Preclinical Development

CEP-28122, a Highly Potent and Selective Orally Active Inhibitor of Anaplastic Lymphoma Kinase with Antitumor Activity in Experimental Models of Human Cancers

Mangeng Cheng1, Matthew R. Quail1, Diane E. Gingrich1, Gregory R. Ott1, Lihui Lu1, Weihua Wan1, Mark S. Alborn1, Thelma S. Angeles1, Lisa D. Aimone1, Flavio Cristofani2, Rodolfo Machiorlatti2, Cristina Abele2, Mark A. Ator1, Bruce D. Dorsey1, Giorgio Inghirami2, and Bruce A. Ruggeri1

Abstract

Anaplastic lymphoma kinase (ALK) is constitutively activated in a number of human cancer types due to chromosomal translocations, point mutations, and gene amplification and has emerged as an excellent molecular target for cancer therapy. Here we report the identification and preclinical characterization of CEP-28122, a highly potent and selective orally active ALK inhibitor. CEP-28122 is a potent inhibitor of recombinant ALK activity and cellular ALK tyrosine phosphorylation. It induced concentration-dependent growth inhibition/cytotoxicity of ALK-positive anaplastic large-cell lymphoma (ALCL), non-small cell lung cancer (NSCLC), and neuroblastoma cells, and displayed dose-dependent inhibition of ALK tyrosine phosphorylation in tumor xenografts in mice, with substantial target inhibition (>90%) for more than 12 hours following single oral dosing at 30 mg/kg. Dose-dependent antitumor activity was observed in ALK-positive ALCL, NSCLC, and neuroblastoma tumor xenografts in mice administered CEP-28122 orally, with complete/near complete tumor regressions observed following treatment at doses of 30 mg/kg twice daily or higher. Treatment of mice bearing Sup-M2 tumor xenografts for 4 weeks and primary human ALCL tumor grafts for 2 weeks at 55 or 100 mg/kg twice daily led to sustained tumor regression in all mice, with no tumor reemergence for more than 60 days postcessation of treatment. Conversely, CEP-28122 displayed marginal antitumor activity against ALK-negative human tumor xenografts under the same dosing regimens. Administration of CEP-28122 was well tolerated in mice and rats. In summary, CEP-28122 is a highly potent and selective orally active ALK inhibitor with a favorable pharmaceutical and pharmacokinetic profile and robust and selective pharmacologic efficacy against ALK-positive human cancer cells and tumor xenograft models in mice. Mol Cancer Ther; 1–10. ©2011 AACR.

Introduction

Anaplastic lymphoma kinase (ALK) is an orphan receptor tyrosine kinase originally identified as part of the nucleophosmin (NPM)-ALK fusion gene in anaplastic large-cell lymphoma (ALCL) with a t(2;5) chromosomal translocation. ALK belongs to the insulin receptor tyrosine kinase superfamily and its expression is mainly restricted to the central and peripheral nervous systems, implicating a potential role in the physiologic development and function of the nervous system (1, 2). Although ALK knockout mice possess a full life span and have no overt abnormalities, behavioral and neurochemical alterations were observed, suggesting that ALK may function in the adult brain to regulate the function of the frontal cortex and hippocampus and may be a target for psychiatric indications, such as schizophrenia and depression (3).

Although the physiologic role of the ALK receptor has not been well defined, involvement of ALK in the oncogenesis of various human cancers has been well documented and characterized. Besides NPM-ALK, various other ALK fusion genes have been detected in ALCL, inflammatory myofibroblastic tumor, diffuse large B-cell lymphoma, systemic histiocytosis, and most notably, in non-small cell lung cancer (NSCLC), resulting in the generation of oncogenic ALK fusion proteins with constitutive phosphorylation/activation of ALK (1, 4, 5). Recently, it has also been reported that germline mutations in ALK are the cause of most hereditary neuroblastoma cases, and ALK activation by mutation and/or gene

References


Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Mangeng Cheng, In Vitro Pharmacology, Merck Research Laboratory, BMB11-138, 33 Avenue Louis Pasteur, Boston, MA 02115. Phone: 617-992-3080; Fax: 617-992-2487; E-mail: mangeng.cheng@merck.com

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amplification is functionally relevant in high-risk sporadic neuroblastoma (6–10). Collectively, these findings indicate that ALK is a potential major therapeutic target for specific human cancers.

