The ALK/ROS1 Inhibitor PF-06463922 Overcomes Primary Resistance to Crizotinib in ALK-Driven Neuroblastoma

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ABSTRACT

Neuroblastomas harboring activating point mutations in anaplastic lymphoma kinase (ALK) are differentially sensitive to the ALK inhibitor crizotinib, with certain mutations conferring intrinsic crizotinib resistance. To overcome this clinical obstacle, our goal was to identify inhibitors with improved potency that can target intractable ALK variants such as F1174L. We find that PF-06463922 has high potency across ALK variants and inhibits ALK more effectively than crizotinib in vitro. Most importantly, PF-06463922 induces complete tumor regression in both crizotinib-resistant and crizotinib-sensitive xenograft mouse models of neuroblastoma, as well as in patient-derived xenografts harboring the crizotinib-resistant F1174L or F1245C mutations. These studies demonstrate that PF-06463922 has the potential to overcome crizotinib resistance and exerts unprecedented activity as a single targeted agent against F1174L and F1245C ALK-mutated xenograft tumors, while also inducing responses in an R1275Q xenograft model. Taken together, these results provide the rationale to move PF-06463922 into clinical trials for treatment of patients with ALK-mutated neuroblastoma.

SIGNIFICANCE: The next-generation ALK/ROS1 inhibitor PF-06463922 exerts unparalleled activity in ALK-driven neuroblastoma models with primary crizotinib resistance. Our biochemical and in vivo data provide the preclinical rationale for fast-tracking the development of this agent in children with relapsed/refractory ALK-mutant neuroblastoma. Cancer Discov; 6(1); 96–107. ©2015 AACR.

See related commentary by Versteeg and George, p. 20.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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INTRODUCTION

Neuroblastoma is a devastating pediatric cancer of the autonomic nervous system, accounting for 12% of childhood cancer deaths (1). Despite major enhancements in treatment approaches, including integration of immunotherapeutic strategies (2), the cure rate for patients with high-risk neuroblastoma remains unsatisfactory. Several recent findings have positioned the anaplastic lymphoma kinase (ALK) receptor tyrosine kinase as the only tractable oncogene product for targeted therapy in neuroblastoma. Germline and somatic aberrations in the gene encoding ALK are implicated in approximately 8% of all neuroblastomas (3–6). Within the high-risk subset of neuroblastoma patients, the overall frequency of ALK aberration at diagnosis is 14% (10% point mutations and 4% amplification) and correlates with inferior outcome (7). Additional ALK mutations at relapse have also been reported (8, 9). The activating mutations are found at several sites in the tyrosine kinase domain of full-length ALK (7), including three hotspots (R1275, F1174, and F1245). These findings motivated a phase I trial of the ATP-competitive ALK/MET/ROS1 inhibitor crizotinib (PF-02341066) in children with refractory neuroblastoma or other malignancies driven by ALK rearrangements, such as anaplastic large-cell lymphoma (ALCL) and inflammatory myofibroblastic tumors (IMT; ref. 10). Results from this trial underscored the importance of ALK across histologically diverse tumors, but recorded less frequent responses in neuroblastoma than in ALK-rearranged tumors—highlighting likely differences between therapeutic targeting of full-length ALK in neuroblastoma and of cytoplasmic ALK fusion proteins in ALCL, IMTs, and lung cancer. Parallel preclinical work has further revealed differential sensitivity to crizotinib for the most common ALK variants observed in neuroblastoma (11–13), with F1174L-mutated cells being resistant when compared with those expressing R1275Q-mutated ALK. Despite real-time integration of these findings in the clinic, and a recommended phase II dose of crizotinib in pediatric patients that is nearly twice the adult maximum tolerated dose (10), these studies emphasize the need to identify an optimal inhibitor for direct ALK kinase inhibition in neuroblastoma in order to maximize clinical benefit.

