SKLB610: A Novel Potential Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases Inhibits Angiogenesis and Tumor Growth *in Vivo*

Zhi-Xing Cao^{a,*}, Ren-Lin Zheng^{a,*}, Hong-Jun Lin^{a,*}, Shi-Dong Luo^a, Yan Zhou^b, You-Zhi Xu^a, Xiu-Xiu Zeng^a, Zhao Wang^a, Li-Na Zhou^a, Yong-qiu Mao^a, Li Yang^a, Yu-Quan Wei^a, Luo-Ting Yu^a, Sheng-Yong Yang^a and Ying-Lan Zhao^a

^aState Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, ^bDepartment of Geriatric, West China Hospital, West China Medical School, Sichuan University, Chengdu, *These authors contributed equally to this work

Key Words

SKLB610 • Vascular endothelial growth factor receptor • Angiogenesis • Tumor growth

Abstract

Antagonizing angiogenesis-related receptor tyrosine kinase is a promising therapeutic strategy in oncology. In present study, we designed and synthesized a novel vascular endothelial growth factor receptor (VEGFR) inhibitor N-methyl-4-(4-(3-(trifluoromethyl) benzamido) phenoxy) picolinamide SKLB610 that potently suppresses human tumor angiogenesis. SKLB610 inhibited angiogenesis-related tyrosine kinase VEGFR2, fibroblast growth factor receptor 2 (FGFR2) and platelet-derived growth factor receptor (PDGFR) at rate of 97%, 65% and 55%, respectively, at concentration of 10 μ M in biochemical kinase assays. *In vitro*, SKLB610 showed more selective inhibition of VEGF-stimulated human umbilical vein endothelial cells (HUVECs) proliferation, and this proliferation inhibitory effect was associated with decreased phosphorylation of VEGFR2 and p42/44 mitogen-activated protein kinase (p42/44 MAPK). Antiangiogenic evaluation showed that SKLB610 inhibited the HUVECs capillary-tube formation on

Matrigel *in vitro* and the sub-intestinal vein formation of zebrafish *in vivo*. Moreover, SKLB610 inhibited a panel of human cancer cells proliferation in a concentration-dependent manner and human non-small cell lung cancer cell line A549 and human colorectal cancer cell line HCT116 were most sensitive to SKLB610 treatment. *In vivo*, chronic intraperitoneally administration of SKLB610 at dose of 50mg/kg/d resulted in significant inhibition in the growth of established human A549 and HCT116 tumor xenografts in nude mice without exhibit toxicity. Histological analysis showed significant reductions in intratumoral microvessel density (CD31 staining) of 43–55% relative to controls depending on the specific tumor xenografts. In conclusion, the present study demonstrated that SKLB610 exhibited its antitumor activity as a multi-targeted inhibitor with more potent inhibition of VEGFR2 activity. Its potential to be a candidate of anticancer agent is worth being further investigated.

Copyright © 2011 S. Karger AG, Basel

Introduction

Angiogenesis, the formation of new vessels from an existing vascular network, play an important role in

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
1015-8987/11/0275-0565\$38.00/0

Accessible online at:
www.karger.com/cpb

Ying-Lan Zhao

State Key Laboratory of Biotherapy and Cancer Center, West China Hospital
West China Medical School, Sichuan University, #1 Keyuan Road 4,
Gaopeng Street, High Technological Development Zone, Chengdu 610041 (China)
Tel. +86-28-85164063, Fax +86-28-85164060, E-Mail alancenxb@sina.com

growth and metastatic dissemination of most solid tumors in human [1-3]. Tumor-associated vasculature are a hallmark of a variety of human and rodent tumors *in vivo* and correlated with tumor growth rate, tumor metastatic potential and poorer patient prognosis in a variety of malignancies [4-7].

Numerous growth factors and cytokines were involved in neovascularization as well as enhanced vascular permeability. Among them, the vascular endothelial growth factor (VEGF) family (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E) and their corresponding receptors (VEGFR1, VEGFR2 and VEGFR3) appears to play a predominant and indispensable role in regulating the angiogenic and lymphangiogenic processes, as well as the induction of vascular permeability and inflammation [3, 8-10]. Gene knock-out experiments for VEGF [11] and its receptors [12] have highlighted the pivotal role of the VEGF/VEGFR system in the development of the embryonic vascular system and block VEGF/VEGFR system using anti-VEGF siRNA obviously suppressed angiogenesis and tumor growth *in vivo* [13]. Moreover, various different studies demonstrated the importance of VEGF receptors in the various solid tumor growths by interfering with the VEGF/VEGFR system in adult animals [14-17]. In all of VEGFRs VEGFR2 is the major stimulator which exerts their mitogenic, chemotactic and permeabilizing effects on the host endothelial cells and vasculature through VEGFs [18-19]. Its expression is low in normal tissues and up-regulated on vascular endothelial cells during embryonic and tumor angiogenesis. In tumor cells, VEGFR2 is strongly autophosphorylated by increased expression of VEGFs and mediates a serial of mitogenic and survival downstream responses including mitogen-activated protein kinase (MAPK) pathway PI3K/Akt pathway survival signaling pathway and so on [20].