Pharmacologic studies using small molecule ALK inhibitors have provided preclinical validation of inhibiting ALK kinase activity for the treatment of ALCL, NSCLC, and neuroblastoma (11–15). The recently reported marked clinical activity of the ALK and c-Met inhibitor, Xalkori (crizotinib), in clinical trials of EML4-ALK–positive NSCLC patients and ALK-positive ALCL and IMT patients has validated ALK as a therapeutic target for various ALK-positive human cancers (16–19).

Xalkori was recently granted accelerated approval by the U.S. Food and Drug Administration (FDA) for the treatment of patients with locally advanced or metastatic NSCLC that is ALK-positive as detected by an FDA-approved companion diagnostic test, on the basis of positive results from 2 single-arm trials (20). Because Xalkori was originally developed as a c-Met inhibitor, its in vitro and in vivo activity against ALK is modest (11), and several resistant mutations have recently been reported (21–23). Therefore, the development of more potent and selective ALK inhibitors is needed.

Here we report the identification and preclinical characterization of CEP-28122, a potent and selective orally active ALK inhibitor showing a favorable pharmaceutical and pharmacokinetic profile and robust and selective pharmacologic efficacy against ALK-positive versus ALK-negative human cancer cells in culture and tumor xenografts in mice.

Materials and Methods

Compound synthesis

CEP-28122 was synthesized in the department of Chemistry at Cephalon, Inc. The synthesis routes for CEP-28122 have been described elsewhere (Gingrich and colleagues; submitted manuscript). For all in vitro experiments with CEP-28122, either the free base or the mesylate-HCl salt with 97% or more purity was used.

Cell lines, antibodies, and reagents

The NPM-ALK–positive ALCL cell lines, Karpas-299 and Sup-M2, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The ALK-negative human leukemia Toledo, lymphoma HuT-102, colon carcinoma HCT-116, neuroblastoma NB-1691 (containing amplified MYCN), the EML4-ALK–positive NSCLC cell line NCI-H2228, and negative NSCLC NCI-H1650 were purchased from American Type Culture Collections. NCI-H3122 cells were provided by Dr. Roberto Polakiewicz of Cell Signaling Technology. NB-1 cells (with wild type ALK gene amplification) were purchased from Japan Health Sciences Foundation, Osaka, Japan. SH-SY5Y (containing the ALK activating mutation F1174L) and NB-1643 (containing the ALK-activating mutation R1275Q) cells were kindly provided by Dr. Mossé of Children Hospital of Philadelphia. All cell lines were cultured in RPMI medium (catalog no. 10-040; Mediatech Inc.) supplemented with 10% FBS (catalog no. SH3007003; Hyclone Laboratory Inc.). Upon arrival, each cell line was expanded and the cells were frozen down in multiple vials at earliest passage as possible. Each cell line was never continuously passaged in culture for more than 2 to 3 months, and after that, a new vial of frozen cells was thawed. The ALK gene status in each cell line (chromosomal translocation, point mutations, gene amplification, or wild type) was evaluated by reverse transcriptase PCR and sequencing, and ALK expression and phosphorylation in each cell line were tested by immunoblotting. No other authentication was done for these cell lines.

The rabbit phospho-NPM-ALK (Y664; catalog no. 3341), ALK antibody (catalog no. 3342), and the STAT3 and phospho-STAT3 (Y705) antibodies (catalog no. 9132 and catalog no. 9145) were purchased from Cell Signaling Technology, and the mouse ALK antibody (catalog no. 35-4300) was obtained from Invitrogen. ERK1/2 and phospho-ERK1/2 antibodies (catalog nos. SC-94 and SC-7383), and AKT and phospho-AKT antibodies (catalog nos. SC-8312 and SC-7985-R) were purchased from Santa Cruz Biotech.

The CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay Kit (catalog no. G5430) and the Apo-ONE Homogenous Caspase 3/7 Assay Kit (catalog no. G7791) were purchased from Promega.

Animals

Severe combined immunodeficient (SCID)/beige or nu/nu mice (6- to 8-week-old female) were maintained 5 per cage in microisolator units on a standard laboratory diet (Teklad Labchow). For primary human lymphoma studies, NOD.Cg-Pkdcrscl/l2rg[tm1Wjl]/Sj (NSG, Jackson Laboratories) mice were used. Animals were housed under humidity- and temperature-controlled conditions and the light/dark cycle was set at 12-hour intervals, maintained under specified and opportunistic pathogen-free conditions. Mice were quarantined at least 1 week before experimental manipulation. All animal studies were conducted under protocol approved by the Institutional Animal Care and Use Committee of Cephalon, Inc. or by University of Turin Ethical Committee.

