SKLB1002, a Novel Potent Inhibitor of VEGF Receptor 2 Signaling, Inhibits Angiogenesis and Tumor Growth In Vivo

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Abstract

Purpose: VEGF receptor 2 (VEGFR2) inhibitors, as efficient antiangiogenesis agents, have been applied in the cancer treatment. However, currently most of these anticancer drugs suffer some adverse effects. Discovery of novel VEGFR2 inhibitors as anticancer drug candidates is still needed.

Experimental Design: In this investigation, we adopted a restricted de novo design method to design VEGFR2 inhibitors. We selected the most potent compound SKLB1002 and analyzed its inhibitory effects on human umbilical vein endothelial cells (HUVEC) in vitro. Tumor xenografts in zebrafish and athymic mice were used to examine the in vivo activity of SKLB1002.

Results: The use of the restricted de novo design method indeed led to a new potent VEGFR2 inhibitor, SKLB1002, which could significantly inhibit HUVEC proliferation, migration, invasion, and tube formation. Western blot analysis was conducted, which indicated that SKLB1002 inhibited VEGF-induced phosphorylation of VEGFR2 kinase and the downstream protein kinases including extracellular signal-regulated kinase, focal adhesion kinase, and Src. In vivo zebrafish model experiments showed that SKLB1002 remarkably blocked the formation of intersegmental vessels in zebrafish embryos. It was further found to inhibit a new microvasculature in zebrafish embryos induced by inoculated tumor cells. Finally, compared with the solvent control, administration of 100 mg/kg/d SKLB1002 reached more than 60% inhibition against human tumor xenografts in athymic mice. The antiangiogenic effect was indicated by CD31 immunohistochemical staining and alginate-encapsulated tumor cell assay.

Conclusions: Our findings suggest that SKLB1002 inhibits angiogenesis and may be a potential drug candidate in anticancer therapy. Clin Cancer Res; 17(13); 4439–50. ©2011 AACR.

Introduction

Angiogenesis is a complex process including endothelial cell proliferation, migration, basement membrane degeneration, and new tube formation. It is required for a variety of physiologic processes such as development and reproduction. However, angiogenesis also plays important roles in some disease states, typically cancer (1–4). The new blood vessels grow and infiltrate into the tumor, providing it with essential nutrients and oxygen, and a route for tumor metastasis (5, 6). Thus, antiangiogenesis has been thought as one of the most important anticancer therapies. Compared with chemotherapy directed at cancer cells, which often rapidly mutate and acquire "drug resistance" to treatment, the antiangiogenic therapy is obviously advantageous.

Numerous growth factors and cytokines are involved during tumor neovascularization. Of all the known angiogenic molecules, VEGF is the key mediator that promotes angiogenesis (7–9). VEGF exerts its biological effects by binding to and activating its receptors. Among them, VEGF receptor 2 (VEGFR2) is a major receptor transducing VEGF-induced signaling in endothelial cells. Activation of VEGFR2 leads to phosphorylation of specific downstream signal transduction mediators, including extracellular signal-regulated kinases (ERK), protein kinase C, focal adhesion kinase (FAK), phosphoinositide 3-kinase, and Akt. Signaling from VEGFR2 is necessary for the execution of VEGF-stimulated proliferation, migration, and sprouting of cultured endothelial cells in vitro and angiogenesis in tumor (10–13). Therefore, VEGFR2 has been recognized as the most important target for the antiangiogenesis therapy of...
Translational Relevance

Inhibition of VEGF signaling is a promising therapeutic approach for tumors by targeting tumor-induced angiogenesis. This may be accomplished by abrogating the kinase activity of VEGF receptor 2 (VEGFR2), which plays a critical role in mediating VEGF-induced signaling in endothelial cells. In the current study, a restricted de novo design method was used to construct new molecules that targeted VEGFR2, which indeed led to a new potent and specific VEGFR2 inhibitor, SKLB1002, which showed dose-dependent inhibitory activity in human umbilical vein endothelial cells and human tumor xenografts in athymic mice with limited toxicity. Moreover, the current study provided the potential technique for high-throughput antiangiogenic drug discovery, which was shown for the first time by using computer-aided drug design and tumor xenograft model in a whole vertebrate system.

