SKLB1206, a novel orally available multi-kinase inhibitor targeting EGFR activating and T790M mutants, ErbB2, ErbB4 and VEGFR2, displays potent antitumor activity both in vitro and in vivo

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Abstract

Anti-epidermal growth factor receptor (EGFR) treatment has been successfully applied in clinical cancer therapy. However, the clinical efficacy of first-generation reversible EGFR inhibitors, such as gefitinib and erlotinib, is limited by the development of drug-resistant mutations, including the gatekeeper T790M mutation and upregulation of alternative signaling pathways. Second-generation irreversible EGFR inhibitors that were designed to overcome the drug resistance due to the T790M mutation have thus far had limited success. Here we report a novel reversible EGFR inhibitor, SKLB1206, which has potent activity against EGFR with gefitinib-sensitive and resistant (T790M) mutations. In addition, SKLB1206 has also considerable inhibition potency against some other related onco-kinases, including ErbB2, ErbB4 and vascular endothelial growth factor receptor 2 (VEGFR2). SKLB1206 exhibited highly anti-proliferative activity against a range of EGFR mutant cell lines, including gefitinib-sensitive and resistant cell lines, and EGFR or ErbB2-overexpressing cell lines. SKLB1206 also showed a potent anti-angiogenesis effect in vitro, in a zebrafish embryonic angiogenesis assay, and in an alginate-encapsulate tumor cell assay. In vivo, oral administration of SKLB1206 demonstrated complete tumor regression in gefitinib-sensitive HCC827 and PC-9 xenograft models, and showed a considerable antitumor effect on the gefitinib-resistant H1975 model as well as other EGFR/ErbB2-overexpressing or -dependent tumor models including A431, LoVo and N87 established in athymic mice. SKLB1206 also showed a very good oral bioavailability (50.1%). Collectively, these
preclinical evaluations may support clinical development of SKLB1206 for cancers with EGFR activating/resistance mutations or EGFR/ErbB2 overexpressed.
Introduction

Mutations or overexpression of epidermal growth factor receptor (EGFR) have been associated with a variety of human malignancies, including lung, colorectal, prostate, head and neck cancers (1-3). The EGFR kinase has thus been thought as an important drug target for treating these types of cancers. Currently, numerous small molecular EGFR inhibitors have also been reported, of which the most successful examples are gefitinib (Iressa) and erlotinib (Tarceva), which have been approved for clinically treating non-small cell lung cancer (NSCLC) (4-6). Gefitinib and erlotinib belong to the first-generation reversible and selective EGFR inhibitors. Clinical studies show that the two drugs are effective in 10~20% of NSCLC patients, whose tumor cells harbor EGFR mutations that occur in either exon 19 characterized by in-frame deletions of amino-acids 747-750, or exon 21 resulting in L858R substitutions. Unfortunately, NSCLC with drug-sensitive EGFR mutations that initially respond to gefitinib or erlotinib eventually develop acquired resistance. Approximately half of cases are a single secondary mutation in EGFR exon 20 that leads to T790M substitutions (7-9). The limited response rate (primary resistance) and acquired resistance (secondary resistance) constitute the main challenges in current anti-cancer therapy targeting EGFR.

Several strategies could be used to conquer the current drug resistance to EGFR inhibitors, including directly acting on EGFR T790M mutation, concomitant inhibition of EGFR and other related receptor tyrosine kinases (RTKs), and simultaneous interference of angiogenesis (10-12). Currently, second-generation
irreversible EGFR inhibitors have been designed to directly target the EGFR T790M mutation (13-15). However, except for very few irreversible inhibitors such as BIBW2992 that is currently in Phase III clinical trial (16), most of the irreversible inhibitors have thus far shown limited clinical efficacy, which has been mainly linked to decreased binding velocity to the mutant kinase (17). In this respect, reversible inhibitors would be more superior or at least another better option compared with irreversible counterparts. Secondly concomitant inhibition of other RTKs that are tightly associated with EGFR has been demonstrated to be helpful for overcoming the drug resistance, for example, the other ErbB family members. Numerous studies have indicated that interactions between ErbB family members play an important role in human malignancies (18-20). Preclinical experiments have shown that ErbB receptors act synergistically to transform NIH3T3 cells (21, 22), and some human cancers that overexpress both EGFR and ErbB2 have a poorer prognosis than cancers that overexpress either receptor alone (23, 24). Therefore, it is expected that simultaneous inhibition of other ErbB family members in addition to EGFR may lead to the enhancement of therapeutic efficacy of EGFR inhibitors and reduce the drug resistance. Thirdly, additional antiangiogenesis activity would help to accentuate the antitumor effects of EGFR inhibitors since angiogenesis plays important roles in the sustained growth and metastasis of tumors through providing nutrients and oxygen to tumor tissues (25). Indeed, several recent studies have shown that dual inhibition of EGFR and vascular endothelial growth factor receptor 2 (VEGFR2), a key mediator of angiogenesis, displayed obvious antitumor supra-additive effects on human head
and neck and colon cancers, including decreased tumor vascularity and increased tumor and endothelial cell apoptosis compared with sole inhibition of EGFR (26-28). Ideally, simultaneous use of these strategies is expected to be able to provide improved and/or broader spectrum anti-cancer efficacy versus selective EGFR inhibitors and better overcome the drug resistance.

