A preclinical evaluation of a novel multikinase inhibitor, SKLB-329, as a therapeutic agent against hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is a serious life-threatening malignant disease of liver. Molecular targeted therapies are considered a promising strategy for the treatment of HCC. Sorafenib is the first, and so far the only targeted drug approved by the US Food and Drug Administration (FDA) for clinical therapy of HCC. Despite being effective in some HCC patients, some demerits of sorafenib in the treatment of HCC, such as modest survival benefits, and drug resistance, have also been reported, which highlights the unmet medical need among patients with HCC. Here, we report a novel multikinase inhibitor discovered by us, SKLB-329, which potently inhibits angiogenesis-related kinases including VEGFR1/2/3, and FGFR2, and the Src kinase. SKLB-329 significantly inhibited endothelial cell growth, migration, invasion and tube formation. It showed potent anti-angiogenic activity in a transgenic zebrafish model. Moreover, SKLB-329 could efficiently restrain the proliferation of HCC cells through down-regulation of Src-mediated FAK and Stat3 activity. In vivo, oral administration of SKLB-329 considerably suppressed the tumor growth in HCC xenograft models (HepG2 and SMMC7721) in a dose-dependent manner. In all of the in vitro and in vivo assays of this investigation, sorafenib was used as a positive control, and in most assays SKLB-329 exhibited a higher potency compared with the positive control. In addition, SKLB-329 also bears favorable pharmacokinetic properties. Collectively, the results of preclinical studies presented here demonstrate that SKLB-329 is a promising drug candidate for HCC treatment.

Hepatocellular carcinoma (HCC) is one of the most common malignances worldwide with ~630 thousands new cases reported per year.1 Unfortunately, most HCC patients present with tumor in advanced and/or incurable stages, making them ineligible for curative therapies such as surgical resection or liver transplantation. For these HCC patients, systemic treatments are the main option. Nevertheless, chemotherapy with conventional cytotoxic agents is often very toxic and fairly ineffective.1,2 The lack of effective systemic therapy for HCC patients has changed with the approval of the first molecular targeted agent sorafenib for clinical use in advanced HCC by the US FDA.3 Sorafenib is a multikinase inhibitor and mainly targets angiogenesis and RAF/MEK/ERK signaling cascade. Despite being effective in some HCC patients, some demerits for sorafenib, such as modest survival benefits, and drug resistance, have also been reported,4–6 which highlights the unmet medical need among patients with HCC. In development of molecular targeted agents, the selection of targets is critical. Currently, a number of targets have been explored as potential therapeutic targets for HCC.

Key words: SKLB-329, hepatocellular carcinoma, multi-kinase inhibitor, Src, anti-angiogenesis

Abbreviations: EBM-2: endothelial cell basal medium-2; EGF: epidermal growth factor; ERK: extracellular signal-regulated kinase; FAK: focal adhesion kinase; FGFR: fibroblast growth factor receptor; HCC: hepatocellular carcinoma; HUVEC: human umbilical vein endothelial cell; MAPK: mitogen-activated protein kinase; Stat3: signal transducer and activator of transcription 3; VEGFR: vascular endothelial growth factor receptor

Additional Supporting Information may be found in the online version of this article.

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What’s new?
Targeted therapy is a potentially effective means of treating hepatocellular carcinoma (HCC), but only a single targeted agent, sorafenib, is FDA-approved, and its clinical benefits are limited. In the interest of meeting the need for the identification of new drug candidates, the present report describes the preclinical investigation of a novel multikinase inhibitor, SKLB-329. In HCC xenografts, SKLB-329 was found to possess anti-angiogenic activity and to exert anti-tumor effects that were more potent than those of sorafenib. SKLB-329 anti-tumor activity was associated with the inhibition of HCC cell proliferation via blockade of Src/FAK and Src/Stat3 signalings.