Recombinant ALK kinase assay

An in vitro recombinant ALK kinase assay was carried out using a modification of the ELISA described for trkA (24). Briefly, 96-well microtiter plates were coated with 10 μg/mL of substrate (recombinant human PLC-γ/GST). The kinase reaction mixture consisting of 20 mmol/L HEPES, pH 7.2, 1 μmol/L ATP, 5 mmol/L MnCl₂, 0.1% bovine serum albumin (BSA), 2.5% dimethyl sulfoxide, and test compound (various concentrations) was added to the plate. Recombinant GST-ALK (30 ng/mL) was added and the reaction was allowed to proceed for 15 minutes at 37°C. Detection of the phosphorylated product was done by adding Eu-N1-labeled PT66 antibody (catalog no.
AD0041; PerkinElmer). Incubation at 37°C proceeded for 1 hour, followed by addition of enhancement solution (catalog no. 1244-105; PerkinElmer). Fluorescence was measured using the time-resolved fluorescence (TRF) protocol on the EnVision 2102 (or 2104) Multilabel Plate Reader (PerkinElmer). Data analysis was done using ActivityBase (IDBS). IC50 values were calculated by plotting percent inhibition versus log10 of the concentration of compound and fitting to the nonlinear regression sigmoidal dose–response (variable slope) equation in XLFit (IDBS).

**Immunoblot analysis**

Immunoblotting of phospho- and total ALK as well as the downstream targets was carried out according to the protocols provided by the antibody suppliers. In brief, after treatment, cells were lysed in FRAK lysis buffer [10 mmol/L Tris, pH 7.5, 1% Triton X-100, 50 mmol/L sodium chloride, 2 mmol/L sodium fluoride, 2 mmol/L sodium pyrophosphate, 0.1% BSA, plus freshly prepared 1 mmol/L activated sodium vanadate, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride, protease inhibitors cocktail III (1:100 dilution, catalog no. 539134; Calbiochem)]. After brief sonication, the lysates were cleared by centrifugation, mixed with sample buffer, and subjected to SDS-PAGE. Following transfer to membranes, the membranes were blotted with individual primary and secondary antibodies, washed in TBS/0.2% Tween, and protein bands visualized with Enhanced Chemiluminescence. The individual bands of phospho- and total NPM-ALK were scanned and quantified with the Gel-Pro Analyzer software (Media Cybernetics, Inc.).

**Cell growth inhibition and caspase activation 3/7 assay**

Living cells were measured with the CellTiter 96 nonradioactive cell proliferation assay (MTS assay) kit. In brief, the cells were seeded on 96-well plates and 48 to 72 hours after compound treatment, equal volume of reagents from the kit was added to the culture medium. After incubation for 1 to 4 hours, the plates were measured with a plate reader and the relative cell numbers were calculated on the basis of the standard curve.

Caspase 3/7 activity was measured with an Apo-one homogenous caspase 3/7 assay kit. Briefly, the cells seeded on 96-well plates were treated with compounds for 16 hours. The reaction reagents from the kit were added to the culture medium and after incubation, the plates were...
measured with a florescence plate reader for the relative caspase 3/7 activity.

**Pharmacodynamic studies**

Exponentially growing cells were implanted subcutaneously to the left flank of each mouse. The mice were monitored and when the tumor xenograft volumes reached approximately 300 to 500 mm$^3$, mice received a single oral administration of either vehicle PEG-400 or CEP-28122 formulated in vehicle. At indicated time points postdosing, the mice were sacrificed, the blood was collected and centrifuged, and the plasma was collected. The tumors were excised and disrupted with a hand-held tissue blender in completed FRAK lysis buffer without Triton X-100. After brief sonication, the lysates were cleared by centrifugation, mixed with sample buffer, and subjected to SDS-PAGE for ALK immunoblotting as described above. The individual bands of phospho-and total NPM-ALK were scanned and quantified with the Gel Pro Analyzer software (Media Cybernetics, Inc.). The relative NPM-ALK tyrosine phosphorylation (phospho-NPM-ALK/NPM-ALK ratio) of each sample was calculated, with the average value of vehicle-treated sample(s) as 100. The compound levels in plasma and tumor lysates were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS).