From above improved understanding of angiogenesis mechanisms in tumorigenesis, the anti-angiogenesis therapies via the VEGF/VEGFR kinase axes become a promising and well-validated therapeutic approach for suppression of pathological neovascularization and, in particular, for inhibiting tumor growth [21-24]. Small molecule inhibitors of VEGFR2 kinases, such as vandetanib, sorafenib and sunitinib were demonstrated to be able to inhibit tumor growth in animal and human [25-27]. Moreover, there are more small molecule inhibitors of VEGFR2, such as ABT-869, AG-013736, PTK787 and AZD2171 are currently under clinical evaluation [28-31].

Our aim was to develop a small molecular compound that potently and selectively blocks the VEGF/VEGFR

Fig. 1. Chemical structure of SKLB-610

system, suitable for the chronic therapy of VEGF-dependent pathological neovascularization. The present study described a small-molecule compound SKLB610, a novel and fully synthetic multi-targeted inhibitor with more potent inhibition of VEGFR2 activity, which has potential antitumor and antiangiogenesis activity. This compound inhibited angiogenesis both *in vitro* and *in vivo*. SKLB610 showed dose-dependent inhibitory activity in human tumor xenografts in mice and it was well tolerated. Taken together, our data suggest that SKLB610 functions as a novel multi-target inhibitor and reduction of tumor growth and angiogenesis.

Materials and Methods

Preparation of SKLB610

SKLB610, N-methyl-4-(4-(3-(trifluoromethyl)benzamido)phenoxy)picolinamide, was synthesized in the State Key Laboratory of Biotherapy, Sichuan University (Sichuan, China) and its structural formula was shown in Fig. 1. For all *in vitro* assays, SKLB610 was prepared initially as a 40 mM stock solution in DMSO and diluted in the relevant assay media. For *in vivo* studies, SKLB610 was dissolved in 25% (v/v) aqueous Cremophor EL/ethanol (50:50; Sigma Cremophor EL, 100% ethyl alcohol) and administered at a dose of 12.5, 25 and 50mg/kg, respectively.

Materials

Cell count kit-8 (CCK-8) was purchased from Tokyo, Japan. Dimethyl sulfoxide (DMSO), Cremophor E1 and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). The primary antibodies for AKT/p-AKT, VEGFR2, p44/42 MAPK/p-p44/42 MAPK were purchased from Cell Signaling Technology (Beverly, MA); antibodies for p-VEGFR2 were from Santa Cruz Biotechnology Co. (Santa Cruz, CA). Human umbilical cord was supported by Department of Gynecology and Obstetrics, West China Second Hospital, Sichuan University (China, Sichuan). All the chemicals employed in this study were of analytic pure and were of culture grade.

Kinase assay

The inhibitory ability of SKLB610 on kinase activity was determined using labeled γ - P^{33} ATP. Peptide as kinase substrates were monitored by labeled phosphate. Briefly, 10mU human VEGFR2, FGFR2, PDGFR- β EGFR, PI3K, Aurora-A, CDK2/cyclinE and CDK6/cyclinD3 are incubated with 25 μ L solution containing SKLB610 or vehicle (8mM MOPS pH 7.0, 0.2mM EDTA, 10mM Mg Acetate, 10 μ M [γ -33P-ATP] and their own peptide as substrate. The reaction is initiated by the addition of the MgATP mix. After incubation for 40 minutes, the reaction is stopped by the addition of 5 μ L of a 3% phosphoric acid solution. 10 μ L of the reaction is then spotted onto a P30 filtermat and washed three times for 5 minutes in 75mM phosphoric acid and once in methanol prior to drying. Finally, kinase activity was detected using scintillation counting method.

Cell culture

The human lung cancer cell line A549 and H446, human colorectal carcinoma cell line HCT116 and SW480, human hepatocellular carcinoma cell line HepG-2, human epithelial carcinoma cell line Hela, human prostate cancer DU145, human mammary carcinoma cell line MDA-MB-231, human epidermoid carcinoma cell line A431, human Burkitt's lymphoma cell line Raji, human melanoma cell line A375, human acute myeloid leukemia cell line MV4-11 were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 or DMEM containing 10% fetal bovine serum (v/v) in 5% CO₂ at 37° C.

Cell viability assay

The effect of SKLB610 on cell viability was evaluated by CCK-8 assay (Tokyo, Japan) according to the manufacturers' instructions. Briefly, cell were seeded in 96-well plates (2~4 $\times$ 10³ cells/well) for 24h, followed by SKLB610 treatment at concentrations of 1.25-40 μ M for 48h. After the culture period, the supernatant fluid was removed and medium contains 10% CCK-8 reagent was added for 2~4 h incubation at 37° C. The light absorptions (OD) were measured at 450 nm with SpectraMAX M5 microplate spectrophotometer (Molecular Devices).

Endothelial Cell viability assay

The effect of SKLB610 on human umbilical vein endothelial cells (HUVECs) proliferation at the presence and absence of growth factors was evaluated using CCK-8 assay. HUVECs isolated from umbilical cords were seeded in 96-well plates (2 $\times$ 10³ cells/well) and incubated for 24 hours. After starved with 1% serum medium for 12 hours, cells were incubated with SKLB 610 for 1 hour before adding recombinant human VEGF165 (40 ng/ml) or bFGF (30 U/ml), control groups include vehicles alone, VEGF or bFGF alone. No factor stimulated inhibition activity of SKLB610 also were detected as above (cell viability assay) with 20%FCS DMEM medium. After 48 hours of incubation, cell viability was evaluated using CCK-8 assay as described above.

