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

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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 ALCL, NSCLC and neuroblastoma cells, and displayed dose-dependent inhibition of ALK tyrosine phosphorylation in tumor xenografts in mice, with substantial target inhibition (>90%) for > 12 h following single oral dosing at 30 mg/kg. Dose-dependent anti-tumor 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 b.i.d. or higher. Treatment of mice bearing Sup-M2 tumor xenografts for 4 weeks and primary human ALCL tumorgrafts for 2 weeks at 55 or 100 mg/kg b.i.d. led to sustained tumor regression in all mice, with no tumor re-emergence for > 60 days post cessation of treatment. Conversely, CEP-28122 displayed marginal anti-tumor 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
pharmacological efficacy against ALK-positive human cancer cells and tumor xenograft models in mice.
Introduction

ALK is an orphan receptor tyrosine kinase originally identified as part of the nucleophosmin (NPM)-ALK fusion gene in 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 physiological 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). While the physiological 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 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.

Pharmacological 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, 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).

Crizotinib was recently granted accelerated approval by 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, based on the positive results from two single-arm trials (20). Since crizotinib 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 demonstrating a favorable pharmaceutical and pharmacokinetic profile and robust and selective pharmacological 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. (West Chester, PA). The synthesis routes for CEP-28122 have been described elsewhere (24). For all in vivo experiments with CEP-28122, either the free base or the mesylate-HCl salt with ≥97% purity was used.

Cell lines, Antibodies and Reagents

The NPM-ALK-positive ALCL cell lines, Karpas-299 and Sup-M2, were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Heidelberg, Germany). The ALK-negative human leukemia Toledo, lymphoma HuT-102, colon carcinoma HCT-116, neuroblastoma NB-1691 (containing amplified MYCN), and the EML4-ALK-positive NSCLC cell lines, NCI-H2228, NCI-H3122 and negative NSCLC NCI-H1650 were purchased from American Type Culture Collections (ATCC, Manassas, VA). 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 (Cat# 10-040, Mediatech Inc, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Cat# SH3007003, Hyclone Laboratory Inc, Logan, UT). 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 2-3 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 RT-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) (Cat# 3341), ALK antibody (Cat# 3342), and the STAT3 and phospho-STAT3 (Y705) antibodies (Cat# 9132 and Cat# 9145) were purchased from Cell Signaling Technology (Beverly, MA), and the mouse ALK antibody (Cat# 35-4300) was obtained from Invitrogen. ERK1/2 and phospho-ERK1/2 antibodies (Cat# SC-94 and SC-7383), and AKT and phospho-AKT antibodies (Cat# SC-8312 and SC-7985-R) were purchased from Santa Cruz Biotech (Santa Cruz, CA).

The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Cat# G5430) and the Apo-ONE® Homogenous Caspase 3/7 Assay kit (Cat# G7791) were purchased from Promega (Madison, WI).

**Animals**

Scid/Beige or nu/nu mice (6-8 week-old female) were maintained 5/cage in microisolator units on a standard laboratory diet (Teklad Labchow, Harlan Teklad, Madison, WI). For primary human lymphoma studies NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG, Jackson Laboratories, Bar Harbor, Maine, MA) 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 one week prior to experimental manipulation. All animal studies were conducted under protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Cephalon, Inc. or by University of Turin Ethical Committee.

**Recombinant ALK kinase assay:**
An in vitro recombinant ALK kinase assay was performed using a modification of the ELISA described for trkA (25). 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 mM Hepes, pH 7.2, 1 μM ATP, 5 mM MnCl₂, 0.1% BSA, 2.5% DMSO, 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 min at 37°C. Detection of the phosphorylated product was performed by adding Eu-N1 labeled PT66 antibody (Cat# AD0041, PerkinElmer, Waltham, MA). Incubation at 37°C proceeded for 1 hour, followed by addition of enhancement solution (Cat# 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 performed using ActivityBase (IDBS, Guilford, UK). 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, Guilford, UK).