Our previous studies indicated that the relative crizotinib sensitivities of ALK variants may simply reflect their ATP-binding affinities, with reduced $K_{M,ATP}$ (higher ATP-binding affinity) predicting crizotinib resistance for F1174L-mutated ALK, for example (11). We explored two strategies to overcome this problem. In the first, we tested a series of second-generation ALK kinase inhibitors, anticipating that their structural differences might allow some to show selective (or improved)
inhibition of F1174L-mutated ALK, as has been suggested for other ALK variants (14). As we failed to find any inhibitors with these characteristics, our second approach was to investigate the impact of increasing drug-binding affinity to all ALK variants—made possible by using the highly potent ALK/ROS1 inhibitor PF-06463922 (15, 16). We hypothesized that a drug with substantially increased ALK-binding affinity might be capable of inhibiting all ALK variants at readily achievable drug concentrations, and that this feature might negate the effects of primary resistance mutations. We show here that PF-06463922 has substantially greater potency than crizotinib as an ALK inhibitor in vitro, across all neuroblastoma mutations tested. Most importantly, PF-06463922 also showed excellent activity against all full-length oncogenic ALK variants tested in preclinical models of ALK-driven neuroblastoma where crizotinib fails. Through biochemical, cell-based, xenograft and patient-derived xenograft (PDX) studies, we demonstrate that PF-06463922 has unprecedented activity as a single agent against ALK mutants, including those with primary crizotinib resistance, thus positioning PF-06463922 as the lead ALK inhibitor for clinical development in ALK-driven neuroblastoma.

RESULTS

In our earlier studies, we were able to recapitulate the differential crizotinib sensitivity of F1174L and R1275Q ALK variants in vitro, using kinase assays (11). We also showed that the transforming ability of ALK variants correlates most closely with in vitro $k_{\text{cat}}$ values for the unphosphorylated kinase domain of the receptor (7), implying that activation of this species is key for oncogenesis. Unfortunately, a preliminary screen through a series of second-generation ALK inhibitors failed to identify any inhibitor that differed significantly from crizotinib in its relative ability to inhibit R1275Q, F1174L, and F1245C variants of the unphosphorylated ALK tyrosine kinase domain (ALK-TKD) in vitro. The ALK/ROS1 inhibitor PF-06463922 stood out starkly in these assays, however, by yielding uniquely low IC$_{50}$ values. As previously pointed out (16), PF-06463922 is more potent against wild-type ALK than crizotinib, alectinib, or ceritinib by 4-fold or more when $K_i$ values are compared. In our in vitro experiments, PF-06463922 gave IC$_{50}$ values for inhibition of F1174L- and F1245C-mutated ALK that were significantly lower than those seen with crizotinib even for the crizotinib-sensitive R1275Q variant (Fig. 1A and B). Indeed, measured IC$_{50}$ values for PF-06463922 were approximately 5-fold lower than those for crizotinib for all variants and were actually limited in this assay by the concentration of kinase protein required to measure activity of unphosphorylated ALK-TKD (50 nmol/L). Estimated $K_i$ values from our data using the equations of Cha (17) are less than 0.2 nmol/L for inhibition of mutated ALK-TKD by PF-06463922, compared with 6 to 10 nmol/L for crizotinib inhibition, $K_i$ values consistent with previous reports using different methods and different sets of mutations (15, 16). Thus, PF-06463922 is a very potent inhibitor for multiple mutated full-length ALK variants seen in neuroblastoma, as also reported for acquired resistance mutations seen in cytoplasmic ALK fusion proteins in lung cancer and elsewhere (15, 16, 18). We hypothesized that this significantly increased potency and/or affinity of PF-06463922, compared with other ALK inhibitors in development, might be sufficient to negate the consequences of differential sensitivity of ALK variants to kinase inhibitors.

**PF-06463922 Induces Complete Tumor Regression in Patient-Derived and Cell Line–Derived Xenografts with and without Primary Resistance to Crizotinib**

The data in Fig. 1 suggest that F1245C-mutated ALK should resemble F1174L-mutated ALK in its primary resistance to crizotinib—consistent with limited clinical data (10)—but that tumors driven by all three hotspot ALK variants should respond to PF-06463922. To test these hypotheses, we compared in vivo efficacies of PF-06463922 and crizotinib in crizotinib-naive PDXs harboring F1174L or F1245C ALK mutations (COG-N-453x and Felix-PDX, respectively), as well as cell line–derived xenografts utilizing SH-SY5Y cells.

