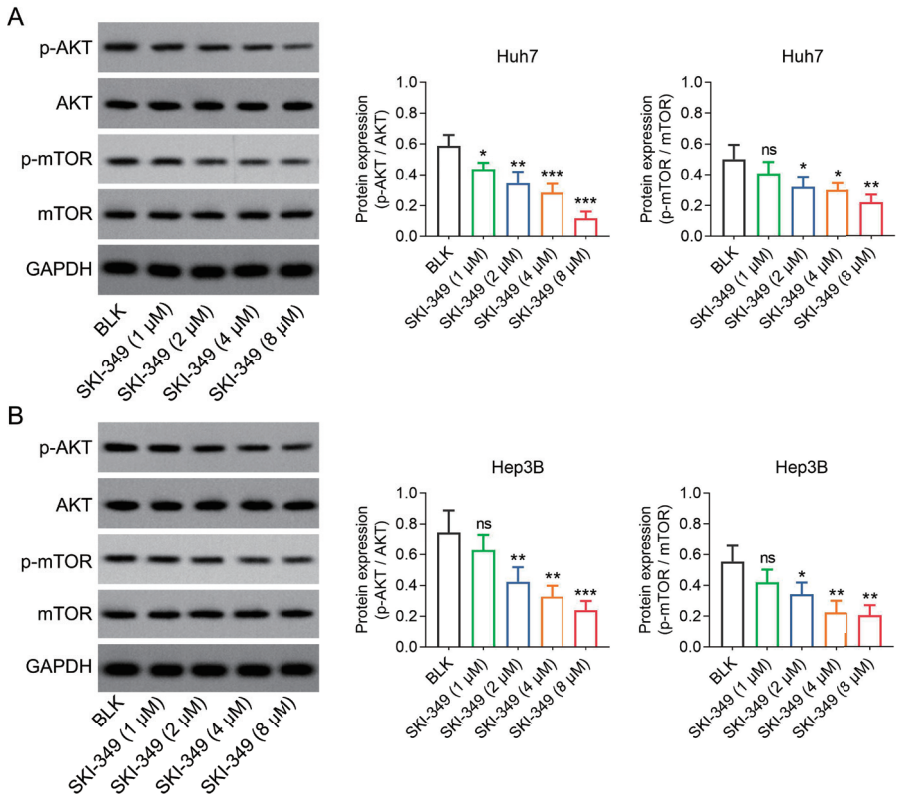

Fig. 5. Effect of SKI-349 on AKT/mTOR signaling in HCC cell lines.
SKI-349 reduced the ratio of p-AKT to AKT and that of p-mTOR to mTOR in the Huh7 (A) and Hep3B (B) cell lines. ns: no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. blank control (BLK); n = 3.

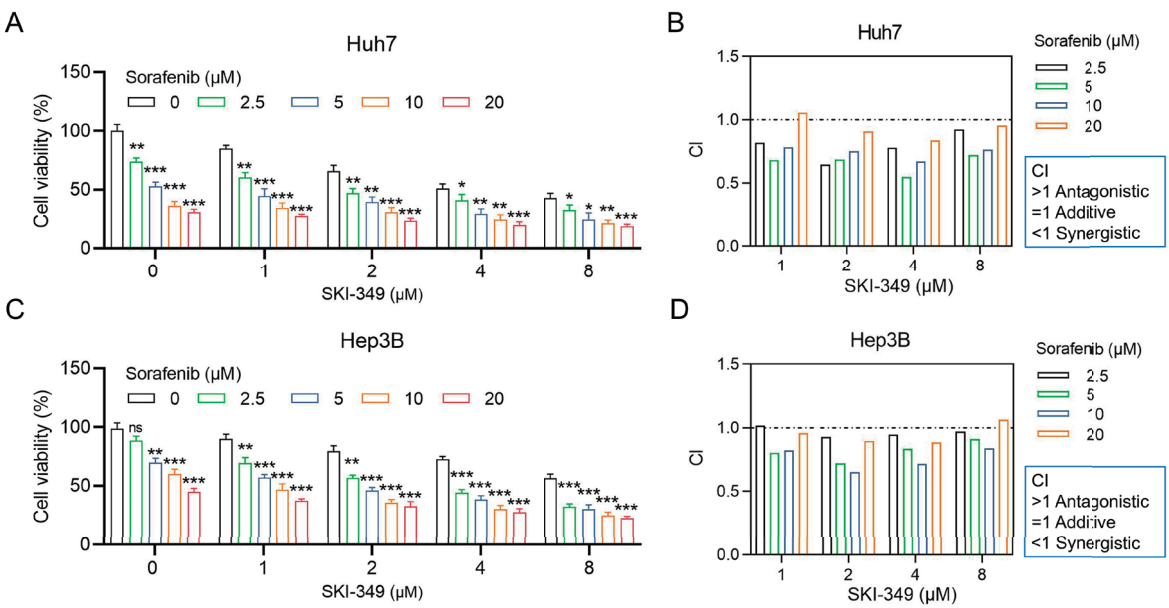

Fig. 6. Synergistic effect between SKI-349 and sorafenib in HCC cell lines.
The combination of SKI-349 and sorafenib reduced cell viability (A) and exhibited synergistic cytotoxic effects (B) in the Huh7 cell line. The combination of SKI-349 and sorafenib reduced cell viability (C) and showed synergistic cytotoxic effects (D) in the Hep3B cell line. ns, no significance; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, *, **, and *** meant vs. 0 μM sorafenib-treated-cells in each group; n = 3. The CI values > 1 were antagonistic, and CI values < 1 were considered synergistic.

tions of 2.5-20 μM reduced cell viability in a concentration-dependent manner vs. SKI-349 sole treatment in the Huh7 cell line (all $P < 0.05$) (Fig. 6A). SKI-349 had a synergistic effect with sorafenib in the Huh7 cell line (Fig. 6B).