demonstrated to be associated with HCC. However, very specific targets for HCC have not been identified, which might be due to the fact that HCC is a biologically heterogeneous malignant disease. In this case, an optimal choice is multtarget drugs, which have been demonstrated to be superior to single-target ones in other cancer types. 7–9 Among targets associated with HCC, receptor tyrosine kinases related to angiogenesis, such as VEGFR, and FGFR, could be one of the most important targets because HCC is a highly vascular tumor. 10,11 Another important target is the nonreceptor tyrosine kinase Src. Src plays a broad and crucial role in activating PI3K/Akt, Ras/Raf/MAPK, JAK/Stats and SFK/FAK/p130CAS signaling pathways; these pathways are associated with cellular proliferation, survival, adhesion, migration and invasion, as well as angiogenesis. 12–16 Recent studies have demonstrated that activation of Src is highly related to the early stage phenotype of HCC, and Src could be an independent prognostic marker of HCC patient. 17–19 These results highlight Src as a potential target for HCC therapy. We therefore hypothesized that agents that simultaneously target angiogenesis and Src might exhibit improved anti-HCC efficacy. Thus, we performed a rational drug design study and lead optimization (related investigations will be reported elsewhere), and obtained a novel compound termed SKLB-329 (Fig. 1a), which potently inhibits VEGFR1/2/3, FGFR2 and Src. SKLB-329 displayed a more potent anti-tumor activity than sorafenib in HCC xenograft models. In this account, we report the preclinical assessment results of SKLB-329.

Material and Methods
Compounds
The 1-(4-((1H-pyrazolo[3,4-d]pyrimidin-4-yl)oxy)-2-fluorophenyl)-3-(4-chloro-3-(trifluoromethyl)phenyl)urea (SKLB-329, Fig. 1a) was synthesized at the State Key Laboratory of Biotherapy, Sichuan University (details for the synthesis of SKLB-329 see Supporting Information). Sorafenib was obtained from commercial source. A stock solution of SKLB-329 for all assays in vitro was prepared in DMSO and then diluted in optimal medium. The final concentration of DMSO in the incubation mixture did not exceed 0.1% (v/v) in each experiment.

Kinase inhibition assays
Kinase inhibition assays were performed by KinaseProfiler service (Millipore, USA). SKLB-329 (0.001–10 μM) or vehicle was incubated with protein kinases in reaction solution containing 8 mM 3-(N-morpholino)propanesulfonic acid (MOPS, pH 7.0), 0.2 mM EDTA, 0.33 mg mL<sup>−1</sup> myelin basic protein, 10 mM Mg acetate, 10 μM [γ<sup>−32</sup>P-ATP], and corresponding peptide as substrate. After incubation for 40 min at room temperature, the reaction was stopped by 3% phosphoric acid solution, and 10 μL of the reaction solution was spotted onto a P30 filtermat. The samples were then washed 3 times with 75 mM phosphoric acid and once in methanol before detecting the kinase activity by scintillation counting.

Cells and cell culture conditions
Human cancer cell lines and normal liver cell line (HL-7702) used in this investigation were obtained from American Type Culture Collection (ATCC, Rockville, MD) and National Platform of Experimental Cell Resources for Sci-Tech (China), respectively, and were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS (Gibco, Eggenstein, Germany), 100 U mL<sup>−1</sup> penicillin (Sigma–Aldrich, USA), and 100 μg mL<sup>−1</sup> streptomycin (Sigma–Aldrich, USA). All cell lines were maintained at 37°C, surrounded by a humidified atmosphere of 5% CO<sub>2</sub>, and passed for <6 months after receipt or resuscitation. Human umbilical vein endothelial cell (HUVEC) was isolated from human umbilical cord veins according to a standard procedure and grown in EGM-2 medium (EBM2 medium containing EndoGRO<sup>TM</sup> VEGF supplement kit, Millipore, USA). HUVECs at passage 2–6 were used for all related experiments.

Cell viability and proliferation assays
Cell viability was measured using MTT assay. The cell lines were seeded in 96-well plates at 2500–4000 cells/well (depending on cell type) in DMEM supplemented with 10% FBS and incubated overnight. The cells were treated with drugs in different concentrations for 72 hr in serum-containing media, and then 20 μL of MTT solution (5 mg mL<sup>−1</sup>, Sigma, USA) was added to each well. After incubation for 2–4 hr at 37°C, the formazan crystals were dissolved with 50 μL of acidified SDS (20%, w/v). Absorbance was determined at 570 nm on Multiskan MK3 (Thermo Scientific, USA) the next day. Each assay was performed in three replicates and all experiments were repeated at least twice. For the HUVEC growth inhibition assay, the cells seeded in 96-well plates (1 × 10<sup>4</sup> per well) were starved overnight in...
EBM2 medium and incubated with SKLB-329 for 45 min. Then 50 ng mL\(^{-1}\) growth factor (VEGF, bFGF or EGF) or 5% FBS was introduced into the assays and the cells were continued to incubate for 72 hr. The subsequent procedures were performed as described above.