cancer. Currently, there have been many reports of VEGFR2 inhibitors, including 2 marketed drugs, namely, vandetanib and sunitinib (14–16). However, most of these VEGFR2 inhibitors suffer some adverse effects in clinical use or clinical trials, which prompts that discovery of more VEGFR2 inhibitors as anticancer drugs is still needed at present.

In an effort to discover more potent VEGFR2 inhibitors, we adopted a restricted de novo design strategy for VEGFR2 inhibitors; a detailed description about this strategy can be found in our previous study (17). By using this strategy, we designed a series of inhibitors derived from quinazoline. SKLB1002 (Fig. 1A) was selected from these compounds and displayed potent and specific inhibition of VEGFR2 tyrosine kinase activity in vitro. The purpose here was to further examine its antiangiogenesis potency in vitro and in vivo. Our results clearly indicated that SKLB1002 inhibited VEGF-induced VEGFR2 phosphorylation and activation of various downstream signaling substrates that were responsible for endothelial cell viability. According to this molecular mechanism, SKLB1002 significantly inhibited endothelial cell proliferation, migration, invasion, and tube formation. By using xenograft models in zebrafish and athymic mouse, we determined that SKLB1002 prolonged tyrosine kinase inhibition and antagonized tumor angiogenesis but caused few toxic effects in the host. Taken together, our data suggest that SKLB1002 functions as a new potent and specific VEGFR2 inhibitor and suppresses angiogenesis during tumor growth.

Materials and Methods

Cell lines

Human colon cancer SW620 cells, hepatic cancer HepG2 cells, and melanoma B16-F10 cells were obtained from American Type Culture Collection. The normal human liver cell line L-02 was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). They were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins by a standard procedure, as previously described, and grown in EBM-2 medium with SingleQuots (Lonza) containing VEGF and other growth factors (18). HUVECs at passages 3 to 8 were used for all experiments.

General computational methods

A restricted de novo design method was used to construct new molecules. In this approach, quinazoline was taken as a general support-nog. Various fragments were allowed to grow at position 4 of quinazoline if their chemical structures and physicochemical properties conformed to the requirements of the hydrophobic pocket. Molecular docking experiments and scoring-ranking operations were carried out on the created molecules, which were suitable for assessing how the molecules fit the kinase active site. The de novo design and docking experiments were carried out by using AutoLudi and LigandFit in Discovery studio2.5, respectively. The scoring functions adopted here were Ludi Energy Estimate 1 and Chemscore.

Kinase inhibition assays

Kinase inhibition was measured by the use of radiometric assays conducted by Kinase Profiler service (Millipore). Briefly, in the presence or absence of SKLB1002, VGF2 (5–10 μM) was incubated in 25-μL reaction solution containing 8 mmol/L 3-(N-morpholino)propanesulfonic 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. After incubation for 40 minutes at room temperature, the reaction was stopped and 10 μL of the reaction solution was then spotted onto a P30 filtermat and washed 3 times for 5 minutes in 75 mmol/L phosphoric acid and once in methanol prior to scintillation counting.

Preparation of SKLB1002

For all in vitro assays and zebrafish studies, SKLB1002 was prepared initially as a 20 mmol/L stock solution in dimethylsulfoxide (DMSO). Stock solution was diluted in the relevant assay media, and 0.1% DMSO served as a vehicle control. For studies in athymic mice, SKLB1002 was suspended in 35% (v/v) polyethylene glycol solution containing 5% (v/v) DMSO and dosed at 0.1 mL/10 g of body weight.