Here we report a novel reversible EGFR inhibitor, named SKLB1206 that is a purine derivative, which was obtained by utilizing the technology of computer aided drug discovery (CADD) and lead optimization (related investigations will be reported elsewhere). SKLB1206 exhibits high inhibition potency against EGFR with gefitinib-sensitive or -resistance (T790M) mutations. In addition, SKLB1206 has also considerable inhibition activity against some other related onco-kinases, including ErbB2, ErbB4 and VEGFR2. SKLB1206 displayed potent antitumor effect both in vitro and in vivo and excellent oral bioavailability which provided a solid base for further clinical development of SKLB1206.
Materials and Methods

Cell culture and regents

Cell lines were acquired from the American Type Culture Collection except specifically mentioned. All the cell lines were maintained in the designated medium (Supplementary Table S1) supplemented with 10% FBS (Gibco) and passaged for less than 6 months after receipt or resuscitation. No further authentication was done for tumor cell lines. Recombinant human EGF was purchased from Prospec Company. Recombinant human VEGF<sub>165</sub> and bFGF were obtained from PeproTech Company. SKLB1206 was synthesized at the State Key Laboratory of Biotherapy, Sichuan University. Gefitinib, Sunitinib, and BIBW2992 were obtained from commercial sources. For all in vitro assays and zebrafish studies, a 10 mM stock solution of each compound was prepared in DMSO and diluted in optimal assay buffers or culture medium.

Kinase inhibition assay

The IC<sub>50</sub> values of SKLB1206 for kinase inhibition in vitro were measured by the use of radiometric assays performed by Kinase Profiler service provided by Millipore as described in detail in the Supplementary Methods.

Cell proliferation assay

Cell proliferation assay was performed as previously described (29). The IC<sub>50</sub> values were calculated by GraphPad Prism 5.01 software.

Colony formation assay

HCC827 cells were seeded in 6-well plate at a density of 2000 per well. These
cells were treated with 0.001 μM of SKLB1206 or gefitinib in the next day. After treatment for 10 days, cells were stained by crystal violet for 10 minutes.

**Western blot analysis**

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

**Growth factor-mediated endothelial cell proliferation assay**

The endothelial cell proliferation assay was carried out as previously described (30).

**In vitro capillary-like tube formation assay**

The tube formation assay was performed as described previously (31).

**Migration assay**

Migration assay was done following the method reported previously (32) and is described in detail in Supplementary Methods.

**Transwell invasion assay**

The cell invasion assay was performed as described previously (33) with some modifications and is described in detail in Supplementary Methods.

**In vivo live fluorescent zebrafish assay**

The transgenic zebrafish (FLK-1:EGFP) embryos were grown and maintained according to the protocols described in ref (34). After 15 h of fertilization, the embryos were treated with indicated concentrations of SKLB1206. Sunitinib as a
positive control and the DMSO control were also included. After incubation overnight, zebrafish were anesthetized and a fluorescent image of each embryo was captured using the fluorescence microscope (Carl Zeiss Microimaging Inc).

