SKLB1206, a Novel Orally Available Multikinase 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 oncokinases, including ErbB2, ErbB4, and VEGF receptor 2 (VEGFR2). SKLB1206 exhibited highly antiproliferative 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 antiangiogenesis effect in vitro, in a zebrafish embryonic angiogenesis assay, and in an alginate-encapsulate tumor cell assay. In vivo, oral administration of SKLB1206 showed 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, and 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-molecule 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; refs. 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% to 20% of patients with NSCLC, whose tumor cells harbor EGFR mutations that occur in either exon 19 characterized by in-frame deletions of amino acids 747 to 750 or exon 21 resulting in L858R substitutions. Unfortunately, NSCLCs with drug-sensitive EGFR mutations that initially respond to gefitinib or erlotinib eventually develop acquired resistance. Approximately, half of the 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 (RTK), 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 are 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 than irreversible counterparts. Second, concomitant inhibition of other RTKs that are tightly associated with EGFR has been shown 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. Third, additional antiangiogenesis activity would help to accentuate the antitumor effects of EGFR inhibitors because 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 VEGF 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 anticancer 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 using 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 -resistant (T790M) mutations. In addition, SKLB1206 has also considerable inhibition activity against some other related oncokinases, 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 reagents

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 VEGF165 and basic fibroblast growth factor were obtained from PeproTech Company. SKLB1206 was synthesized at the State Key Laboratory of Biotherapy, Sichuan University, Sichuan, China. Gefitinib, sunitinib, and BIBW2992 were obtained from commercial sources. For all in vitro assays and zebrafish studies, a 10 mmol/L stock solution of each compound was prepared in dimethyl sulfoxide (DMSO) and diluted in optimal assay buffers or culture medium.

Kinase inhibition assay

The IC50 values of SKLB1206 for kinase inhibition in vitro were measured by the use of radiometric assays conducted by Kinase Profiler service provided by Millipore as described in detail in the Supplementary Methods.

Cell proliferation assay

Cell proliferation assay was conducted as previously described (29). The IC50 values were calculated by GraphPad Prism 5.01 software (GraphPad Software Inc.).

Colony formation assay

HCC827 cells were seeded in 6-well plate at a density of 2,000 per well. These cells were treated with 0.001 μmol/L of SKLB1206 or gefitinib the next day. After treatment for 10 days, cells were stained with crystal violet for 10 minutes.

Western blot analysis

Cells lysates were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore). All antibodies were purchased from Cell Signaling Technology. Specific proteins were detected using 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 conducted 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 conducted 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 the work of Kimmel and colleagues (34). After 15 hours 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.).

Subcutaneous xenograft models

All animal experiments carried out were approved by the Animal Care and Use Committee of Sichuan University. Tumor xenograft models were established by subcutaneously injecting 100 μL tumor cell suspension (between 5 × 10^6 and 1 × 10^7 cells) into the right flank of the animals. Mice were randomized into groups of 6 to 7 before treatment at a point when tumors reached a volume of 0.1 to 0.3 cm³. SKLB1206, gefitinib, or BBW2992 was suspended in 1% (v/v) solution of polyoxyethylene sorbitan monooleate (Tween-80) in deionized water. Animals were given SKLB1206 (2–50 mg/kg), gefitinib (100 mg/kg), BBW2992 (20 mg/kg), or vehicle once daily 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 is presented in Supplementary Methods.

Pharmacokinetic assessments

Blood from Sprague-Dawley 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/mass spectrometry.

Alginate-encapsulate tumor cell assay

The alginate-encapsulate tumor cell assay was conducted as described previously (35).

TUNEL detection

The analysis of apoptotic cells in the tumor tissue was conducted by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using an apoptotic cell detection kit (Roche). Images of the sections were taken by a fluorescence microscope. The apoptotic 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 eosin using standard methods. Immunohistochemical staining was conducted using antibodies from Cell Signaling Technology. CD31 (BD Bioscience) staining was conducted using frozen sections of tissue embedded in optimum cutting temperature (36).