Proliferation assay

Cell proliferation was measured using MTT assay as previously described (19). Various cells including HUVECs, L-02, B16-F10, HepG2, and SW620 were treated with indicated concentrations of SKLB1002 for 24 hours. Vandetanib and sunitinib (Sigma) served as positive controls. Each assay was replicated 3 times.
Wound healing assay

Monolayer HUVECs were wounded by scratching with pipette tips and washed with PBS. Fresh EGM2 medium containing vehicle or different concentrations of SKLB1002 was added to the scratched monolayers. Images were taken by an OLYMPUS digital camera after 24 hours. The migrated cells were quantified by manual counting, and the percentage of inhibition was expressed using untreated cells at 100%. Vandetanib and sunitinib served as positive controls.

Transwell invasion assay

Invasion assay was done as described previously (20). Briefly, the filter of the Transwell plate (Millipore) was coated with 50 μL Matrigel (BD Biosciences). After Matrigel polymerization, the bottom chambers were filled with EGM-2 medium containing various growth factors and the top chambers were seeded with 100 μL EBM-2 medium (without growth factors) and HUVECs (2 × 10^4 cells per well). The top chamber contained vehicle or various concentrations of SKLB1002. Cells were allowed to migrate for 24 hours. Nonmigrated cells were scraped with a cotton swab, and migrated cells were fixed with 100% methanol and stained with 0.05% crystal violet. The cells were quantified by manual counting and photographed under a light microscope. The percentage of migrated cells inhibited by SKLB1002 was expressed on the basis of vehicle control wells, and vandetanib and sunitinib served as positive controls.

Tube formation assay

The tube formation assay was conducted as described previously (21). After polymerization at 37°C for 30 minutes, HUVECs suspended in EBM-2 medium were seeded onto the Matrigel. They were then treated with SKLB1002, vandetanib, sunitinib, or vehicle. After 6 hours, cells were photographed with a digital camera attached to an inverted microscope.

Western blot analysis

To determine the effects of SKLB1002 on VEGFR2-dependent signaling cascade, subconfluent HUVECs were serum-starved overnight and incubated with SKLB1002 for 90 minutes, followed by 50 ng/mL VEGF_165 treatment for 10 minutes. Cells were lysed with buffer containing 1% Triton X-100, 1% deoxycholate, and proteinase inhibitor cocktail (Sigma). Protein concentrations were determined using a modified Lowry protein assay kit.
Drug studies in zebrafish

FLK-1 promoter EGFP transgenic zebrafish (FLK-1:EGFP) was used in all of our experiments. We used 30 embryos per experimental group in our study, and each experiment was carried out in 3 independent replicates. Embryos were maintained in Holtfreter's solution in a humidified incubator at 28°C (22). The bright and consistent fluorescence of blood vessels in the zebrafish embryos suggested that they could provide an ideal tool for testing antiangiogenesis drugs. Fifteen hours postfertilization (hpf), zebrafish embryos were incubated overnight with 2.5 μmol/L SKLB1002, vandetanib, sunitinib, or vehicle. At 30 hpf, zebrafish were anesthetized with 0.01% tricaine and imaged under a fluorescence microscope (Carl Zeiss Micro imaging Inc.) equipped with a AxioCam MRc5 digital CCD camera (Carl Zeiss Microimaging Inc.).

Histone H3 phosphorylation may initiate at different phases of the cell division in different organisms during both mitosis and meiosis (23). In this study, normal cell proliferation in the zebrafish embryos was observed using an antibody to phosphorylated histone-3 (Millipore). Briefly, zebrafish embryos at 15 hpf were incubated overnight with 2.5 μmol/L SKLB1002 or vehicle. At 24 hpf, zebrafish embryos were fixed in 4% formaldehyde solution for 4 hours at 4°C and permeabilized in aceton at −20°C for 10 minutes. Subsequently, zebrafish embryos were processed for immunofluorescence by using anti-phosphorylated histone-3 antibody (1:1,000) and TRITC-conjugated antibodies (red label; 1:2,000). In this way, the initiation of intersegmental vessels sprouting and normal cell proliferation in the zebrafish embryos could be simultaneously observed using a fluorescence microscope.

