A Preclinical Evaluation of SKLB261, a Multikinase Inhibitor of EGFR/Src/VEGFR2, as a Therapeutic Agent against Pancreatic Cancer

Youli Pan¹, Mingwu Zheng¹, Lei Zhong¹, Jiao Yang¹, Shu Zhou¹, Ya Qin¹, Rong Xiang², Yuzong Chen³, and Sheng-Yong Yang¹

Abstract

The clinical prognosis of pancreatic cancer remains rather disappointing despite tremendous efforts in exploring medical treatments in the past two decades. Development of more effective treatment strategies is still desperately needed to improve outcomes in patients with pancreatic cancer. SKLB261 is a multikinase inhibitor obtained recently through a lead optimization. In this investigation, we shall evaluate its anti–pancreatic cancer effects both in vitro and in vivo. SKLB261 is a multikinase inhibitor potently inhibiting EGFR, Src, and VEGFR2 kinases. It could significantly inhibit cell proliferation, migration, and invasion, and induce apoptosis in cellular assays of human pancreatic cancer cells that are sensitive or resistant to dasatinib and/or gemcitabine. Western blot analysis showed that SKLB261 inhibited the activation of EGFR and Src kinases as well as their downstream signaling proteins, including FAK, ERK, and STAT3. SKLB261 also showed potent antiangiogenic effects in transgenic zebrafish models. In vivo, SKLB261 displayed more potent antitumor activities than dasatinib, gemcitabine, or erlotinib in pancreatic cancer xenografts, including BxPC-3, PANC-1, AsPC-1, and HPAC. Furthermore, mice receiving SKLB261 therapy showed significant survival advantage compared with vehicle-treated and gemcitabine-treated groups in an experimental metastasis model of pancreatic cancer. These data, together with the good pharmacokinetic properties and low toxicity of this compound, provide a rationale for the ongoing clinical evaluation of SKLB261 in the treatment of pancreatic cancer. Mol Cancer Ther; 14(2); 407–18. ©2014 AACR.

Introduction

Pancreatic cancer is one of the most intractable cancers (1) and remains the fourth leading cause of cancer-related deaths in the United States (2). Despite tremendous efforts in exploring medical treatments in the past two decades, the overall 5-year survival rate is still lower than 5% (3). Currently, gemcitabine, a deoxycytosine analogue, represents the standard of care for advanced pancreatic cancer but only prolongs patient survival for few weeks (4). Moreover, even with notable improvements in surgical techniques and chemotherapeutic approaches, nearly all early-stage patients who undergo resection also eventually succumb to recurrent and/or metastatic tumor growth (5–7). Therefore, development of more effective treatment strategies that can prevent tumor growth and metastasis is desperately needed to improve outcomes in patients with pancreatic cancer.

Recent advances in understanding of the critical molecular events that promote this disease may facilitate the development of effective therapeutics modalities. In 2008, detailed and global genomic analyses revealed that pancreatic cancers contain an average of 63 genetic alterations or abnormalities, defining a core set of 12 cellular signaling pathways and processes that are each genetically altered in 67% to 100% of the tumors (8). Further studies have also demonstrated that the interaction between pancreatic tumor and host stroma such as vascular endothelial cells, pericytes, fibroblasts, and immune cells adds to the complexity of altered cellular signaling pathways (9, 10). Thus, agents targeting multiple tumor-associated pathways have been expected to benefit the clinical therapeutic effect of pancreatic cancer.

