Insights into ALK-Driven Cancers Revealed through Development of Novel ALK Tyrosine Kinase Inhibitors

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Abstract

Aberrant forms of the anaplastic lymphoma kinase (ALK) have been implicated in the pathogenesis of multiple human cancers, where ALK represents a rational therapeutic target in these settings. In this study, we report the identification and biological characterization of X-376 and X-396, two potent and highly specific ALK small molecule tyrosine kinase inhibitors (TKIs). In Ambit kinome screens, cell growth inhibition studies, and surrogate kinase assays, X-376 and X-396 were more potent inhibitors of ALK but less potent inhibitors of MET compared to PF-02341066 (PF-1066), an ALK/MET dual TKI currently in clinical trials. Both X-376 and X-396 displayed potent antitumor activity in vivo with favorable pharmacokinetic and toxicity profiles. Similar levels of drug sensitivity were displayed by the three most common ALK fusion proteins in lung cancer (EML4-ALK variants E13;A20, E20;A20, and E6b;A20) as well as a KIF5B–ALK fusion protein. Moreover, X-396 could potently inhibit ALK kinases engineered with two point mutations associated with acquired resistance to PF-1066, L196M, and C1156Y, when engineered into an E13;A20 fusion variant. Finally, X-396 displayed synergistic growth inhibitory activity when combined with the mTOR inhibitor rapamycin. Our findings offer preclinical proof-of-concept for use of these novel agents to improve therapeutic outcomes of patients with mutant ALK-driven malignancies. Cancer Res; 71(14): 4920–31. ©2011 AACR.

Introduction

The anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is aberrant in a variety of malignancies. ALK was originally discovered in anaplastic large cell lymphoma (ALCL) as part of a chromosomal translocation t(2,5), which fuses the C-terminal kinase domain of ALK encoded on chromosome 2p23 to the N-terminus of nucleophosmin (NPM) on chromosome 5q35 (1). Subsequently, a variety of ALK fusion proteins have been found in multiple malignancies, including inflammatory myofibroblastic tumor (IMT; ref. 2) and non–small cell lung cancer (NSCLC; refs. 3–10). All ALK fusions tested biologically to date have showed gain of function properties (1, 3, 4, 7). Activating mutations in wild-type ALK have also been identified in both familial and sporadic neuroblastoma. Most of these activating mutations occur within the tyrosine kinase domain and are transforming in vitro and in vivo (11–14).

Importantly, the activity of cancer-specific ALK variants is required for tumor maintenance. Thus, ALK mutants can serve as "Achilles heels" to be exploited therapeutically. Multiple preclinical studies have shown that specific small molecule ALK tyrosine kinase inhibitors (TKIs) can delay tumor growth and/or induce tumor regression in xenograft and transgenic models (3–5, 15–19).

Based on such promising preclinical studies, ALK inhibitors have recently entered into clinical trials. The first agent in humans is PF-02341066 (crizotinib, Pfizer; hereafter referred to as "PF-1066"), an orally available small molecule ATP-mimetic compound. PF-1066 was originally designed as a MET inhibitor but was recognized to have "off-target" anti-ALK activity (17). Strikingly, in a Phase I study, patients with ALK fusion positive NSCLC showed a 57% radiographic response rate (20). By contrast, chemotherapy response rates are <10% in previously treated patients with unselected NSCLC (21). A Phase III trial randomizing patients to crizotinib (PF-1066) versus standard chemotherapy after disease progression on first-line treatment is now ongoing for patients with ALK fusion positive NSCLC.

Here, we report identification of X-376 and X-396, novel, more potent and specific ALK TKIs with potential therapeutic relevance. We compare the effectiveness of these "second-generation" TKIs versus PF-1066 both in vitro and in vivo. We show the antitumor activity of these compounds against multiple ALK variants found in NSCLC, including 2 point

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mutations in the ALK tyrosine kinase domain which have been associated with acquired resistance to PF-1066. Finally, we show that these ALK TKIs display synergistic antitumor activity when combined with the mTOR inhibitor, rapamycin.

Materials and Methods

Compounds

X-376 and X-396 were synthesized according to procedures published in WO 2009/154769 and dissolved in DMSO. PF-1066 (ChemieTek) and TAE-684 (Selleck Chemicals) were dissolved in DMSO. Rapamycin (Catalog No. 9904, Cell Signaling) was dissolved in methanol. Ambit KINOMEscans (Ambit Biosciences) were performed for each ALK TKI against 96 distinct kinase targets using methods previously described (22, 23). Compounds (PF-1066, X-376, X-396) were formulated in 0.5% HPMC, 0.4% Tween80, 99.1% DI water for all in vivo studies (PK, efficacy, toxicity).