For the HCC cell proliferation assay, cells were seeded in 96-well plates and treated with 20 μM SKLB-329 the next day. After exposure to the drug-containing medium for 24 hr, EdU incorporation assay was conducted on the cells following the manufacturer’s instruction (RIBOBIO, China), and the pictures were captured using ArrayScan VTI HCS reader (Thermo Scientific, USA).

**Colony formation assay**

Cells were seeded in six-well plates at a density of 5,000 per well and treated with vehicle or SKLB-329 the next day. The
PBS and stained with 50–70% ethanol overnight. The cells were then washed with cold PBS and stained with 50 μg mL⁻¹ propidium iodide containing 100 μg mL⁻¹ RNase, and 0.1% Triton X-100. The cell cycle profiles were determined on a FACS Calibur flow cytometer (Becton Dickinson, USA) and analyzed using the ModFit LT 3.2 software (Verity Software House, USA).

**Wound healing assay**

HUVECs were cultured to confluence in 24-well plates and wounded using a sterilized yellow pipette tip to make a straight scratch. Cells were rinsed with sterile PBS gently, and then PBS was replaced with EGM2 medium containing vehicle, sorafenib or SKLB-329. Pictures were taken by an OLYMPUS digital camera attached to a light microscope after 18 hr.

**Transwell invasion assay**

Transwell invasion assay was done as described previously. In brief, 50 μL per well of diluted Matrigel (BD Biosciences, USA) was added to the Transwell compartments (Millipore, USA), which have been inserted into a 24-well plate. HUVECs were suspended in EBM-2 medium (without growth factors) 1 hr later and seeded in the upper chamber (3 × 10⁵/100 μL). Afterward, another 100 μL medium containing vehicle, sorafenib or SKLB-329 was added to each upper chamber. The lower compartments were filled with 500 μL EGM-2 medium (EBM-2 medium supplemented with various growth factors). After incubation for 24 hr at 37°C, the migrated cells were fixed with methanol and stained with 0.05% crystal violet for 15 min, followed by rinsing twice with PBS. The cells were photographed under a light microscope (Leica, Germany).

**Tube formation assay**

The tube formation assay was conducted as described previously. Briefly, 50 μL per well of Matrigel was added to the 96-well plate and incubated for 30 min at 37°C to allow the gel to solidify. HUVECs were harvested and seeded on the matrigel for 10 min, followed by treatment with vehicle, sorafenib or serial dilutions of SKLB-329. Cells were photographed with an OLYMPUS digital camera 6 hr later.

**Angiogenesis in live fluorescent zebrafish assay**

Anti-angiogenesis activity of SKLB-329 and sorafenib was assessed in transgenic zebrafish (FLK-1: EGFP) according to the protocol reported previously. Zebrafish embryos at the 13-somite stage (30 embryos per group) were incubated overnight with vehicle, sorafenib or SKLB-329. Then zebrafish were anesthetized and imaged using a fluorescence microscope (Carl Zeiss Microimaging, Germany).

**Western blot analysis**

For the HUVEC immunoblot studies, subconfluent cells were serum starved overnight in EBM2 medium, and then incubated with vehicle, sorafenib, or SKLB-329 for 2 hr, followed by treatment with 50 ng mL⁻¹ recombinant human VEGF or bFGF (Peprotech, USA) for 10 min. The cells were lysed in RIPA buffer (Beyotime, China) containing Roche protease inhibitor cocktail, and the protein concentrations were determined by the Bradford method. Proteins were separated by gel electrophoresis on 5–10% SDS-PAGE gels and probed with specific antibodies (Cell Signaling Technology, USA) including anti-VEGFR2, anti-pVEGFR2 Tyr1175, anti-FAK, anti-pFAK Tyr925, anti-Src, anti-pSrc Tyr416, anti-AKT, anti-pAKT Ser473, anti-ERK, anti-pERK Thr202/Tyr204 and anti-β-actin. All of the antibodies were used at a 1:1,000 dilution, and the horseradish peroxidase-coupled secondary antibodies (Zhong Shan Golden Bridge Bio-technology, China) were used at 1:5,000.