**Immunoblot analysis**

Immunoblotting of phospho- and total ALK, and 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 mM Tris, pH 7.5, 1% Triton X-100, 50 mM sodium chloride, 20 mM sodium fluoride, 2 mM sodium pyrophosphate, 0.1% BSA, plus freshly prepared 1 mM activated sodium vanadate, 1 mM DTT, and 1 mM PMSF, protease inhibitors cocktail III (1:100 dilution, Cat# 539134, Calbiochem, La Jolla, CA)]. 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, Bethesda, MD).

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

Living cells were measured with the Celltiter 96 non-radioactive cell proliferation assay (MTS assay) kit. In brief, the cells were seeded on 96 well plates and 48 hrs after compound treatment, equal volume of reagents from the kit were added to the culture medium. After incubation for 1-4 hours, the plates were measured with a plate reader and the relative cell numbers were calculated based on 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 24 hrs. 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 300-500 mm³, mice received a single oral administration of either vehicle PEG-400 or CEP-28122 formulated in vehicle. At indicated time points post dosing, the mice were sacrificed and 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, Bethesda, MD). The relative NPM-ALK tyrosine phosphorylation (phospho-NPM-ALK/NPM-ALK ratio) of each sample at indicated time points was then calculated, with the value of vehicle-treated sample as 100. The compound levels in plasma and tumor lysates were measured by LC-MS/MS.

**Anti-tumor efficacy studies**

Tumor-bearing mice were randomized into different treatment groups (8-10 mice/group) and administered orally either vehicle (PEG-400, or dH2O) or CEP-28122 formulated in vehicle at indicated doses (expressed as mg/kg equivalents of free base) and with indicated dosing frequency, with 100 μL per dosing volume. The length (L) and width (W) of each tumor was measured with a vernier caliper and the mouse body weight was determined every 2-3 days. The tumor volumes were then calculated with the formula of $0.5236 \times W \times (L+W)/2$. Statistical analyses of tumor volumes and mouse body weight were carried out using the Mann-Whitney Rank Sum Test. Plasma and tumor samples were obtained at 2 hours post final dose, and the compound levels in plasma and tumor lysates were measured by LC-MS/MS.
Results and Discussion

Potent and selective ALK inhibitory activity of CEP-28122

CEP-28122, a diamidopyrimidine derivative (Figure 1A), is a potent, selective and oral bioavailable ALK inhibitor. In an enzyme-based TRF assay, the IC\textsubscript{50} of CEP-28122 for recombinant ALK kinase activity was 1.9 ± 0.5 nM (Table 1). The kinase selectivity of CEP-28122 was evaluated using the Millipore Kinase Profiler\textsuperscript{TM} in which the inhibitory activity of CEP-28122 against a panel of 259 protein kinases was measured at 1 µM. CEP-28122 exhibited no- to-weak inhibition against the majority of the kinases tested at 1 µM and only 15 kinases showed > 90% inhibition at 1 µM (See Table S1). Except for Rsk2, 3 and 4 (IC\textsubscript{50} values range 7-19 nM), the IC\textsubscript{50} value for any other kinase is at least 10-fold higher than the IC\textsubscript{50} value for ALK (Table 1). These results suggest 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\textsubscript{50} values of 20-30 nM (Figure 1B). Phosphorylated Tyr664 of NPM-ALK (equivalent to Tyr1604 of full length ALK) is required for the interaction with PLC\textsubscript{γ} and activation of PLC\textsubscript{γ} by NPM-ALK is a crucial step for its mitogenic activity and is important in the pathogenesis of anaplastic lymphomas (1). Similarly, CEP-28122 inhibited EML4-ALK tyrosine phosphorylation in NSCLC NCI-H2228 and NCI-H3122 cells and inhibited full-length ALK receptor tyrosine phosphorylation in neuroblastoma cell line NB-1 cells, in a concentration-dependent manner with similar potency (Figure 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-M2-1 cells (Figure 1D), and AKT and ERK1/2 but not Stat-3 in NB-1 cells (Figure 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 support 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 -3000 nM) growth inhibition of NPM-ALK positive Karpas-299 and Sup-M2 cells in culture, associated with concentration-related caspase 3/7 activation (Figure 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 3000 nM (Figure 2A).