Figure 1. PF-06463922 exhibits superior inhibitory activity against all tested ALK-tyrosine kinase domain (TKD) variants. In vitro inhibition profiles of purified unphosphorylated ALK-TKD protein (at 50 nmol/L) harboring the noted mutations with crizotinib (A) and PF-06463922 (B). Inhibitor concentration was varied from 0 to 25,600 nmol/L, with ATP present at 2 mmol/L, and substrate peptide was held at 0.5 mmol/L, as described previously (11). Results are plotted as mean ± SD for at least three independent experiments. As discussed in the text, the IC$_{50}$ values obtained in this experimental format correspond to $K_i$ values in the 6 to 10 nmol/L range for crizotinib and the 0.2 nmol/L range for PF-06463922. WT, wild-type.
PF-06463922 Overcomes Primary Resistance in Neuroblastoma

Phosphorylation activity, we used immunoblotting to analyze pALK levels in PF-06463922 Efficiently Inhibits ALK to S2D. Thus, xenografts harboring the (COG-N-453x), 7.1 weeks (Felix-PDX), 5 weeks (SH-SY5Y), 10 mg/kg/day PF-06463922 remained in complete remission observation and palpation. Mice that had been treated with mals were further monitored for tumor progression by duration of treatment in all xenografts (red curves in Fig. 2; weeks) and sustained complete tumor regression for the PF-06463922 (5 mg/kg b.i.d.) resulted in rapid (within 2–3 weeks) and sustained complete tumor regression for the duration of treatment in all xenografts (red curves in Fig. 2; Table 1). After PF-06463922 treatment was stopped, animals were further monitored for tumor progression by observation and palpation. Mice that had been treated with 10 mg/kg/day PF-06463922 remained in complete remission with no discernible tumor growth for a further 4.8 weeks (COG-N-453x), 7.1 weeks (Felix-PDX), 5 weeks (SH-SY5Y), and 4 weeks (NB-1643), as shown in Supplementary Fig. S2A to S2D. Thus, xenografts harboring the de novo crizotinib-resistant ALK F1174L mutation exhibited unprecedented antitumor responses to single-agent ALK inhibition therapy with PF-06463922, as did a PDX harboring the F1245C mutation.

Given these rapid and complete responses to 10 mg/kg/day PF-06463922 shown in Fig. 2, we also evaluated a lower dose of 3 mg/kg/day (1.5 mg/kg b.i.d.) in both the Felix-PDX (F1245C) and SH-SY5Y (F1174L) xenografts. As shown in Supplementary Fig. S3A and S3B and Table 2, this lower dose of PF-06463922 was still substantially more effective than a 33-fold higher dose of crizotinib. In the SH-SY5Y xenograft (Supplementary Fig. S3B), 3 mg/kg/day PF-06463922 induced complete regression within 6 weeks of treatment (compared with 2–3 weeks for 10 mg/kg/day PF-06463922). The lower dose of PF-06463922 caused significant growth delay in Felix-PDX (Supplementary Fig. S3A), with tumors remaining palpable throughout treatment and showing little further growth after approximately 4 weeks. PF-06463922 at both dosing levels significantly prolonged event-free survival (EFS) relative to control and crizotinib arms in SH-SY5Y and Felix xenografts (Supplementary Fig. S3 and Table 2).

PF-06463922 Efficiently Inhibits ALK Phosphorylation In Vivo

To compare PF-06463922 and crizotinib over a broader range of ALK-driven tumors, we assessed their abilities to inhibit growth of a range of cell lines in vitro, employing a CellTiter-Glo luminescent cell viability assay. As shown in Fig. 4 and Supplementary Table S1, IC_{50} values measured for PF-06463922 in neuroblastoma cell lines dependent on R1275Q-mutated ALK were 51-fold (NB-1643) and 19-fold (LAN-5) lower than those measured for crizotinib (Fig. 4A and B), which themselves were approximately 310 nmol/L. Importantly, IC_{50} values for PF-06463922 inhibition of cell lines driven by the ALK-resistant F1174L ALK variant were also much lower—falling in the range of 12.7 nmol/L to 26.6 nmol/L, which is approximately 30-fold lower than measured for crizotinib (Fig. 4C–F and Supplementary Table S1). A similar difference was also seen for Felix cells, driven by the F1245C variant (Fig. 4G). Whereas a small (~3-fold) difference in IC_{50} appeared to be sufficient to explain the relative crizotinib resistance of tumors and cell lines driven by F1174L-mutated ALK in neuroblastoma compared with those driven by R1275Q (11), the overall higher potency of PF-06463922 appears to render such differences irrelevant, so that both tumor cell lines (Fig. 4) and xenografts (Fig. 2) driven by the F1174L variant are sensitive to PF-06463922. The same appears to be true for the F1245C variant. Similar results were seen for NB-1 cells, driven by amplified wild-type ALK, with IC_{50} values of 256 nmol/L and 5.2 nmol/L for crizotinib and PF-06463922, respectively (Fig. 4H).