For the HepG2 and PLC/PRF/5 Western blot assays, cells were incubated for 24 hr in medium containing vehicle, SKLB-329, or sorafenib, and then lysed in RIPA buffer. Western blots were done on whole-cell extracts as described above.

**In vivo antitumor effects**

All animal studies were conducted according to the guidelines of the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China). Female Balb/c athymic mice were purchased from Chinese Academy of Medical Science (Beijing, China). Nearly 5 × 10⁶ tumor cells were implanted subcutaneously into the hind flank region of mice, and tumors were allowed to reach a size of ~200 mm³ before initiation of treatment. The solvent containing 10% Cremophor EL (Sigma, USA), 10% ethanol, and 80% water was used as vehicle. Tumor sizes were monitored twice-weekly with vernier caliper and calculated as length × width²/2. Studies were typically terminated when tumors in vehicle-treated animals reached an average size of 1,500 mm³. Inhibition rate of tumor growth was calculated as 100 × [1 − (tumor volume final − tumor volume initial) for the compound-treated group]/[(tumor volume final − tumor volume initial) for the vehicle-treated group].

**Immunohistochemistry**

Tumor-bearing mice were given vehicle or SKLB-329 (15 mg kg⁻¹) once daily by oral gavage, and parafin-embedded tumor sections were made after harvesting the tumors which had been treated for 2 weeks. For immunohistochemistry studies, antigen retrieval was performed on tumor sections and the following antibodies were used: phospho-Src (Cell Signaling Technology, 1:100), phospho-FAK (Cell Signaling Technology, 1:100), phospho-Stat3 (Cell Signaling Technology, 1:200). A DAKO polymer secondary antibody system (Dako Envision +K4007) was used for secondary detection,
and the sections were counterstained with Carazzi’s hematoxylin. Additionally, Rat anti-mouse CD31 antibody (BD Biosciences, USA) and anti-Ki67 antibody (Thermo Fisher Scientific, USA) were used to determine vessel density and cell proliferation following the manufacturer’s protocol, respectively. Images were captured using an Olympus digital camera.

Pharmacokinetic assessments
The pharmacokinetic properties of SKLB-329 were investigated in male Sprague–Dawley rats (Chinese Academy of Medical Science, Beijing, China). A catheter was surgically placed into the jugular vein of the rats, and the animals were fasted overnight, followed by receiving SKLB-329 or sorafenib as a single dose of 20 mg kg⁻¹ by oral gavage. Serial plasma samples were collected at 0.17, 0.5, 1, 2, 4, 6, 8, 12, 24, 35, 48, 60, and 72 hr postdose. The plasma concentrations were determined by high performance liquid chromatography (Waters 2695–2998, USA). Noncompartmental pharmacokinetic parameters were fitted using DAS software (Enterprise Version 2.0, Mathematical Pharmacology Professional Committee of China).

Statistical analysis
SPSS 13.0 was used to evaluate the statistical significance of differences between the means. Data were analyzed by the Student t test and ANOVA. Differences were considered significant if p < 0.05.

Results
Kinase inhibition potency of SKLB-329
The kinase inhibition potency of SKLB-329 against various human protein kinases were measured with gold-standard ³²P radiolabeled technology. The results are shown in Table 1. SKLB-329 potently inhibited VEGFR1/2/3 with IC₅₀ values of 5, 18, and 5 nM, respectively, which are more potent than that of sorafenib (Corresponding IC₅₀ values for sorafenib are 26, 90, and 20 nM, respectively).²³⁻²⁵ It showed a considerable potency against FGFR2 and Src with IC₅₀ values of 26 and 18 nM, respectively. Though sorafenib has some activity against FGFR2 and Src, its potencies are very weak (IC₅₀: 825 nM for FGFR2, 390 nM for Src).²³⁻²⁴ SKLB-329 also showed potency for several selected kinases including Flt3 (IC₅₀: 59 nM), PDGFRα (IC₅₀: 499 nM), PDGFRβ (IC₅₀: 151 nM), EphA2 (IC₅₀: 341 nM), EphB2 (IC₅₀: 158 nM), c-Kit (IC₅₀: 745 nM) and Met (IC₅₀: 3095 nM). For other selected 10 kinases, SKLB-329 showed very weak or no activity (IC₅₀ > 10 μM).