**S.c. xenograft models**

All animal experiments were performed approved by the Animal Care and Use Committee of Sichuan University. Tumor xenograft models were established by s.c. injecting 100 μL tumor cell suspension (between $5 \times 10^6$ and $1 \times 10^7$ cells) into the right flank of the animals. Mice were randomized into groups of 6-7 before treatment at a point when tumors reached a volume of 0.1-0.3 cm$^3$. SKLB1206, gefitinib, or BIBW2992 was suspended in 1% (v/v) solution of polyxyethylene sorbitan monooleate (Tween 80 ) in deionized water. Animals were given SKLB1206 (2-50 mg/kg), gefitinib (100 mg/kg), BIBW2992 (20 mg/kg), or vehicle once daily (qd) by oral gavage. Tumors were measured twice weekly using calipers, and the volume was calculated using the following formula: length × width$^2$ × 0.52. The complete summary of tumor xenograft models are presented in Supplementary Methods.

**Pharmacokinetic assessments**

Blood from SD rats dosed with SKLB1206 was collected in EDTA-containing tubes and the plasma was isolated by the centrifugation. Plasma concentrations of SKLB1206 were determined by liquid chromatography tandem mass spectrometry (LC/MS).

**Alginate-encapsulate tumor cell assay**

The alginate-encapsulate tumor cell assay was performed as described previously
TUNEL detection

The analysis of apoptotic cells in the tumor tissue was performed by TUNEL staining using an apoptotic cell detection kit (Roche). Images of the sections were taken by a fluorescence microscope. The Apoptosis index was calculated by dividing the number of TUNEL-positive cells by the total number of cells in the field.

Immunohistochemistry

Paraffin-embedded sections of tumors were stained with Hematoxylin and esosin using standard methods. Immunohistochemical staining was performed using antibodies from Cell Signaling Technology. CD31 (BD Bioscience) staining was performed using frozen sections of tissue embedded in OCT.

Statistical analysis

SPSS 11.5 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by Student’s t test and ANOVA. P value < 0.05 was considered statistically significant.

Results

The kinase inhibition profile of SKLB1206 against recombinant human protein kinases

The structure of SKLB1206 and gefitinib is displayed in Fig. 1A and the kinase inhibition profile of SKLB1206 against a panel of kinases is shown in Supplementary Table S2. SKLB1206 potently inhibited wild-type (WT) EGFR, EGFR L858R, and
L858R/T790M mutants with IC_{50} values of 0.005 μM, 0.005 μM, and 0.046 μM, respectively. This compound also inhibited ErbB2, ErbB4, and VEGFR2 with moderate activity (IC_{50}s: 0.9 μM, 0.3 μM, and 0.03 μM, respectively), but only weakly inhibited AXL, EPHB4, FLT3, and MERTK (IC_{50}s: 1.5 μM, 2.4 μM, 2.7 μM, and 2.5 μM, respectively). SKLB1026 displayed almost no inhibition activity to other 44 selected protein kinases. All of these demonstrate that SKLB1206 is a potent EGFR (both activating and drug-resistance mutations) inhibitor with good kinase spectrum selectivity.

**Inhibitions of tumor cell growth and colony formation in vitro**

The anti-viability activity of SKLB1206 against various tumor cell lines was measured using MTT method (Table 1 and Supplementary Fig. S1). SKLB1206 displayed exceptionally potent inhibition activity against gefitinib-sensitive NSCLC cell lines HCC827 and PC-9 (IC_{50}s: 0.0008 μM and 0.001 μM, respectively, see Fig. 1B), which is about 5-fold more potent than gefitinib. It also showed good inhibition potency against gefitinib-resistant NSCLC cell lines H1975 (see Fig. 1B) and H820 (IC_{50}s: 1.1 μM and 0.67 μM, respectively). To cell lines, in which EGFR or ErbB2 is overexpressed and to which gefitinib showed moderate inhibition activity, including H292, Calu-3, BT474, FaDu, and N87, SKLB1206 displayed relatively higher potency than gefitinib. To those cell lines, in which EGFR or ErbB2 is overexpressed but on which gefitinib had minimal growth inhibitory effect, including SK-BR-3, MDA-MB-468, LoVo, and A431, SKLB1206 showed moderate anti-proliferative activity. By contrast, those KRAS mutant cell lines including A549, H460,
MDA-MB-231, SW480, and HCT116 were resistant to both SKLB1206 and gefitinib (IC$_{50}$ > 1 μM). Similarly, in MCF-7 cell line containing PI3K mutation and HepG2 cell line overexpressing Aurora B, both SKLB1206 and gefitinib displayed minimal growth inhibitory effect (IC$_{50}$ > 4 μM). These data exclude the possibility that the anti-viability activity of SKLB1206 is due to its cell toxicity. Collectively, these results clearly indicate that SKLB1206 has improved antitumor potency to a broader spectrum of tumor cells compared with gefitinib.

