Preclinical Evaluation of a Novel Orally Available SRC/Raf/VEGFR2 Inhibitor, SKLB646, in the Treatment of Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is the most aggressive and deadly breast cancer subtype. To date, chemotherapy is the only systemic therapy and prognosis remains poor. Herein, we report the preclinical evaluation of SKLB646 in the treatment of TNBC; SKLB646 is a novel multiple kinase inhibitor developed by us recently. This compound potently inhibited SRC and VEGFR2 with IC50 values of 0.002 µmol/L and 0.012 µmol/L, respectively. It also considerably inhibited B-Raf and C-Raf with IC50 values of 0.022 and 0.019 µmol/L, respectively. It exhibited significant antiproliferation and anti-viability activities against TNBC cell lines. Studies of mechanism of action indicated that SKLB646 inhibited the activation of SRC signaling and blocked the MAPK signaling through inhibiting the Raf kinases. Interestingly, SKLB646 dose dependently downregulated the expression of Fra1, a transcriptional factor that plays a critical role in the epithelial-to-mesenchymal transition. In addition, SKLB646 could inhibit HUVEC proliferation, migration, and invasion. It effectively blocked the formation of intersegmental vessels in zebrafish embryos and displayed considerable antiangiogenic effects in the tumor-induced neovascularization zebrafish model. In TNBC xenograft models, SKLB646 suppressed the tumor growth in a dose-dependent manner. Moreover, SKLB646 could remarkably inhibit TNBC cell migration and invasion in vitro. Furthermore, in an experimental lung metastasis model, the overall survival time of groups treated with SKLB646 was much longer compared with the control-, dasatinib-, and paclitaxel-treated groups. In a preliminary pharmacokinetic study, SKLB646 showed good pharmacokinetic properties. Taken together, the preclinical data show that SKLB646 could be a promising lead compound for the treatment of TNBC. Mol Cancer Ther; 15(3): 1–13. ©2015 AACR.

Introduction

Triple-negative breast cancer (TNBC), phenotypically characterized by a lack of expression of estrogen receptor (ER), progesterone receptor (PR), and the HER2, is likely the most aggressive and deadly breast cancer subtype (1, 2). Different from other subtypes of breast cancer, TNBC cannot benefit from hormonal therapies or treatments targeted against HER2. Chemotherapy is the only systemic therapy for TNBC and prognosis remains poor (3, 4). Because targeted therapies have the advantage of maximizing efficacy while often reducing toxicity, research and development of such kind of drugs. Nevertheless, the progress is still very limited (1, 7). Reasons leading to this situation are complicated, but the refractoriness of TNBC and no validated specific biomarker could be the most important reasons.

Recent molecular pathologic studies have improved our understanding of the molecular alterations involved in tumor initiation and development of TNBC (8, 9). Some potential targets for the treatment of TNBC have been suggested. Among these targets, SRC and Raf are of particular importance. SRC is a non-receptor tyrosine kinase and has been revealed to be a critical regulator of a large number of intracellular signaling pathway (10). Abnormal activation or amplification of SRC has been detected in TNBC and demonstrated to play a role in proliferation, migration, and invasion of breast cancer cell lines (11, 12). Furthermore, a number of recent studies have shown that dysregulation of SRC is strongly associated with tumor metastasis and poor prognosis in TNBC (13, 14). SRC therefore represents a rational molecular target for TNBC. Raf kinases are a family of serine/threonine-specific protein kinases, which are one of the key components of the MAPK signaling cascade (15). The Cancer Genome Atlas (TCGA) analysis has determined that about 80% of basal-like TNBC have some degree of genomic amplification or activation of major components of the MAPK signaling network (16). Though both Raf and MEK are the important central nodes in the MAPK pathway, targeted drugs against SRC have thus attracted great attention in the past decade (3, 6). Many pharmaceutical companies and drug research institutes have been involved in the research and development of such kind of drugs. Nevertheless, the progress is still very limited (1, 7). Reasons leading to this situation are complicated, but the refractoriness of TNBC and no validated specific biomarker could be the most important reasons.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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pathway, MEK inhibitors in TNBC could induce the activation of several upstream receptor tyrosine kinases through negative feedback, which can reactivate ERK and lead to drug resistance (17). All of these suggest that Raf other than MEK is a better target for the MAPK pathway–targeting therapy of TNBC.

Angiogenesis is one of the main hallmarks of solid tumors, which is essential for the progression of primary and metastatic tumors, including TNBC (18). Targeting tumor angiogenesis is a well-established approach for cancer treatment. VEGFR2 is a key mediator of angiogenesis. High expression of VEGFR2 was found in tumor tissues of TNBC patients, and was also linked to poor prognosis in TNBC (19). Therefore, VEGFR2 could be taken as another rational target for the treatment of TNBC.