Computational modeling

A model of X-376 in ALK was generated using the DS ViewerPro 5.0 program from Accelrys Inc. The X-ray crystal structure of PF-1066 in the MET kinase domain (PDB code: 2WGJ) was used as a starting point.

Cell culture

All cell lines were maintained in a humidified incubator with 5% CO₂ at 37°C. The human lung adenocarcinoma cell lines H3122, H2228, PC-9, H2030, and HCC78 have been described previously (5, 24) and were verified to harbor their reported genetic alterations by direct sequencing of DNA or cDNA. The human anaplastic lymphoma cell line was a generous gift from Dr. S. Morris of St. Jude Children’s Research Hospital (1). The human gastric carcinoma cell line, MKN-45, and the human hepatocellular carcinoma cell line, HepG2, have been described previously (27, 28). These cell lines were maintained in RPMI 1640 medium (Mediatech, Inc.) supplemented with 10% FBS (Sigma-Aldrich) and penicillin-strep-to-mycin (Mediatech, Inc.) to final concentrations 100 U/mL and 100 µg/mL, respectively. The human neuroblastoma cell line SH-SY5Y (ATCC) was grown in a 1:1 mixture of EMEM and F12 (both from Mediatech, Inc.) supplemented with 10% FBS and pen-strep. The human hepatocellular carcinoma cell line HepG2 (ATCC) was grown in EMEM supplemented with 10% FBS and pen-strep. 293 cells (ATCC) were cultivated in DMEM (Mediatech) supplemented with 10% FBS. 293 cells were transfected with various ALK cDNAs using Superfect reagent (Qiagen) according to the manufacturer’s instructions.

Ba/F3 cell line generation

EML4-ALK E13A20 was cloned from pMA-3FLAG-EML4-ALK E13A20 into the retroviral pBabe-puro backbone. The L1196M and C1156Y mutations were introduced into the cDNA encoding the E13;A20 variant using site-directed mutagenesis (Stratagene) with mutant-specific primers according to the manufacturer’s instructions. Ba/F3 cell lines were established as described previously using purified IL-3 at 10 ng/mL and puromycin at 1.5 µg/mL (29). Transgene expression was assessed by Sanger sequencing and immunoblotting.

Antibodies and immunoblotting

The following antibodies were obtained from Cell Signaling Technology: ALK (Catalog No. 3333), phospho-ALK tyrosine 1604 (Catalog No. 3341), phospho-ALK tyrosine 1278/1282/1283 (Catalog No. 3983), ribosomal protein S6 (Catalog No. 2317), phospho-S6 (Catalog No. 5364), phospho-ERK tyrosine 202/204 (Catalog No. 9101), ERK (Catalog No. 9102), phospho- AKT serine 473 (Catalog No. 9271), AKT (Catalog No. 9272), HRP-conjugated anti-mouse (Catalog No. 7076), and HRP-conjugated anti-rabbit (Catalog No. 7074). The Flag M2 antibody (Catalog No. F1804) and the actin antibody (Catalog No. A2066) were purchased from Sigma-Aldrich.

For immunoblotting, cells were harvested, washed in PBS, and lysed in 50 mmol/L Tris-HCl, pH 8.0/150 mmol/L sodium chloride/5 mmol/L magnesium chloride/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/40 mmol/L sodium fluoride/1 mmol/L sodium orthovanadate and complete protease inhibitors (Roche Diagnostics). Lysates were subjected to SDS-PAGE followed by blotting with the indicated antibodies and detection by Western Lightening ECL reagent (Perkin Elmer). Images were quantified using a Bio-Rad Gel Doc XR and Image Lab software (Bio-Rad).

Cell viability and apoptosis assay

For viability experiments, cells were seeded in 96-well plates at 25% to 33% confluency and exposed to drugs alone or in combination the following day. At 72 hours after drug addition, Cell Titer Blue Reagent (Promega) was added and fluorescence was measured on a Spectramax spectrophotometer (Molecular Devices) according to the manufacturer's instructions. All experimental points were set up in hexuplicate replicates and were performed at least 2 independent times. IC₅₀s were calculated using GraphPad Prism version 5 for Windows. The curves were fit using a nonlinear regression model with a log (inhibitor) versus response formula.

For apoptosis experiments, cells were seeded in 12 well plates at 25% confluency and treated in triplicate with ALK TKI. At 72 hours after drug addition, cells were collected, washed in PBS, and stained with annexin V and propidium iodide according to the manufacturer's instructions (Vybrant Apoptosis Assay Kit, Invitrogen). Data were collected on a FACScanto II (BD Biosciences) and processed using WinList flow cytometry analysis software (Verity Software).