Colony formation assay was further performed to visually assess the anti-viability activity of SKLB1206. Fig. 1C presents the cell survival state of HCC827 after exposure to 0.001 μM of SKLB1206. The same concentration of gefitinib was also used for the comparison. Obviously, SKLB1206 completely blocked the formation of colonies while gefitinib only decreased the clonogenic survival of HCC827 cells compared with the control group.

Inhibition of ErbB receptor autophosphorylation and inactivation of downstream signaling proteins in cell cultures

The ability of SKLB1206 to inhibit the activation of EGFR, ErbB-2, and downstream signaling proteins in intact cells was assessed by Western blot analysis. In gefitinib-sensitive HCC827 cell line, SKLB1206 inhibited EGFR phosphorylation at lower concentrations of drug with an estimated IC$_{50}$ value of 0.003 μM compared with gefitinib with an estimated IC$_{50}$ value of 0.01 μM (Fig. 2A). This was accompanied by corresponding inhibition of the downstream targets, AKT and ERK. Furthermore, in EGF-stimulated A431 cell line, SKLB1206 inhibited EGF-dependent
phosphorylation of EGFR and AKT with IC₅₀ value of 0.1 μM as potently as gefitinib (Fig. 2B). However, SKLB1206 reduced ERK phosphorylation more effectively than gefitinib, possibly reflecting their difference in the growth inhibition of A431 cell line. To assess the effect of SKLB1206 on the phosphorylation of ErbB2, a classical ErbB2-overexpressing cell line, BT474, was used. SKLB1206 displayed good inhibition potency against the ErbB2 phosphorylation with an estimated IC₅₀ value of 1 μM, still superior to gefitinib (Fig. 2C).

**Anti-angiogenesis effect of SKLB1206**

To evaluate the anti-angiogenesis effect of SKLB1206, the anti-proliferative ability of SKLB1206 against human umbilical vein endothelial cell (HUVEC) was first assessed by MTT assay. SKLB1206 showed a good anti-proliferative activity against VEGF and EGF-stimulated HUVEC with IC₅₀ values of 0.102 μM and 0.310 μM, respectively (Supplementary Fig. S2A). Then the inhibitory efficacy of SKLB1206 to HUVEC migration, invasion, and tube formation, which are indispensable for angiogenesis, was examined. In wound-healing assay, we found that SKLB1206 significantly inhibited the migration of HUVECs in a dose-dependent manner and the migration ability of HUVEC was inhibited by about 70% in the presence of 1.25 μM of SKLB1206 (Fig. 3A and Supplementary Fig. S2B). In addition, in the transwell assay assessing the invasion ability of HUVECs, 1 μM of SKLB1206 inhibited almost all invasion activities of HUVECs (Fig. 3A and Supplementary Fig. S2B). Furthermore, we investigated the effect of SKLB1206 on the ability of endothelial cell tube formation. 0.625 μM of SKLB1206 inhibited tube
formation of HUVECs by 50% and 2.5 μM potently blocked the tube formation of HUVECs (Fig. 3A and Supplementary Fig. S2B). Taken together, these data indicate that SKLB1206 can inhibit angiogenesis in vitro.

In order to test the anti-angiogenesis ability of SKLB1206 in vivo, we examined the effect of SKLB1206 on embryonic angiogenesis in zebrafish. Treatment of live fish embryos with SKLB1206 completely blocked the formation of intersegmental vessel at the concentration of 5 μM while preserving fluorescence in the doral aorta and major cranial vessels, and 2.5 μM of sunitinib showed the similar effect (Fig. 3B). In the presence of 1.25 μM or 2.5 μM of SKLB1206, the formation of intersegmental vessel was significantly inhibited compared with vehicle control group, exhibiting a dose-dependent inhibition pattern.

Finally, to mimic better the process of angiogenesis induced by tumor cells in vivo and determine the inhibitory effect of SKLB1206 on it, we performed an alginate-encapsulate tumor cell assay. In SKLB1206-treated mice, new blood vessels in alginate beads were apparently reduced and FITC-dextran uptake was significantly decreased when compared with control group (Fig. 3C). Collectively, based on the above results, we can conclude that SKLB1206 efficiently inhibited angiogenesis both in vitro and in vivo.