| Table 1. In vitro kinase inhibition profile of SKLB-329 |
|-----------------------------------------------------|
| **Biochemical activity**   | IC₅₀ (nM) |
|---------------------------|----------|
| VEGFR-1/2/3 (h)           | 5, 18, 5 |
| FGFR2 (h)                 | 26       |
| Src (h)                   | 18       |
| Fli3 (h)                  | 59       |
| EphA2 (h)                 | 341      |
| EphB2 (h)                 | 158      |
| cKit (h)                  | 745      |
| PDGFRα (h)                | 499      |
| PDGFRβ (h)                | 151      |
| Met (h)                   | 3095     |
| c-raf (h)                 | >10,000  |
| FAK (h)                   | >10,000  |
| EGFR (h)                  | >10,000  |
| ErbB2 (h)                 | >10,000  |
| Lck (h)                   | >10,000  |
| aurora A (h)              | >10,000  |
| aurora B (h)              | >10,000  |
| DLK (h)                   | >10,000  |
| PLK1 (h)                  | >10,000  |
| IGF1R (h)                 | >10,000  |

SKLB-329 inhibits HUVEC growth, migration, invasion and tube formation
To assess the anti-angiogenesis effects of SKLB-329 in vitro, the inhibitory activity of SKLB-329 against various growth factors-induced growth of HUVECs was measured by MTT assay. SKLB-329 showed a strong inhibitory effect on VEGF or bFGF-stimulated cells, and the VEGF-stimulated cells seemed more sensitive to SKLB-329 compared with bFGF-stimulated cells (Fig. 1b). SKLB-329 displayed a very weak anti-viability effect on EGF or FBS stimulated HUVECs. These results indicated that SKLB-329 inhibited endothelial cells growth mainly through the suppression of VEGF and FGF signaling.

Angiogenesis arising from endothelial cells in the pre-existing vessels is a complex process, in which cell migration and invasion are both pivotal steps. The inhibitory effect of SKLB-329 on HUVEC migration was evaluated using wound healing assay. After exposure to SKLB-329 in various concentrations for 18 hr, the number of migrating cells was significantly diminished by SKLB-329 as compared with vehicle (Fig. 1c); the IC₅₀ was about 2.5 μM. In transwell invasion assay, treatment of cells with 2.5–10 μM SKLB-329 strongly inhibited invasion by 40.2–84.4% (Fig. 1d). Tube formation of endothelial cells is also indispensable for angiogenesis. We used Matrigel-based tube formation assay to assess the effect of SKLB-329 and found that it dose-dependently inhibited the ability of HUVEC to assemble into branched capillary-like structures, with inhibition rates of 31.6, 68.4, and 85.4% at concentrations of 2.5, 5, and 10 μM, respectively (Fig. 1e).

As a positive control, sorafenib also showed comparable suppression effects in all functional assays. Taken together, these data demonstrated that SKLB-329 could effectively inhibit angiogenesis in vitro.
SKLB-329 inhibits embryonic angiogenesis in zebrafish

Transgenic zebrafish assays were adopted to further assess the anti-angiogenesis effect of SKLB-329. As shown in Figure 2a, SKLB-329 could dose-dependently inhibit the intersegmental blood vessel growth. A concentration of 1.25 μM could lead to entire blockade of the intersegmental blood vessel growth, and 0.625 μM SKLB-329 resulted in the intersegmental blood vessel growth suppressed by 65.4%. By comparison, a complete inhibition of the intersegmental blood vessel growth needed a concentration of 2.5 μM for sorafenib, and 1.25 μM sorafenib led to a suppression of 52.7% (Fig. 2b). These results showed that SKLB-329 could effectively restrain angiogenesis in vivo and had higher potency of anti-angiogenesis than sorafenib.