Three other interesting observations emerge from this study. First, the difference in IC_{50} values for crizotinib and PF-06463922 was even greater (>100-fold) for the NCI-H3122 lung cancer cell line driven by the EML4–ALK fusion (Fig. 4I and Supplementary Table S1), suggesting that the presence of the TKD in the intact receptor might slightly limit the potency of PF-06463922. Second, in agreement with previous studies (16), PF-06463922 was less toxic than crizotinib in cell lines that are not dependent on ALK for growth (Fig. 4J and K), consistent with its high selectivity (15). Third, PF-06463922 treatment did not cause complete growth inhibition in cell lines with R1275Q, F1174L, or F1245C mutations, whereas crizotinib did. One possible explanation for
Figure 2. PF-06463922 induces complete tumor regression in PDX and xenograft models of crizotinib-resistant and crizotinib-sensitive neuroblastoma: A, COG-N-453 x PDX (F1174L); B, Felix-PDX (F1245C); C, SH-SY5Y (F1174L); D, NB-1643 (R1275Q). Subcutaneously implanted neuroblastoma tumors were monitored in CB17 SCID mice treated with 10 mg/kg/day PF-06463922 (solid red line and squares), 100 mg/kg/day crizotinib (dashed blue line and triangles), or vehicle (solid black line and diamonds). Data are shown only for the duration of treatment in each case. Study end points for survival analysis (right) are shown using Kaplan–Meier curves (median ± SEM; n = 10 for each data point). A mixed-effects linear model was used to analyze the statistical significance of tumor growth delay. Kaplan–Meier curves for EFS were compared using a log-rank test, with P < 0.05 taken as indicating significance (see Table 1). Additional EFS data for 4 to 7 weeks beyond the end of treatment are plotted in Supplementary Fig. S2.
Table 1. Statistical analysis for in vivo efficacy studies in PDXs from COG-N-453x and Felix cells, and xenografts from SH-SY5Y and NB-1643 cells

<table>
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<tr>
<th>PDX or xenograft (mutation)</th>
<th>ALK status</th>
<th>MYCN status</th>
<th>Treatment</th>
<th>Tumor volume</th>
<th>EFS</th>
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<tr>
<td></td>
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<td>P value vs. crizotinib</td>
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<td>&lt;0.0001</td>
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<tr>
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<td>Crizotinib</td>
<td>PF-06463922</td>
<td>&lt;0.0001</td>
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Table 2. Statistical analysis for Felix-PDX and SH-SY5Y in vivo efficacy studies testing two doses of PF-06463922 (10 mg/kg/day and 3 mg/kg/day)

<table>
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<tr>
<th>Xenograft/ PDX</th>
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<th>MYCN status</th>
<th>Treatment</th>
<th>Tumor volume</th>
<th>EFS</th>
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<td></td>
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<td></td>
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<td>P value vs. vehicle control</td>
<td>P value vs. crizotinib</td>
</tr>
<tr>
<td>Felix-PDX</td>
<td>Mutated: F1245C Nonamplified</td>
<td>Crizotinib</td>
<td>PF-06463922 (3 mg/kg/day)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<td></td>
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<td></td>
<td>PF-06463922 (10 mg/kg/day)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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<tr>
<td>SH-SY5Y</td>
<td>Mutated: F1174L Nonamplified</td>
<td>Crizotinib</td>
<td>PF-06463922 (3 mg/kg/day)</td>
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<td>&lt;0.0001</td>
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<td>PF-06463922 (10 mg/kg/day)</td>
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cancer line (IC_{50} = 2.8 nmol/L). Zou and colleagues (16) recently reported PF-06463922 IC_{50} values in the 2 to 3 nmol/L range for cells driven by EML4-ALK fusions, but higher values (similar to those seen for neuroblastoma cells) in the cells expressing mutated EML4-ALK. In all cases, however, the superior potency of PF-06463922 was such that the IC_{50} values were sufficient to predict clinical efficacy.