**Antitumor efficacy of SKLB1206 in relevant human tumor xenograft models**

A total of six relevant human tumor xenograft models were used, including two gefitinib-sensitive NSCLC HCC827 and PC-9 tumor models bearing EGFR activating mutation, one gefitinib-resistant NSCLC H1975 tumor model harboring T790M
mutation in EGFR, one wild-type EGFR-overexpressing A431 epidermoid carcinoma xenograft model, one wild-type EGFR-driven LoVo colon carcinoma xenograft model, and one ErbB2-overexpressing N87 gastric carcinoma xenograft model.

In the HCC827 tumor model, SKLB1206 at all dose levels markedly induced tumor regression and 20 mg/kg of SKLB1206 displayed complete tumor regression in all treated mice within a week as effectively as gefitinib did at the dose of 100 mg/kg (Fig. 4 and Supplementary Fig. S3A). Even 2 mg/kg of SKLB1206 resulted in almost complete tumor regression at the end of treatment (Fig. 4). In another EGFR activating mutation tumor, PC-9 xenograft model, SKLB1206 also significantly induced tumor regression at the indicated doses even with a low dose of 5 mg/kg (Fig. 4). Similar results were observed when treatment with gefitinib at the dose of 100 mg/kg. To determine whether SKLB1206 can overcome the disadvantage of gefitinib against EGFR T790M mutation tumor, the H1975 tumor xenograft model was established in nude mice. Encouragingly, oral administration of SKLB1206 substantially inhibited tumor growth in a dose-dependent manner (Fig. 4 and Supplementary Fig. S3A), with tumor growth inhibition reaching 65% and 72% for the doses of 25 mg/kg and 50 mg/kg compared with the control group, respectively. BIBW2992 as a positive control had the tumor growth inhibition of 90% at 20 mg/kg (Supplementary Table S3). However, treatment with gefitinib even at 100 mg/kg only produced a marginal effect on tumor growth (23% inhibition). These results show that SKLB1206 is, to a great extent, capable of overcoming the acquired drug-resistance of gefitinib. Furthermore, because SKLB1206 was also active against WT EGFR
kinase and EGFR-overexpressing tumor cell lines in vitro, the effect of this compound on A431 tumor xenograft model was studied. Daily oral administration with SKLB1206 between 12.5 mg/kg and 50 mg/kg for 18 days resulted in tumor inhibition in a dose-dependent manner, with 50 mg/kg exhibiting partial tumor regression and 12.5 mg/kg showing complete inhibition of the tumor growth (inhibition rate: ~100%) (Fig. 4 and Supplementary Fig. S3A). Additionally, the anti-tumor efficacy of SKLB1206 was further studied in LoVo tumor model, which has been recognized as a WT EGFR-driven model showing high basal EGFR phosphorylation (37, 38). SKLB1206 suppressed tumor growth when administrated to animals between 12.5 mg/kg (89% inhibition, day 21) and 50 mg/kg (105% inhibition, day 21) (Fig. 4). Finally, growth of the N87 gastric tumor, in which ErbB2 gene is amplified and overexpressed, was completely inhibited by SKLB1206 at doses of 25 mg/kg or higher (Fig. 4), suggesting that SKLB1206 was also active against ErbB2-overexpressing tumor xenograft model. All antitumor data generated from these six models are summarized in Supplementary Table S3. In all of the above tumor models, only minor weight loss was associated with the high dose (50 mg/kg) group for SKLB1206, which recovered with continued treatment. However, no significant weight loss was observed in other treatment groups compared with the control group.

**Mechanism of action of SKLB1206 in human tumor xenograft models**

To determine whether the observed activity in vivo is targeted specifically, immunohistochemistry was first done using tumor tissues isolated from HCC827
tumor model with EGFR activating mutation. In the HCC827 tumor model, we observed a significant decrease in the phosphorylation of EGFR isolated from SKLB1206-treated animals when compared with the control group (Fig. 5A). A significant reduction in the phosphorylation of AKT and ERK was also observed in the treatment group (Fig. 5A), indicating that EGFR-mediated downstream signaling pathway was potently inhibited by SKLB1206 \textit{in vivo}. These tumors exhibited reduced staining of Ki67, implying a significant reduction in proliferating cells in the tumor, and showed more TUNEL-positive cells with dark green fluorescent staining, indicating a significant increased apoptosis in the treatment group when compared with the control group (Fig. 5A and 5B).