Vessel Sprout Formation Assay

The effect of SKLB610 on angiogenesis was tested in an *in vitro* model using Matrigel Matrix containing growth factors

(BD Biosciences, Bedford, MA) as described previously [32]. Briefly, 96-well plate was pre-incubated with serum-free medium diluted Matrigel Matrix (1:1, 100 μ L/well) at 37° C for 1h, then HUVECs (2 $\times$ 10⁴ cells/well) were seeded cultivated for 30 min. After another incubation with SKLB610 for 6h at 37° C, vessel tube formation was quantified from images viewed under an inverse microscope (Zeiss, Axiovert 200, Germany).

Zebrafish embryos expressing green fluorescent protein (GFP)-VEGFR2 were maintained in Holtfreter's solution (60mM NaCl, 2.4mM sodium bicarbonate, 0.8mM CaCl₂, 0.67mM KCl, and 10mM HEPES) in a humidified incubator at 28° C. They were manually dechorionated with injector pinhead at 24h post-fertilization under the dissecting microscope and were seeded 10embryos/well in 24-well plate. After incubated with 5 μ M SKLB610 at 28.5° C for 24h or 48h, sub-intestinal vein were detected using the fluorescence microscope in the blue excitation light.

VEGF-stimulated VEGFR2 autophosphorylation assay

When covered 75~85% of the bottom, HUVECs were starved over night using serum-free medium, followed by SKLB610 treatment for 2h and eventually stimulated with human recombinant VEGF165 (120ng/mL) for 10 min. Cells were lysed in RIPA buffer and cell lysate was detected with immunoblot analysis using anti-phospho-VEGFR2 (Tyr951), anti-VEGFR2, anti-p44/42 MAPK and anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody. All of above antibodies were detected using HRP-conjugated secondary antibodies, and then blots were developed with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) on Amersham Hyperfilm.

Morphological analysis and western blot assay

Tumor cells treated with SKLB610 was further confirmed by morphological analysis and western blot assay. Briefly, after treated with SKLB610 for 48 hours, A549 were fixed with 75% ethanol and then stained with PI solution containing 50 μ g/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100. The nucleus morphological changes were analyzed under fluorescence microscope (Zeiss, Axiovert 200, Germany).

To identify the mechanism of SKLB610 on cell proliferation, we detected the change of AKT/p-AKT using western blot assay. Briefly, A549 cells were treated with SKLB610 in designed concentrations for 48 h, and then cells were lysed in RIPA buffer. Cell lysate was detected with immunoblot analysis using anti-phospho-AKT (Ser473), anti-AKT and horseradish peroxidase (HRP)-conjugated secondary antibodies, and then blots were developed with enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) on Amersham Hyperfilm β -actin was used as a loading control.

Effect of SKLB610 on Tumor Growth In Vivo

A549 and HCT116 tumors were established by injection of 1 $\times$ 10⁷ cells (100 μ L) into the hind flank of female BALB/c nude mice (6~7 weeks old) subcutaneously. When tumors grew up to approximately 100mm³, the mice were randomly divided into different groups (6 mice each group). Mice then received intraperitoneally injection (i.p.) of SKLB610 (12.5 mg/kg, 25 mg/kg and 50 mg/kg, respectively) or vehicle once daily for 30

days. Tumor growth and body weight was measured every three days during the treatment. Tumor volume was calculated using the formula: Tumor volume (mm^3) = $0.5 \times a \times b^2$ where a is the length and b is the width.

Histological analysis for tumor tissue

Female BALB/c nude mice were implanted with HCT116 and A549 cells as described above. After 14 days administration of SKLB610 (50 mg/kg/day, i.p.), mice were killed and tumors were collected for intratumoral microvessel density (MVD) (CD31 detection). Briefly, paraffin sections of tumor tissue (4–8 μm) were incubated with anti-CD31 antibody (1:200) (Santa Cruz Biotechnology, Heidelberg, Germany) over night at 4° C followed biotinylated labeled secondary antibody incubation. Then, HRP-conjugated StreptABComplex were incubated for 30-minutes and diaminobenzidine was used to produce localized and visible staining. Four fields per tumor were scored (in blinded fashion) for tumor microvessel elements at $\times 40$ magnification by two blinded observers, and tumor microvessel densities (*i.e.*, number of microvessel elements/field) were calculated. General tissue morphology and mitotic figures were visualized by H&E staining.

Acute toxicity study in rats

Seven-week-old female and male SD rats weighing 180 to 200 g were used in this study. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

For acute toxicity test, the male and female rats ($n = 10$, respectively) were intraperitoneally administrated SKLB610 with a dose of 3g/kg. Clinical symptoms including mortality, clinical signs, and gross findings were observed once a day for 14 days. On day 14, the rats were sacrificed and examined by necropsy. Gross histological examinations of the major organs were carried out after dissection.

Statistical Analysis

All experiments were performed in triplicate and repeated 3 times. Data were analysed by chi-square test, one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered to be statistically significant when $P < 0.05$

Results

Selective inhibition of protein kinase by SKLB610

Respecting the selective inhibition for angiogenesis, we detected the kinase inhibition of SKLB610 for VEGFR2, FGFR2 and PDGFR β using SPA (scintillation proximity assay) method, SKLB610 inhibited all of the kinases tested. At concentration of 10 μM , SKLB610 inhibited 65% FGFR2 activity and 55% PDGFR β activity, respectively. Relative to PDGFR2 and FGFR2 SKLB610 has more selective inhibition of VEGFR2 which showed 97% inhibition of VEGFR2 activity at 10 μM *in vitro*.