One of the major molecular abnormalities implicated in pancreatic tumorigenesis is the overexpression or excessive activation of EGFR (11), occurring in 30% to 50% of pancreatic cancer cases (12). Recent evidence has also shown that EGFR is required and essential for KRAS-induced pancreatic tumorigenesis (13, 14). Moreover, aberrant EGFR activation is often correlated with poor clinical prognosis (15, 16). Hence, inactivating EGFR activity has been considered to be a promising strategy for clinically treating pancreatic tumors. Similarly, activation of the nonreceptor tyrosine kinase Src is found in about 70% of pancreatic cancers (17). The hyperactivation of Src kinase often leads to deregulation of tumor cell proliferation and survival, induction of tumor angiogenesis, disruption of cell to cell contacts, and promotion of invasiveness and metastasis (18, 19). In addition, prior reports have provided evidence that gemcitabine resistance correlates with increased Src activity, and Src inhibition could overcome
this resistance (20, 21). Recently, Src inhibition with small-molecule inhibitors has demonstrated significant antitumor and antimetastatic activity in preclinical pancreatic tumor models (22, 23). All the compelling evidence supports the Src kinase as an ideal target for therapeutic intervention in pancreatic cancer. In addition, angiogenesis is among the hallmarks of cancer (24, 25), which is essential for the progression of primary and metastatic solid tumors, including pancreatic cancer (26). Targeting tumor angiogenesis is a well-established approach for cancer treatment. Numerous antiangiogenic agents, including bevacizumab, an antivascular VEGF monoclonal antibody (27), and various tyrosine kinase inhibitors (28) have been tested clinically in patients with pancreatic cancer. It is reasonable to hypothesize that agents that can simultaneously attack these targets might have enhanced effects on the therapy of pancreatic cancer. SKLB261 (Fig. 1A) is an orally available multitarget inhibitor potently inhibiting EGFR, Src, and VEGFR2, which was obtained recently through a lead optimization process (29). In this account, we report the preclinical evaluation of SKLB261 as a therapeutic agent against pancreatic cancer. SKLB261 showed considerable antiviability activity across the pancreatic tumor cell lines tested in vitro, including those cell lines sensitive or resistant to dasatinib and/or gemcitabine. SKLB261 displayed more potent antitumor effects than dasatinib, gemcitabine, or erlotinib, in pancreatic cancer xenografts, including BxPC-3, PANC-1, AsPC-1, and HPAC. Further characterization of EGFR, Src, STAT3, and VEGFR2 showed that SKLB261 significantly blocked the activation of these kinases (30). In an experimental metastasis model, the transgenic zebrafish, which was fluorescent-labeled B16 tumor cells, were treated with SKLB261 or other agents for 24 hours, and an enhanced chemiluminescence system (Millipore) was used to detect the embryos. Specific proteins were detected using the enhanced chemiluminescence system (Millipore).

**Materials and Methods**

**Cell culture and reagents**

Human pancreatic cancer cell lines BxPC-3, PANC-1, AsPC-1, MIAPaCa-2, CFPAC-1, HPAC, Capan-1, and Capan-2 were all acquired from the ATCC in 2012. All tumor cell lines were maintained according to the ATCC procedures and considered as originally obtained from commercial sources. SKLB261 and other agents for 24 hours. Then, the proliferation cells were assayed with an EdU-Apollo DNA proliferation detection kit according to the manufacturer's instructions.

**Cell-cycle analysis**

Cells were seeded in 6-well plates. After 24 hours, the cells were treated with SKLB261 or other agents for 24 hours. Then, the cells were incubated with 50 μg/ml propidium iodide (PI) and 0.1% Triton X-100 for 30 minutes in the dark, and analyzed by flow cytometry (FCM).

**Apoptosis analysis by FCM**

The cells were treated with SKLB261 or other agents for 24 hours and harvested. The apoptotic cells were determined using the enhanced chemiluminescence system (Millipore).

**Western blot analysis**

Cell lysates were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore). All antibodies were purchased from Cell Signaling Technology. Specific proteins were detected using the enhanced chemiluminescence system (Millipore).

**Migration assay**

Migration assay was performed as described previously (31) and is described in detail in Supplementary Methods.

**Transwell invasion assay**

The cell invasion assay was performed and described previously (32) with some modifications and is described in detail in Supplementary Methods.

**Drug studies in zebrafish**

The transgenic zebrafish (FLK-1:EGFP) embryos were grown and maintained according to the protocols described in the work of Kimmel and colleagues (33). After 15 hours of fertilization, the embryos were treated with indicated concentrations of SKLB261 or other agents. After incubation overnight, a fluorescent image of each embryo was captured using Fluorescence Microscope (Carl Zeiss MicroImaging Inc.).

We then used a tumor xenograft model in zebrafish, which was reported previously (34), to further evaluate the antiangiogenic effect of SKLB261. Briefly, red fluorescence-labeled B16 tumor cells were implanted into 48 hours postfertilization (hpf) embryos through the perivitel line space. SKLB261 or other agents were added into the fish water at a final concentration of 2.5 μmol/L one day after implantation. On fifth day postimplantation, digital micrographs were taken using a fluorescence microscope.
Pharmacokinetic analyses
Blood from Sprague-Dawley rats dosed with SKLB261 was collected in EDTA-containing tubes and the plasma was isolated by centrifugation. Plasma concentrations of SKLB261 were determined by liquid chromatography tandem mass spectrometry.