As mentioned above, SRC, Raf, and VEGFR2 could be potential targets for the treatment of TNBC. It is reasonable to hypothesize that agents that can simultaneously attack these targets might have enhanced effects on the therapy of TNBC. In a previous study, our lab discovered a series of 3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine derivatives as a new class of SRC inhibitors (20). An expanded kinase inhibition assay to the most active SRC inhibitor (1g in ref. 20) showed that this compound could also inhibit the activation of Raf and VEGFR2. We thus carried out a broad screening for all compounds that can simultaneously inhibit SRC, Raf, and VEGFR2 in the SRC inhibitors we synthesized. Finally, we obtained a compound, N-(3-((4-amino-1-{(1-methylpyriderin-4-yl})-1H- pyrazolo-pyrimidin-3-yl) ethynyl)-4-methylphenyl)-3-(trifluoromethyl) benzamide, named SKLB646 (Fig. 1A), which showed considerably high potency against all the three kinases. The major purpose of this investigation is to perform comprehensive preclinical evaluation to SKLB646, including anti-TNBC activities both in vitro and in vivo, and the mechanism of action, as well as preliminary evaluation of pharmacokinetic properties.

Materials and Methods

Compounds

Compound SKLB646, formulated as N-(3-((4-amino-1-{1-methylpyriderin-4-yl})-1H-pyrazolo-pyrimidin-3-yl) ethynyl)-4-methylphenyl)-3-(trifluoromethyl) benzamide (Fig. 1A), was synthesized at the State Key Laboratory of Biotherapy and Cancer Center, Sichuan University. SKLB646 was purified to >95% purity, as determined by high-performance liquid chromatography (HPLC; details for the chemical synthesis and characterization of SKLB646 see Supplementary Fig. S1 and Supplementary Methods). Dasatinib and paclitaxel were purchased from Nanjing Chemlin Chemical Industry Co., Ltd.

Cells and cell culture conditions

These cell lines (K562, 786-O, A549, Jurkat, HS-5, MCF10A, HEK293, I929, MDA-MB-231, SKBR-3, BT474) were obtained from ATCC between 2007 and 2009. These cell lines (MDA-MB-435, MDA-MB-436, MDA-MB-453, MCF-7, RPMI-8226, H4, SH-SY-5Y, Hela, HepG2, PLC/PRF/5, 4T1, MEF-1, PC9, 22Rv1) were obtained from National Platform of Experimental Cell Resources for Sci-Tech between 2008 and 2012. The cells were grown in RPMI1640 or DMEM containing 10% FBS (v/v), 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich) according to the manufacturer’s guidelines. All cell lines were passaged for less than 6 months. No further authentication was done for these cell lines in the past 6 months. Human umbilical vein endothelial cell (HUVEC) was isolated from human umbilical cord veins according to a standard procedure and grown in EGM-2 medium as described before (21).

Kinase inhibition assay

IC_{50} values of SKLB646 for kinase inhibition in vitro were measured by radiometric assays conducted by Eurofins Pharma Discovery Services UK Limited (22). Briefly, each kinase (5–10 nM) was incubated with different concentrations of SKLB646 in 25 µL indicated reaction solutions, which contained 8 mmol/L 3- (N-morpholino) propa-nesulfonic acid (MOPS), pH 7.0, 0.2 mmol/L EDTA, 0.33 mg/mL myelin basic protein, 10 mmol/L Mg acetate, and γ-[33P] ATP. The reaction was stopped by the addition of 5 µL of 3% phosphoric acid solution after incubation for 40 minutes at room temperature. Ten microliter of the reaction was then spotted onto a P30 Filtermat A and washed three times for 5 minutes in 75 mmol/L phosphoric acid and once in methanol prior to scintillation counting.

Cell proliferation assay

Cell proliferation assays were conducted as previously reported (21). A variety of human TNBC cell lines were treated with indicated concentrations of SKLB646 or other agents for 72 hours and cell viability was measured using MTT assay (Sigma-Aldrich). The IC_{50} values were calculated by GraphPad Prism 6 software.

Colony formation assay

The cells were seeded in 6-well plates at a density of 2,000–5,000 per well. After 24 hours, the cells were incubated with different concentrations of SKLB646 or other agents. The medium containing different concentrations of SKLB646 or other agents was replaced every 4 days. After 12 days of incubation, cells were fixed with methanol and stained with crystal violet.

The EdU incorporation assay

MDA-MB-231 and MDA-MB-435 cell lines were seeded in 96-well plates at the density of 2,000 and 6,000 per well, respectively. The next day, these cells were treated with different concentrations of SKLB646 or other agents for 24 hours. Then, the EdU incorporation assay was conducted following the manufacturer’s instruction (Ribobio).

The apoptosis assay

For apoptosis analysis, an Annexin V–FITC and propidium iodide apoptosis detection kit (Keygene Biotech) and Hoechst 33342 (Beyotime) were used for a direct observation of viable, early apoptotic, and late apoptotic cells. Cells were treated with different concentrations of SKLB646 or other agents for 24 hours. The cells were washed once with PBS. Then, Annexin V–FITC and propidium iodide (PI) were added according to the manufacturer’s instructions, and the samples were incubated in the dark for 15 minutes. After that, the cells were washed again with PBS and stained with Hoechst 33342 at a final concentration of 5 µg/mL for 5 minutes. Pictures were taken by an Olympus digital camera attached to a light microscope.