Fig. 2. SKLB-610 inhibits ligand-dependent phosphorylation of VEGFR2 and MAPK in human endothelial cells (HUVECs). HUVECs were treated with SKLB610 at various concentrations (0–20 μM) for 2 hours and then cells stimulated with VEGF for 10min. The expression of VEGFR2 and p44/42 MAPK was detected by western blotting assay.

Furthermore, in addition to the data presented, no inhibition of enzyme activity was detected when 10 μM of SKLB610 was examined against PI3K, EGFR, Aurora-A, CDK2/cyclinE and CDK6/cyclinD3 (Table 1).

Proliferation inhibitory effect of SKLB610 on HUVECs

The inhibition effect of SKLB610 on growth factor-stimulated (40ng/ml VEGF or 30U/ml bFGF) HUVECs growth was examined using CCK-8 assay. The results showed that SKLB610 have a selective inhibition of VEGF-induced proliferation with an IC₅₀ value of 2.2 μM . SKLB610 selectivity was examined further against proliferation induced by different growth factors. In HUVECs, SKLB610 showed selectivity of 2-fold for inhibition of VEGF-induced proliferation versus bFGF-induced proliferation with an IC₅₀ value of 4.7 μM , whereas the IC₅₀ for non growth factors stimulation is 12.8 μM (Table 3). These results showed that SKLB610 can selectively inhibit VEGFR-dependent proliferation, in contrast to proliferation mediated by bFGF.

SKLB610 inhibited VEGF induced VEGFR2 phosphorylation in HUVECs

Using western blot analysis, we investigated the effects of SKLB610 on the protein levels of VEGFR2, phospho-VEGFR2, p44/42 MAPK and phosphorylated p44/42 MAPK in HUVECs. Consistent with the inhibitory activity of SKLB610 shown against VEGF-stimulated HUVECs proliferation, SKLB610 blocked VEGF-stimulated phosphorylation of VEGFR2 in a dose-

Fig. 3. The anti-angiogenesis effect of SKLB-610. (A) Tube formation analysis *in vitro*: The effect of SKLB-610 on growth factor-stimulated tube formation on the matrigel. Pictures represented the tube forms 6h after different concentrations of SKLB-610 treatment. (B) Zebrafish angiogenesis assay *in vivo*: SKLB610 dosage begin at the 24phf when the embryo have generate essential blood vascular system. SKLB-610 (5 μ M) broke the sub-intestinal vein formation after 24h or 48h drug treatment.

dependent manner after SKLB610 treatment (Fig. 2). The phosphorylation of p44/42 MAPK, a downstream marker of VEGFR2, was inhibited concomitantly. But total VEGFR2 and p44/42 MAPK were unaffected by the SKLB-610 treatment.

Tube formation analysis in vitro

To evaluate the anti-angiogenesis effect of SKLB610, the HUVEC capillary tube formation was evaluated at the presents and absence of SKLB610 *in vitro*. After 6 hours migration on the matrigel, HUVECs rapidly reorganized and subsequently formed tube structures like capillary. However, SKLB610 significant inhibited HUVECs capillary tube formation in a concentration-dependent manner (Fig. 3A). SKLB610 inhibited the formation of vessel-like structures at 2.5 μ M,

Fig. 4. Effects of SKLB-610 on cell apoptosis induction and AKT inhibition. (A) Fluorescence microscopic appearance of PI-stained nuclei of A549 cells after incubation with SKLB610 for 48 hours at varying concentrations. (original magnification 400 $\times$). Apoptotic cells containing condensed and fragmented fluorescent nuclei are visible in SKLB610-treated cells. (B) A549 cells were treated with SKLB610 at various concentrations (0-20 μ M) for 48 hours. The level of AKT was analyzed by Western blotting.

Kinase	inhibition rate (10uM)
VEGFR-2	97%
FGFR2	65%
PDGFR	55%
Aurora-A	30%
PI3K	2%
CDK2/cyclinE	0
EGFR	1%
CDK6/cyclinD3	0

Table 1. SKLB610 inhibition of VEGFR kinase activity and selectivity profile. All these data determined using recombinant human enzyme and detected using scintillation counting method.

and the endothelial cells migration inhibited completely when SKLB610 concentration increased to 10 μ M.

Cell line	Tumor type	IC50 (μM)
A549	human lung cancer	5.7
HepG2	human hepatocellular carcinoma	6.6
A431	human epidermoid carcinoma	11.6
HCT116	human colorectal carcinoma	5.3
SW480	human colorectal carcinoma	8.7
MDA-MB-231	human mammary carcinoma	25.6
Raji	human Burkitt's lymphoma	6.4
A375	human melanoma	10.3
Hela	human epithelial carcinoma	7.9
H446	human lung cancer	8.2
MV4-11	human acute myeloid leukemia	8.2
DU145	human prostate cancer	6.3

Table 2. Proliferation inhibitory effect of SKLB610 on tumor cells: Each cell was treated with various concentrations (0-40 μM) of SKLB610 for 48 hours, respectively. Cell viability was detected by CCK-8 assay.