Subcutaneous xenograft models
All animal experiments carried out were approved by the Animal Care and Use Committee of Sichuan University. Tumor xenograft models were established by subcutaneously injecting 100 μl tumor cell suspension (between 5 x 10^6 and 1 x 10^7) into the right flank of animals. Mice were randomized into groups of 6 to 7 before treatment at a point when tumors reached a volume of 0.1 to 0.4 cm^3. SKLB261, erlotinib, and sunitinib were dissolved in 25% (v/v) PEG400 plus 5% DMSO in deionized water. Dasatinib was suspended in a mixture of propylene glycol/water (50:50). Gemcitabine was dissolved in normal saline. Animals were given SKLB261 (12.5–50 mg/kg), dasatinib (50 mg/kg), erlotinib (100 mg/kg), sunitinib (40 mg/kg), or vehicle once daily by oral gavage. Gemcitabine was administered i.p. twice a week (125 mg/kg). Tumors were measured every 3 days using calipers, and the volume was calculated using the following formula: length x width^2 x 0.5.

Experimental metastasis assay
For the metastasis study, Panc-1 cells (1 x 10^6) were implanted via tail vein injection into 5- to 6-week-old female athymic nude mice that was immediately followed by randomization (n = 8 per group). Individual treatments, starting at the same day of cell injection, with SKLB261 (25–50 mg/kg, p.o.), dasatinib (50 mg/kg, p.o.), erlotinib (100 mg/kg, p.o.), sunitinib (40 mg/kg, p.o.), gemcitabine (125 mg/kg, i.p.), or vehicle control (p.o.) were performed. Survival was determined by observation of animals and tumor burdens in the lungs were examined by the use of hematoxylin and eosin (H&E) staining.

IHC
Paraffin-embedded sections of tumors were stained with H&E using standard methods. IHC staining was conducted using antibodies from Cell Signaling Technology or Abcam. CD31 (BD Bioscience) staining was conducted using frozen sections of tissue embedded in optimal temperature. Quantitative analysis of Ki-67, cleaved caspase-3, p-EGFR, p-Src, p-ERK, p-STAT3, and p-AKT-positive staining as well as vessel numbers was performed with NIH image analysis software, ImageJ. At least four independent regions were evaluated from at least three independent mice per group.

Statistical analysis
GraphPad Prism 5.01 software was used for statistical analysis. The statistical significance of results in all of experiment was determined by the Student t test and ANOVA. P value <0.05 was considered statistically significant.

Results
Enzymatic activities of SKLB261 against recombinant human protein kinases
Enzymatic activities of SKLB261 against a panel of recombinant human protein kinases were measured using the ‘gold standard’ radiometric kinase assay. SKLB261 is a multitarget inhibitor that potently inhibits EGFR and VEGFR2 with IC_{50} values of 0.004 and 0.02 μmol/L, respectively (Supplementary Table S1). It also showed considerable potency against several other onco-kinases, including ERBB2, ERBB4, KIT, Src, SVK, and p38 with moderate potencies (IC_{50} = 0.06, 0.03, 0.54, 0.29, 0.72, and 0.23 μmol/L, respectively), but only weak activity against ALK, MET, JAK1, JAK2, JAK3, JNK2, MNK1, MNK2, PAK, PKCα, PKCβ, PKN1, S6K, and TYK2 (IC_{50} = 2.3, 6.4, 3.3, 1.2, 2.6, 1.5, 6.7, 3.2, 1.1, 6.7, 3.6, 6.1, 9.4, and 6.7 μmol/L, respectively). SKLB261 displayed almost no inhibitory activity against 20 other protein kinases.

Antiproliferative activities of SKLB261 against pancreatic cancer cells in vitro
The in vitro antiproliferative activities of SKLB261 against various pancreatic cancer cell lines were examined by the MTT method. Ten human pancreatic cancer cell lines with varied genetic backgrounds, including BxPC-3, Panc-1, Capan-1, Capan-2, AsPC-1, HPAC, HPAF-II, SW1990, CFPAC-1, and MiaPaCa-2, were chosen in this assay. For comparison, the antiproliferative activities of four typical small-molecule agents, namely dasatinib (an oral multi-BCR/Abi and Src family tyrosine kinase inhibitor), gemcitabine, erlotinib (an oral EGFR tyrosine kinase inhibitor), and sunitinib (an oral PDGFR/VEGFR2/c-Kit kinase inhibitor), were also measured; the four agents are in either clinical use or clinical trials for the treatment of pancreatic cancer. Dose–response curves of these agents are presented in Fig. 1B or Supplementary Fig. S1A and IC_{50} values are given in Supplementary Table S2. SKLB261 showed considerable antiviability activity across the tumor cell lines tested. Among these cell lines, BxPC-3, Capan-1, Capan-2, AsPC-1, HPAC, and SW1990 are relatively sensitive to SKLB261 with IC_{50} values of 0.010, 0.033, 0.025, 0.066, 0.048, and 0.039 μmol/L, respectively. Notably, SKLB261 could efficiently kill the Panc-1 cells (IC_{50} = 0.302 μmol/L), which are resistant to other tested agents (IC_{50} > 10 μmol/L). It also showed very good potency against HPAF-II cells (IC_{50} = 0.330 μmol/L), which are resistant to gemcitabine and sunitinib (IC_{50} > 10 μmol/L).