Flow cytometry for cell-cycle analysis

The cells were treated with different concentrations of SKLB646 or other agents for 48 hours. For cell-cycle analysis, the cells in experimental groups and control group were harvested and washed once with ice-cold PBS. After that, cells were resuspended

Zheng et al.
Figure 1.
Antiproliferative activities of SKLB646 against TNBC in vitro. A, chemical structure of SKLB646. B, the cell viability inhibitory profile of SKLB646 and dasatinib against TNBC cells. C, TNBC cells were seeded in 6-well plates and treated with SKLB646 and dasatinib for 12 days and colonies were stained with crystal violet and quantified. Representative images for TNBC cells treated with 0.3 μmol/L of SKLB646 or dasatinib are shown (left). The percentages of the colony numbers are presented (right). Columns, mean (n = 3); bars, SD. ***, P < 0.001. D, EdU incorporation assay. MDA-MB-231 and MDA-MB-435 cells were treated with indicated agents for 24 hours, followed by incubation with EdU and Hoechst in sequence. The fluorescence of EdU (red) and Hoechst (blue) represent proliferating cells and cell nucleus, respectively. Scale bars, 50 μm for micrographs (left). The percentages of the EdU-positive cells are presented (right). Columns, mean (n = 3); bars, SD. * P < 0.05; **, P < 0.01; ***, P < 0.001.
in 1 mL of 70% ice-cold ethanol and fixed overnight. Fixed cells were washed once with ice-cold PBS and resuspended in 500 μL PI solution (50 μg/mL PI, 0.1% NP-40, 0.1% sodium citrate, 0.1% Triton X-100). All these cells were analyzed by flow cytometry (ESP Elite, Beckman Coulter).

Western blot analysis
MDA-MB-231 and MDA-MB-435 cells were incubated for 20 hours in medium containing different concentrations of SKLB646 or other agents. Cells were lysed with lysis buffer and protein concentrations were determined. Protein was separated by SDS-PAGE gels. After electrophoresis, protein was transferred to polyvinylidene fluoride membranes (Millipore), blocked in TBS-T and 5% non-fat dry milk for 1 hour, and subsequently washed and incubated with TBS-T and the specific antibodies (Cell Signaling Technology) including anti-VEGFR2, anti-pVEGFR2 Tyr972, anti-Src, anti-pSrc Tyr416, anti-FAK, anti-pFAK Tyr925, anti-STAT3, anti-pSTAT3 Tyr705, anti-MEK, anti-pMEK Thr202/Tyr204, anti-ERK, anti-pERK Thr202/Tyr204, anti-STAT3, anti-pSTAT3 Tyr705, anti-ERK, anti-pERK Thr202/Tyr204, anti-Fra 1, anti-p Fra 1 Ser265, anti-cleaved caspase-3, and anti-β-actin for 12 hour at 4°C. After washing with TBS-T, membranes were incubated with a peroxidase-conjugated secondary antibody for 1 hour at 37°C. Signals were detected by chemoluminescence using the Enhanced Chemoluminescence System (Millipore).

Migration assay
Migration assay was done following the method reported previously (23). Briefly, cells were grown in normal growth media to monolayer confluence in 24-well plates. The centers of the cell monolayer were scraped with a sterile 200 μL pipette tip to create a denuded zone (gap) of constant width. Subsequently, floating cells were removed by extensive washing with sterile PBS. After that, cells were cultured with various concentrations of SKLB646 or other agents in normal growth media. Images were taken by Olympus inverted microscope after 18 hours incubation at 37°C. The migrated cells were quantified using the following formula: length × width2 × 0.5.

Transwell invasion assay
The cell invasion assay was performed as described previously (24). In brief, Matrigel (BD Biosciences) diluted 1:3 in serum-free DMEM medium or other kinds of medium was added (50 μL) to the top chamber of 24-well transwell plates (6.5-mm diameter, 10-μm thickness, and 8-μm pore size) and was solidified at 37°C for 30 minutes. A total of 300,000 cells in serum-free media were seeded in the top chambers of 24-well invasion chambers with growth media in the bottom chambers. Immediately, DMEM or other kinds of media with various concentrations of SKLB646 or other agents were added to the top chambers. After 24 hours, invasion was terminated by swabbing the cells in the top chambers with a cotton swab, and the filters were fixed with 4% paraformaldehyde for 15 minutes and stained with crystal violet for 15 minutes. Images were taken using the Olympus microscope. Five independent areas per well were counted and the mean number of migrated cells was calculated.

Transgenic zebrafish
The transgenic zebrafish (FLK: EGFP) assay was conducted according to the protocol reported previously (25). Transgenic zebrafish (FLK: EGFP) were maintained normally (temperature, 28°C; pH 7.2–7.4; 14-hour on and 10-hour off light cycle). The 13-somite stage of zebrafish embryos were incubated overnight with dasatinib and SKLB646, respectively. The image was acquired after the zebrafish was anaesthetized by a fluorescence microscope.