Figure 2. Anti-angiogenesis effect of SKLB-329 in transgenic zebrafish embryos and its anti-angiogenesis mechanism of action. (a) and (b), SKLB-329 inhibited zebrafish embryonic angiogenesis. Brightfield and fluorescent images were taken after treatment with vehicle, sorafenib or SKLB-329, and the length of intersegmental vessels (ISVs) was used for statistics. Scale bars represent 100 μm (left) and 50 μm (right). Column, mean; bars, SD (n = 10; **, p < 0.01 vs. the vehicle; ANOVA). (c) SKLB-329 inhibited VEGFR phosphorylation and activity of its downstream signaling proteins in HUVECs. (d) SKLB-329 inhibited the downstream signaling of FGFR in FGF-stimulated HUVECs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Inhibition of VEGFR and FGFR signalings in HUVECs
The ability of SKLB-329 to inhibit the VEGFR and FGFR signalings in intact cells was evaluated in HUVECs using Western blot analysis. As shown in Figure 2c, the VEGFR signaling was activated upon stimulation by VEGF. This activation could be effectively inhibited by SKLB-329: VEGFR phosphorylation was completely blocked at concentrations higher than 10 nM, and downstream kinases including AKT, extracellular signal-regulated kinase (ERK), FAK and Src were also significantly inhibited with IC50s being about 10 nM. Very similar results were observed in FGF-stimulated HUVECs. As indicated in Figure 2d, FGF stimuli also activated VEGFR and the downstream effector proteins of VEGFR and FGFR, and this activation was significantly suppressed when exposed to SKLB-329. The IC50 value for VEGFR phosphorylation was between 1 and 10 nM, and those for downstream kinases were between 10 and 100 nM. Here one may notice that the downstream kinase Src could not be fully inactivated even at high concentrations (>100 nM) of SKLB-329. One of the possible reasons could be that Src is also regulated by other signaling pathways in addition to VEGFR/FGFR signaling. Collectively, these data suggested that SKLB-329 inhibited angiogenesis mainly through blockade of VEGFR and FGFR signaling pathways.

Inhibition of tumor cell viability and colony formation in vitro
The anti-viability activity of SKLB-329 against HCC cells was measured by MTT assay. SKLB-329 could efficiently inhibit the viability of HCC cells including HepG2, SMMC7721, and PLC/PRF/5, with IC50 values of 15.6, 16.3, and 14.8 μM, respectively (Fig. 3a), which were comparable to those for sorafenib (the corresponding IC50 values were 11.0, 12.2, and 11.1 μM, respectively). In addition, both SKLB-329 and sorafenib showed weaker anti-viability activity on a normal human liver cell line, HL-7702; the IC50 values were 22.8 and 16.9 μM for SKLB-329 and sorafenib, respectively. Colony formation assays were then performed to visually assess the cytoreductive activity. SKLB-329 at a concentration of 10 μM notably decreased the formation of colonies in all the three HCC cell lines, but showed no apparent influence on HL-7702 colonies (Fig. 3b). Collectively, these results indicated that SKLB-329 could efficiently inhibit cell viability and colony formation of HCC cells, but had lower toxicity to normal liver cells and no apparent impact on normal liver cell colonies.

SKLB-329 inhibits HCC cell proliferation via blocking Src/FAK and Src/Stat3 signalings
The anti-proliferation effect of SKLB-329 was examined by Edu cell proliferation assay. As shown in Figure 4a, treatment
with SKLB-329 markedly reduced the number of proliferating cells (red nuclei) compared with the control. Cell cycle studies showed that SKLB-329 dose-dependently induced cell cycle arrest in the G0/G1 phase in all the three HCC cell lines (Fig. 4b). To further study the anti-proliferative mechanism of SKLB-329, changes in the phosphorylation levels of pivotal proteins modulated by Src were detected by Western blot analysis in HepG2 and PLC/PRF/5 HCC cell lines. SKLB-329 effectively inhibited Src phosphorylation, and down-regulated the phosphorylation levels of FAK and Stat3 at concentrations between 3 and 30 μM in HepG2 tumor cells, and between 10 and 30 μM in PLC/PRF/5 tumor cells (Fig. 4c). Nevertheless, there were no changes in the phosphorylation levels of AKT and ERK. These results imply a mechanism of action different from that of sorafenib, which exerts its anti-proliferative activity mainly through inhibition of MAPK signaling.10,26 To further verify that sorafenib uses different mechanisms of action in suppressing HCC cell proliferation, we examined the influence of the two agents on phosphorylation level of MEK in the PLC/PRF/5 cell line. As shown in Figure 4d, there was a significant change in phosphorylated MEK when the cells were treated with sorafenib, but not in the SKLB-329 treatment group. All of these results revealed that SKLB-329 inhibited the growth of HCC cells mainly through the suppression of Src/FAK and Src/Stat3 signalings, but not the MAPK cascade, which is the main target of sorafenib.