Similarly, treatment with CEP-28122 resulted in a concentration-dependent (3-3000 nM) growth inhibition of EML4-ALK-positive NCI-H2228 and NCI-H3122 cells in culture, while over the same concentration range tested, CEP-28122 displayed no or minimal cytotoxicity against EML4-ALK-negative NCI-H1650 cells (Figure 2B).

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 (26), in which no ALK expression and phosphorylation can be detected (therefore defined as an ALK-negative neuroblastoma cell line).

These data suggest 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 anti-tumor 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 (sc) 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 75–80% inhibition of NPM-ALK phosphorylation extending to 12 hours post-administration and 10 mg/kg led to near complete inhibition of NPM-ALK phosphorylation up to 6 hours with 75–80% inhibition observed at 12 hours post-dose. Complete target inhibition was observed extending up to 12 hours post single oral dose administration at 30 mg/kg (Figure 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 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 b.i.d. produced dose-dependent anti-tumor activity in Sup-M2 sc tumor xenografts, with
minimal anti-tumor 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 < 5% of the original tumor volume) following 12 days of treatment with 30 mg/kg CEP-28122 (Figure 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 anti-tumor activity on the growth of ALK-negative HCT-116 human colon carcinoma xenografts in mice at 10 and 30 mg/kg, po b.i.d. (Figure 3C), suggesting that at these doses, the anti-tumor 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 re-emerge upon cessation of CEP-28122 administration. To address this question, longer duration of treatment and follow-up observations were carried out in Sup-M2 sc tumor xenograft models in Scid mice. Mice bearing Sup-M2 sc tumor xenografts were treated with 55 or 100 mg/kg b.i.d. po 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 treatment. 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 re-emergence in any mouse up to 60 days post cessation of CEP-28122 (Figure 3D), suggesting that the Sup-M2 sc tumor xenografts in mice were completely eradicated after 4 weeks of treatment with CEP-28122 at 55 mg/kg or 100 mg/kg.
CEP-28122 displayed dose-related anti-tumor activity in mice bearing EML4-ALK-positive NSCLC NCI-H2228 and NCI-H3122 sc tumor xenografts when administered orally at 30 and 55 mg/kg, b.i.d. (Figure 4A). For NCI-H2228 tumor xenograft models, treatment of CEP-28122 at 30 and 55 mg/kg po, b.i.d. for 12 days resulted in tumor regression. For NCI-H3122 tumor xenograft models, treatment of CEP-28122 po, b.i.d. 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 anti-tumor activity observed in NCI-H2228 tumor xenografts was likely due to the higher tumor distribution of CEP-28122 (data not shown). In contrast, no anti-tumor activity was observed on the growth of the EML4-ALK-negative NSCLC, NCI-H1650, tumor xenografts in mice with the same dosing regimens (Figure 4A).

Administration of CEP-28122 at 30 and 55 mg/kg b.i.d. orally for 14 days led to significant anti-tumor activity with tumor stasis and partial tumor regressions observed in NB-1 sc tumor xenografts in mice (Figure 4B). There was 75% tumor growth inhibition in the 30 mg/kg CEP-28122-treated group and 90% tumor growth inhibition in the 55 mg/kg CEP-28122-treated group at the end of study. In contrast, treatment of CEP-28122 had no effect on the growth of ALK-negative NB-1691 sc tumor xenografts in mice with the same dosing regimens (Figure 4B). These data demonstrate the pharmacologically-selective efficacy of CEP-28122 against specifically defined tumors with constitutively activated ALK.