Red fluorescence-labeled B16 cells were harvested at a concentration of 1 × 10^7 cells/mL. The cell suspensions were implanted into each zebrafish embryo to create a tumor xenograft model in zebrafish (26). One day after implantation, SKLB646 and dasatinib were added directly into the fish water at different final concentrations. The next day, images capture, processing, and adjustment were performed with ZEISS Axiovisio rel. 4.8 software.

Subcutaneous xenograft models
All animal studies were conducted according to the guidelines of the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China). Six-week-old female SCID mice were purchased from HFK Biotechnology Company. Tumor xenograft models were established by subcutaneously injecting 100 μL tumor cell suspension (between 5 × 10^5 and 1 × 10^6) into hind flank region of mice. Tumors were allowed to reach a volume of 150 to 200 mm^3 before initiation of treatment. The solvent containing 5% DMSO, 25% (v/v) PEG400, and 70% water was used as control. Mice were given SKLB646 (10–40 mg/kg), dasatinib (40 mg/kg), or blank solvent once daily by oral gavage. Paclitaxel was administered intravenously once a week (10 mg/kg). Tumor sizes were monitored every 3 days using calipers, and the volume was calculated using the following formula: length × width^2 × 0.5.

Experimental lung metastasis assay
For the metastasis study, MDA-MB-231 cells (1 × 10^6) were implanted via tail vein injection into 5-week-old to 6-week-old female SCID mice that was immediately followed by randomization (n = 6 per group). Individual treatments were performed, starting at the same day of cell injection, with SKLB646 (20 and 40 mg/kg, orally), dasatinib (40 mg/kg, orally), paclitaxel (10 mg/kg), or blank solvent once daily by oral gavage. Paclitaxel was administered intravenously once a week (10 mg/kg). Tumor burdens in the lungs were examined by the use of hematoxylin and eosin (H&E) staining and IHC. Survival data were analyzed using Kaplan–Meier method, and statistical significance was evaluated with a log rank test comparing the survival time of each treatment group with the control group.

IHC and TUNEL protocol
Immunohistochemical staining was conducted using antibodies from Cell Signaling Technology or Abcam, except CD31 (BD Biosciences) and Ki67 (Thermo Fisher Scientific). Cell apoptosis in MDA-MB-231 and MDA-MB-435 xenograft tumors was also determined using TUNEL assays following the manufacturer’s instructions (Promega). The images were captured with a Carl Zeiss digital camera attached to a light microscope. The number of Ki-67, cleaved caspase-3, and TUNEL-positive cells was quantified. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells.

Pharmacokinetic assessments
Pharmacokinetic assessments were conducted as described previously (21). Catheters were surgically placed into the jugular vein of male Sprague-Dawley rats (HFK Biotechnology). The
animals were fasted overnight, followed by orally dosing with 10 mg/kg SKLB646. Blood was collected at 0.17, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60, and 72 hours postdose. The plasma concentrations of SKLB646 were measured by HPLC-mass spectrometry (HPLC-MS). Noncompartmental pharmacokinetic parameters were fitted using DAS software (Enterprise Version 2.0, Mathematical Pharmacology Professional Committee of China).

**Statistical analysis**

GraphPad Prism 6.0 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by the Student t test and ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Enzymatic activities of SKLB646 against recombinant human protein kinases**

Kinase inhibitory activities of SKLB646 against a panel of recombinant human protein kinases were measured by the "gold standard" radiometric kinase assay approach, and the results are presented in Supplementary Table S1. SKLB646 potently inhibited SRC and VEGFR2 with IC₅₀ values of 0.002 μmol/L and 0.12 μmol/L, respectively. It also showed considerable potency in inhibiting B-Raf with IC₅₀ values of 0.022 and 0.019 μmol/L, respectively (Fig. 1A). SKLB646 exhibited moderate activity against several other selected kinases, including PDGFRα, TAK1, and ErbB2. For other selected 30 kinases, SKLB646 displayed very weak or no activity at a concentration of 10 μmol/L (Supplementary Table S1). These results indicated that SKLB646 is a multikinase inhibitor that potently inhibits SRC, VEGFR2, and Raf kinases.

**Antiviability and antiproliferation activities of SKLB646 against TNBC and other cancer cells in vitro**

MTT assays were used to measure the antiviability activities of SKLB646 against TNBC cell lines and a number of other selected cancer cell lines. The results are presented in Supplementary Tables S2 and S3. SKLB646 displayed considerable antiviability activity against TNBC cell lines MDA-MB-231 and MDA-MB-435 with IC₅₀ values of 0.013 μmol/L and 0.080 μmol/L, respectively (Fig. 1B). It also showed good inhibitory potency against other two TNBC cell lines MDA-MB-436 and MDA-MB-453 (IC₅₀ values, 1.1 μmol/L and 0.367 μmol/L, respectively; Fig. 1B). For the other selected cancer cell lines, including BT474, MCF-7, Hela, and HepG2, SKLB646 exhibited very weak inhibitory activity (Supplementary Table S3). Although SKLB646 also displayed some inhibitory activity against noncancer cell lines MCF10A, HS-5, MEF-1, HEK293, and L929 (Supplementary Table S3), the activity was much weaker compared with that for TNBC cell lines MDA-MB-231 and MDA-MB-435. These results indicated that SKLB646 was more sensitive to TNBC cells than to other cancer or noncancer cells tested.