Antitumor efficacy of SKLB-329 in human HCC xenograft models
The in vivo anti-tumor efficacy of SKLB-329 was assessed using HepG2 and SMMC7721 tumor xenograft models. Oral dosing of SKLB-329 at 7.5 and 15 mg kg⁻¹ day⁻¹ slowed down the tumor growth dose-dependently, with tumor growth inhibition rate of 62.0 and 80.2%, respectively, for the HepG2 model, and 57.4 and 77.0%, respectively, for the SMMC7721 model (Fig. 5a). As a positive control, 30 mg kg⁻¹ sorafenib groups showed inhibition rates of 69.8 and 63.2% for the HepG2 and SMMC7721 models, respectively, indicating a slightly weaker antitumor activity compared with those of SKLB-329. No
Figure 5. In vivo pharmacodynamic and pharmacokinetic studies of SKLB-329. (a) and (b), in vivo anti-HCC studies of SKLB-329 against HepG2 and SMMC7721 tumor xenograft models. Tumor volume and body weight were monitored twice weekly. Points, mean tumor volume (mm$^3$) or mean body weight (g); bars, SD. Immunohistochemical staining analysis was used to determine the anti-angiogenesis and anti-proliferative effects of SKLB-329 on HepG2 (c), and SMMC7721 models (d). Scale bars represent 100 μm. (e) The plasma concentration-time curves of SKLB-329 and sorafenib in Sprague–Dawley rats after a single oral dose of 20 mg kg$^{-1}$. Blood was collected at indicated time and the plasma concentration was determined by HPLC. Points, mean; bars, SD; n = 6.
weight loss (Fig. 5b) and pathological changes of major organs (Supporting Information Fig. 1) were observed in all treatment groups during the whole experiments.

**Anti-tumor mechanisms of action of SKLB-329**

To determine the in vivo anti-tumor mechanisms of action of SKLB-329, immunohistochemical staining assays were carried out on the tumor tissues. Figures 5c and 5d show the immunohistochemical analysis results for the tumor tissues in the HepG2 and SMMC7721 models, respectively. Tumor tissues from the SKLB-329 treatment groups all showed a decrease in the phosphorylation levels of Src, FAK and Stat3, as well as in the tumor mitotic index (Ki67) compared with the corresponding control groups (Figs. 5c and 5d), suggesting that SKLB-329 was also able to inhibit the proliferation of tumor cells in vivo. In addition, a significantly reduced microvessel density was also observed in the SKLB-329 treatment groups compared with the control groups (Figs. 5c and 5d), indicating inhibition of angiogenesis. Taken together, SKLB-329 exerts its anti-tumor effects in vivo through inhibition of both tumor angiogenesis and tumor cell proliferation.

**Pharmacokinetic characteristics of SKLB-329**

The pharmacokinetic characteristics of SKLB-329 were assessed on rats following peros administration. The plasma concentration versus time profile is presented in Figure 5e. The key pharmacokinetic parameters calculated are summarized in Supporting Information Table S1. The area under the concentration-time curve (AUC0–72h) is 386.7 μg mL⁻¹ h⁻¹, which is slightly larger than that of sorafenib (336.5 μg mL⁻¹ h⁻¹). SKLB-329 was absorbed very well, achieving a maximum plasma concentration (Cmax) of 14.1 μg mL⁻¹ within about 9.2 hr, and displayed long half-life (t1/2) of 11.7 hr, slow clearance rate (CL) of 0.05 L h⁻¹ kg⁻¹, and small apparent distribution volume (Vss) of 0.8 L kg⁻¹, indicating that SKLB-329 was mainly distributed in vasculature and eliminated slowly from the body.