We then investigated that the implantation of murine tumor cells B16-F10 could trigger an angiogenic response in zebrafish embryos. It was a suitable model for us to study the antiangiogenic effect of SKLB1002. We used red fluorescent dye CM-DiI (Invitrogen Corporation)-labeled tumor cells for easy observation in our zebrafish/tumor xenograft model, which was inspected with a fluorescence microscope provided with a multidimensional acquisition software (Carl Zeiss Microimaging Inc.). Briefly, 48 hpf zebrafish embryos were anesthetized with 0.01% of tricaine and injected with 300 murine B16-F10 melanoma cells per embryo. Cells were resuspended in Hank’s balanced salt medium and directly injected into zebrafish perivitelline space by using an air-driven Cell Tram microinjector (Medical System Corp.). Compounds were added into the incubating water at a concentration of 2.5 μmol/L. 1 day after injection of cells. At 5-day postinoculation (dpi), digital micrographs were taken using a fluorescence microscope as described above.

Xenograft mouse model

Animal studies were conducted in conformity with institutional guide for the care and use of laboratory animals. All mouse protocols were approved by the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China). Six-week-old female athymic (nu/nu) mice were obtained from Chinese Academy of Medical Science (Beijing, China). SW620 and HepG2 tumors were established by s.c. injection of 5 × 10⁶ cells. After 10 days, mice bearing tumors around 100 mm³ were selected and randomized into treatment groups (6 mice per group). The dosing schedules were SKLB1002 100 mg/kg/d, 50 mg/kg/d, or vehicle once a day intraperitoneally. Tumor length and width were determined every 3 days and tumor volume (TV) was calculated using the following formula: TV = length × width² × 0.52. At the end of experiment, mice were sacrificed. Solid tumors were removed and processed for immunohistochemical analysis and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay.

Immunohistochemistry and alginate-encapsulated tumor cell assay

To investigate whether SKLB1002 inhibited tumor growth by suppressing tumor angiogenesis, detection of vessel density in tumor tissue was done as described previously (24). Frozen sections of SW620 tumor xenografts were used to determine vessel density with an anti-CD31 antibody (BD Biosciences).

An alginate-encapsulated assay was conducted as described (25). Briefly, alginate beads containing 5 × 10⁵ tumor cells per bead were formed and implanted s.c. into both dorsal sides of the athymic mice. Then mice were treated with SKLB1002 at 100 mg/kg, 50 mg/kg, or vehicle once a day intraperitoneally for 12 days. At the end of experiment, 0.1 mL of 2% fluorescein isothiocyanate (FITC)–dextran solution (Sigma) was injected i.v. into the lateral tail vein of mice. Alginate beads were removed and photographed within 20 minutes after being exposed surgically. The uptake of FITC–dextran was measured as described (25).

In situ TUNEL

Cell apoptosis in SW620 xenograft tumors was determined using a TUNEL assay following the manufacturer’s instructions (Promega). Three tumors per group were analyzed. The number of TUNEL-positive cells was quantified.
by fluorescence microscopy, and the apoptotic index in 6 random fields per group was counted.

**Toxicity evaluation**
To investigate potential side effects or toxicity on mice during the treatment, they were observed continuously for relevant indexes such as weight loss, diarrhea, anorexia, skin ulceration, and toxic deaths. The tissues of heart, liver, spleen, lung, and kidney were stained with hematoxylin and eosin (H&E).

**Statistical analysis**
SPSS 11.5 was used for statistical analysis. Data were analyzed statistically by using 1-way ANOVA followed by
the Tukey test. Differences were considered significant if $P < 0.05$.