Then, we conducted colony formation assays to further evaluate the antiproliferative activity of SKLB261. We first included in this assay two cell lines, namely BxPC-3 and Panc-1. For both cell lines, SKLB261 significantly inhibited their colony growth even at low concentrations (Fig. 1C). For BxPC-3 cells, in addition to SKLB261, dasatinib, gemcitabine, erlotinib, and sunitinib also showed considerable potency in suppressing the colony growth of BxPC-3. For Panc-1 cells, in addition to SKLB261, only gemcitabine showed high potency in blockade of colony formation. Moreover, we performed EdU assays to visually assess the proliferation of BxPC-3 and Panc-1 cells after treatment with different concentrations of SKLB261. In BxPC-3 cells, SKLB261 at 0.1 or 1 μmol/L exhibited a more potent ability than gemcitabine, erlotinib, and sunitinib in reducing EdU-positive cells (Fig. 1D). When Panc-1 cells were exposed to 1 μmol/L SKLB261 for 24 hours, the EdU-positive cells were greatly reduced relative to the control (Fig. 1D), indicating that DNA replication was blocked. In contrast, other reference compounds, including dasatinib, gemcitabine, erlotinib, and sunitinib, did not induce a significant decrease in the number of EdU-positive cells (Fig. 1D). Because BxPC-3 and Panc-1 cell lines have different mutation status in particular regarding K-ras and p53 (BxPC-3: WT K-ras/mutant...
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p53; PANC-1: mutant K-ras/mutant p53), it is necessary to examine whether the antiproliferative activities of SKLB261 are correlated with the p53 mutation or not. We thus used a third cell line with mutant K-ras/WT p53, namely HPAC, to repeat the above antiproliferation assays. As shown in Supplementary Fig. S1B and S1C, SKLB261 also significantly inhibited colony formation of HPAC tumor cells and reduced EdU-positive cells relative to the control. Taken together, these results suggest that SKLB261 could substantially inhibit pancreatic cancer proliferation in vitro.

Induction of G0–G1 phase arrest and apoptosis by SKLB261 treatment in pancreatic cancer cells in vitro

To examine the mechanisms responsible for SKLB261-mediated cell growth inhibition, we first performed a cell-cycle

Figure 2.
Induction of G0–G1 phase arrest and apoptosis by SKLB261 treatment in pancreatic cancer cells in vitro. A, cells were treated with indicated agents for 24 hours and the cell-cycle distribution was analyzed by FCM. Representative images for BxPC-3 cells treated with 0.1 μmol/L of agents or for PANC-1 cells treated with 1 μmol/L of agents are shown (left). The percentages of the cell cycle are presented (right). Data are shown as mean ± SD (n = 3). B, cells were treated with indicated agents for 24 hours and the level of apoptosis was assessed using the Annexin V–FITC/PI dual-labeling techniques. Representative images for BxPC-3 cells treated with 0.1 μmol/L of agents or for PANC-1 cells treated with 1 μmol/L of agents are shown (left). The apoptotic rate is shown (right). Data are mean ± SD (n = 3).

Figure 1.
Antiproliferative activities of SKLB261 against pancreatic cancer cells in vitro. A, chemical structure of SKLB261. B, cells were treated with indicated agents for 72 hours and cell viability was measured by MTT assay. Every experiment was carried out in triplicate. Points, mean value; bars, SD. C, cells were incubated with various concentrations of indicated agents for 18 days (BxPC-3) or 14 days (PANC-1). Then, cells were stained with crystal violet and quantified. Representative images for BxPC-3 cells treated with 0.1 μmol/L of agents or for PANC-1 cells treated with 1 μmol/L of agents are shown. Columns, mean (n = 3); bars, SD.