**Antitumor efficacy studies**

Tumor-bearing mice were randomized into different treatment groups (8–10 mice per group) and administered orally either vehicle (PEG-400, or dH$_2$O) or CEP-28122 formulated in vehicle at indicated doses (expressed as mg/kg equivalents of free base) and with indicated dosing frequency, with 100 mg/kg/dosage given in the table. The inhibitory activity of CEP-28122 was evaluated using the Millipore Kinase Profiler in which the relative NPM-ALK tyrosine phosphorylation (phospho-NPM-ALK/NPM-ALK ratio) of each sample was calculated, with the average value of vehicle-treated sample(s) as 100. The compound levels in plasma and tumor lysates were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS).

**Results and Discussion**

**Potent and selective ALK inhibitory activity of CEP-28122**

CEP-28122, a diaminopyrimidine derivative (Fig. 1A), is a potent, selective, and oral bioavailable ALK inhibitor. In an enzyme-based TRF assay, the IC$_{50}$ of CEP-28122 for recombinant ALK kinase activity was 1.9 ± 0.5 nmol/L (Table 1). The kinase selectivity of CEP-28122 was evaluated using the Millipore Kinase Profiler in which the inhibitory activity of CEP-28122 was assessed against a panel of 259 protein kinases. CEP-28122 exhibited no- to-weak inhibition against the majority of the kinases tested at 1 μmol/L and only 15 kinases showed more than 90% inhibition at 1 μmol/L (See Supplementary Table S1). Except for Rsk2, 3, and 4 (IC$_{50}$ values range 7–19 nmol/L), the IC$_{50}$ value for any other kinase is at least 10-fold higher than the IC$_{50}$ value for ALK (Table 1). These results suggested that CEP-28122 is a highly potent and selective ALK inhibitor.

In a cellular phosphorylation assay, treatment of NPM-ALK–positive ALCL cells, Sup-M2 and Karpas-299, with CEP-28122 led to concentration-related inhibition of NPM-ALK tyrosine (664) phosphorylation, with calculated cellular IC$_{50}$ values of 20 to 30 nmol/L (Fig. 1B). Phosphorylated Tyr664 of NPM-ALK (equivalent to Tyr1604 of full-length ALK) is required for the interaction with PLC$_\gamma$ and activation of PLC$_\gamma$ by NPM-ALK is a crucial step for its mitogenic activity and is important in the pathogenesis of anaplastic lymphomas (1).

### Table 1. Enzyme inhibitory activity of CEP-28122 on select protein kinases

<table>
<thead>
<tr>
<th>Tyr kinases</th>
<th>IC$_{50}$ (nmol/L)</th>
<th>Ser/Thr kinases</th>
<th>IC$_{50}$ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK$^a$</td>
<td>1.9 ± 0.5</td>
<td>Rsk3</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>Fit4</td>
<td>46 ± 10</td>
<td>Rsk2</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>Fer</td>
<td>84 ± 4</td>
<td>Rsk4</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>Fes</td>
<td>97 ± 35</td>
<td>ARK5</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Fit3</td>
<td>87 ± 35</td>
<td>CHK2</td>
<td>28 ± 7</td>
</tr>
<tr>
<td>FAK</td>
<td>130 ± 15</td>
<td>ACK1</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>TNK2$^a$</td>
<td>138 ± 53</td>
<td>GCK</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>IGF-1R$^a$</td>
<td>255 ± 61</td>
<td>Rsk1</td>
<td>59 ± 10</td>
</tr>
<tr>
<td>PYK2$^a$</td>
<td>414 ± 163</td>
<td>BRK</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>IR$^a$</td>
<td>1,257 ± 492</td>
<td>JNK1α1</td>
<td>109 ± 17</td>
</tr>
<tr>
<td>TYK2$^a$</td>
<td>1,486 ± 503</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK2$^a$</td>
<td>2,037 ± 539</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABL$^a$</td>
<td>&gt;3,000</td>
<td></td>
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<tr>
<td>JAK1$^a$</td>
<td>&gt;3,000</td>
<td></td>
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<tr>
<td>JAK3$^a$</td>
<td>&gt;3,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRC$^a$</td>
<td>&gt;3,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Met$^a$</td>
<td>&gt;10,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The kinase selectivity of CEP-28122 was evaluated using the Millipore Kinase Profiler, a radiometric assay format in which peptide substrates are phosphorylated with [$\gamma$-32P]ATP by the test kinase and the product and substrate are separated by filtration. The inhibitory activity of CEP-28122 against 259 kinases was measured at 1 μmol/L (see Supplementary Table S1). For those kinases inhibited by 90% or more, the IC$_{50}$ values were determined and reported in the table.