Then, the antiproliferation activities of SKLB646 against TNBC cell lines including MDA-MB-231, MDA-MB-435, MDA-MB-436, and MDA-MB-453 were examined by colony-forming assays. As shown in Fig. 1C, SKLB646 markedly inhibited the formation of colonies of MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells at a concentration of 0.3 μmol/L. Dasatinib also inhibited the formation of colonies of these cells but with relatively weaker potency. For MDA-MB-436, SKLB646 moderately decreased the formation of colonies, which were comparable with the effect of dasatinib.

The effects of SKLB646 on the proliferation of MDA-MB-231 and MDA-MB-435 cells were further evaluated by EdU assays. The results, depicted in Fig. 1D, showed that SKLB646 strongly suppressed the DNA replication of MDA-MB-231 and MDA-MB-435 cells at concentrations of 0.1 μmol/L and 1 μmol/L. However, only a slight decrease in the number of EdU-positive cells in the dasatinib-treated groups was observed compared with that in the control group, indicating a weak influence on the cell proliferation. Collectively, these results demonstrated that SKLB646 could substantially inhibit TNBC cell proliferation in vitro.

**SKLB646 induced TNBC cell apoptosis and cell-cycle arrest in vitro**

 Annexin V/PI staining was used to investigate the proapoptosis ability of SKLB646. As shown in Fig. 2A, SKLB646 induced an obvious apoptosis in cultured MDA-MB-231 and MDA-MB-435 cells at concentrations larger than 0.03 μmol/L. In contrast, dasatinib just showed a weak or moderate effect on the apoptosis of MDA-MB-231 and MDA-MB-435 cells.

The influence of SKLB646 on the cell cycle was detected by flow cytometry. As shown in Fig. 2B, after incubation with a series of concentrations of SKLB646, significant accumulation of cells in G₀–G₁ phase was observed in MDA-MB-231 and MDA-MB-435 cells. The number of cells in S and G₂–M phase correspondingly decreased. These results indicated that SKLB646 could cause TNBC cell arrest in G₀–G₁ phase. Meanwhile, dasatinib also arrested MDA-MB-231 and MDA-MB-435 cells in G₀–G₁ phase but moderately or weakly.

**SKLB646 inhibited the activation of multiple signaling pathways in intact TNBC cells**

Western blot analysis was performed to assess the ability of SKLB646 to inhibit important signaling pathways in MDA-MB-231 and MDA-MB-435 cells. As shown in Fig. 3A and B, SKLB646 effectively inhibited SRC phosphorylation in both MDA-MB-231 and MDA-MB-435 cells in a concentration-dependent manner. Consistent with the SRC inhibition, the activation of downstream signaling proteins FAK and STAT3 was significantly inhibited. It was also observed that SKLB646 dose dependently inhibited the phosphorylation of MEK and ERK, indicating an inhibition to the MAPK signaling. Furthermore, SKLB646 was found to significantly inhibit both the expression and phosphorylation of Fra1 at concentrations larger than 0.01 μmol/L; Fra1 has been demonstrated to be a key regulator of epithelial-to-mesenchymal transition (EMT; ref. 27), which is one of the important factors responsible for the tumor metastasis. Though dasatinib showed similar effect on the phosphorylation of SRC, it just displayed very weak effects on the MAPK signaling and Fra1. In addition, SKLB646 could increase the level of cleaved caspase-3 in a dose-dependent manner, indicating induction of apoptosis (28). Dasatinib also caused a weaker increase in caspase-3 activity.

The results were consistent with those observed in Fig. 2A.

**Antiangiogenic activity of SKLB646 in cultured HUVECs and transgenic zebrafish models**

We first examined the influences of SKLB646 on the proliferation, migration, and invasion of cultured HUVECs. In an MTT assay, SKLB646 showed a considerable suppression effect on the HUVEC proliferation in a dose-dependent manner...
Supplementary Fig. S2). As shown in Fig. 4A, a wound-healing assay indicated that 0.3 μmol/L SKLB646 significantly inhibited the migration of HUVECs. In a transwell invasion assay, treatment of cells with 0.3 μmol/L SKLB646 suppressed invasion by 72.2%. In both wound-healing assays and transwell invasion assays, SKLB646 also displayed inhibition effect in a dose-dependent manner (Fig. 4B). In the three experiments above, dasatinib also showed similar effects. Western blot analysis was then carried out to understand the molecular mechanisms. As shown in Fig. 4C, SKLB646 at concentrations larger than 0.1 μmol/L markedly abolished the phosphorylation of VEGFR and SRC. The phosphorylation of FAK, MEK and ERK was also considerably inhibited at the same concentrations. Dasatinib showed the similar effects on SRC and FAK, but weak effects on VEGFR, MEK, and ERK. These results implied that the effects of SKLB646 on the proliferation, migration and invasion of
HUVECs may be due to the integrative inhibition of VEGFR, SRC, and MAPK signaling. However, dasatinib displayed its effects on the proliferation, migration, and invasion of HUVECs mainly through the inhibition of SRC signaling.