Inhibition of NIH 3T3 Cell Transformation by Mutated ALK

To compare the effects of the two ALK inhibitors on mutated ALK variants all in a common cell background, we also analyzed their ability to inhibit ALK-mediated transformation of NIH 3T3 cells in focus assays, as described previously (7). As shown in Fig. 6A–C, PF-06463922 was substantially more effective than crizotinib at blocking transformation/focus formation induced by ALK with any of the three hotspot mutations. IC_{50} values were consistently 5- to 10-fold lower for PF-06463922 than for crizotinib, consistent with experiments in all other in vitro and in vivo contexts described here. It is interesting, however, that NIH 3T3 cells expressing the R1275Q variant were significantly more sensitive to both drugs than cells expressing F1174L- or F1245C-mutated ALK. Indeed, the IC_{50} values for inhibition of focus formation/focus formation induced by ALK with any of the three hotspot mutations. IC_{50} values were consistently 5- to 10-fold lower for PF-06463922 than for crizotinib, consistently higher values (similar to those seen for neuroblastoma cells) in the cells expressing mutated EML4-ALK. In all cases, however, the superior potency of PF-06463922 was such that the IC_{50} values were sufficient to predict clinical efficacy.

DISCUSSION

Although our work, and that of others, has positioned ALK as the first tractable oncogene for targeted therapy in neuroblastoma, it is now clear that not all ALK mutations impart oncogenic dependency (7, 12). Moreover, susceptibility of ALK-driven tumors and cell lines to crizotinib differs according to the underlying mutation (11, 21), with mutations at two of the three hotspots (F1174 and F1245) apparently causing primary resistance to ALK kinase inhibition with crizotinib (7, 11). Consistent with this, whereas marked antitumor activity was observed in the Children’s Oncology Group (COG) phase I trial of crizotinib in pediatric patients with diseases caused by ALK translocations, far fewer objective responses were seen in patients with ALK-mutated neuroblastoma (10). We therefore sought alternative ALK inhibition strategies to overcome this intrinsic resistance. Here, we report that the superior potency of PF-06463922 as an ALK kinase inhibitor delivers impressive activity across a range of in vitro and in vivo preclinical studies in which significant resistance is detected with all other inhibitors that we have tested, including crizotinib (11). Effective doses of PF-06463922 were 10- to 30-fold lower than those required for crizotinib in these preclinical studies. In particular, we show that PF-06463922 has notable and unparalleled single-agent antitumor activity (even at 3 mg/kg/day) in PDX models of ALK-mutated neuroblastoma for which the underlying ALK mutations were known to be associated with primary resistance to crizotinib. Moreover, treatment with PF-06463922 abrogates pALK more completely than crizotinib in xenograft tumors harboring the crizotinib-sensitive R1275Q mutation (in NB-1643 xenografts), and promotes sustained complete regressions without subsequent tumor regrowth. With the caveat that the mouse models used here are xenografts, rather than transgenic or orthotopic models, these data indicate that PF-06463922 will have much broader utility against ALK-driven neuroblastoma than any other ALK inhibitor that we have investigated, largely as a result of the substantially increased affinity for its target.

PF-06463922 is currently being investigated in a phase I/II clinical trial (NCT01970865) for patients with advanced non-small cell lung carcinoma (NSCLC) harboring ALK or ROS1 molecular alterations (16). Although most patients with ALK-translocated NSCLC derive substantial clinical benefit from crizotinib, some of these patients derive no benefit due to intrinsic resistance, and others derive initial benefit...
PF-06463922 Overcomes Primary Resistance in Neuroblastoma