**P < 0.05; ***P < 0.001; ns, not significant, versus the vehicle control. C, cells were treated with indicated agents for 24 hours. Then, cells were lysed and the proteins were analyzed by Western blot analysis.
analysis. As shown in Fig. 2A, exposure of BxPC-3 cells to the growth suppressive concentrations of SKLB261 led to significant accumulation of cells in G0–G1 phase. For example, the percentage of cells in G0–G1 phase was increased to 70.66% (the corresponding value of control was 53.56%) after treatment with 0.1 μmol/L SKLB261 for 24 hours. Similar results were obtained after treatment with 0.1 μmol/L dasatinib. In contrast, gemcitabine, erlotinib, and sunitinib at the concentration of 0.1 μmol/L just had a slight effect on the percentage of cells in G0–G1 phase. In the PANC-1 cells, SKLB261 showed a more potent ability than dasatinib, gemcitabine, erlotinib, and sunitinib in arresting cells in G0–G1 phase (Fig. 2A). For example, SKLB261 treatment increased the cell numbers in G0–G1 from 42.58% (control) to 80.35% (1 μmol/L SKLB261 treated for 24 hours).

We next investigated whether SKLB261 treatment could induce apoptosis by the method of Annexin V/propidium idodide (PI) staining. Exposure of BxPC-3 and PANC-1 cells to SKLB261 for 24 hours led to significant increases of Annexin V+ populations in a concentration-dependent manner (Fig. 2B), indicating that SKLB261 significantly induced apoptosis in the two cell lines. Dasatinib, gemcitabine, erlotinib, and sunitinib also induced considerable apoptosis in BxPC-3 cells at a concentration of 0.1 μmol/L, but not in PANC-1 cells even at a concentration of 1 μmol/L.

Furthermore, the cell-cycle arrest and induction of apoptosis by SKLB261 treatment were confirmed by analyzing the expression levels of cyclin D1 and caspase-3. As expected, SKLB261 led to decreased levels of cyclin D1 and pro-caspase-3 and increased levels of cleaved caspase-3 in both BxPC-3 and PANC-1 cell lines (Fig. 2C), indicating cell-cycle arrest and induction of apoptosis. Similar results were obtained for gemcitabine, erlotinib, and dasatinib in BxPC-3 cells, but not in PANC-1 cells.

To further investigate the potential mechanisms underpinning the antiproliferative activity of SKLB261, we assessed its ability to inhibit important kinases and signal pathways involved in tumor cell survival, proliferation, migration, and invasion in intact BxPC-3 and PANC-1 cells by Western blot analysis. In BxPC-3 cells, SKLB261 inhibited phosphorylation of EGFR, Src, FAK, and ERK in a concentration-dependent manner, but did not significantly decrease the AKT phosphorylation even at a concentration of 10 μmol/L (Fig. 3A). Similar results were obtained for erlotinib and dasatinib. The lack of significant AKT phosphorylation probably reflects that AKT is not key mediators of EGFR and Src functions in BxPC-3 cells. In PANC-1 cells, SKLB261 dose dependently inhibited the phosphorylation of EGFR, Src, FAK, ERK, and AKT (Fig. 3B). In contrast, dasatinib did not inhibit the phosphorylation of EGFR, AKT, and ERK despite its significant inhibition of Src and FAK phosphorylation. Significantly, gemcitabine appeared to increase the phosphorylation of EGFR and ERK in both BxPC-3 and PANC-1 cells at a concentration of 10 μmol/L, which is consistent with a previous report (35). Of note, SKLB261 reduced the STAT3 phosphorylation in a concentration-dependent manner in both BxPC-3 cells and PANC-1 cells, which is reported to be one of the mechanisms of resistance to Src inhibition in pancreatic cancer cells (36).

To test the antiangiogenic effect of SKLB261, we used the FLK Transgenic zebrafish (FLK-1:EGFP), which provides a rapid and visual model for the evaluation of antiangiogenic action in vivo. First, the antiangiogenic effect was examined on zebrafish embryos. Treatment of live fish embryos with SKLB261 strongly inhibited the formation of intersegmental vessels in a dose-dependent manner.

**Figure 3.** Blockade of the activation of multiple downstream signaling pathways by SKLB261 treatment in pancreatic cancer cells. A, BxPC-3 cells were treated with SKLB261, gemcitabine, erlotinib, or dasatinib for 24 hours. Then, cells were lysed and the proteins were analyzed by Western blot analysis. B, PANC-1 cells were treated with SKLB261, gemcitabine, erlotinib, or dasatinib for 24 hours. Then, cells were lysed and the proteins were analyzed by Western blot analysis.
manner, with the intersegmental vessels almost completely blocked at the concentration of 2.5 μmol/L (Fig. 4A and B). No obvious effect was detected for other agents tested except sunitinib, which showed very similar effect as SKLB261 (Fig. 4A and B).