$^a$The IC$_{50}$ values for additional protein kinases were generated in house using TRF-based assays similar to that used for ALK (described in Materials and Methods).

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tyrosine phosphorylation in neuroblastoma cell line NB-1 cells, in a concentration-dependent manner with similar potency (Fig. 1C). ALK inhibition in human cancer cells resulted in substantial suppression of phosphorylation of putative downstream effectors of ALK, including Stat-3, Akt, and ERK1/2 in Sup-M2 cells (Fig. 1D), and Akt and ERK1/2 but not Stat-3 in NB-1 cells (Supplementary Fig. S1), indicating that the downstream signaling pathways mediated by individual ALK fusion protein or ALK receptor could vary among different types of cancers. In contrast, no such effects were observed in ALK-negative human cancer cell lines treated with CEP-28122 (data not shown). These data further supported the conclusion that CEP-28122 is a selective ALK inhibitor.

**ALK inhibition-dependent cytotoxicity in vitro**

Treatment with CEP-28122 led to concentration-dependent (3–3,000 nmol/L) growth inhibition of NPM-ALK–positive Karpas-299 and Sup-M2 cells in culture, associated with concentration-related caspase 3/7 activation (Fig. 2A). The activity of growth inhibition and caspase activation are consistent with the cellular inhibition of NPM-ALK phosphorylation. In contrast, CEP-28122 had no-to-marginal growth inhibition and did not induce significant caspase 3/7 activation in ALK-negative leukemia Toledo and lymphoma HuT-102 cells at concentrations up to 3,000 nmol/L (Fig. 2A).

Similarly, treatment with CEP-28122 resulted in a concentration-dependent (3–3,000 nmol/L) growth inhibition of EML4-ALK–positive NCI-H2228 and H3122) and EML4-ALK–negative (NCI-H1650) NSCLC cells (B) and neuroblastoma cell lines (C) seeded in 96-well cell culture plates were treated with CEP-28122 for 72 hours and the living cells were measured with CellTiter 96 AQ<sub>−</sub>non-Radioactive Cell Proliferation Assay as described in Materials and Methods. Relative living cell numbers of each sample as compared with vehicle-treated samples were then calculated and reported as the average ± SEM from 2 to 3 individual experiments.

CEP-28122 induced significant growth inhibition of human neuroblastoma cell lines with detectable activated ALK receptor, such as NB-1 cells with gene-amplified WT ALK receptor, SH-SY5Y, and NB-1643 cells with the activating mutations of ALK receptor, L1174L and R1275Q, respectively. In contrast, CEEP-28122 had no significant effects on the growth and survival of NB-1691 cells, a MYCN-amplified chemoresistant neuroblastoma cell line.
with WT ALK receptor gene (25), in which no ALK expression and phosphorylation can be detected (therefore defined as an ALK-negative neuroblastoma cell line).

These data suggested that at the concentrations tested, CEP-28122 exerts growth inhibition and cytotoxicity on ALK-positive human cancer cells mainly through inhibiting ALK kinase activity.

**Pharmacodynamics and antitumor efficacy in vivo**

Dose-dependent inhibition (in terms of both the degree and duration of inhibition) of NPM-ALK tyrosine (664) phosphorylation in NPM-ALK–positive ALCL subcutaneous tumor xenografts in SCID mice was detected following oral administration of CEP-28122. A single oral dose of CEP-28122 at 3 mg/kg led to approximately 75% to 80% inhibition of NPM-ALK phosphorylation extending to 12 hours postadministration and 10 mg/kg led to near complete inhibition of NPM-ALK phosphorylation up to 6 hours, with 75% to 80% inhibition observed at 12 hours postdose. Complete target inhibition was observed extending up to 12 hours post single oral dose administration at 30 mg/kg (Fig. 3A). The degree of NPM-ALK phosphorylation inhibition was consistent with CEP-28122 levels in tumor xenografts based on the calculated cellular activity of CEP-28122 in murine plasma, suggesting that the target inhibition in tumor xenografts was likely due to direct inhibitory effects exerted by CEP-28122.