Expression constructs

cDNAs for EML4-ALK E13A20 (variant 1), EML4-ALK E20; A20 (variant 2), EML4-ALK E6bA20 (variant 3b), and KIF5B-ALK were synthesized by Geneart. The cDNAs were subcloned into a 3Flag-CMV vector (Sigma-Aldrich). The L1196M and C1156Y mutations were introduced into the cDNA encoding the E13A20 variant using site-directed mutagenesis (Stratagene) with mutant specific primers according to the manufacturer’s instructions. The cDNAs were fully re-sequenced in each case to ensure that no additional mutations were introduced. A cDNA encoding MET was obtained from Origene.
**Xenograft studies**

Nude mice (nu/nu; Harlan Laboratories) were used for in vivo studies and were cared for in accordance with guidelines approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee and Research Animal Resource Center. Eight-week-old female mice were injected s.c. with 5 million H3122 cells together with matrigel. Once tumors reached an average volume of 290 mm³, the tumors were randomized and dosed via oral gavage with either PF-1066, X-376, or X-396 at the indicated doses. A uniform volume for administration (200 µL) was used for each group. Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula: length × width² × 0.52. Body weight was also assessed twice weekly. The experiment was terminated after 3 weeks of treatment. Tumor samples were collected at either 2 or 8 weeks. Each sample was cut in halves; half was preserved in 10% neutral buffered formalin and half was flash frozen in liquid nitrogen and stored at −80°C.

**Pharmacokinetic and toxicity studies**

Nude mice (nu/nu; Harlan Laboratories) were injected with H3122 cells as earlier. Once tumors reached an average volume of 450 mm³, a total of 27 athymic mice harboring H3122 tumors were randomized and dosed via oral gavage with either 50 mg/kg X-376, 25 mg/kg X-396, or the control vehicle. Two, five, and fifteen hours after the single treatment (3 tumors/timepoint/group), mice were killed and serum was collected for assessment of drug concentration using an LC-MS-based bioanalytical method by the DMPK group of the Scripps Research Institute (Jupiter, Florida).

To determine if PF-1066, X-376, and X-396 can cross the blood–brain–barrier (BBB), each compound was dosed to 3 male C57BL/6j mice (The Jackson Laboratory) at their respective efficacious doses (PF-1066: 50 mg/kg, X-376 50 mg/kg, and X-396 25 mg/kg). Two hours after dosing, mice were killed. Blood and brain tissues were collected and analyzed for drug concentration. The bioanalysis was done at the Scripps Research Institute as described.

For toxicity studies, the test article or vehicle was administered to all groups orally once a day for 10 consecutive days at a dose volume of 10 mL/kg. Observations for morbidity, mortality, injury, and the availability of food and water were conducted twice daily for all animals. Clinical observations were conducted twice daily. Body weights were measured and recorded every other day during the study. Food consumption was measured and recorded daily during the study. Blood samples for clinical pathology evaluations were collected from all animals on day 11. Blood samples from an additional 2 animals/sex/group (minus vehicle group) were collected for determination of test article plasma concentrations at designated time points on day 10. The toxicokinetic parameters were determined for the test articles from concentration–time data in the test species. At study termination, necropsy examinations were performed, organ weights were recorded, and selected tissues were microscopically examined. Studies were done in accordance with the current International Conference on harmonization harmonized tripartite guidelines, and the protocol was approved by the Medicilon Institutional Animal Care and Use Committee (Shanghai, China).

**Results**

**X-376 and X-396 are aminopyrazidine-based kinase inhibitors with increased in vitro potency against ALK compared to PF-1066**

Based on protein structure modeling of ALK, we designed a series of small molecule inhibitors that we hypothesized would have greater potency and specificity against ALK. Although PF-1066 is an aminopyrazidine-based small molecule inhibitor, the lead compounds we identified, X-376 and X-396, are aminopyrazidine-based structures. These agents all share a common hydrophobic 2,6-dichloro-3-fluoro-phenylethoxy group as well as a similar kinase hinge binding group compared to PF-1066, but differ significantly in their side chains (Fig. 1A). We next performed KINOMEscan (Ambit Biosciences) analysis to test the specificity of the ALK TKIs. Briefly, KINOMEscan is a competition binding assay that quantitatively measures the ability of a test compound to compete with an immobilized, active-site directed ligand (22, 23). Initial KINOMEscan screens on racemic forms of PF-1066 and X-376 showed different but overlapping binding constant specificity profiles (Fig. 1B). Supplementary Table S1 summarizes the differential ability of these agents to inhibit the activity of 96 kinases; the vast majority of kinases are minimally affected. Additional Ambit-based dose–response profiling suggested that X-376 and X-396 were approximately 10-fold more potent against ALK but less specific for MET compared to PF-1066 (Table 1; ref. 17).