**Discussion**

The anti-angiogenesis therapy has been established as an efficacious strategy for the treatment of solid tumors. The success of sorafenib in treating HCC could be one of the most convincing proofs. SKLB-329 reported here is also a small molecule anti-angiogenesis agent. In terms of the potency of anti-angiogenesis, SKLB-329 is more potent than sorafenib. For example, in biochemical assays, SKLB-329 displayed higher activities in inhibiting receptor tyrosine kinases associated with angiogenesis, including VEGFR1/2/3, FGFR2; the IC50 values for SKLB-329 against VEGFR1/2/3 and FGFR2 are 5, 18, 5 and 26 nM, respectively, and those for sorafenib are 26, 90, 20 and 825 nM, respectively. In functional assays, such as HUVEC assays and zebrafish embryonic assay, SKLB-329 also exhibited higher or comparable potency compared with sorafenib. Here, it is necessary mentioning that though SKLB-329 has low nanomolar enzymatic activities (IC50s) against VEGFR1/2/3 and FGFR2, and almost ablated phosphorylation of VEGFR in HUVEC at nanomolar range (10–1,000 nM), inhibition of HUVEC migration, invasion, and tube formation still required much higher concentrations (2.5–10 μM, Figs. 1c–1d). The most plausible explanation is the significant binding of SKLB-329 with proteins in the medium which shifts potency into micromolar range because extracts for western blots were prepared from serum starved HUVECs whereas all phenotypic assays were performed in a presence of serum. In addition, various growth factors contained in the medium could also play a role since these growth factors might activate angiogenesis-related signaling pathways, which may partially compensate the inhibition action of SKLB-329.

Another attractive point for SKLB-329 is its ability to inhibit Src. Src plays critical roles in cell morphology, adhesion, differentiation, proliferation, survival, migration and invasion. Activation of Src can stimulate a variety of downstream signaling pathways, such as PI3K/Akt, Ras/Raf/MAPK, JAK/Stats and SFK/FAK/p130CAS cascades; these signaling pathways play crucial roles in regulating the development of cancer. Over-expression or abnormal activation of Src protein kinase was often detected in a wide variety of human cancers, including tumors of the breast, prostate, pancreas, lung, ovarian, colon and brain. Though Src is also expressed in the normal tissues of these organs, the expression level is much lower than that in tumors. Src was thus recognized as a good target for these cancers. Src inhibitors or their combination with other agents have exerted efficacy in the treatment of some of these cancers. Recently, a pathological analysis conducted by Lau and colleagues indicated a high level of Src in most HCC cases, but a low level in normal liver and in chronic persistent hepatitis caused by chronic hepatitis C. Further studies have shown that the prognosis of HCC patients with a high level of Src is significantly worse than that of patients with a low level of Src. Thus has thus also been considered an attractive target for HCC. Some small molecule Src inhibitors indeed showed strong anti-HCC effect in vitro. SKLB-329 is a good Src inhibitor with an IC50 value of 18 nM. In cellular assays, SKLB-329 could efficiently inhibit HCC cell proliferation and arrest the cell cycle in the G0-G1 phase. Further studies showed that the anti-proliferation effect of SKLB-329 was mainly due to the blockade of Src/FAK and Src/Stat3 signaling cascades, but not PI3K/PTEN/Akt or Ras/Raf/MAPK signaling pathway. Again, it is worth mentioning that though SKLB-329 has low nanomolar IC50 against Src, effective concentrations in inhibition of HCC cell viability and proliferation were still in micromolar range (10–20 μM, Figs. 3 and 4). A possible reason could be that the growth of HCC cells is regulated by multiple signaling pathways, and Src kinase is not the only pivotal regulatory molecule driving HCC cell survival and proliferation.
In HCC xenograft models, SKLB-329 exhibited more potent anti-tumor activity than sorafenib. Studies of mechanisms of action showed that SKLB-329 inhibited both tumor angiogenesis and HCC cell proliferation in tumor tissues. Though it is difficult to exactly differentiate how much of the observed in vivo effects of SKLB-329 are due to anti-angiogenesis effects and how much due to its tumor cell suppression, roughly speaking, anti-angiogenesis effects should play more important roles on the anti-tumor efficacy; this speculation was based on biochemical and functional properties of SKLB-329.

In conclusion, SKLB-329 is a novel multikinase inhibitor that potently inhibits VEGFR1/2/3, FGFR2, and Src. It showed significant activity in inhibiting angiogenesis and considerable potency in suppressing tumor cell proliferation both in vitro and in vivo. SKLB-329 has the convenience of oral administration, favorable pharmacokinetic properties. Taken together, the results of preclinical evaluation of SKLB-329 support the use of SKLB-329 as a promising candidate for clinical studies in patients with HCC.

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