### Results

#### Design, synthesis, and evaluation of SKLB1002

Seventy-five novel molecules were designed with the use of the proposed restricted de novo design approach. A total of 15 molecules, which were ranked at the top 20% of the created molecules according to values of the Ludi energy estimate 1, were chemically synthesized, followed by in vitro kinase inhibition assays (Supplementary Table S1). Among all the tested compounds, 6,7-dimethoxy-4-(5-methyl-1,3,4-thiadiazol-2-ylthio)quinazoline (SKLB1002) was found to be the most potent one. SKLB1002 showed potent inhibition of VEGFR2 kinase activity ($IC_{50} = 32\text{ nmol/L}$), which was similar with the positive drugs vandetanib ($IC_{50} = 40\text{ nmol/L}$) and sunitinib ($IC_{50} = 9\text{ nmol/L}$).

Figure 1 presents a possible binding mode of SKLB1002 in the VEGFR2 active site. The quinazoline ring locates at the front of the ATP binding pocket, where it is sandwiched between 2 loops in the N- and C-lobes of the catalytic domain. As anticipated, the quinazoline ring of SKLB1002 is oriented with its N1 atom hydrogen bonded to the Cys919 residue in the hinge region of VEGFR2. The N4 atom in thiadiazole moiety of SKLB1002 forms another interaction with gatekeeper residues: an N (thiadiazole)--O--H (Thr916) hydrogen bonding interaction. Furthermore, the quinazoline ring forms an important interaction with hinge region residues: an aromatic-aromatic face-to-face $\pi--\pi$ interaction with the benzene ring in the side chain of Phe918. SKLB1002 also forms hydrophobic interaction with residues Leu840, Ala866, Phe918, Gly922, Leu1035, and Phe1047 in ATP binding pocket of VEGFR2.

SKLB1002 shows strikingly lower cytotoxicity than vandetanib and sunitinib

L-02 cell strain was established from normal human liver and considered to have extensive application in cytotoxicity assay (26). To determine the cytotoxicity of SKLB1002 on normal human cells, we conducted MTT assay in L-02 cells after administration with indicated concentrations of compounds. As shown in Supplementary Figure S2B, SKLB1002 effectively induces cell growth inhibition of L-02 cells at concentrations over 150 $\mu$mol/L. In contrast, vandetanib and sunitinib sufficiently inhibited L-02 cells only at concentrations of 40.9 and 30.1 $\mu$mol/L, respectively.

SKLB1002 inhibits HUVEC proliferation, migration, invasion, and tube formation

To assess the antiangiogenic activity of SKLB1002 in vitro, its inhibitory effects on VEGF-induced proliferation of endothelial cells were first evaluated by MTT assay. SKLB1002 significantly inhibited endothelial cell proliferation with an $IC_{50}$ value of 11.9 $\mu$mol/L. However, vandetanib and sunitinib inhibited cell viability at a much lower concentration with an $IC_{50}$ value of 3.8 $\mu$mol/L and 1.9 $\mu$mol/L, respectively (Supplementary Fig. S2A).

Cell migration and invasion are essential for endothelial cells in angiogenesis. We carried out wound
healing assays to investigate the effects of SKLB1002 on cell migration and found that 2.5 μmol/L SKLB1002 obviously inhibited the migration of HUVECs (Fig. 2A). We also conducted Transwell invasion assays to evaluate the ability of HUVECs to pass through the Matrigel and membrane barrier of the Transwell in the presence of vehicle or various concentrations of SKLB1002. Data showed that SKLB1002 significantly inhibited the invasion properties of endothelial cells in a dose-dependent manner (Fig. 2B).

Although angiogenesis is a complex process of several kinds of cells, tube formation of endothelial cells is one of the key steps. HUVECs plated on the surface of Matrigel formed capillary-like structures in the vehicle group within 6 hours. However, treatment with SKLB1002 dose dependently inhibited the tube formation (Fig. 2C).

As shown in Figure 2 and Supplementary Figure S2, we indicated that SKLB1002 had similar antiangiogenic efficacy in HUVEC migration, invasion, and tube formation but strikingly lower cytotoxicity in comparison with the positive drugs vandetanib and sunitinib.