Next, transgenic Tg (flk1:EGFP) zebrafish experiments were used to examine the *in vivo* antiangiogenic activity of SKLB646. As shown in Fig. 4D and E, the angiogenesis of intersegmental vessels was significantly inhibited in embryos treated with 5 μmol/L and 10 μmol/L SKLB646. While dasatinib just showed very weak effect on the angiogenesis of intersegmental vessels at the concentration of 10 μmol/L.

A tumor-induced neovascularization zebrafish model was further conducted to evaluate the effect of SKLB646 on angiogenesis *in vivo* (26). In the control group, endothelial cells were found to penetrate into the tumor tissue by two days after the implantation of tumor cells in zebrafish embryos, hence sustaining the tumor growth. However, treatment with SKLB646 at concentrations larger than 2.5 μmol/L significantly inhibited the neovascularization and tumor growth (Fig. 4F). In contrast, no evident effect on the neovascularization or tumor growth was observed in the group treated with 10 μmol/L dasatinib when compared with the control group. Collectively, these experiments indicated that SKLB646 possesses a considerable antiangiogenic activity.

### In vivo antitumor activity and mechanisms of action of SKLB646 in TNBC xenograft models

The *in vivo* antitumor activities of SKLB646 were evaluated with mouse subcutaneous xenograft models of MDA-MB-231 and MDA-MB-435. SCID mice were treated by oral medication daily for 18 days with 10, 20, and 40 mg/kg of SKLB646. Dasatinib (40 mg/kg/d administered orally and once daily) and paclitaxel (intravenous injection at a dose of 10 mg/kg once a week) were used as positive controls. The tumor volumes were measured every three days. In the MDA-MB-231 model, tumor growth was inhibited by SKLB646 in a dose-dependent manner with a tumor inhibition rate of >90% at a dose of 40 mg/kg (Fig. 5A). The final average tumor volume of 40 mg/kg SKLB646 group was smaller than those treated with dasatinib or paclitaxel (*P* < 0.01; Fig. 5A). In the MDA-MB-435 model, SKLB646 also displayed considerable tumor inhibitory ability with 40 mg/kg SKLB646 exhibiting a tumor inhibition rate of >60% (Fig. 5B). The final average tumor volume of 40 mg/kg SKLB646 group was also smaller than those treated with dasatinib or paclitaxel (*P* < 0.01; Fig. 5B). During the period of treatment, there was no significant decrease in weights (Supplementary Fig. S3A and S3B).

To understand the antitumor mechanisms of action of SKLB646, immunohistochemical analysis of tumor tissues resected from experimental animals were performed. As shown in Fig. 5C and Supplementary Fig. S4, for both MDA-MB-231 and MDA-MB-435 models, it was found that SKLB646 evidently reduced the phosphorylation of SRC, MEK, and ERK. As a consequence, the percentage of Ki67-expressing cells in viable tumor tissue was considerably lower following SKLB646 treatment, indicating a suppression of tumor cell proliferation. Furthermore, the deep color of cleaved caspase-3 stain (Fig. 5C and Supplementary Fig. S5) together with TUNEL assays (Supplementary Fig. S4) indicated that SKLB646 induced apoptosis in the tumor tissues. In addition, we evaluated the effect of SKLB646 on neovascularization using antibodies of p-VEGFR2 and CD31, which are the markers of angiogenesis. The results showed an obvious reduction of tumor microvessel density in tumors from experimental animals treated with SKLB646.

Although dasatinib also downregulated the phosphorylation of SRC, it did not effectively suppress the activation of MAPK signaling, which agrees with the *in vitro* result. Dasatinib showed similar but relatively weak antiproliferation and proapoptosis activities. Immunohistochemical detection of p-VEGFR2 and CD31 revealed that dasatinib slightly reduced the microvessel density compared with the control groups. As another positive control, paclitaxel did not obviously inhibit SRC and MAPK signaling pathways in both models, although it also led to a moderate decrease in the levels of expression of Ki-67 and increase in the number of cleaved caspase-3-positive cells. Furthermore, after the treatment of paclitaxel, the number of p-VEGFR2 or CD31-positive cells around vessel both remained constant compared with the control group.