Statistical analysis

SPSS 11.5 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by the Student t test and ANOVA. P 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 IC₅₀ values of 0.005, 0.005, and 0.046 μmol/L, respectively. This compound also inhibited ErbB2, ErbB4, and VEGFR2 with moderate activity (IC₅₀ values, 0.9, 0.3, and 0.03 μmol/L, respectively), but only weakly inhibited AXL, EPHB4, FLT3, and MERTK (IC₅₀ values, 1.5, 2.4, 2.7, and 2.5 μmol/L, respectively). SKLB1206 displayed almost no inhibition activity to other 44 selected protein kinases. All of these show 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 antiviability activity of SKLB1206 against various tumor cell lines was measured using MTT method (Table I and Supplementary Fig. S1). SKLB1206 displayed exceptionally potent inhibition activity against gefitinib-sensitive NSCLC cell lines HCC827 and PC-9 (IC₅₀ values, 0.0008 and 0.001 μmol/L, 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 (IC₅₀ values, 1.1 and 0.67 μmol/L, respectively). In cell lines, in which EGFR or ErbB2 is overexpressed and in which gefitinib showed moderate inhibition activity, including H292, Calu-3, BT474, FaDu, and N87, SKLB1206 displayed relatively higher potency than gefitinib. In those cell lines, in which EGFR or ErbB2 is overexpressed but gefitinib had minimal growth-inhibitory effect, including SK-BR-3, MDA-MB-468, LoVo, and A431, SKLB1206 showed moderate antiproliferative activity. In contrast, those KRAS-mutant cell lines including A549, H460, MDA-MB-231, SW480, and HCT116 were resistant to both SKLB1206 and gefitinib (IC₅₀ > 1 μmol/L). Similarly, in MCF-7 cell line containing phosphoinositide 3-kinase (PI3K) mutation and HepG2 cell line overexpressing Aurora B, both SKLB1206 and gefitinib displayed minimal growth-inhibitory effect (IC₅₀ > 4 μmol/L). These
data exclude the possibility that the antiviability 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 conducted to visually assess the antiviability activity of SKLB1206. Figure 1C presents the cell survival state of HCC827 after exposure to 0.001 μmol/L of SKLB1206. The same concentration of gefitinib was also used for the comparison. Obviously, SKLB1206 completely blocked the formation of colonies whereas 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 IC50 value of 0.003 μmol/L compared with gefitinib with an estimated IC50 value of 0.01 μmol/L (Fig. 2A). This was accompanied by corresponding inhibition of the downstream targets, AKT and extracellular signal–regulated kinase (ERK). Furthermore, in EGF-stimulated A431 cell line, SKLB1206 inhibited EGF-dependent phosphorylation of EGFR and AKT with IC50 value of 0.1 μmol/L 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 μmol/L, still superior to gefitinib (Fig. 2C).
Antiangiogenesis effect of SKLB1206

To evaluate the antiangiogenesis effect of SKLB1206, the antiproliferative ability of SKLB1206 against human umbilical vein endothelial cells (HUVEC) was first assessed by MTT assay. SKLB1206 showed a good anti-proliferative activity against VEGF and EGF-stimulated HUVECs with IC\textsubscript{50} values of 0.102 and 0.310 \textmu mol/L, 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 \textmu mol/L of SKLB1206 (Fig. 3A and Supplementary Fig. S2B). In addition, in the Transwell assay assessing the invasion ability of HUVECs, 1 \textmu mol/L of SKLB1206 inhibited almost all invasion activities of HUVECs (Fig. 3A and Supplementary Fig. S2B). In addition, in the Transwell assay assessing the invasion ability of HUVECs, 1 \textmu mol/L 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. A solution of 0.625 \textmu mol/L of SKLB1206 inhibited tube formation of HUVECs by 50% and 2.5 \textmu mol/L 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.