Next, we used a tumor xenograft model in zebrafish to further assess the antiangiogenic effect of SKLB261. In the control group, endothelial cells had effectively penetrated into the tumor tissue and efficiently orchestrated the primary vascular network 5 days after implantation into the zebrafish embryos (Fig. 4C), thus sustaining the tumor growth. Nevertheless, treatment with 2.5 μmol/L SKLB261 or sunitinib significantly inhibited the neovascularization and tumor growth (Fig. 4C and D). In contrast, no significant effect on the neovascularization or tumor growth was observed in the group treated with 2.5 μmol/L dasatinib, erlotinib, or gemcitabine when compared with the control group. Taken together, these experiments indicate that SKLB261 possesses a notable antiangiogenic activity.

Pharmacokinetic characteristics of SKLB261

To determine the pharmacokinetic (PK) characteristics of SKLB261 in vivo, SKLB261 was orally administrated to male Sprague–Dawley rats and the plasma concentration of SKLB261 was measured. The pharmacokinetic parameters of SKLB261 at a dose of 20 mg/kg are summarized in Supplementary Table S3. After oral administration at a single dose of 20 mg/kg, SKLB261 displayed a clearance of 9.72 L/hour/kg, with a T1/2 value of 4.86 hours. The steady-state volume (Vss) was much larger than the volume of total body water (0.67 L/kg), suggesting extravascular distribution. Furthermore, the absorption of SKLB261 was relatively quick and it reached the maximum concentration in the plasma (Cmax, 0.23 mg/L) at 1 to 3 hours. In addition, we did not know whether PK was linear up to 50 mg/kg p.o. which was the following highest dose used to evaluate the antitumor activity of SKLB261 in pancreatic tumor xenograft models because to obtain the absolute oral bioavailability of SKLB261 in male rats, SKLB261 was administrated both intravenously and p.o. at the same dose but SKLB261 at a dose of 50 mg/kg was not suitable for intravenous administration due to poor drug solubility at that dose, resulting in no design of an oral administration dose of 50 mg/kg in this pharmacokinetic experiment.

Antitumor activity and mechanisms of action of SKLB261 in vivo

To evaluate the antitumor activity of SKLB261 in vivo, we used four human pancreatic tumor xenograft models, including BxPC-3, PANC-1, AsPC-1, and HPAC models. Among them, BxPC-3, AsPC-1, and HPAC cells were sensitive to SKLB261 treatment in vitro (IC50 < 0.1 μmol/L), and PANC-1 cells exhibited resistance to both dasatinib and gemcitabine treatment in MIT assay (IC50 > 10 μmol/L) but retained moderate sensitivity to SKLB261 in vitro (IC50 = 0.3 μmol/L). In all the models, three dose groups, including 12.5, 25, and 50 mg/kg/d, were set for SKLB261 and one dose group for each of the other agents (50 mg/kg/d for dasatinib, 100 mg/kg/d for erlotinib, 125 mg/kg/twice a week for gemcitabine, and 40 mg/kg/d for sunitinib).

In all of the four models, SKLB261 showed significant antitumor activities in a dose-dependent manner (Fig. 5A and B and...
SKLB261 potently inhibited EGFR, Src, ERK 1/2, and STAT3 and PANC-1 tumor models. As depicted in Fig. 5C and D, groups, no significant weight loss was observed when compared with the control group (Supplementary Fig. S2B).