Consistent with the above pharmacodynamic data, oral administration of CEP-28122 twice daily produced dose-dependent antitumor activity in Sup-M2 subcutaneous tumor xenografts, with minimal antitumor activity at 3 mg/kg, tumor stasis observed following 12-day treatment with 10 mg/kg CEP-28122, and complete/near complete tumor regression (defined as the tumor volume at the end of treatment less than 5% of the original tumor volume) following 12 days of treatment with 30 mg/kg CEP-28122 (Fig. 3B). CEP-28122 was well tolerated with no overt toxicity and no significant compound-related body weight loss of mice (data not shown).

In contrast, CEP-28122 had no antitumor activity on the growth of ALK-negative HCT-116 human colon carcinoma xenografts in mice at 10 and 30 mg/kg, per oral dose twice daily (Fig. 3C), suggesting that at these doses, the antitumor activity of CEP-28122 in NPM-ALK–positive Sup-M2 tumor models is due to sustained NPM-ALK inhibition in tumors.

However, it was not clear from the short-term (12-day) studies whether the tumor regression was permanent or
the tumors would reemerge upon cessation of CEP-28122 administration. To address this question, longer duration of treatment and follow-up observations were carried out in Sup-M2 subcutaneous tumor xenograft models in SCID mice. Mice bearing Sup-M2 subcutaneous tumor xenografts were treated with 55 or 100 mg/kg twice daily per oral CEP-28122 for 4 weeks. By the end of the dosing, complete tumor regression was observed in 100% of mice. Except for the vehicle-treated group, the mice were observed for 60 more days after the cessation of CEP-28122. In both the 55 and 100 mg/kg CEP-28122–treated groups, sustained tumor regression was observed in 100% of the mice, with no tumor reemergence in any mouse up to 60 days postcessation of CEP-28122 (Fig. 3D), suggesting that the Sup-M2 subcutaneous tumor xenografts in mice were completely eradicated after 4 weeks of treatment with CEP-28122 at 55 or 100 mg/kg.

CEP-28122 displayed dose-related antitumor activity in mice bearing EML4-ALK-positive NSCLC NCI-H2228 and NCI-H3122 subcutaneous tumor xenografts when administered orally at 30 and 55 mg/kg, twice daily (Fig. 4A). For NCI-H2228 tumor xenograft models, treatment of CEP-28122 at 30 and 55 mg/kg per oral, twice daily for 12 days resulted in tumor regression. For NCI-H3122 tumor xenograft models, treatment of CEP-28122 per oral, twice daily for 12 days at 30 mg/kg resulted in significant tumor growth inhibition and at 55 mg/kg led to tumor stasis and partial tumor regression. The superior antitumor activity observed in NCI-H2228 tumor xenografts was likely due to the higher tumor distribution of CEP-28122 (data not shown). In contrast, no antitumor activity was observed on the growth of the EML4-ALK–negative NSCLC, NCI-H1650, tumor xenografts in mice with the same dosing regimens (Fig. 4A).

Administration of CEP-28122 at 30 and 55 mg/kg twice daily orally for 14 days led to significant antitumor activity with tumor stasis and partial tumor regressions observed in NB-1 subcutaneous tumor xenografts.
The antitumor efficacy of CEP-28122 on human primary NPM-ALK–positive ALCL tumor grafts were also evaluated and compared with that of conventional chemotherapeutical agents. The transplanted primary tumors partially responded to doxorubicin (10 mg/kg, i.v.), resulting in partial tumor regression. However, the treatment of doxorubicin was associated with overt toxicity (Fig. 5A). Treatment of primary tumor grafts with CEP-28122, 100 mg/kg, led to tumor regression with 1 to 2 days and complete tumor regressions in all mice after 2 weeks of treatment, regardless of the original tumor size (Fig. 5B). No reemerging tumor was observed in any mouse up to 30 days (Fig. 5B) and even 60 days (not shown) postcessation of CEP-28122 dosing, suggesting that the transplanted human primary ALCL tumor grafts in mice were completely eradicated after only 2 weeks of treatment with CEP-28122. Of note, the tumors were equally sensitive to CEP-28122 treatment after initial treatment of doxorubicin (Fig. 5A). In contrast to doxorubicin, CEP-28122 treatment was well tolerated with no overt toxicity.