### Effect of SKLB646 on antimetastasis of TNBC *in vitro* and *in vivo*

To investigate the effect of SKLB646 on antimetastasis of TNBC, we first conducted wound-healing assays and transwell assays with cultured MDA-MB-231 and MDA-MB-435 cells. As shown...
in Fig. 6A, treatment of MDA-MB-231 cells with 0.01, 0.03, 0.1, and 0.3 μmol/L SKLB646 decreased cell migration by 25.1%, 55%, 70.4%, and 80.1%, respectively, indicating that SKLB646 reduced cell motility in a dose-dependent manner. Transwell assays showed that cell invasion properties of MDA-MB-231 cells were decreased significantly in response to SKLB646.
Preclinical Studies of SKLB646 against TNBC

Figure 5.
SKLB646 inhibited tumor growth in MDA-MB-231 and MDA-MB-435 xenograft models. A, mice implanted with MDA-MB-231 cells were treated when the tumor grew to about 200 mm³. Animals (at least 6 per group) were treated with solvent control, SKLB646 at doses of 10, 20, and 40 mg/kg/day, or dasatinib at a dose of 40 mg/kg/day or paclitaxel at a dose of 10 mg/kg/week. B, mice implanted with MDA-MB-435 cells were treated when the tumor grew to about 200 mm³. Animals (at least 6 per group) were treated with solvent control, SKLB646 at doses of 10, 20, and 40 mg/kg/day, or dasatinib at a dose of 40 mg/kg/day or paclitaxel at a dose of 10 mg/kg/week. Points, mean tumor volumes and bars, SD. **, P < 0.01; ***, P < 0.001.

C, mechanisms of the actions of SKLB646 in MDA-MB-231 and MDA-MB-435 xenograft. Tumor tissues were taken out for immunohistochemical stain to determine the inhibition of signal pathways and the induction of antiproliferation, apoptosis, and antiangiogenesis in vivo at the end of the experiments. Scale bars, 50 μm for micrographs. C-CASP3, cleaved caspase-3.

(Fig. 6B): 0.1 and 0.3 μmol/L SKLB646 decreased MDA-MB-231 cell invasion by 45.4% and 85.2%, respectively. Similar inhibition results for both assays were obtained in MDA-MB-435 after treated with SKLB646. Dasatinib also displayed a considerable ability to block the migration and invasion of MDA-MB-231 and MDA-MB-435 cells.
Figure 6.
SKLB646 suppressed the activity of invasion and metastasis against TNBC in vitro and in vivo. A, MDA-MB-231 and MDA-MB-435 were treated with SKLB646 and dasatinib for migration assays. These assays were performed as described before. Scale bars, 100 μm (left). The percentages of the migrated cells are presented (right). Columns, mean (n = 3); bars, SD. **, P < 0.01; ***, P < 0.001. B, MDA-MB-231 and MDA-MB-435 were treated with SKLB646 and dasatinib for invasion assays. These assays were performed as described before. Scale bars, 100 μm (left). The percentages of the invaded cells are presented (right). Columns, mean (n = 3); bars, SD. ***, P < 0.001. C, graph of the experimental lung metastasis assay. Kaplan–Meier survival curve was diagrammed for every treatment group. *, P < 0.05; **, P < 0.01; ***, P < 0.001. D, the plasma concentration-time curves of SKLB646 in male Sprague–Dawley rats after a single oral dose of 10 mg/kg. Points, mean plasma concentrations; bars, SD; n = 6. E, hematoxylin and eosin (H&E) staining and IHC were used to analyze for lungs taken from different treatment group mice after implantation for 30 days. Pictures were taken with low (200×) and high (400×) magnification. Scale bars, 100 μm and 50 μm for low (200×) and high (400×) magnification, respectively.
We further evaluated the in vivo antimitastatic efficacy of SKLB646 in a survival model, in which MDA-MB-231 cells were injected intravenously. As shown in Fig. 6C, the median survival time (MST) for the control mice was 40 days. Daily oral treatment with 20 and 40 mg/kg/day SKLB646 for 30 days significantly prolonged MST to 71 days and 82 days, respectively. These results were much better than those achieved following daily oral administration of 40 mg/kg dasatinib and weekly intravenously administration of 10 mg/kg paclitaxel, in which the MSTs were 50.5 days and 56 days, respectively.

To understand whether there is some association between the prolonged MST and the reduction in lung metastases, host mice were sacrificed on day 30 after cell injection through caudal vein upon the same administration as above. Intact lungs from mice were examined by hematoxylin and eosin (H&E) stain and immunohistochemical stain (IHC) for Vimentin. Mice treated with SKLB646 showed dramatic reduction in the size of metastatic lung nodules compared with that of control group, dasatinib group, and paclitaxel group (Fig. 6E).

Pharmacokinetics characteristics of SKLB646

To evaluate the pharmacokinetic characteristics of SKLB646 in vivo, a single oral dose of 10 mg/kg SKLB646 was administrated to male Sprague-Dawley rats. The plasma concentration of SKLB646 at every time point was measured, and the plasma concentration versus time profile was presented in Fig. 6D. The pharmacokinetic parameters of SKLB646 were summarized in Supplementary Table S4. The results of pharmacokinetic studies showed that SKLB646 was absorbed well, achieving a maximum plasma concentration ($C_{\text{max}}$) of 0.328 mg/L at about 4 hours, and displayed a half-life ($t_{1/2}$) of approximately 15.171 hours, with a clearance rate of 2.307 L/h/kg. In addition, the steady-state volume ($V_{\text{ss}}$; 50.507 L/kg) of SKLB646 markedly exceeded the volume of total body water of human (0.67/kg), showing a certain portion of the dose distributed into tissues. These data indicated that SKLB646 had good pharmacokinetic properties.