SKLB1002 inhibits VEGFR2 signaling pathway

VEGFR2 phosphorylation leads to the activation of various downstream signaling substrates that are responsible for endothelial cell migration and tube formation. To investigate whether SKLB1002 inhibited VEGFR2 and its downstream signaling, we screened some essential kinases involved in VEGFR2 signaling pathway. As shown in Figure 3A, 10 μmol/L SKLB1002 significantly suppressed the phosphorylation of VEGFR2, ERK, FAK, and Src induced by VEGF, which suggested that SKLB1002 exerted its antiangiogenic function by directly targeting VEGFR2 on the surface of endothelial cells and further antagonizing VEGFR2-mediated downstream signaling cascade. Consistent with previous reports (27, 28), SW620 expressed moderate VEGFR2, which was not sufficient to lead to the activation of various downstream signaling substrates that were responsible for cell proliferation.

**SKLB1002 inhibits embryonic angiogenesis in zebrafish**

Fifteen-hpf zebrafish embryos were incubated overnight with SKLB1002, vandetanib, sunitinib, or vehicle, zebrafish were anesthetized with 0.01% tricaine, and a digital image of each embryo was captured using the fluorescence microscope. Intersegmental blood vessel growth was greatly inhibited in the 2.5 μmol/L SKLB1002 group compared with vehicle treated and untreated embryos (Fig. 4A).
We also observed a slight decrease in body length of zebrafish embryos, as the inhibition of developmental angiogenesis had moderate effects on the growth of the embryo.

To evaluate whether SKLB1002 affected proliferation of normal cells in the zebrafish embryos, we used the antibody of phosphorylated histone-3 to observe normal cell proliferation in the zebrafish embryos treated with SKLB1002 or vehicle, respectively. Nine hours after drug administration, zebrafish embryos were processed for immunofluorescence. We found that SKLB1002 strongly antagonized the initiation of intersegmental vessels sprouting with no or least impact on normal cell proliferation in the zebrafish embryos (Supplementary Fig. S4A).

**SKLB1002 inhibits tumor-induced angiogenesis in zebrafish**

Red fluorescence-labeled B16-F10 melanoma cells were resuspended in Hank’s balanced salt medium and micro-injected in zebrafish embryos at 48 hpf through the perivitelline space between the periderm and the yolk. By 5 days after the implantation in zebrafish, endothelial cells had filled the inner space of the xenografted tumor in control group and built up its primary vascular network. However, at the same time, minimal angiogenic effect was observed in the embryos treated with 2.5 μmol/L SKLB1002, vandetanib, or sunitinib (Fig. 4B).

**SKLB1002 inhibits tumor growth in vivo**

We used 2 xenograft tumor models to investigate the effect of SKLB1002 on tumor growth. Athymic mice bearing SW620 or HepG2 xenografts were treated daily for 3 weeks with SKLB1002. As shown in Figure 5, we found that 100 mg/kg SKLB1002 significantly suppressed TV and caused more than 60% inhibition of tumor growth compared with vehicle-treated mice.

To evaluate the health status of mice treated with SKLB1002, weight of mice was monitored once every 3 days throughout the whole experiment. As shown in Supplementary Figure S5, no significant differences in weights were found among the 3 groups. No adverse effects in other gross measures such as skin ulcerations or toxic death were observed in SKLB1002 group. Furthermore, toxic pathologic changes in liver, lungs, kidneys, spleen, and heart were not detected by microscopic examination (Fig. 5E). These data determined that the inhibition of tumor growth was not attributable to systemic toxicity.

**SKLB1002 inhibits tumor angiogenesis in vivo**

Immunohistochemical anti-CD31 staining of the tumor tissue from SKLB1002-treated mice showed significantly decreased microvessel density compared with vehicle groups (Fig. 6B). In addition, inhibition of angiogenesis could also be detected in the alginate-encapsulated tumor cell assay. New blood vessels in alginate beads from SKLB1002-treated mice were apparently...
sparse. Besides, FITC–dextran uptake of mice treated with SKLB1002 was significantly decreased when compared with control groups (Fig. 6A). These results suggested that tumor angiogenesis was inhibited in mice treated with SKLB1002, which participated in suppression of tumor growth.