To attempt to explain the increased potency for X-376 and X-396 against ALK on a structural level, we generated a model of X-376 within the ATP binding pocket of the ALK tyrosine kinase domain (Fig. 1C and D). This model was based on the crystal structure of PF-1066 in the MET kinase domain (PDB code: 2WGJ). The hydrophobic interaction, especially the aromatic stacking, between Y1269 and the dichloroflorophenyl moiety, is conserved between PF-1066 and X-376 (Fig. 1D). However, for hydrogen bonds with hinge residues (E1197 and M1199), X-376 may be able to form 2 additional hydrogen bonds (C and D) compared to PF-1066. The hydrogen bond distances are 2.08 A, 1.95 A, 2.45 A, and 2.66 A for A, B, C, and D, respectively, in this model. Although the hydrogen bonds C and D are weaker than A and B, they may still contribute to the binding of X-376, thereby further increasing this compound’s affinity for ALK.

**X-376 and X-396 are more potent ALK inhibitors than PF-1066**

To compare and contrast the in vitro effects of X-376 and X-396 versus PF-1066, we tested the ability of all 3 agents to inhibit the growth of 4 different cancer cell lines known to harbor ALK fusions (Table 1). In H3122 lung cancer cell harboring EML4-ALK E13;A20 (variant 1), X-376 and X-396 were 3- and 10-fold more potent, respectively, than PF-1066 (IC50 of PF-1066...
180 nmol/L, X-376 77 nmol/L, X-396 15 nmol/L; Fig. 2A and Table 2). Similar results were obtained with H2228 lung cancer cells (Fig. 2B), SUDHL-1 lymphoma cells (Fig. 2C), and SY5Y neuroblastoma cells (Fig. 2D), which harbor an EML4-ALK E6a/b;A20 (variant 3a/b) fusion, an NPM-ALK fusion, and an activating point mutation within the ALK kinase domain (F1174L; ref. 12), respectively. In H3122 cells, the relative decrease in cell growth seen with X-376 treatment correlated with increased apoptosis, as assessed by fluorescence-activated cell sorting for Annexin V and propidium iodide (Fig. 2E).

By contrast, the ALK inhibitors did not significantly inhibit the growth of cells driven by other mutant kinases. For example, KRAS mutant (H2030) and EGFR mutant (PC-9) lung cancer cell lines were insensitive to treatment at low

Table 1. In vitro potency and selectivity of ALK TKIs

<table>
<thead>
<tr>
<th>Compound</th>
<th>ALK IC50 (nmol/L)</th>
<th>MET IC50 (nmol/L)</th>
</tr>
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<tbody>
<tr>
<td>PF-1066</td>
<td>4.5</td>
<td>0.51</td>
</tr>
<tr>
<td>X-376</td>
<td>0.61</td>
<td>0.69</td>
</tr>
<tr>
<td>X-396</td>
<td>&lt;0.4</td>
<td>0.74</td>
</tr>
</tbody>
</table>

The degree of inhibition from Kinomescan is shown for each protein.
nanomolar concentrations of drug (Table 2; Fig. S1B and C). HCC78 cells, which contain a ROS1 fusion protein, have displayed moderate sensitivity to another ALK TKI, TAE684, which has “off-target” anti-ROS activity (30). Our Ambit screen showed that the racemic forms of both PF-1066 and X-376 inhibited ROS1 in vitro (IC50 PF-1066 1.7 nmol/L; X-376: 19 nmol/L; Supplementary Table S1). However, we found HCC78 cells to be only modestly sensitive to PF-1066.
measure for potential hepatotoxicity (31). This assay is often used as a surrogate measure. Two hundred and ninety-three cells were transfected with an expression plasmid encoding flag-tagged EML4-ALK E13;A20 cDNAs and treated with PF-1066, X-376, or X-396. Immunoblotting of lysates showed that all 3 compounds inhibited the activity of ALK in a concentration-dependent manner. Consistent with data from the growth inhibition assays, X-376 and X-396 led to greater reductions in phospho-ALK at lower concentrations of drug (Fig. 2F). This was accompanied by corresponding inhibition of the downstream targets, ERK and AKT. We also found that phosphorylation of the mTOR substrate, ribosomal protein S6, was inhibited with ALK TKI treatment (Fig. 2G). Collectively, these data show that X-376 and X-396 are more potent ALK inhibitors than PF-1066.