To understand better the mechanism of SKLB1206-mediated antitumor efficacy in different tumor models, we also performed immunohistochemistry analysis in WT EGFR A431 tumor model. As depicted in Fig. 5C, SKLB1206 showed significant inhibition of EGFR and ErbB2 phosphorylation compared with the control group. A significant decrease in Ki67 expression was also observed in SKLB1206-treated group (Fig. 5C). In addition, the increased apoptosis observed in SKLB1206-treated groups compared with the control group was dose-dependent, with nearly 50% apoptotic index for the dose of 50 mg/kg (Supplementary Fig. S3B and S3C). Moreover, due to the potent inhibition of VEGFR2 tyrosine kinase and angiogenesis \textit{in vitro}, the effect of SKLB1206 on A431 tumor angiogenesis \textit{in vivo} was evaluated by immunohistochemical staining of the tumor blood vessel. We observed that SKLB1206-treated mice showed significantly decreased microvessel density
compared with the control group (Fig. 5D and Supplementary Fig. S3D), suggesting that the antitumor activity of SKLB1206 in A431 model should be, at least to some extent, attributed to its inhibition of angiogenesis. Taken together, these results clearly demonstrate that SKLB1206 inhibits specific molecular targets in vivo to block proliferation, induce apoptosis and inhibit tumor angiogenesis in human tumor xenograft model.

**Pharmacokinetic characteristics of SKLB1206**

To account better for the antitumor activity observed in the human tumor xenograft models, pharmacokinetic parameters of SKLB1206 following IV and PO administration to male rats were measured, which results are summarized in Supplementary Table S4. The plasma concentration versus time profile of SKLB1206 is shown in Supplementary Fig. S4. After IV at the dose of 20 mg/kg, SKLB1206 displayed a clearance of 38.7 mL/min/kg, with a T_{1/2} of 5.7 h. The V_{ss} (18.4 L/kg) was much larger than the volume of total body water (0.67 L/kg), suggesting extravascular distribution. Following oral administration at a single dose of 60 mg/kg, the absorption of SKLB1206 was relatively quick, which reach the maximum concentration in the plasma (C_{max}, 1.05 μg/ml) at 2-6 h. The absolute oral bioavailability of SKLB1206 was 50.1% after an oral dose of 60 mg/kg.

**Discussion**

Although numerous potential anti-cancer targets have been reported, EGFR is one of the few targets that have been confirmed clinically. Unfortunately, despite the
benefits of EGFR inhibitors gefitinib and erlotinib in clinical practice, most, if not all, patients ultimately develop acquired resistance to these agents (7, 39). The gatekeeper T790M mutation in EGFR, like the analogous T315I mutation in ABL, is one of the most difficult drug-resistant mutations to overcome at present (40-42). In recent years, irreversible EGFR inhibitors have been suggested to combat this type of drug-resistance; the irreversible EGFR inhibitors carry a Michael acceptor functional group and irreversibly alkylate a cysteine (C797) in the ATP binding site of EGFR. However, most of the quinazoline-based irreversible inhibitors have thus far achieved limited success, which may be related to their inherent weaknesses including relatively large toxicity and decreased binding velocity to the mutant kinase (17). SKLB1206 reported here is a reversible EGFR inhibitor. SKLB1206 not only targets the EGFR-activating mutations, but also inhibits efficiently EGFR T790M mutation with IC_{50} = 0.046 μM. In addition, SKLB1206 also inhibits other related protein kinases, including ErbB2 (IC_{50}: 0.9 μM), ErbB4 (IC_{50}: 0.3 μM), and VEGFR2 (IC_{50}: 0.03 μM). This multiple target inhibition is expected to be able to lead to the enhancement of the efficacy of SKLB1206 through additive and/or synergistic effect. And it also could help to overcome the drug-resistance. In the cell line-based assay, SKLB1206 indeed displayed much higher proliferative inhibition potency to HCC827 and PC-9 than gefitinib, although enzymatic inhibition potency of SKLB1206 is comparable with that of gefitinib. The proliferative inhibition to cell lines harboring T790M mutation (H1975 and H820), and overexpressing ErbB2 (BT474), suggests that SKLB1206 has an ability to overcome the drug-resistance (both acquired and de
Anti-angiogenesis is another promising approach to anti-cancer therapy (25). Angiogenesis is a complex process including endothelial cell proliferation, migration, invasion, and tube formation (43). VEGFR2 is a major mediator of angiogenesis through VEGF-induced signaling in endothelial cell (44). As SKLB1206 is also a potent inhibitor of VEGFR2 tyrosine kinase, we evaluated the effect of SKLB1206 on anti-angiogenesis by using various methods, including inhibition assays of proliferation, migration, invasion, and tube formation of HUVEC, as well as a zebrafish embryonic angiogenesis assay and an alginate-encapsulate tumor cell assay. All of these assays validated the anti-angiogenesis effect of SKLB1206.