To elucidate the mechanisms of SKLB261-mediated antitumor efficacy in vivo, we conducted IHC analysis in the BxPC-3 and PANC-1 tumor models. As depicted in Fig. 5C and D, SKLB261 potently inhibited EGFR, Src, ERK 1/2, and STAT3 phosphorylation, and led to a substantial decrease in tumor cell proliferation (Ki-67–positive cells) and a significant increase in apoptosis (cleaved caspase-3–positive cells) when compared with the control group in the BxPC-3 tumor model. Gemcitabine seemed to increase EGFR and ERK 1/2 phosphorylation in vivo, which was consistent with the results obtained in vitro. Strikingly, no significant effect on AKT phosphorylation was observed after treatment with these five agents relative to vehicle-treated group (Fig. 5C and D). In the relatively resistant PANC-1 tumor model, tumor tissues from SKLB261-treated mice had significantly reduced EGFR, Src, ERK 1/2, STAT3, and AKT phosphorylation, and resulted in increased necrosis and apoptosis (cleaved caspase-3–positive cells) as well as reduced tumor cell proliferation (Ki-67–positive cells) compared with tumors from vehicle-treated mice (Supplementary Fig. S3A and S3B). Gemcitabine and sunitinib treatment did not significantly inhibit EGFR, Src, ERK, and AKT phosphorylation compared with the control, although they led to increased necrosis and reduced Ki-67–positive cells (Supplementary Fig. S3A and S3B). We also observed no notable induction of apoptosis and inhibition of ERK, STAT3, and AKT phosphorylation in tumors treated with dasatinib and erlotinib, although dasatinib significantly suppressed tumor cell proliferation (Supplementary Fig. S3A and S3B). Moreover, we observed that SKLB261-treated tumors showed significantly reduced microvessel density compared with vehicle-treated tumors in both BxPC-3 and PANC-1 tumor models (Supplementary Fig. S4), indicating that the antitumor activity of SKLB261 in pancreatic tumor models may be partly attributed to its inhibition of tumor angiogenesis. To further determine whether reduced microvessel density after 7 days of SKLB261 treatment was one of the cause of the antitumor activity and not a consequence due to its tumor growth inhibition, we have performed an in vivo dose response and kinetic analysis after oral administration of SKLB261 at a dose of 50 mg/kg in a representative BxPC-3 tumor xenograft model. As shown in Supplementary Fig. S5A and S5B, we found a reduction in microvessel density and a statistically significant decrease in microvessel density in the SKLB261-treated group compared with the control group after SKLB261 treatment for 3 and 5 days, respectively. However, at these time points, there was no statistically significant difference in tumor volume between the treatment group and the control group, thus indicating that the antiangiogenesis activity of SKLB261 could partly account for the antitumor efficacy. Taken together, these results showed that SKLB261 inhibited specific molecular targets in vivo, thus blocking proliferation and inducing apoptosis, and it also inhibited tumor angiogenesis in human pancreatic tumor xenograft models.

Antimetastatic activities of SKLB261 against pancreatic cancer cells in vitro and in vivo

We further assessed the effect of SKLB261 on the blockade of tumor metastasis. Here, BxPC-3, PANC-1, and HPAC were chosen as examples because they all possess metastatic ability (37, 38). First, wound-healing assays were used to evaluate the migration ability of BxPC-3, PANC-1, and HPAC cells. SKLB261 significantly inhibited the migration of these cells in a dose-dependent manner (Fig. 6A and B and Supplementary Fig. S6A and S6B), with the migration inhibition rate reaching about 92%, 85%, and 55% in the presence of 1 μmol/L SKLB261, respectively. For BxPC-3 cells, dasatinib showed the similar effect as SKLB261 and erlotinib was less potent than SKLB261 in inhibiting cell migration. For PANC-1 cells, dasatinib also displayed the similar effect as SKLB261 and sunitinib exhibited relatively weaker potency relative to SKLB261 at blocking PANC-1 cell migration. In contrast, both gemcitabine and erlotinib just had slight effect on the migration of PANC-1 cells even at higher concentrations (>1 μmol/L). For HPAC cells, only SKLB261 and dasatinib showed high potency in blockade of cell migration. Then, Transwell assays were performed to assess the invasion ability of tumor cells. For three cell lines, SKLB261 potently blocked the invasion of these cells. Of note, 1 μmol/L SKLB261 had the invasion inhibition rate of 90%, 70%, and 92% relative to the control for BxPC-3, PANC-1, and HPAC cells, respectively. Dasatinib showed the similar effect as SKLB261 and other agents, including gemcitabine, erlotinib, and sunitinib, were much less potent than SKLB261 in blocking the invasion ability of these cells (Fig. 6A and B and Supplementary Fig. S6A and S6B). These results showed that SKLB261 could efficiently inhibit the migration and invasion of tumor cells in vitro.

Finally, we examined the effect of SKLB261 on the metastasis-related survival by intravenous PANC-1 cell lung metastasis assays. As shown in Fig. 6C, metastasis-related mortality began at day 46 in the vehicle-treated mouse group, and all of the mice in the control group were dead at day 53. SKLB261 treatment significantly prolonged animal survival compared with the control group, which extended the median survival from 51.5 to 70 days at the dose of 50 mg/kg/d (Fig. 6C and Supplementary Figure S6C).
Mice administrated with sunitinib, dasatinib, or erlotinib, at the indicated dose also showed a considerable prolongation of survival compared with vehicle-treated mice (Fig. 6C and Supplementary Table S4). However, gemcitabine treatment in this study showed no survival benefit relative to the control group. In addition, examination of intact lungs from mice which died in every group by H&E staining confirmed that those mice developed macrometastasis in the lungs (Fig. 6D).