X-376 and X-396 display less activity against MET than PF-1066
PF-1066 has higher binding affinity for MET versus ALK in Ambit KINOMEscan assays (17). By contrast, X-376 and X-396 displayed similar binding constants for both kinases (Table 1). To assess if such differences in binding correlated with less activity against MET in cells, we determined the ability of X-376 and X-396 to inhibit the growth of MKN-45 gastric carcinoma cells, which are known to be highly sensitive to MET inhibitors (27). Compared to X-376 and X-396, PF-1066 was 3-fold more potent against MKN-45 cells (IC\textsubscript{50}: PF-1066 51 nmol/L, X-376 150 nmol/L, X-396 156 nmol/L; Fig. S2A and Table 2). Consistent with these data, PF-1066 more effectively reduced MET autophosphorylation in surrogate kinase assays (17). By contrast, X-376 and X-396 displayed similar binding constants for both kinases (Supplementary Table S2). Thus, X-376 and X-396 are both more potent and more specific inhibitors of ALK compared to PF-1066.

Antitumor activity and pharmacokinetic properties of X-376 and X-396 in vivo
We next examined the effects of X-376 and X-396 in vivo against H3122 xenografts. A pharmacokinetic study revealed that both X-376 and X-396 showed substantial bioavailability and moderate half-lives in vivo (Supplementary Table S2). Based on these data, we selected twice a day dosing for initial efficacy studies. We treated nude mice harboring H3122 xenografts with either X-376 at 50 mg/kg bid or X-396 at 25 mg/kg bid. Both agents significantly delayed the growth of tumors compared to vehicle alone (Fig. 3A). The efficacy was similar to that seen with PF-1066 dosed at 50 mg/kg bid (Fig. S3). Additional doses and schedules of X-376 and X-396

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>IC\textsubscript{50} (\textmu mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3122</td>
<td>NSCLC, adenocarcinoma (EML4-ALK E13;A20)</td>
<td>0.180</td>
</tr>
<tr>
<td>H2228</td>
<td>NSCLC, adenocarcinoma (EML4-ALK E6a/b; A20)</td>
<td>0.150</td>
</tr>
<tr>
<td>SUDHL-1</td>
<td>ALCL (NPM-ALK)</td>
<td>0.073</td>
</tr>
<tr>
<td>SYSY</td>
<td>Neuroblastoma (ALK F1174L)</td>
<td>0.338</td>
</tr>
<tr>
<td>MKN-45</td>
<td>Gastric adenocarcinoma (MET dependent)</td>
<td>0.051</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatocellular carcinoma</td>
<td>14.502</td>
</tr>
<tr>
<td>H2030</td>
<td>NSCLC, adenocarcinoma (KRAS G12C)</td>
<td>2.474</td>
</tr>
<tr>
<td>PC-9</td>
<td>NSCLC, adenocarcinoma (EGFR exon 19 del)</td>
<td>1.670</td>
</tr>
<tr>
<td>HCC78</td>
<td>NSCLC, adenocarcinoma (SLC34A2-ROS fusion)</td>
<td>1.355</td>
</tr>
<tr>
<td>Ba/F3</td>
<td>+ EML4-ALK E13;A20 WT</td>
<td>0.250</td>
</tr>
<tr>
<td></td>
<td>+ EML4-ALK E13;A20 L1196M</td>
<td>1.815</td>
</tr>
<tr>
<td></td>
<td>+ EML4-ALK E13;A20 C1156Y</td>
<td>0.458</td>
</tr>
</tbody>
</table>

Cell lines were treated with ALK TKIs for 72 hours. Cell titer blue assays were performed to assess cell proliferation. IC\textsubscript{50} values were calculated using GraphPad Prism software with a nonlinear regression. Each experiment was performed at least 2 times. NT, not tested.
have not yet been examined. However, based on our toxicity studies, we show that X-396 dosed at 25 mg/kg is probably well below the maximum tolerated dose, suggesting that higher doses and greater efficacy can be achieved.

At the doses used in these xenograft experiments (PF-1066: 50 mg/kg bid; X376: 50 mg/kg bid; X-396 25 mg/kg bid), plasma levels inversely correlated with cellular potency. For example, although PF-1066 and X-396 were the least and most potent ALK inhibitors, respectively, the plasma level at 2 hours after dosing was highest for PF-1066 (10.19 μmol/L), lower for X-376 (6.27 μmol/L), and lowest for X-396 (2.28 μmol/L; Supplementary Table S2). The plasma levels followed the same trend at 5 and 15 hours after dosing, respectively. These findings suggest that inhibitors such as PF-1066 may need to be administered at higher doses to achieve the same efficacy of the more potent compounds.