Treatment factors	IC50 (μM)
VEGF(40 ng/ml) +1%FCS	2.2
bFGF (30U/ml) +1%FCS	4.7
20%FCS	12.8

Table 3. Inhibitory effect of SKLB610 on the proliferation of endothelial cells. Data was from various concentrations (0-40 μM) treatment of SKLB610 for 48 hours, respectively. Cell viability was detected by CCK-8 assay.

Zebrafish angiogenesis assay in vivo

During zebrafish embryonic development, the sub-intestinal veins sprouting initiate at 10 hpf (hours post-fertilization) and reached its highest density at 72 hpf. SKLB610 administration was started from 24 hpf and we detected the sub-intestinal veins at 48 hpf and 72 hpf. The result showed SKLB610 displayed significant inhibition of zebrafish sub-intestinal intersegmental vessels formation (Fig. 3B). On the whole, endothelial cells can't migrate orderly in the intersomitic region and form integrity vessels, which indicating the presence of anti-angiogenic activation of SKLB610 *in vivo*.

Growth-inhibitory activity of SKLB610 on tumor cell lines

A panel of cancer cell lines was used to investigate the growth inhibitory properties of SKLB610 and the IC50 values determined from three independent experiments. Most IC50 against different tumor cells were ranged from 5-10μM. A comparison between the twelve cell lines

examined showed that A549 and HCT116 cells were by far the more sensitive to SKLB610 treatment than the other cell lines with IC50 5.7μM and 5.3μM, respectively (Table 2).

The mechanism of SKLB610 on cell viability

Fluorescence microscopic examination of PI-stained cell was performed to confirm the apoptosis induction effect of SKLB610. After PI-staining cells cultured in the control medium retained their normal size and shape, kept proliferating character and full covered the bottom of plate by 48 hours. However, treatment with SKLB610 resulted in morphological changes characteristic for apoptosis: a brightly red-fluorescent condensed nuclei (intact or fragmented) by fluorescence microscopy of PI-stained nuclei, reduction of cell volume, condensation of nuclear chromatin, nuclear fragmentation and the change was concentration-dependent (Fig. 4A).

Furthermore, we investigated whether AKT, which is known to be very important in cell growth and survival, were involved in SKLB610-mediated proliferation inhibitory effect. Our result showed SKLB610 clearly reduced phosphorylated AKT in a concentration-dependent manner (Fig. 4B), and the total level of AKT was not visibly changed significantly. This data indicated that SKLB610 can inhibit AKT activity.

Effect on tumor Growth of SKLB-610 in Nude Mouse Models

To study the antitumor activity of SKLB610 *in vivo*, A549 or HCT116 xenograft-bearing mice were treated with SKLB610 at the dose of 12.5 mg/kg, 25 mg/kg and 50 mg/kg, respectively. From the results (Fig. 5A), we found that the tumor growth of the SKLB610 group become significant slowed 10 days after treatment and the growth inhibition display significant dose-dependent property. After 30 days treatment, SKLB610 exhibited a significantly antitumor activity in inhibiting tumor progress compared with control. The inhibition rate of tumor volume in SKLB610-treated groups was 70.2% (50 mg/kg) for A549 and 77.1% (50 mg/kg) for HCT116, respectively. Compared with the control, there were statistically significant difference among tumor volumes of treatment groups ($p < 0.05$). Moreover, SKLB610 treatment was well tolerated. After treatment with SKLB610 for 30 days, body weight of the mice were statisticed, no significant difference was observed among the drug treatment and vehicle groups (Fig. 5B), which proved the tolerance of mice to the SKLB610. Moreover, no adverse effects on clinical condition were observed.

Fig. 5. SKLB-610 inhibits tumor xenograft model growth of human non-small-cell lung cancer model (A549) and human colon cancer model (HCT116) and was well tolerated. (A) Xenografts were established s.c. in BALB/c nude mice and SKLB610 administration were started when the solid tumor grow up to approximately 100 mm³. Once daily i.p administration of SKLB610 (50 mg/kg, 25 mg/kg, 12.5 mg/kg) or vehicle was continued for 30 days. Significant differences among tumor volumes of treatment was observed ($p < 0.05$). (B) After 30 days SKLB610 treatment, the body weight of the drug and vehicle groups were statisted, and there were no significant difference among the groups.

Fig. 6. SKLB610 inhibits neovessels genetic *in vivo*. (A) Histological sections of control and SKLB610 (50 mg/kg/day)-treated A549 tumors and HCT116 tumors stained with CD31. A significant reduction in CD31 (endothelial cell) staining was evident after SKLB610 treatment. (B) Mean CD31-positive area was determined by morphometric image analysis after 14 days treatment of SKLB610.

Acute toxicity test

Mortality, clinical signs and body weight of SD rats were measured for 14 days and no obvious changes of these signals were observed throughout the 14 days observation period. Moreover, histopathological, serum biochemistry and hematological analysis did not show any significant pathological changes (data not shown). Based on these results, we can conclude that no observed

adverse effect level (NOAEL) value of SKLB610 could be greater than 3g/kg in rat.