Additionally, sorafenib at concentrations of 5-20 μM decreased cell viability with concentration gradient effects compared to blank control in the Hep3B cell line (all $P < 0.05$). SKI-349 at concentrations of 1-8 μM combined with sorafenib at concentrations of 2.5-20 μM dose-dependently reduced cell viability vs. SKI-349 sole treatment in the Hep3B cell line (all $P < 0.05$) (Fig. 6C). The CI values of SKI-349 and sorafenib exhibited that they had synergistic cytotoxic effects in the Hep3B cell line (Fig. 6D).

Discussion

SPHK1 and SPHK2 have been deemed to be involved in the progression of HCC, and their inhibitors may be an important treatment strategy to manage HCC (Liu et al. 2016; Cai et al. 2017; Satyananda et al. 2021; Liu et al. 2022). Notably, SKI-349 is a new-disclosed dual SPHK1/2 inhibitor developed by the modification of the linker region between the substituted phenyl rings of the SKI-178 chemotype, which has an enhanced inhibitory effect on SPHK1/2 and higher cytotoxic potency vs. SKI-178 (Hengst et al. 2020). However, the role of SKI-349 in HCC remains unknown. In our study, SKI-349 inhibited SPHK1 and SPHK2 activity in HCC cell lines. Meanwhile, SKI-349 suppressed cell viability, proliferation, and invasion, and promoted apoptosis in HCC cell lines. The possible reasons were as follows: (1) SKI-349 might perform similar functions as SKI-178, which competed for the SPH binding site in SPHK1/2, thus suppressing SPHK1 and SPHK2 activity in HCC cell lines (Hengst et al. 2017). (2) SKI-349 inhibited the production of S1P by suppressing SPHK1 and SPHK2 activity, which reduced HCC cell viability, proliferation, and invasion (Nagahashi et al. 2014; Hengst et al. 2020; Satyananda et al. 2021; Yokota et al. 2021). (3) SKI-349 might induce HCC cell apoptosis by elevating ceramide levels and declining S1P levels (LeBlanc et al. 2015).

The AKT/mTOR signaling pathway is considered to be an oncogenic signal transduction pathway that participates in the progression of various cancers (Tewari et al. 2022; Yu et al. 2022). Notably, one study illustrates that S1P receptor 2 activates the AKT/mTOR signaling pathway to increase HCC cell proliferation (Wang et al. 2023). Another study also observes that the overexpression of SPHK1 promotes renal cell carcinoma progression via activating the AKT/mTOR signaling pathway (Xu et al. 2018). Therefore, it is hypothesized that the AKT/mTOR signaling pathway may be the downstream pathway of SKI-349 in HCC cell lines. Our study found that SKI-349 inhibited the AKT/mTOR signaling pathway in HCC cell lines, which might be due to the fact that: (1) SKI-349 inhibited SPHK, which led to ceramide accumulation in HCC cells and activated phosphatases (including protein phosphatase 1 and protein phosphatase 2A), thus inactivating the AKT/mTOR cascade (Lin et al. 2006). (2) As mentioned above, SKI-349 inhibited SPHK1 and SPHK2 activity (Hengst et al. 2020); meanwhile, the decrease of SPHK1 and SPHK2 activity reduced S1P and further inhibit AKT/mTOR cascade reaction in HCC cell lines (Yuan et al. 2022). Thus, SKI-349 was hypothesized to inhibit the AKT/mTOR signaling pathway by suppressing SPHK1- and SPHK2-regulated S1P. However, this hypothesis was required to be further verified by future studies.

Sorafenib is an effective first-line drug that promotes apoptosis, inhibits angiogenesis and suppresses tumor cell proliferation in HCC (Tang et al. 2020). Notably, one previous study reveals that SPHK inhibitor ABC294640 in combination with sorafenib produces synergistic anti-HCC effects (Beljanski et al. 2011). Thus, our study explored the effect of SKI-349 combined with sorafenib in HCC cell lines, which suggested that SKI-349 had a synergistic effect with sorafenib to kill HCC cells. The possible explanation for the result might include that: (1) According to the above-mentioned content, SKI-349 suppressed the AKT/mTOR signaling pathway in HCC cell lines, which might increase the sensitivity of tumor cells to sorafenib (Sun et al. 2022). (2) SKI-349 might lead to the increase in the activity of caspase 3/7 (Beljanski et al. 2011); meanwhile, sorafenib induced cell apoptosis by the cleavage of caspases 3/7 (Schult et al. 2010). Thus, the synergistic effects between SKI-349 and sorafenib might be related to the elevation in caspase 3/7 activation, while further studies were required for verification (Beljanski et al. 2011). Notably, although our study revealed the synergistic anti-tumor effect of SKI-349 combined with sorafenib in HCC cell lines, future studies were required to validate this effect in animal experiments.

In conclusion, SKI-349 inhibits cell viability, invasion, and the AKT/mTOR signaling pathway; moreover, it synergizes with sorafenib to kill tumor cells in HCC cell lines, which is a potential therapeutic choice for HCC. However, the underlying mechanisms by which SKI-349 and sorafenib produce a synergistic cytotoxic effect needs to be further explored in future studies.

Conflict of Interest

The authors declare no conflict of interest.

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