**Sustained complete tumor regression of human primary tumorgrafts**

The anti-tumor efficacy of CEP-28122 on human primary NPM-ALK positive ALCL tumorgrafts were also evaluated and compared with that of conventional
chemotherapeutical agents. The transplanted primary tumorgrafts partially responded to doxorubicin (10 mg/kg, iv), resulting in partial tumor regression. However, the treatment of doxorubicin was associated with overt toxicity (Figure 5A). Treatment of primary tumorgrafts with CEP-28122, 100 mg/kg, led to tumor regression with 1-2 days and complete tumor regressions in all mice after 2-week’s treatment, regardless of the original tumor size (Figure 5B). No re-emerging tumor was observed in any mouse up to 30 days (Figure 5B) and even 60 days (not shown) post cessation of CEP-28122 dosing, suggesting that the transplanted human primary ALCL tumorgrafts in mice were completely eradicated after only 2 weeks of treatment with CEP-28122. Of note, the tumorgrafts were equally sensitive to CEP-28122 treatment after initial treatment of doxorubicin (Figure 5A). In contrast to doxorubicin, CEP-28122 treatment was well tolerated with no overt toxicity.

Crizotinib 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 crizotinib 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 gate-keeper mutation because it is one of the most frequently reported mutants commonly associated with kinase inhibitor resistance, including crizotinib (22, 27-29).

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 (27).
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 (30). 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 (31). Indeed, other ALK secondary mutations and alternative pathway activation have been reported for lung cancer cells resistant to crizotinib (22-23, 32). 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 crizotinib against ALK is modest (11, 33). In our own experiments, a single oral dose of crizotinib at 30 mg/kg achieved only 65-70% target inhibition in Karpas-299 tumor xenografts, despite 5-6 µM (2737 ng/g) compound levels detected in tumor xenografts (Figure S2). Therefore, it is expected that high dose and extremely high tissue levels of crizotinib are required to achieve substantial target inhibition leading to clinical efficacy. Indeed, administration of crizotinib at 25 mg/kg had no significant anti-tumor efficacy and at 50 mg/kg led to only partial regression of Karpas-299 tumor xenografts in mice. Although crizotinib dosed at 100 mg/kg led to complete tumor regression, some of the tumors did re-emerge 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 75-80% inhibition of NPM-ALK phosphorylation in tumor xenografts, with compounds levels in tumors of about 0.5 µM (257 ng/g) (see Figure 3A), suggesting CEP-28122 is at least 10 times more potent in term of inhibition of ALK phosphorylation in tumors than crizotinib. More importantly, CEP-28122 dosed at 55 mg/kg or higher orally led to sustained complete tumor regression with no re-emerging tumor observed after cessation of treatment.

In conclusion, CEP-28122 is a potent and selective orally active ALK inhibitor demonstrating a favorable pharmaceutical and pharmacokinetic profile and robust and selective pharmacological 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 1-receptor (IGF-1R) and c-Met. Since ALK expression in normal adult tissues is very limited, a selective ALK inhibitor like 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.
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    Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to


Table 1. Enzyme inhibitory activity of CEP-28122 on select protein kinases. The kinase selectivity of CEP-28122 was evaluated using the Millipore Kinase Profiler™, a radiometric assay format in which peptide substrates are phosphorylated with [γ-33P]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 µM (see table S1). For those kinases inhibited by ≥ 90%, the IC₅₀ values were determined and reported in the table. *The IC₅₀ values for additional protein kinases were generated in house using TRF-based assays similar to that employed for ALK (described in Materials and Methods).

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<tr>
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Figure Legends

Figure 1. A. Chemical structure of CEP-28122. B. Sup-M2 and Karpas-299 cells were treated with CEP-28122 at the indicated concentrations for 2 hours and phospho (Y664)- and total NPM-ALK were detected by immunoblot analysis as described Materials and Methods. The relative NPM-ALK tyrosine phosphorylation of each sample is presented
as the mean ± standard error of mean (SEM) from 2-3 independent experiments. C. NSCLC NCI-H2228 and NCI-H3122 cells and neuroblastoma NB-1 cells were treated with CEP-28122 at the indicated concentrations for 2 hours and phospho- and total EML4-ALK or ALK receptor were detected by immunoblot analysis as described Materials and Methods. The relative EML4-ALK or ALK receptor tyrosine phosphorylation of each sample is presented as the mean ± SEM from 2-3 independent experiments. D. Sup-M2 cells were treated with CEP-28122 at the indicated concentrations for 2 hours and phospho- and total ALK, Stat3, Akt and ERK1/2 were detected by immunoblot analysis as described in Materials and Methods.