Clinical studies have shown that sole use of drugs that directly target tumor cells might lead to acquired drug-resistance with a relatively large possibility (7, 39, 45). On the other hand, recent investigations have also indicated that sole anti-angiogenesis treatment could have an unintended effect of promoting tumor metastasis (46, 47). Nevertheless, combined anti-angiogenesis treatment with drugs directly attacking cancer cells might lead to beneficial clinical effects, which has been demonstrated by numerous investigations (26, 27, 29). SKLB1206 is such a compound with combined pan-ErbB/VEGF receptor inhibitory activities in the same molecule, implying improved antitumor efficacy and broadened application possibilities just like ZD6474 evaluated in xenograft models of EGFR inhibitor resistance (29).

Although SKLB1206 has shown good potency in vitro in both suppressing tumor
cells through inhibition of EGFR and related protein kinases, and anti-angiogenesis by
VEGFR2 inhibition, one still cannot exactly differentiate how much of the observed
in vivo effects of SKLB1206 are due to its tumor cell suppression and how much due
to anti-angiogenic effects. Nevertheless, some qualitative conclusions can still be
drawn from the experiment outcomes. To the tumor models with activating-mutant
EGFR including HCC827 and PC-9, the direct tumor suppression may play the
primary role on the anti-tumor efficacy from the observations that these two tumor
cell lines are highly sensitive to SKLB1206 with IC_{50} values at a very low nanomolar
level in the cell viability inhibition assay, and a low dose of 5 mg/kg can induce tumor
regression in the two tumor models. Furthermore, nearly complete inhibition of EGFR,
ERK and AKT phosphorylation in vivo along with reduced expression of Ki67 and
increased apoptosis in tumor cell after SKLB1206 treatment for only 3 days confirms
this speculation. In the wild type EGFR-overexpressing A431 tumor model, the direct
inhibition of tumor growth and the indirect inhibition of tumor angiogenesis may
jointly account for the anti-tumor efficacy, since both the anti-proliferation and
anti-angiogenesis effects can be observed (see Fig. 5C and 5D) in the shrunk tumor
with SKLB1206 treated. To the LoVo and N87 tumor models, a similar mechanism
like A431 can be reasonably expected since LoVo and N87 cell lines showed the same
level of sensitivity to SKLB1206 as A431. In the H1975 tumor model, although both
the anti-proliferation and anti-angiogenesis effects may contribute to the anti-tumor
potency, the anti-angiogenesis effect may play a more important role which can be
deduced from the following facts: (1) the plasma peak concentration (C_{max}) of
SKLB1206 is 2.37 μM (1.05 μg/mL), which is larger than the IC₅₀ value of SKLB1206 against H1975 cell line; (2) an IC₅₀ value of 1.1 μM of SKLB1206 against H1975 cell line indicates that H1975 is not so sensitive to SKLB1206; (3) a very similar anti-angiogenesis effect of SKLB1206 on the H1975 tumor model was observed by immunohistochemical analysis with anti-CD31 antibody (data not shown).

In conclusion, our studies show that SKLB1206 is a potent reversible kinase inhibitor of EGFR with not only gefitinib-sensitive mutations but also gefitinib-resistance (T790M) mutation. In addition, SKLB1206 also potently inhibits several kinase targets that are closely associated with tumor growth and angiogenesis, as well as drug-resistance. Indeed, SKLB1206 displayed improved anti-tumor efficacy compared with gefitinib both in vitro and in vivo. The broader spectrum of anti-tumor, especially the ability of overcoming the drug-resistance, together with the good pharmacokinetic properties establishes a favorable preclinical profile of SKLB1206, which may support SKLB1206 as a good candidate for clinical studies in cancer patients with EGFR mutations or overexpressed.
Acknowledgments

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Table 1  IC₅₀ values (μM) for inhibition of cell growth by 72h treatment with SKLB1206 or Gefitinib

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Cell line</th>
<th>Characteristics</th>
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<th>Gefitinib</th>
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<td>NSCLC</td>
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Figure Legends

Figure 1. Inhibition of tumor cell growth and colony formation by SKLB1206 in vitro. A, chemical structure of SKLB1206 and Gefitinib. B, cells were treated with SKLB1206 or gefitinib for 72 h and cell viability was determined by MTT assay. Every experiment was carried out in triplicate. Points, mean values; bars, SD. C, HCC827 cells were incubated with gefitinib or SKLB1206 for ten days and stained by crystal violet.