Immunohistochemistry assay

In addition to measuring changes in tumor volume, the potent effects of SKLB610 on microvasculature inhibition was evaluated based on histological and anti-CD31 immunohistochemical analysis of tumors resected

from treated and untreated animals in HCT116 and A549 models. Compared with vehicle-treated controls, reduced tumor MVD which is taken to be an indication of antiangiogenic activity in tumor tissue was observed. Compared to control, 43% and 55.6% MVD reduction, respectively, were detected in HCT116 and A549 models (Fig. 6). This result indicated antiangiogenic activity of SKLB610 even *in vivo* consistent with results that SKLB610 inhibited HUVEC proliferation *in vitro*.

Discussion

The role of angiogenesis has been proved the key steps of the tumor progression, and inhibition of growth factor receptor activation and intracellular growth factor-activated signal transduction pathways has been demonstrated as a promising strategy for anticancer therapies [33-36]. In various growth factors and receptors, VEGF and its receptor VEGFR2 have been well established as important mediators in endothelial cell proliferation, migration, invasion, and differentiation during angiogenesis [37]. Moreover, the expression of VEGF is strongly correlated with tumor progression and poor prognosis in malignancies, therefore, VEGF and VEGFR2 was considered a valuable target for cancer therapy [24]. In this respect, various selective small molecule inhibitors of the VEGF-VEGFR pathway and antibodies to both the growth factor and receptor have been successfully tested as anticancer drugs *in vitro* and in preclinical models and a number are now in late stage clinical development.

Our group engaged in the design, screen and synthesis of novel antiangiogenesis agents targeted on VEGFR2 as potential new anticancer agents. SKLB610 was obtained from the structural modification of 4- (4-benzamidophenoxy) picolinamide with a potential strategies to found a anti-tumor small compound. The chemical structure of SKLB610 is novel and its structure is different from VEGFR inhibitors in clinical use. We already got the SKLB610 patent from State Intellectual Property Office the People's Republic of China (patent number: ZL 200810304730.5). In enzymatic assays using recombinant protein kinases, SKLB610 has been shown to be a multi-target inhibitor of the tyrosine kinases. It is most potent against VEGFR2 (inhibit rate 97%), and exhibits slightly weaker inhibitor of FGFR2 (inhibit rate 65%) and PDGFR (inhibit rate 55%). Under the same assays conditions, protein kinases tested that belong to other families such as PI3K was almost not inhibited by

SKLB610. Because endothelial cell proliferation play a important role in process of angiogenesis which is forming new blood vessels from the preexisting vessels, and VEGFR2 has the predominant role in mediating VEGF signal transduction in endothelial cells and angiogenesis, we then detected the effect of SKLB610 on VEGFR-VEGFR mediated endothelial cells proliferation and angiogenesis. Our results showed that SKLB610 selectively inhibited VEGF-mediated HUVEC proliferation. Moreover, SKLB610 significantly inhibited VEGF-induced VEGFR2 phosphorylation in HUVECs that naturally express VEGFR2. The results from enzyme assay and cellular assay demonstrated that VEGFR2 has the predominant role in SKLB610 mediated endothelial cells proliferation inhibitory effect. Moreover, SKLB610 also showed weaker inhibitory effect in bFGF-mediated proliferation compared with non growth factors stimulation. The FGF and FGFR have been found to be associated with multiple biological activities, including proliferation, differentiation, motility, and transforming activities of endothelial cells and angiogenesis [34]. Therefore, inhibition of the fibroblast growth factors and its receptor could potentially play a part role in SKLB610 anti-angiogenesis. SKLB610 is a multi-targeted inhibitor with more potent inhibition of VEGFR2 activity.

As above results showed SKLB610 can lead to a disruption of neovascularization, we further confirmed that SKLB610 can obviously inhibit the sub-intestinal veins formation of zebrafish *in vivo* and inhibits growth-factors induced two-dimensional tube formation in an *in vitro* angiogenesis assay. All of these indicated the presence of anti-tumor and angiogenesis activation of SKLB610.

Inhibition of VEGF signaling is being pursued avidly as a therapeutic strategy in oncology, to inhibit angiogenesis, neovascular survival, and vascular permeability in tumors. We therefore investigated the inhibitory effect of SKLB610 on tumor growth *in vivo*. Doses of 50 mg/kg/day SKLB610 were examined for activity in established histologically distinct (colon and lung) human tumor xenograft models in athymic mice. SKLB610 inhibited tumor growth at inhibitory rate of 70.2% and 77.1% for colon (HCT116) and lung (A549) cancer, respectively. Furthermore, to investigate temporal changes in tumor vessel growth and survival following SKLB610 treatment, the vascular density of A549 lung tumor and HCT116 colon tumor xenograft model was examined after SKLB610 treatment. Compare with control, the vascular density in SKLB610-treated tumors revealed a 55.6% reduction in A549 tumor model and a 43% reduction in HCT116 tumor model following 14 days

treatment. The antitumor profile and antiangiogenesis effect observed with SKLB610 treatment *in vivo* is consistent with the effect on tumor vasculature, a common growth-limiting target in colon and lung cancer.

Relate to tumor inhibition, SKLB610 not only showed cancer cell vitality inhibition *in vitro*, but also have an inhibition activity for tumor growth *in vivo*. When it was tested in cellular assays, SKLB610 dose-dependently inhibited cell growth and viability in series tumor cells. The fluorescence microscopic examination and western blotting assay showed that SKLB610 can induce apoptosis and inhibited AKT phosphorylation. Therefore, besides angiogenesis, SKLB610 might exhibit its anti-tumor effect via inhibited cancer cell proliferation directly.