Figure 2. A. MTS assays: NPM-ALK-positive ALCL (Karpas-299 and Sup-M2) cells and ALK-negative lymphoma HuT-102 and leukemia Toledo cells in 96-well cell culture plates were treated with CEP-28122 for 48 hours and the living cells were measured with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay as described in Materials and Methods. Relative living cell numbers of each sample as compared to vehicle-treated samples were then calculated, and reported as the average ± SEM from 2-3 individual experiments; Caspase 3/7 assays: the cells in 96-well cell culture plates were treated with CEP-28122 for 16 hours and the caspase 3/7 activity was measured with the Apo-ONE® Homogeneous Caspase 3/7 Assay kit as described in Materials and Methods. The net caspase 3/7 activity of each sample was calculated by subtracting the values of vehicle-treated samples and reported as the average ± SEM from 2-3 individual experiments. B and C. EML4-ALK-positive (NCI-H2228 and H3122) and –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® AQueous Non-Radioactive Cell Proliferation Assay as described in Materials and Methods. Relative living cell numbers of each sample as compared to vehicle-treated samples were then calculated, and reported as the average ± SEM from 2-3 individual experiments;

Figure 3. A. Scid mice bearing Sup-M2 sc tumor xenografts were administered CEP-28122 at 3, 10 or 30 mg/kg, po. P-NPM-ALK and total NPM-ALK in tumor samples were detected by immunoblotting and relative NPM-ALK phosphorylation was calculated as described in Materials and Methods and presented as the mean ± standard error of mean (SEM). The compound levels in plasma and tumor lysates were measured by LC-MS/MS and presented as the mean ± SEM. B and C. Scid mice bearing Sup-M2 (B) or HCT116 (C) sc tumor xenografts were administered either vehicle or CEP-28122 orally at indicated doses, b.i.d. Tumor sizes were measured and recorded every two to three days, and the tumor volumes were calculated as described in Materials and Methods. Tumor volumes are presented as mean ± SEM. Statistical analyses of tumor volumes and mouse body weight were carried out with the Mann-Whitney Rank Sum Test and P values less than 0.05 or 0.01 were considered significant and labeled on graphs. D. Scid mice bearing Sup-M2 sc tumor xenografts were administered either vehicle or CEP-28122 orally at indicated doses, b.i.d. for 24 days and the mice were monitored for additional 60 days. Tumor sizes were measured and recorded every two to three days, and the tumor volumes were calculated as described in Materials and Methods. The tumor volumes are presented as mean ± SEM.

Figure 4. Scid mice bearing NCI-H2228, NCI-H3122 and NCI-1650 (A) or NB-1 and NB-1691 (B) sc tumor xenografts were administered either vehicle or CEP-28122 orally
at indicated doses, b.i.d. Tumor sizes were measured and recorded every two to three days, and the tumor volumes were calculated as described in Materials and Methods. Tumor volumes are presented as mean ± SEM. Statistical analyses of tumor volumes and mouse body weight were carried out with the Mann-Whitney Rank Sum Test and P values less than 0.05 or 0.01 were considered significant and labeled on graphs.

Figure 5. NSG mice transplanted with human primary ALK-positive ALCL tumorgrafts were administered either doxorubicin, 10 mg/kg, iv or CEP-28122 orally, 100 mg/kg, b.i.d., or doxorubicin following by CEP-28122. Tumor diameters were measured and recorded every day, presented as mean ± SEM (A) or individual value (B).
Figure 1. CEP-28122 is a potent ALK inhibitor
Figure 2. ALK Inhibition-dependent cytotoxicity
Figure 3. Pharmacodynamics and anti-tumor efficacy in ALCL tumor xenografts
Figure 4. ALK inhibition dependent anti-tumor efficacy in NSCLC and neuroblastoma
Figure 5. Sustained tumor regression of human primary ALCL tumogrfts
Molecular Cancer Therapeutics

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

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