To test the antiangiogenesis 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 interssegmental vessel at the concentration of 5 \textmu mol/L while preserving fluorescence in the dorsal aorta and major cranial vessels, and 2.5 \textmu mol/L of sunitinib showed the similar effect (Fig. 3B). In the presence of 1.25 or 2.5 \textmu mol/L of SKLB1206, the formation of interssegmental 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 conducted an alginate-encapsulate tumor cell assay. In SKLB1206-treated mice, new blood vessels in alginate beads were apparently reduced and fluorescein isothiocyanate (FITC)-dextran uptake was significantly decreased when compared with control group (Fig. 3C). Collectively, on the basis of 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 6 relevant human tumor xenograft models were used, including 2 gefitinib-sensitive NSCLCs, 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 Table 1.

<table>
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<tr>
<th>Cancer type</th>
<th>Cell line</th>
<th>Characteristics</th>
<th>SKLB1206</th>
<th>Gefitinib</th>
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<tr>
<td>NSCLC</td>
<td>HCC827</td>
<td>Del E746_A750 (EGFR mutant)</td>
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<td>KRAS mutation (G13D)</td>
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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 xeno-graft 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 after 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 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 wild-type 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 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). In addition, the antitumor efficacy of SKLB1206 was further studied in LoVo tumor model, which has been recognized as a wild-type 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 6 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 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, B).

To better understand the mechanism of SKLB1206-mediated antitumor efficacy in different tumor models, we also conducted immunohistochemical analysis in...
wild-type 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, because of the potent inhibition of VEGFR2 tyrosine kinase and angiogenesis in vitro, the effect of SKLB1206 on A431 tumor angiogenesis 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. 5C and Supplementary Fig. S3D), suggesting 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 show 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 intravenous and per os administration to male rats were measured, the results of which are summarized in Supplementary Table S4. The plasma concentration versus time profile of SKLB1206 is shown in Supplementary Fig. S4. After intravenous injection 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 hours. The steady-state volume ($V_{ss}$; 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 reached the maximum concentration in the plasma ($C_{max}$, 1.05 mg/ml) at 2 to 6 hours. The absolute oral bioavailability of SKLB1206 was 50.1% after an oral dose of 60 mg/kg.

Discussion

Although numerous potential anticancer 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.

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 orally 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.
SKLB1206 at the dose of 25 mg/kg. Scale bars represent 50 μm. DAPI, 4',6-diamidino-2-phenylindole.}

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 μmol/L. In addition, SKLB1206 also inhibits other related protein kinases, including ErbB2 (IC_{50}, 0.9 μmol/L), ErbB4 (IC_{50}, 0.3 μmol/L), and VEGFR2 (IC_{50}, 0.03 μmol/L). 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 novo).

Antiangiogenesis is another promising approach to anticancer therapy (25). Angiogenesis is a complex process involving 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 antiangiogenesis 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 antiangiogenesis 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 antiangiogenesis treatment could have an unintended effect of promoting tumor metastasis (46, 47). Nevertheless, combined antiangiogenesis treatment with drugs directly attacking cancer cells might lead to beneficial clinical effects, which has been shown 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 anti-tumor 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 antiangiogenesis 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 antiangiogenic effects. Nevertheless, some qualitative conclusions can still be drawn from the experiment outcomes. To the tumor models with EGFR-activating mutant including HCC827 and PC-9, the direct tumor suppression may play the primary role on the antitumor efficacy from the observations that these 2 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 2 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 antitumor efficacy because both the antiproliferation and antiangiogenesis effects can be observed (see Fig. 5C and D) in the shrunk tumor with SKLB1206 treated. To the LoVo and N87 tumor models, a similar mechanism like A431 can be reasonably expected because LoVo and N87 cell lines showed the same level of sensitivity to SKLB1206 as A431. In the H1975 tumor model, although both the antiproliferation and antiangiogenesis effects may contribute to the antitumor potency, the antiangiogenesis effect may play a more important role, which can be deduced from the following facts: (i) the plasma peak concentration (C_max) of SKLB1206 is 2.37 μmol/L (1.05 μg/mL), which is larger than the IC50 value of SKLB1206 against H1975 cell line; (ii) an IC50 value of 1.1 μmol/L of SKLB1206 against H1975 cell line indicates that H1975 is not so sensitive to SKLB1206; and (iii) a very similar antiangiogenesis 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-resistant (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 antitumor, 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 patients with cancer with EGFR mutations or overexpressed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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