Figure 2. Inhibition of ErbB receptor autophosphorylation and inactivation of downstream signaling proteins in cell cultures by SKLB1206. A, HCC827 cells were treated with SKLB1206 or gefitinib for 6 h. Cells were lysed and the proteins were analyzed by Western blot. B, Serum-starved A431 cells were treated for 3 h with the SKLB1206 or gefitinib, followed by the addition of EGF (100 ng/ml) for 15 min. Protein extracts were analyzed by Western blot. C, BT474 cells were incubated with SKLB1206 or gefitinib for 6 h and then the proteins were analyzed by western blot.

Figure 3. Anti-angiogenesis effect of SKLB1206. A, upper, HUVECs were treated with SKLB1206 and migration assays were performed as described in Materials and Methods. Scale bars represent 100 μM. Middle, the bottom chamber of the transwell was filled with M200 complete media whereas the Matrigel layer of the top chambers were seeded with HUVECs in the presence or absence of SKLB1206. Invasion was allowed to proceed for 24 h. Scale bars represent 50 μM. Lower, HUVECs (2×10⁴)
and SKLB1206 were added on Matrigel layers. After 6 h of incubation, HUVEC tube-like formation was assessed with an inverted photomicroscope. Scale bars represent 100 μM. B, fluorescent images of 15 h postfertilization zebrafish treated overnight with 0.1% DMSO (vehicle control) or indicated concentrations of SKLB1206. Sunitinib as a positive control was also included. Scale bars represent 200 μM (left) and 50 μM (right). C, vascularization of alginate implants. SKLB1206 was administrated p.o. once daily for 12 days after injection of the alginate beads. On study day 12, beads were surgically removed and FITC-dextran was quantified as described in Materials and Methods. Column, mean; bars, SD (n=6; *, P<0.05 versus the control, ANOVA).

**Figure 4.** *In vivo* antitumor efficacy of SKLB1206 against HCC827, PC-9, H1975, A431, LoVo, and N87 tumor xenograft models. Animals were given SKLB1206 or gefitinib p.o. once daily (qd) at the indicated dose levels or vehicle alone over the designated treatment schedule when tumors reached the determined size. In all models, the animal weight and tumor volume were monitored twice weekly. Tumor volume was measured using Vernier calipers on the indicated days. Points, mean tumor volume (mm$^3$); bars, SD.

**Figure 5.** Mechanism of action of SKLB1206 in human tumor xenograft models. A and B, animals bearing HCC827 tumor xenograft were treated with SKLB1206 at 20 mg/kg or vehicle for 3 days. At the study day 3 at 4 h postadministration of
SKLB1206, mice were humanly euthanized, and tumors were isolated for further immunohistochemical analysis and TUNEL detection. Scale bars for A and B represent 50 μM and 100 μM, respectively. C, A431 tumors from mice treated with vehicle or SKLB1206 (50 mg/kg) were harvested on study day 6. The tissues were processed for immunohistochemistry analysis. Scale bars represent 50 μM. D, frozen sections of A431 tumors were tested by immunohistochemical analysis with anti-CD31 antibody. Tumors were established (200 mm³) before 9 days of continuous therapy with vehicle or SKLB1206 at the dose of 25 mg/kg. Scale bars represent 50 μM.
Figure 1
## Figure 2

### A

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Figure 3

A

B

Control
SKLB1206 (1.25 μM)
SKLB1206 (2.5 μM)
SKLB1206 (5 μM)
Sunitinib (2.5 μM)

C

Control
SKLB1206 (25 mg/kg)

FITC-dextran (µg/head)
Figure 4
Figure 5

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SKLB1206, a novel orally available multi-kinase inhibitor targeting EGFR activating and T790M mutants, ErbB2, ErbB4 and VEGFR2, displays potent antitumor activity both in vitro and in vivo

Youli Pan, Yong Xu, Shan Feng, et al.

Mol Cancer Ther  Published OnlineFirst February 8, 2012.

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