In the xenograft experiments, both X-376 and X-396 appeared well-tolerated in vivo. Mouse weight was unaffected by either treatment (Fig. 3B). Drug-treated mice appeared healthy and did not display any signs of compound-related toxicity. To further assess potential side effects of X-376 and X-396, we performed additional systemic toxicity and toxicokinetic studies in Sprague–Dawley (SD) rats. Following 10 days of repeated oral administration of X-376 at 25, 50, and 100 mg/kg or X-396 at 20, 40, and 80 mg/kg in SD rats, all animals survived to study termination. The no significant toxicity (NST) levels were determined to be 50 mg/kg for X-376 and 80 mg/kg for X-396. The only statistically significant
Because PF-1066 at the selected Phase 3 dose achieves a plasma concentration in brain tissues compared to that in plasma. Brain penetration was defined as the ratio of compound tissues were collected and analyzed for drug concentration. Doses (PF-1066: 50 mg/kg, X-376 50 mg/kg, and X-396 25 mg/kg) were administered to 3 male C57BL/6J mice at their respective efficacious doses. We treated with ALK TKIs, we examined the penetration of PF-1066, X-376, and X-396 across the BBB in mice. Each drug was dosed to mice. Two hours after dosing, mice were killed. Blood and brain tissues were collected and analyzed for drug concentration. Brain penetration was defined as the ratio of compound concentration in brain tissues compared to that in plasma. We found that X-396 had comparable brain penetration to PF-1066, whereas X-376 had less (Supplementary Table S3). Taken together, these data suggest that X-396 is able to inhibit ALK effectively at doses far below those that are well tolerated.

**Penetration of ALK TKIs in the brain**

In EGFR mutant lung cancer, patients with acquired resistance to EGFR TKIs may relapse in the brain. One contributing factor is that the dose of drug in the brain is only one hundredth that in the blood. In anticipation of a similar phenomenon occurring in patients with ALK fusion positive lung cancers treated with ALK TKIs, we examined the penetration of PF-1066, X-376, and X-396 across the BBB in mice. Each drug was dosed to 3 male C57BL/6J mice at their respective efficacious doses (PF-1066: 50 mg/kg, X-376 50 mg/kg, and X-396 25 mg/kg). Two hours after dosing, mice were killed. Blood and brain tissues were collected and analyzed for drug concentration. Brain penetration was defined as the ratio of compound concentration in brain tissues compared to that in plasma. We found that X-396 had comparable brain penetration to PF-1066, whereas X-376 had less (Supplementary Table S3). Because PF-1066 at the selected Phase 3 dose achieves a plasma level of ~500 nmol/L, projection of results from this animal study to humans would suggest that the concentration of PF-1066 in human brain would be only ~80 nmol/L (16% of plasma level), which is below the IC_{50} (180 nmol/L) for inhibiting H3122 cell growth (Supplementary Table S4). By contrast, if X-396 were also to achieve a plasma level of ~500 nmol/L in humans, its brain concentration would be ~65 nmol/L (13% of plasma level), which is significantly higher than its IC_{50} of 15 nmol/L (Supplementary Table S4). These data suggest that drugs such as X-396 could lead to higher efficacy against ALK-fusion positive brain metastases. However, further studies with additional sampling times will be required to most accurately assess intracranial drug levels.

**X-376 and X-396 are effective against multiple ALK variants found in NSCLC, including ALK mutations associated with acquired resistance to PF-1066**

To date, at least 11 different ALK rearrangements have been reported in NSCLC (3–10). Although all contain the ALK kinase domain starting at the region encoded by exon 20, most contain variable portions of EML4 at the N-terminus. Another fusion involves KIF5B (7). Whether these variants display differential sensitivity to ALK TKIs is unknown but could influence the outcome of clinical trials with ALK inhibitors. We therefore compared the efficacy of X-376 against 4 different ALK variants in surrogate kinase assays. 293 cells were transiently transfected with cDNAs encoding Flag-tagged versions of 4 ALK rearrangements: 3 of the most common EML4-ALK variants, EML4-ALK E13A20 (variant 1), EML4-ALK E20A20 (variant 2), EML4-ALK E6bA20 (variant 3b; refs. 6, 34), and KIF5B-ALK. After treatment with X-376, cell extracts were subjected to immunoblotting with antibodies against phospho-ALK and Flag. All fusions displayed dose-dependent inhibition of ALK kinase activity (Fig. 4A). Based on quantitative examination of the immunoblotting results (data not shown), no major differences in sensitivity were observed among the 4 variants in these in vitro surrogate-kinase assays. However, additional studies in cell lines where these ALK variants are the oncogenic "drivers" are needed to establish more subtle differences.