In addition, we found that SKLB610 did not exhibit toxicity. During 30 days treatment, SKLB610 treatment was well tolerated and no adverse effects on clinical condition. No observed adverse effect level (NOAEL) value of SKLB610 could be greater than 3g/kg in rat. However, in preclinical safety evaluation of VEGFR inhibitors such as sorafenib which are in clinical use, its maximal tolerance dose (MTD) is 500mg/kg in rat, which is 17% of SKLB610's NOAEL. The acute toxicities were observed in the gastrointestinal tract (soft feces and vomiting) and in the liver (AST and ALT were increased). In the case of another VEGFR2 inhibitor sunitinib, its MTD was 500mg/kg. The body weights were decreased dose dependently to maximums of 47% and 21% in 500mg/kg in males and females mice, respectively. ALT was increase by almost 10 fold over control in 3/4 500mg/kg females (<http://www.accessdata.fda.gov/drugsatfda>). The above observation suggested the preclinical safety of SKLB610 is much higher than VEGFR inhibitors which were in clinical use. Based on above observation, we can conclude that SKLB610 has excellent safety feature.

In future studies, it will be promising to optimize the structures of the SKLB610 compounds for anti-angiogenesis and anti-tumor growth *in vivo*. There must be compounds inhibiting angiogenesis at lower concentrations than these current drugs. In summary, SKLB610 is a potent anti-angiogenesis compound. It is a multi-targeted inhibitor that is focused on the VEGFR2, FGFR2, and PDGFR. The effect of SKLB610 on tumor growth in nude mice bearing tumor xenografts indicated its potential bioactivity *in vivo*. Finally, SKLB610 might provide a relatively nontoxic adjuvant treatment for tumor therapy, or its therapeutic alliance use will show its greater value for clinical use.

Abbreviations

VEGF (Vascular endothelial growth factor); VEGFR (Vascular endothelial growth factor receptor); MAPK (Mitogen-activated protein kinase); PI3K (Phosphatidylinositol 3 kinase); PDGFR (Platelet-derived growth factor receptor); bFGF (Vasic fibroblast growth factor); FGFR2 (Fibroblast growth factor receptor 2; SKLB610 (N-methyl-4-(4-(3-(trifluoromethyl)benzamido)phenoxy)picolinamide); GFP (Green fluorescent protein); HUVECs (Human umbilical vein endothelial cells); MVD (Microvessal density); DMSO (Dimethyl sulfoxide).

Acknowledgements

This work was supported by National S&T Major project (2009ZX09103-133, 2009ZX09103-132 and 2011ZX09102-001-013).

References

- Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. *Nature* 2000;407:249-257.
- Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353-364.
- Bergers G, Benjamin LE: Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003;3:401-410.
- Folkman J: Anti-Angiogenesis: New Concept for Therapy of Solid Tumors. *Ann Surg* 1972;175:409-416.
- Norrby K: Angiogenesis: New aspects relating to its initiation and control. *APMIS* 1997;105:417-437.
- Lund EL, Spang-Thomsen M, Skovgaard-Poulsen H, Kristjansen PEG: Tumor angiogenesis - a new therapeutic target in gliomas. *Acta Neurol Scand* 1998;97:52-62.
- Kerbel RS: Molecular origins of cancer: Tumor angiogenesis. *New Engl J Med* 2008;358:2039-2049.
- Klagsbrun M, D'Amore PA: Vascular Endothelial Growth Factor and its Receptors. *Cytokine Grow Factor Rev* 1996;7:259-270.
- McMahon G: VEGF Receptor Signaling in Tumor Angiogenesis. *Oncologist* 2000;5:3-10.