Reduced neovascular growth induces more apoptosis in vivo

We next analyzed the effect of SKLB1002 on apoptosis in the SW620 xenograft tumors by TUNEL staining. TUNEL-positive cells were counted only in regions of intact tumor in such a way that the central necrosis typically observed in xenografts did not interfere with quantification of apoptotic cells. Representative fields from each group were shown, which clearly indicated the higher rate of apoptosis in mice treated with SKLB1002. The number of apoptotic cells in 6 random fields from 3 different tumors in each group was counted, and the apoptotic index is shown in Figure 6C.

Discussion

We have designed and identified a new small molecule, SKLB1002, as a potent inhibitor of VEGFR2. The compound is an ATP-competitive inhibitor of VEGFR2 with IC₅₀ of 32 nmol/L. Our work indicated inhibitory effects of SKLB1002 on HUVEC migration, invasion, and tube formation in a concentration-dependent manner. Notably, the inhibition of VEGF signaling by SKLB1002 occurs at concentrations below those that show significant direct effects on the normal growth of endothelial cell, as the compound exhibited an IC₅₀ of 11.9 μmol/L against VEGF-induced HUVECs in the MTT assay. We showed that 2.5 μM SKLB1002 was sufficient to obviously block capillary-like structure formation and migration in vitro.

VEGFR2 signaling is essential for the functions of vascular endothelial cells. Tyr1175 is the major autophosphorylation site within VEGFR2, and its phosphorylation initiates the downstream signaling events to endothelial cells (29). Phosphorylated Tyr1175 of VEGFR2 mediates...
activation of the MAPK/ERK cascade and proliferation of endothelial cells. It has also been linked to VEGF-induced activation of Src, which regulates vascular permeability and cell migration (30–31). Other signaling molecules that have been indicated in VEGF-induced migration through VEGFR2 include FAK and its substrate paxillin, which are involved in focal adhesion turnover during cell migration (32–34). In our study, by directly blocking VEGFR2 phosphorylation, SKLB1002 subsequently blocked the activation of ERK, FAK, and Src signaling pathway and inhibited cellular activities.

Figure 6. SKLB1002 inhibited tumor angiogenesis and induced tumor apoptosis in vivo. A, vascularization of alginate implants. Alginate beads containing 5 × 10⁴ tumor cells per bead were implanted s.c. into the backs of mice. Mice were then treated with vehicle control or SKLB1002 once a day intraperitoneally for 12 days. Beads were surgically removed 12 days later, and FITC–dextran was quantified. FITC–dextran uptake of beads from SKLB1002-treated mice showed a significant decrease compared with the vehicle group (n = 6; ANOVA; **, P < 0.01). B, SKLB1002 significantly inhibited tumor vessels in SW620 tumor xenografts. Frozen sections of SW620 tumor tissue were tested by immunohistochemical analysis with anti-CD31 antibody. Representative tumor vasculature from vehicle- or SKLB1002-treated mice was shown. The density of microvessel was calculated in each group (n = 5; ANOVA; **, P < 0.01). C, apoptosis was measured on paraffin-embedded SW620 tumor sections by TUNEL staining. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells. The treatment with SKLB1002 resulted in significantly increased apoptosis in a dose-dependent manner versus vehicle control (n = 6; ANOVA; **, P < 0.01).