We next examined the efficacy of X-396 against 2 point mutations in EML4-ALK recently identified in patients with acquired resistance to PF-1066, L1196M, and C1156Y (35). L1196 of ALK corresponds to the "gatekeeper" site, and C1156 is positioned adjacent to the N-terminal helix αC (35). To determine how the L1196M and C1156Y mutations affect ALK fusion proteins, we individually introduced the specific mutations into EML4-ALK E13A20 cDNAs and performed surrogate kinase assays as described. Introduction of L1196M and C11561 into the EML4-ALK fusion protein led to a greater baseline level of phosphorylation (Fig. 4B and C), suggesting that these mutations lead to increased kinase activity. As expected, the L1196M and C1156Y mutants did reduce the sensitivity of the wild-type ALK fusion protein to both PF-1066 and X-396, however, the degree of reduction was less for X-396.

To expand on these results from the surrogate kinase assays, we evaluated the biological impact of each mutation on drug sensitivity in Ba/F3 cells. Expression of EML4-ALK E13A20 WT, L1196M, and C1156Y (Fig. 4E) each led to IL3 independent growth of Ba/F3 cells. In accord with our data above from tumor cell lines with ALK genomic alterations, X-396 was ~10 fold more potent than PF-1066 in inhibiting the growth of Ba/F3 cells expressing EML4-ALK E13A20 wild type (IC_{50} for WT: PF-1066 250 nmol/L, X-396 22 nmol/L; Fig. 4E and Table 2). As expected, the presence of the L1196M and C1156Y point mutants reduced the sensitivity of the EML4-ALK fusion to both PF-1066 and X-396 (Fig. 4E). However, the IC_{50} values for X-396 against the L1196M and C1156Y mutants were still in the ~100 nmol/L. These data suggest that X-396 has the potential to overcome these second-site drug resistance mutants.

**Combined ALK/mTOR inhibition enhances growth inhibition of ALK fusion positive lung cancer cell lines**

As shown earlier, treatment of H3122 cells with ALK TKIs led to a decrease in phosphorylation of the mTOR substrate, ribosomal protein S6 (Fig. 2G). Previous studies in NPM-ALK positive lymphoma cell lines have shown that the mTOR inhibitor, rapamycin, decreases proliferation and increases apoptosis in ALK fusion positive lymphoma cells (36). Given these 2 observations, we sought to determine if mTOR
Figure 4. Effects of ALK TKIs against various ALK fusion variants and the EML4-ALK L1196M and C1156Y point mutants. A, 293 cells were transiently transfected with expression plasmids encoding various ALK fusions. At 48 hours posttransfection, the cells were treated with increasing amounts of X-376 for 2 hours. Lysates were subjected to immunoblotting with antibodies specific for the indicated proteins. B–C, 293 cells were transiently transfected with 3Flag-EML4-ALK E13;A20 WT alongside 3Flag-EML4-ALK E13;A20 L1196M (B) or 3Flag-EML4-ALK E13;A20 C1156Y (C). At 48 hours post transfection, the cells were treated with increasing amounts of the indicated ALK-TKI for 2 hours. Lysates were subjected to immunoblotting with antibodies specific for the indicated proteins. The exposure of the pALK blot was selected to highlight the difference in baseline phosphorylation between wild type and mutants. D, Western blot showing EML4-ALK E13;A20 WT, L1196M, and C1156Y expression in Ba/F3 cell lines. E, Ba/F3 cells expressing EML4-ALK E13;A20 WT, L1196M, or C1156Y were treated with ALK TKIs or vehicle for 72 hours. Cell titer blue assays were performed to assess growth inhibition. Each point represents hexuplicate replicates. Data are presented as the percentage of viable cells compared to control (vehicle only treated) cells. See Materials and Methods for details.
inhibition could synergize with ALK inhibition in EML4-ALK fusion positive NSCLC cell lines. Rapamycin alone caused a small but reproducible decrease in the proliferation of H3122 and H2228 cells (Fig. S4). Strikingly, when rapamycin was combined with X-396, we observed a synergistic decrease of cell growth in both H3122 (Fig. 5A) and in H2228 (Fig. 5B) cells. Drug synergism was assessed by both the Chou-Talalay and Mix-Low Methods (Supplementary Table S5 and Fig. S5; refs. 37, 38). Consistent with these data, phosphorylation of the mTOR substrate, S6, was decreased in the presence of rapamycin and was further diminished in the presence of both rapamycin and X-396 (Fig. 5C). Overall, these data suggest that cotreatment with ALK and mTOR inhibitors may represent a strategy to increase the therapeutic efficacy for patients with ALK fusion positive lung cancer.