- 10 Roy H, Bhardwaj S, Yla-Herttuala S: Biology of vascular endothelial growth factors. *FEBS Lett* 2006;580:2879-2887.
- 11 Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435-439.
- 12 Schuh AC, Faloon P, Hu QL, Bhimani M, Choi K: *In vitro* hematopoietic and endothelial potential of flk-1(-/-) embryonic stem cells and embryos. *P Natl Acad Sci USA* 1999;96:2159-2164.
- 13 Murata N, Takashima Y, Toyoshima K, Yamamoto M, Okada H: Anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice. *J Control Release* 2008;126:246-254.
- 14 Sini P, Samarzija I, Baffert F, Littlewood-Evans A, Schnell C, Theuer A, Christian S, Boos A, Hess-Stumpp H, Foekens JA, Setyono-Han B, Wood J, Hynes NE: Inhibition of multiple vascular endothelial growth factor receptors (VEGFR) blocks lymph node metastases but inhibition of VEGFR-2 is sufficient to sensitize tumor cells to platinum-based chemotherapeutics. *Cancer Res* 2008;68:1581-1592.
- 15 Amino N, Ideyama Y, Yamano M, Kuromitsu S, Tajinda K, Samizu K, Hisamichi H, Matsuhisa A, Shirasuna K, Kudoh M, Shibasaki M: YM-359445, an orally bioavailable vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor, has highly potent antitumor activity against established tumors. *Clin Cancer Res* 2006;12:1630-1638.
- 16 Korc M: Pathways for aberrant angiogenesis in pancreatic cancer. *Mol Cancer* 2003;2:1-8.
- 17 Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS: Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. *Blood* 2001;98:1904-1913.
- 18 Gille H, Kowalski J, Li B, LeCouter J, Moffat B, Zioncheck TF, Pelletier N, Ferrara N: Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and VEGFR2 (VEGFR-2) - A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem* 2001;276:3222-3230.
- 19 Zeng HY, Sanyal S, Mukhopadhyay D: Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (VEGFR2) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *J Biol Chem* 2001;276:32714-32719.
- 20 Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D, Warren RS, Donner DB: Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. *J Biol Chem* 2000;275:5096-5103.
- 21 Kiselyov A, Balakin KV, Tkachenko SE: VEGF/VEGFR signalling as a target for inhibiting angiogenesis. *Expert Opin Inv Drug* 2007;16:83-107.
- 22 Spannuth WA, Sood AK, Coleman RL: Angiogenesis as a strategic target for ovarian cancer therapy. *Nat Clin Pract Oncol* 2008;5:194-204.
- 23 Wakelee ECH: Role of Anti-angiogenesis Agents in Treating NSCLC: Focus on Bevacizumab and VEGFR Tyrosine Kinase Inhibitors. *Curr Treat Options Oncol* 2007;8:15-27.
- 24 Ferrara N: Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* 2004;9:2-10.
- 25 Morabito A, Piccirillo MC, Falasconi F, De Feo G, Del Giudice A, Bryce J, Di Maio M, De Maio E, Normanno N, Perrone F: Vandetanib (ZD6474), a Dual Inhibitor of Vascular Endothelial Growth Factor Receptor (VEGFR) and Epidermal Growth Factor Receptor (EGFR) Tyrosine Kinases: Current Status and Future Directions. *Oncologist* 2009;14:378-390.
- 26 Wilhelm S, , Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S: Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 2006;5:835-844.
- 27 Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, Schreck RE, Abrams TJ, Ngai TJ, Lee LB, Murray LJ, Carver J, Chan E, Moss KG, Haznedar JO, Sukbuntherng J, Blake RA, Sun L, Tang C, Miller T, Shirazian S, McMahon G, Cherrington JM: *In vivo* antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: Determination of a pharmacokinetic/pharmacodynamic relationship. *Clin Cancer Res* 2003;9:327-337.
- 28 Wong CI, Koh TS, Soo R, Hartono S, Thng CH, McKeegan E, Yong WP, Chen CS, Lee SC, Wong J, Lim R, Sukri N, Lim SE, Ong AB, Steinberg J, Gupta N, Pradhan R, Humerickhouse R, Goh BC: Phase I and Biomarker Study of ABT-869, a Multiple Receptor Tyrosine Kinase Inhibitor, in Patients With Refractory Solid Malignancies. *J Clin Oncol* 2009;27:4718-4726.
- 29 Schiller JH, Larson T, Ou SH, Limentani S, Sandler A, Vokes E, Kim S, Liau K, Bycott P, Olszanski AJ, von Pawel J: Efficacy and Safety of Axitinib in Patients With Advanced Non-Small-Cell Lung Cancer: Results From a Phase II Study. *J Clin Oncol* 2009;27:3836-3841.
- 30 Brandes AA, Stupp R, Hau P, Lacombe D, Gorlia T, Tosoni A, Mirimanoff RO, Kros JM, van den Bent MJ: EORTC study 26041-22041: Phase I/II study on concomitant and adjuvant temozolomide (TMZ) and radiotherapy (RT) with PTK787/ZK222584 (PTK/ZK) in newly diagnosed glioblastoma. *Eur J Cancer* 2010;46:348-54.
- 31 Batchelor TT, Duda DG, di Tomaso E, Ancukiewicz M, Plotkin SR, Gerstner E, Eichler AF, Drappatz J, Hochberg FH, Benner T, Louis DN, Cohen KS, Chea H, Exarhopoulos A, Loeffler JS, Moses MA, Ivy P, Sorensen AG, Wen PY, Jain RK: Phase II Study of Cediranib, an Oral Pan-Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor, in Patients With Recurrent Glioblastoma. *J Clin Oncol* 2010;28:2817-2823.
- 32 Wu HT, Lin SH, Chen YH: Inhibition of cell proliferation and *in vitro* markers of angiogenesis by indole-3-carbinol, a major indole metabolite present in cruciferous vegetables. *J Agr Food Chem* 2005;53:5164-5169.
- 33 Zou HY, Li Q, Lee JH, McDonnell SR, Yamazaki S, Koudriakova TB, Alton G, Cui JJ, Kung PP, Nambu MD, Los G, Bender SL, Mroczkowski B, Christensen JG: An orally available small-molecule inhibitor of c-met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. *Cancer Res* 2007;67:4408-4417.
- 34 Ribatti D, Vacca A, Rusnati M, Presta M: The discovery of basic fibroblast growth factor/fibroblast growth factor-2 and its role in haematological malignancies. *Cytokine Growth Factor Rev* 2007;18:327-34.
- 35 Krause DS, Van Etten RA: Tyrosine kinases as targets for cancer therapy. *New Engl J Med* 2005;353:172-187.
- 36 Le Tourneau C, Faivre S, Raymond E: New developments in multitargeted therapy for patients with solid tumours. *Cancer Treat Rev* 2008;34:37-48.
- 37 Prior BM, Yang HT, Terjung RL: What makes vessels grow with exercise training? *J Appl Physiol* 2004;97:1119-1128.