Zebrafish are beginning to be used in the drug discovery process and can be a useful and cost-effective alternative to some expensive, labor-intensive mammalian models (36). Pathologic angiogenesis has been considered as an important therapeutic target in several major diseases. Zebrafish offer a potential tool for antiangiogenic drug development in a whole vertebrate system (37–39). In zebrafish, formation of intersegmental vessels is considered to represent capillary sprouting during mammalian development whereas the axial vessels correspond to arteries and veins. Disruption of VEGFR2 signaling impairs intersegmental sprouting angiogenesis (40). In this assay, we used transgenic zebrafish with fluorescent blood vessels to facilitate image analysis and found that angiogenic intersegmental blood vessel growth was inhibited in zebrafish embryos treated with 2.5 μmol/L SKLB1002. Importantly, by detecting cells during proliferation in zebrafish embryos treated by SKLB1002 or vehicle, we determined that SKLB1002 did not affect normal cell proliferation and its antiangiogenic activity was not due to broad cytotoxic effects. Recently, some studies have reported that the implantation of murine or human tumor cells could elicit an angiogenic response in zebrafish embryos. The tumor xenograft model in the zebrafish is suitable both for studying the effect of antiangiogenic compounds and for the identification of genes involved in tumor angiogenesis (41, 42). In this study, we used murine B16-F10 melanoma cells to build
tumor xenograft model in zebrafish embryos and found that B16-F10 cell induced the rapid formation of tumor microvasculature. We found low concentration of SKLB1002 could remarkably suppress the tumor angiogenesis in the xenografted tumor mass. Furthermore, in combination with computer-aided drug design, tumor xenograft model in zebrafish offers the potential for high-throughput antiangiogenic drug discovery in a whole vertebrate system. Briefly, this model can be used to screen compound library built by the restricted de novo design strategy for antiangiogenic compounds.

SW620 is a human carcinoma tumor cell line and highly tumorigenic in athymic mice (43). In our established SW620 tumor xenograft models in athymic mice, tumor growth was significantly inhibited by SKLB1002 administration (100 mg/kg/d) with an inhibitory rate of 67%. Inhibition of angiogenesis was observed both in mouse tumor tissue and in analginate-encapsulated tumor cell assay. Similar to results of a previous report (44), owing to reduced neovascular growth, more apoptotic cells could be visualized in the tumor tissues treated with SKLB1002 than in control group by in situ TUNEL assay.

Unlike those widely used anticancer drugs that have adverse effects or severe cytotoxicity to induce cell apoptosis in modern cancer chemotherapy, various small molecule VEGFR tyrosine kinase inhibitors have been identified and developed to block tumor angiogenesis and metastasis formation (4, 45). Initially, these agents were expected to be active without causing toxicities or resistance because of the genetic stability of endothelial cells. However, contrary to initial expectations, VEGFR2 inhibitors could cause major side effects in recent clinical experiences (46–48). The possible causes of side effects induced by VEGFR2 inhibitors are numerous, but the main cause is their poor target selectivity (49, 50). Thus, understanding the molecular mechanisms involved in the toxicity of angiogenesis inhibition should allow more specific and more potent inhibitors to be developed. Following the proposed strategy of designing specific kinase inhibitors, the restricted de novo design method was adopted to construct new molecules that targeted VEGFR2, which led to the identification of SKLB1002. The high selectivity of SKLB1002 was evidenced by the lack of activity against a variety of other kinases tested (Supplementary Table S2). SKLB1002 (100 mg/kg/d) did not affect the body weight of the mice but showed significant inhibitory effects on solid tumor growth and tumor angiogenesis. No adverse effects in other gross measures such as diarrhea, anorexia, skin ulceration, bleeding, and toxic deaths were observed in SKLB1002-treated group. Furthermore, toxic pathologic changes in liver, lungs, kidneys, spleen, and heart were not found by microscopic examination. Thus, we assume that SKLB1002 may be a novel anticancer agent with limited toxicity.

Taken together, our studies indicate that SKLB1002 is a potent and specific inhibitor of tumor angiogenesis by targeting the VEGFR2 signaling pathways. Further structural modifications of SKLB1002 are still underway. SKLB1002 and its derivates are promising candidates as antiangiogenic and anticancer drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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