Discussion

TNBC has been thought as an extremely aggressive and refractory malignancy (29). Currently, no targeted drug is approved to use in the treatment of TNBC in clinic (30). Though some targeted drugs have entered clinical trials, most of them just showed very limited efficacy against TNBC. For example, dasatinib, a SRC family kinase inhibitor, has been assessed in phase II clinical trial. As a single agent, dasatinib exhibited moderate efficacy in TNBC patients and the clinical response rate was just 9.3% (4/43; ref. 31). Another agent, sorafenib, a VEGFR/Raf inhibitor, has been reported to have modest activity as a single agent in metastatic breast cancer patients, which contained some TNBC patients (32). Interestingly, a recent study has demonstrated that combined use of dasatinib and sorafenib displayed an enhanced efficacy compared with sole use of either dasatinib or sorafenib in inhibiting TNBC cells (33). This study prompted an advantage of use agents concurrently targeting multi-targets in the treatment of TNBC. SKLB646 is such a compound that potently inhibits SRC, Raf, and VEGFR2. It displayed potent antiproliferation and antiviability activities in cultured TNBC cells. In MDA-MB-231 and MDA-MB-435 xenograft models, SKLB646 exhibited considerable antitumor activity; it showed a little higher potency than dasatinib and paclitaxel at the doses used in this study.

Studies of mechanisms of action indicated that SKLB646 suppressed the tumor cell proliferation and induced apoptosis, which are mainly through the inhibition of SRC and MAPK signaling. SKLB646 also displayed considerable antiangiogenesis activity, which is due to the inhibition of VEGFR2. However, it is difficult to exactly differentiate how much of the in vivo antitumor efficacy of SKLB646 was resulted from the tumor cell killing effects, and how much from the antiangiogenic effects.

Our data in this investigation have clearly indicated that SKLB646 is able to efficiently inhibit SRC, Raf, and VEGFR2 both in vitro and in vivo. It is also possible that this compound could potently inhibit other kinases (off-target) due to the highly conservative structure of the kinases. The potential off-target effects of this compound might also contribute to the antitumor efficacy. To address this issue, further studies are necessary, including a kinase profiling assay against all the human kinase.

As mentioned above, a number of studies have demonstrated that SRC, Raf, and VEGFR2 could be potential targets for the treatment of TNBC (13, 15, 34, 35). The SRC/Raf inhibitor, SKLB646, indeed showed a nice selective inhibition of TNBC cell lines bearing an overexpression of SRC and mutant Raf, MD-MB-231, and MD-MB-435 (36, 37). Here, VEGFR2 is not considered because, in general, it is associated with the angiogenesis of solid tumors and has little effect on the tumor cell proliferation. For cell lines that weakly responded or did not respond to SKLB646, most of them (such as BT474, MCF-7, Jurkat, 22Rv1, MEF-1, L929 and Hela, see Supplementary Table S3) harbor a relative low expression of VEGFR2 and a wild type of Raf gene (38–40). HepG2 is one exception that bears an overexpression of SRC and mutant Raf (41, 42), but weakly responded to SKLB646. A possible reason could be that the survival of HepG2 is not dependent on the SRC and MAPK signaling. However, regarding whether SRC/Raf/VEGFR2 could represent relevant biomarkers for tumors that might respond to SKLB646, we cannot rush to a conclusion just from the cell line data in this study, and studies on a large number of clinical specimens are needed.

A significant risk of developing metastases in TNBC is one of main causes responsible for a poor prognosis (43). For example, TNBC has been demonstrated to be much more easy to metastasize to viscera (44), particularly to the lungs, than other subtypes of breast cancer (45). SKLB646 displayed considerable antimitastatic activities in vitro. In the TNBC cell-derived experimental lung metastasis model, SKLB646 prolonged the overall survival time of host mice and reduced the size of metastatic lung nodules. Studies of mechanisms of action indicated that SKLB646 effectively inhibited the expression and phosphorylation of Fra1, which has been thought as a key regulator of EMT; EMT is very important for metastatic behavior. Even so, the detailed mechanisms of antimitastasis of SKLB646 still need an in-depth study in future.

In conclusion, a comprehensive preclinical evaluation of SKLB646 in the treatment of TNBC has been carried out. SKLB646 is a novel multikinase inhibitor that poletantly inhibits SRC, Raf, and VEGFR2. It displayed potent antitumor activities both in vitro and in vivo. This compound also showed considerable ability to inhibit the migration and invasion of TNBC. In the TNBC cell–derived experimental lung metastasis model, SKLB646 evidently prolonged the overall survival time. Collectively, all the data provided here indicate that SKLB646 could be a promising lead compound for the treatment of TNBC and deserves further studies.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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