Table S4). Mice administrated with sunitinib, dasatinib, or erlotinib, at the indicated dose also showed a considerable prolongation of survival compared with vehicle-treated mice (Fig. 6C and Supplementary Table S4). However, gemcitabine treatment in this study showed no survival benefit relative to the control group. In addition, examination of intact lungs from mice which died in every group by H&E staining confirmed that those mice developed macrometastasis in the lungs (Fig. 6D).
Discussion

Pancreatic cancer is a biologically heterogeneous malignant disease with multiple genes or signaling pathways involved in the tumorigenesis and progression (8, 39, 40). Of these genes, EGFR and Src are among the most relevant genes that are associated with not only tumorigenesis and progression (8, 39, 40), but also drug resistance to conventional chemotherapy (20, 41). Besides, the progressive growth and metastasis of neoplasms, including pancreatic cancer, depend, in part, on angiogenesis (26). Thus, agents that simultaneously target EGFR and Src as well as angiogenesis could contribute to enhancing the therapeutic effects of pancreatic cancer. SKLB261 is such an agent that potently inhibits EGFR and Src as well as VEGFR2, an important regulator of angiogenesis. SKLB261 indeed showed significant anti-pancreatic cancer activities both in vitro and in vivo.

SKLB261 has at least two advantages in the treatment of pancreatic cancer. The first one is that it can not only kill the tumor cells, but also block the tumor angiogenesis. This could be used to interpret why SKLB261 has a comparable antiproliferative activity against BxPC-3, AsPC-1, and HPAC cells as gemcitabine and/or dasatinib in cellular assays, but has a superior in vitro antitumor potency in BxPC-3, AsPC-1, and HPAC xenograft models. The second one is the ability of overcoming drug resistance of SKLB261. For example, PANC-1 is a typical drug-resistant pancreatic cancer cell line, which showed an obvious resistance to gemcitabine, dasatinib, erlotinib, and sunitinib in cellular assays (IC_{50} > 10 \mu mol/L). Nevertheless, SKLB261 exhibited a considerable antiproliferative potency against PANC-1 cells with an IC_{50} value of 0.302 \mu mol/L. It also showed a very good antitumor activity in the PANC-1 xenograft model with a tumor inhibitory rate of 104% at a dose of 50 mg/kg, but the other tested agents just showed a poor or marginal antitumor activity.

Another attractive point of SKLB261 is its potency in inhibiting the activation of STAT3. STAT3 is a member of the STAT family of cytoplasmic transcription factors (42). It has been well established that STAT3 activation regulates oncogenic signaling in many tumor types and leads to increased cell survival, proliferation, and tumor growth (43–46). Furthermore, recent studies have also shown that activated STAT3 is a biomarker of resistance to Src inhibition with dasatinib treatment in resistant PANC-1 cells (32, 36). Consistent with these reports, we found that dasatinib did not inhibit the STAT3 phosphorylation in PANC-1 cells in vitro. Gemcitabine and erlotinib only showed a modest effect on phosphorylation of STAT3. However, SKLB261 potently inhibited STAT3 activation. This could be another important cause that contributed to the better performance of SKLB261 in the treatment of pancreatic cancer.

In conclusion, SKLB261 is a novel multitarget inhibitor that potently inhibits EGFR, Src, and VEGFR2. It showed significant activity in suppressing tumor cell proliferation both in vitro and in vivo, and inhibiting angiogenesis in zebrafish and tumor models. SKLB261 has the convenience of oral administration, and favorable pharmacokinetic properties. Taken together, the results of preclinical evaluation of SKLB261 support the use of SKLB261 as a promising candidate for clinical studies in patients with pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Pan, L. Zhong, R. Xiang
Development of methodology: Y. Pan, S. Zhou, R. Xiang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Pan, M. Zheng, L. Zhong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Pan
Writing, review, and/or revision of the manuscript: Y. Pan, M. Zheng, S.-Y. Yang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Pan, L. Zhong, J. Yang, S. Zhou, Y. Qin, Y. Chen
Study supervision: Y. Pan, Y. Qin, Y. Chen

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Youli Pan, Mingwu Zheng, Lei Zhong, et al.

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