**Figure 4.** PF-06463922 inhibits viability of neuroblastoma cell lines at concentrations corresponding to clinically relevant doses (≤100 nmol/L). Dose-response curves for the effects of crizotinib (dashed blue curves) and PF-06463922 (red curves) on cell viability are shown for a series of neuroblastoma cell lines driven by R1275Q-mutated ALK (A and B), F1174L-mutated ALK (C–F), F1245C-mutated ALK (G), and amplified wild-type ALK (H). NCI-H3122 cells, driven by an ALK translocation, are shown for comparison (I), as well as two cell lines that are not ALK dependent (J and K). Data points are plotted as means ± SD for at least three different experiments, with blue triangles for crizotinib and red squares for PF-06463922. The IC50 curves for individual experiments are also plotted, with mean IC50 values obtained over the three experiments listed in Supplementary Table S1. RLU, relative light unit; WT, wild-type.

but subsequently acquire resistance through secondary ALK mutations or other mechanisms (22). PF-06463922 was discovered using structure-guided efforts to maintain potency across a range of resistance mutations and to optimize physicochemical properties (15). It is a potent macrocyclic ALK inhibitor with good absorption, distribution, metabolism, and excretion, as well as a low propensity for P-glycoprotein-mediated efflux and considerably improved central nervous system penetration. PF-06463922 was also designed and optimized to penetrate the blood–brain barrier and has shown strong distribution in the central nervous system in preclinical studies (15). This is also likely to be a highly...
**Figure 5.** PF-06463922 displays on-target activity in neuroblastoma and NSCLC cells. A–C, NB-1643 (A), SH-SY5Y (B), and NCI-H3122 (C) cells were treated with increasing concentrations of crizotinib or PF-06463922. Levels of ALK autophosphorylation at Y1278 in the kinase activation loop were analyzed by Western blotting 24 hours after treatment, as described in Methods.

<table>
<thead>
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<tr>
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<td>–</td>
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<tr>
<td>PF-06463922</td>
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<tr>
<td>ALK pY1278</td>
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<tr>
<td>ALK (total)</td>
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<td>Actin</td>
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<tr>
<th>B</th>
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<td>ALK pY1278</td>
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<td>ALK (total)</td>
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<td>Actin</td>
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<th>C</th>
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<td>ALK (total)</td>
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<td>Actin</td>
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favorable attribute for use of PF-06463922 in neuroblastoma that recurs at this site (23).

Our results show that PF-06463922 inhibits various onco-
genically mutated ALK variants with subnanomolar $K_i$ values in vitro, and with IC$_{50}$ values in the 10 nmol/L range in cell viability assays. As in very recently reported preclinical studies of ALK-driven lung cancers (16), the activity of PF-06463922 did not vary significantly depending on the underlying ALK mutation—arguing that it will have a very broad potency in ALK-driven neuroblastomas, as well as in lung cancer. The in vivo responses to PF-06463922 that we report are unprec-
edented in ALK-driven neuroblastoma models and argue for the expedited clinical development of PF-06463922 in this disease. As is typically the case with single-agent therapies against oncogene-addicted cancers, acquired resistance to PF-06463922 is likely to arise, and future studies will need to identify the mechanisms of resistance. It is important to note, however, that the major challenge for targeted therapy.
PF-06463922 Overcomes Primary Resistance in Neuroblastoma

![Graph of ALK transformation](image)

**Figure 6.** PF-06463922 is more effective than crizotinib in inhibiting NIH-3T3 cell transformation by mutated ALK variants. Inhibition of NIH-3T3 cell transformation in focus-formation assays is shown for transfections with ALK harboring the R1275Q mutation (A), the F1174L mutation (B), and the F1245C mutation (C). The individual inhibition curves are shown for 3 to 4 experiments in each case for both crizotinib (blue dashed curves) and PF-06463922 (red solid curves), with data points from all experiments superimposed on the plots. Mean IC_{50} values (±SD) for crizotinib and PF-06463922, respectively, were 7.5 nmol/L and 0.6 nmol/L for R1275Q, 3.2 nmol/L and 2.8 nmol/L for F1174L, and 13.8 nmol/L and 3.0 nmol/L for F1245C.

in ALK-driven neuroblastoma has been overcoming de novo resistance, and our data show that this is achieved with PF-06463922. We are therefore now poised to fast-track the development of PF-06463922 to maximize clinical benefit in the subset of patients with ALK-driven neuroblastoma.