Xalkori is the most advanced small molecule ALK inhibitor recently being granted accelerated approval by FDA for the treatment of patients with locally advanced or metastatic ALK-positive NSCLC. Although marked activity of Xalkori has been observed in clinical trials of various ALK-positive human cancers, some patients do develop resistance and several drug-resistant mutations have been reported (21–23).

Currently, most efforts to combat resistance to crizotinib have been focused on targeting the gatekeeper mutation, because it is one of the most frequently reported mutants commonly associated with kinase inhibitor resistance, including Xalkori (23, 26–28). For example, CH5424802 was reported to be a potent, selective, and orally available ALK inhibitor with potent
activity against ALK gatekeeper mutation L1196M and blocking EML4-ALK-L1196M–driven cell growth and recently entered phase I clinical trials (26). At this moment, the clinical efficacy of newly developed ALK inhibitors targeting the gatekeeper mutant L1196M of ALK still remains to be determined. Moreover, preclinical activity may not always translate into clinical efficacy. For example, irreversible epidermal growth factor receptor (EGFR) kinase inhibitors, such as neratinib (HKI-272), display potent activity against the gatekeeper mutations of EGFR; but to date, limited clinical efficacy has been achieved with these inhibitors (29). The poor clinical result could be due to dose-limiting toxicities associated with wild-type EGFR inhibition, but also reflect the fact that most resistant tumors are heterogeneous and other factors, besides the gatekeeper mutation, could coexist and contribute to the resistance phenotype (30). Indeed, other ALK secondary mutations and alternative pathway activation have been reported for lung cancer cells resistant to crizotinib (22, 23, 31). In these cases, an inhibitor specifically targeting the gatekeeper mutant may not be sufficient to overcome kinase inhibitor resistance. The in vitro and in vivo activity of Xalkori against ALK is modest (11, 32). In our own experiments, a single oral dose of Xalkori at 30 mg/kg achieved only 65% to 70% target inhibition in Karpas-299 tumor xenografts, despite 5 to 6 μmol/L (2,737 ng/g) compound levels detected in tumor xenografts (Supplementary Fig. S2). Therefore, it is expected that high dose and extremely high tissue levels of Xalkori are required to achieve substantial target inhibition leading to clinical efficacy. Indeed, administration of Xalkori at 25 mg/kg had no significant antitumor efficacy and at 50 mg/kg led to only partial regression of Karpas-299 tumor xenografts in mice. Although Xalkori dosed at 100 mg/kg led to complete tumor regression, some of the tumors did reemerge after cessation of treatment (11). We have observed in experimental tumor models that constant maintenance of a high degree of target inhibition in tumors is critical to maintain efficacy and suppress the development of resistance. Therefore, in addition to developing new ALK inhibitors active against the resistant mutants, another strategy may be to develop highly potent and selective ALK inhibitors, with more favorable pharmaceutical properties capable of providing a wider therapeutic window. These types of ALK inhibitors may not only provide greater efficacy in the clinic but also likely decrease the incidence of development of resistance by tumors. CEP-28122 dosed at 3 mg/kg resulted in 75% to 80% inhibition of NPM-ALK phosphorylation in tumor xenografts, with compounds levels in tumors of about 0.5 μmol/L (257 ng/g; see Fig. 3A), suggesting CEP-28122 is at least 10 times more potent in term of inhibition of ALK phosphorylation in tumors than Xalkori. More importantly, CEP-28122 dosed at 55 mg/kg or higher orally led to sustained complete tumor regression with no reemerging tumor observed after cessation of treatment.

In conclusion, CEP-28122 is a potent and selective orally active ALK inhibitor showing a favorable pharmaceutical and pharmacokinetic profile and robust and selective pharmacologic efficacy against ALK-positive human cancer cells in culture and tumor xenografts in mice, without significant effect on ALK-negative cells and tumors in those assays. CEP-28122 is highly selective for ALK among various types of tyrosine kinases, including insulin receptor (IR), insulin-like growth factor I-receptor (IGF-IR) and c-Met. Because ALK expression in normal adult tissues is very limited, a selective ALK inhibitor such as CEP-28122 would be expected to exhibit a wide therapeutic window in patients with ALK-activated cancers by providing a much higher exposure than that of the efficacious dose.

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

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