Discussion

The advent of targeted therapies for specific cohorts of tumors defined by molecular criteria holds promise for improving therapeutic outcomes of cancer patients. Mutant ALK represents 1 clinically relevant molecular marker, found in a diverse spectrum of malignancies, including ALCL, NSCLC, IMT, and neuroblastoma. Constitutive activation of ALK, either through oncogenic fusions or point mutation, not only initiates tumorigenesis in these cancers but also renders them sensitive to treatment with ALK small molecule inhibitors. The first ALK inhibitor in clinical trials, PF-1066, has showed promising results in NSCLCs harboring ALK fusions (20). However, despite displaying impressive radiographic regressions, patients have already showed disease progression on drug. Thus, novel agents and drug combinations need to be developed to optimize the care of patients with tumors harboring ALK genomic alterations.

To this end, we report here identification and characterization of X-376 and X-396, 2 potent and highly specific ALK small molecule TKIs. In multiple different experiments, including Ambit kinome screens, in vitro growth inhibition studies using oncogene-dependent cancer cell lines, and surrogate kinase assays, X-376 and X-396 were more potent inhibitors of ALK.

Figure 5. The mTOR inhibitor, rapamycin, is synergistic with X-396. H3122 (A) and H2228 (B) were treated with rapamycin alone, X-396 alone, rapamycin plus X-396 at the indicated concentrations for 72 hours. Cell titer blue assays were performed to assess proliferation as per methods. Data are presented as the percentage of viable cells compared to control (vehicle only treated) cells. P values were calculated using the Wilcoxon rank-sum test. C, H3122 cells were treated with rapamycin alone, X-396 alone, or rapamycin plus X-396 at the indicated concentrations for 1 hour. Lysates were subjected to immunoblotting with antibodies specific for the indicated proteins.
Both PF-1066 and X-396, however, the IC50 values for X-396, albeit at higher concentrations required to block autophosphorylation was significantly diminished by X-396, whose disease initially responded to therapy have already developed progressive disease. Mechanisms of ‘acquired resistance’ to ALK TKIs are just beginning to be elucidated. Recently, Choi and colleagues reported the finding of L1196M and C1156Y point mutations in a patient with lung cancer who developed acquired resistance to PF-1066 (35). L1196, corresponds to the ‘gatekeeper’ site in ALK. Mutations at this site alter drug binding (39) and are commonly found in tumor cells from cancer patients with acquired resistance to kinase inhibitors. C1156 is positioned adjacent to the N-terminal helix OC (35). The structural mechanism for how mutation at this site confers resistance to ALK TKIs remains to be elucidated. When the ALK L1196M and C1156Y mutations were introduced into the EML4-ALK E13;A20 fusion, kinase activity, as assessed by ALK autophosphorylation, was not inhibited by up to 300 nmol/L of PF-1066. By contrast, ALK autophosphorylation was significantly diminished by X-396, albeit at higher concentrations required to block autophosphorylation of the wild-type fusion. These results were recapitulated in Ba/F3 cells where introduction of the L1196M and C1156Y point mutants reduced the sensitivity of cells to both PF-1066 and X-396, however, the IC50 values for X-396 against these mutants were still ~100 nmol/L or less.

Because both of these point mutations have been found in clinical samples from patients with acquired resistance to ALK TKIs, treatment with X-396 may represent one strategy to overcome them. In addition, our brain drug penetration studies suggest that X-396 can achieve high enough concentrations to treat intra-cerebral metastases effectively should they arise in patients.

Finally, an emerging paradigm in the design and implementation of targeted agents is the combination of such agents to improve therapeutic efficacy. The serine–threonine protein kinase, mTOR, plays an important role in protein translation and cell growth and has been implicated as a therapeutic target in a variety of malignancies. Previous studies in NPM-ALK positive lymphoma cell lines have shown that the mTOR inhibitor, rapamycin, decreases proliferation and increases apoptosis in ALK fusion positive lymphoma cells (36). Interestingly, recent studies performed in an EML4-ALK mouse model showed only modest antitumor efficacy with combined PI3K/mTOR and MEK inhibition (40). However, the combination of an ALK inhibitor plus an mTOR inhibitor was not evaluated. Our results show that this combination can synergistically decrease cell proliferation in 2 different EML4-ALK lung cancer cell lines. These data indicate that mTOR signaling may play critical roles in ALK fusion signaling in general, which is currently not well characterized. Clinically, these data also suggest that trials of ALK TKIs in combination with rapamycin or analogous agents should be considered to enhance the antitumor efficacy of ALK inhibitors.

**Disclosure of Potential Conflicts of Interest**

C. Liang is cofounder and the chief scientific officer of Xovery. The other authors disclosed no potential conflicts of interest.

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**References**


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