**METHODS**

**Studies of Recombinant ALK-TKD**

Protein expression, purification, and analysis of kinase activity (and inhibition) were performed exactly as described by Bresler and colleagues (7), using unphosphorylated ALK-TKD. Kinase assays monitored incorporation of \( ^{32}P \) from \( γ-^{32}P \) ATP at 25°C. Enzyme concentration was fixed at 50 nmol/L, and peptide and ATP concentrations were held fixed at 0.5 nmol/L and 2 mmol/L, respectively. All data were normalized to the no-inhibitor reaction after subtracting background counts from no-enzyme reaction, and were fitted to \( \log_{10}{IC_{50}} \) (inhibitor) versus response nonlinear-regression curve to find IC_{50}, using GraphPad Prism 5.0. Triplicate experiments were run, and mean values are reported with standard deviation.

**In Vivo Efficacy Studies**

The Felix and COG-N-453x PDXs and the SH-SYSY and NB-1643 cell line–derived xenografts were implanted subcutaneously into the right flanks of female CB17 SCID mice (Taconic Biosciences). PDX tumors were established in the laboratory of Dr. C. Patrick Reynolds and obtained through the Children’s Oncology Group Cell Culture and Xenograft Repository. For single-agent treatment arms, mice were randomized into statistically identical cohorts at a median minimum of 6 weeks. Vehicle control mice were given 0.5% methylcellulose (Clinard) and volume was calculated by spheroid formula \( \pi d^3/6 \), where \( d \) represents the mean diameter. According to the Children’s Hospital of Philadelphia (CHOP) IACUC protocol #IAC-15-000643, tumor size was measured two to three times a week starting tumor volume of 200 to 300 mm^3 with 10 mice per group. Two groups other than the vehicle group. If a mouse was sacrificed or removed for biologic or biochemical analysis, the tumor volume was considered missing on the days afterward. EFS curves were estimated using the Kaplan-Meier method and were compared using the log-rank test. Event includes death and tumor size exceeding 3 cm^3 (mouse sacrificed). Mice that were removed for biologic or biochemical analysis were considered censored at the time of removal.

**Cell Lines and Reagents**

All neuroblastoma cell lines [SH-SYSY, KELLY, 415-IMDM, NBSD, Felix, NB-1643, LAN-5, NB-1, NB-EB1, and SK-N-BE(2)C] were obtained in March 2013 from the CHOP cell bank, which is routinely genotyped by short tandem repeat (STR) analysis and tested for Mycoplasma. Annual genotyping (AmpFLSTR Identifiler Kit) of these lines and a single nucleotide polymorphism array analysis (Illumina H550) were performed to ensure maintenance of cell identity. The NSCLC cell line NCI-H3122 was generously provided by Jeffrey Engelman (Massachusetts General Hospital, Boston, MA) in March 2014. Cell lines were maintained at 37°C and 5% CO2 and cultured in RPMI-1640 media supplemented with 10% FBS, 1% l-glutamine, 1% penicillin/streptomycin, and 0.1% gentamycin (as per provider protocol, gentamycin was not used for NCI-H3122). Felix, which was established from the same postmortem sample as Felix-PDX, was grown in Iscove’s modified Dulbecco’s medium (IMDM) base media with 20% FBS, 1% l-glutamine, and 1% insulin, transferrin, and selenium (ITS; Sigma-Aldrich).

**Statistical Analysis of In Vivo Studies**

A linear mixed-effects model was used to test the difference in the rate of tumor volume change over time between different groups. The model included group, day, and group-by-day interaction as fixed effects and included a random intercept and a random slope for each mouse. A significant group-by-day interaction would suggest that the tumor volume changes at different rates for the comparison groups. The model used vehicle group as the reference group and created separate group indicators and interaction terms for other groups. Appropriate contrast statements were created to compare the two groups other than the vehicle group. If a mouse was sacrificed or removed for biologic or biochemical analysis, the tumor volume was considered missing on the days afterward.

**In Vitro Pharmacologic Growth Inhibition**

Crizotinib and PF-06463922 were provided by Pfizer, Inc. The drugs were reconstituted in 100% DMSO to a stock concentration of 10 mmol/L and stored at −80°C. Pfizer, Inc., verified the concentrations of the stocks by mass spectrometry. Cells were plated at a density of 3,000 cells per well in triplicate in black clear-bottom tissue
culture-treated 96-well plates (VWR) and treated 24 hours later with a 4-log dose range of inhibitor and a DMSO control. To ensure optimal solvation, serial drug dilutions were performed in DMSO and then diluted further in starving media (0.04% FBS in RPMI or IMDM base media) before treatment. Plates were incubated for 120 hours before measuring ATP levels using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer’s protocol. Luminescence was measured three times per plate with a GloMax-Multi Microplate Multimode Reader (Promega) to produce an average reading for each well. Viability assays were repeated three times per cell line. To generate IC50 values, the data were analyzed as follows: luminescence readings were normalized to media-only background wells and expressed as percentages of the DMSO-treated control wells. GraphPad Prism 5.0 was used to plot percent luminescence against inhibitor concentration (logarithmic scale). IC50 values and dose-response curves were then generated using the log10(inhibitor) versus response nonlinear regression, defining IC50 as the concentration that achieves half-maximal effect where complete killing was not observed.

Focus-Formation Assays

For focus-formation assays, full-length ALK variants were subcloned into the MigR1 vector (BioPioneer, Inc.). Low-passage NIH 3T3 cells (typically <15) at approximately 60% to 70% confluence were transfected with full-length mutated ALK constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were then allowed to recover for 2 days and were transferred to 6-well plates. Each well was then topped off with medium containing DMSO as vehicle control or the appropriate concentration of the inhibitor in DMSO (covering 4–5 logs). Medium (DMEM with GlutaMAX with 5% calf serum) was then changed every 3 days with a fresh inhibitor.

Focus-Formation Assays

Focus formation typically took 10 to 14 days. Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 and then stained with 0.05% crystal violet in distilled water for 30 minutes in order to count foci. The number of foci per well was normalized to the result seen in the DMSO control well. The half-maximal inhibitory concentration (IC50) was then determined from nonlinear regression analysis of a log10(inhibitor) versus response curve using GraphPad Prism 5.0. Each independent experiment was performed in triplicate at least, and mean values are reported with standard deviation. Western blot analysis in parallel with transfections showed similar levels of expression for the different mutants.

Western Blot Analysis

Between 2 × 106 and 3 × 106 cells were plated in 100 × 20 mm tissue culture dishes, allowed to attach for 24 hours, treated, and harvested on ice. Pellets were washed twice with ice-cold PBS and stored at −80°C until lysis. Cells were lysed with hypotonic lysis (10 mmol/L Tris/HCl, 10 mmol/L NaCl, 1% Triton, 1 mmol/L EGTA, pH 7.4) buffer with 1% protease/phosphatase inhibitor cocktail (Cell Signaling Technology), incubated on ice for at least 15 minutes, and then spun in a microcentrifuge at top speed at 4°C for 5 minutes. Protein from the supernatant was quantified using the Bicinchoninic Acid (BCA) Assay (Thermo Scientific). Lysates (50 μg protein) were separated on NuPAGE Novex 4% to 12% Bis-Tris gels (Life Technologies) at 130 volts, then transferred to polyvinylidene difluoride membranes (Millipore) at 4°C at 30 volts. Membranes were blocked in 5% milk or 5% bovine serum albumin fraction V, heat shock (Roche) for phospho-proteins in 1× Tris-buffered saline and Tween 20 (TBST) for 45 minutes at room temperature, and then incubated with primary antibodies overnight at 4°C. All primary antibodies were diluted 1:1,000, except for the β-actin antibodies (1:5,000). ALK pY1604, ALK pY1278, and ALK antibodies were purchased from Cell Signaling Technology, and the β-actin antibody was purchased from Santa Cruz Biotechnology. Membranes were washed for 10 minutes three times in 1× TBST before a 45-minute incubation at room temperature with the secondary antibody. Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Cell Signaling Technology and diluted 1:5,000 in 5% milk in 1× TBST. Membranes were washed again three times before developing. If necessary, membranes were stripped with a 0.1 mol/L glycine, 0.5% Tween 20 (pH 2.5) buffer for two 15-minute incubations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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REFERENCES

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