Tackling Crizotinib Resistance: The Pathway from Drug Discovery to the Pediatric Clinic
Elizabeth R. Tucker, Laura S. Danielson, Paolo Innocenti, and Louis Chesler

Abstract
Neuroblastoma is a childhood malignancy that has not yet benefitted from the rapid progress in the development of small-molecule therapeutics for cancer. An opportunity to take advantage of pharmaceutical innovation in this area arose when the identification of ALK fusion proteins in non–small cell lung cancer (NSCLC) occurred in parallel to the discovery of point mutations of ALK in neuroblastomas. ALK is now known to be a marker of poor outcome in neuroblastoma, and therefore, urgent development of specific ALK inhibitors to treat this devastating disease is a necessity. However, the translation of small molecules from adult directly into pediatric practice has thus far been challenging, due to mutation-specific structural variances in the ALK kinase domain. We discuss how the most recent structural and biological characterizations of ALK are directing preclinical and clinical studies of ALK inhibitors for both NSCLC and neuroblastoma.

Introduction
The ALK gene is found on chromosome 2p23 and encodes a receptor tyrosine kinase originally identified in anaplastic large-cell lymphoma, where it occurs as an oncogenic protein with nucleophosmin due to the chromosomal translocation t (2;5) (1). ALK protein consists of 1,620 amino acids with an extracellular domain, a transmembrane sequence, and intracellular region containing juxtamembrane and kinase domains (2). ALK came into the limelight following the discovery that in roughly 5% of non–small cell lung cancers (NSCLC), it exists as an oncoprotein, due to a translocation event that results in the fusion of the echinoderm microtubule-associated protein-like 4 (EML4) gene (3). In the childhood malignancy, neuroblastoma, for which the current treatment of intermediate and high-risk disease depends upon intensive multimodal therapy, mutations of ALK can occur, allowing constitutive phosphorylation and activation of downstream signaling. The differential activity of each individual ALK mutant found in neuroblastoma impacts upon the sensitivity of this signaling pathway to structurally different ALK inhibitors (4). This strongly suggests that genomically stratified medicine will be crucial to improve the outcome of this subset of children with neuroblastoma.

Crizotinib Resistance in NSCLC
The rapid FDA approval of the first-generation ALK inhibitor crizotinib for first-line treatment of ALK-positive NSCLC in 2011 was a milestone in the history of clinical development of small-molecule inhibitors to treat cancer. However, resistance to crizotinib was anticipated, and between 2010 and 2013 no fewer than 40 patent applications were received for the next generation of ALK inhibitors. These have widely varying structural motifs, and currently seven are in clinical trials for adult disease. Not necessarily anticipated, however, was the variety of mechanisms of drug resistance seen in relapsing patients (5). The first documented crizotinib resistance mutation in NSCLC is the "gatekeeper" mutation, L1196M (Fig. 1B;i; ref. 6). However, there are multiple additional ALK mutations that can occur in response to crizotinib treatment, together accounting for about 30% of the resistance cases seen in NSCLC (6, 7). This large number of reported secondary mutations in the ALK kinase domain following crizotinib treatment poses a great challenge to those working in the drug-discovery field, as it is apparent that no two mutations will result in the same sensitivity to a particular non-crizotinib ALK inhibitor. The work done by Ceccon and colleagues (8) was among the first to illustrate this point through the use of ALK-positive cell lines grown in increasing concentrations of crizotinib. Subsequently, cells acquired either a substitution at L1196, which conferred resistance to crizotinib, but not to AP26113 and NVP-TAE694, or the neuroblastoma-associated I1171N mutation, which was resistant to all tested inhibitors.

The Neuroblastoma Story
In 2008, a series of primary mutations in full-length ALK were identified in neuroblastoma, the pediatric malignancy that originates in the neural crest (9–12). It is now clear that three “hotspot” residues in the kinase domain account for 85% of mutations (R1275, F1174, and F1245), each of which renders ALK constitutively activated (Fig. 1B;ii; ref. 4). Substitutions at R1275 are oncogenic drivers in the majority of familial neuroblastomas, and the results of the phase I study of crizotinib in pediatric patients suggest that these patients will benefit from treatment with this inhibitor (9–11, 13). In contrast, the most predominant change in sporadic neuroblastoma, F1174L, defines relative resistance to crizotinib in the clinical setting (12, 13). The
largest cohort of neuroblastoma patients studied to date has found that not only mutations of \textit{ALK}, but also any \textit{ALK} aberration, including copy-number gain or amplification, are a marker of worse overall survival, underlining the urgent need to evaluate \textit{ALK} inhibitors for the treatment of this group of patients (4). \textit{ALK} mutations may also be acquired upon disease relapse; therefore, it is anticipated that even more patients could potentially benefit from therapeutic ALK inhibition (14).

The most common somatic \textit{ALK} mutation, F1174L, has been extensively characterized as a mutation that is transforming \textit{in vitro} (12), and that can accelerate the development of neuroblastoma tumors in animal models, in conjunction with expression of the most common neuroblastoma genetic aberration, \textit{MYCN} (15–17). The characterization of the signaling pathways activated downstream of \textit{ALK} in neuroblastoma has shown that there is overlap with those described in the context of adult \textit{ALK} tumors (Fig. 1A). Important mediators of cell proliferation and survival activated following \textit{ALK} phosphorylation are the Ras/Raf/MEK/ERK1/2/5 pathway, the JAK/STAT (Janus activated kinase/signal transducer and activator of transcription) pathway, the PI3K (phosphatidylinositol 3-kinase)/Akt pathway, and the PLC (phospholipase C)-γ pathway (Fig. 1A; refs. 15, 18, 19).

Following the recognition that children with aberrations of both \textit{ALK} and \textit{MYCN} have an especially poor outcome, it became a priority to understand the nature of the synergism between these two oncoproteins. Both the zebrafish and the \textit{ALK}F1174L; DBHCre mouse models of neuroblastoma have shown that tumor development is either dependent upon or greatly enhanced with \textit{coexpression} of \textit{Mycn} (16, 17). \textit{In vitro} studies have also shown that constitutively activated \textit{ALK} upregulates the transcription of \textit{Mycn} (20). Our \textit{Th-ALK}F1174L/\textit{MYCN} transgenic model of high-risk neuroblastoma conclusively provided the \textit{in vivo} evidence of a cooperative relationship between \textit{MYCN} and \textit{ALK}, and further demonstrated the downstream signaling pathways activated due to an \textit{ALK} mutation (15). It is essential to fully characterize the complex signaling pathways maintaining cellular proliferation in mutant \textit{ALK} neuroblastoma, because if single-agent inhibition of \textit{ALK} is insufficient to treat this disease, combinational therapeutic targeting can be explored.
Therapeutic Inhibition of ALK to Treat Neuroblastoma

Efforts to produce next-generation ALK inhibitors have been directed toward designing molecules that target the EML4-ALK mutant variants found in crizotinib-resistant NSCLC, including the gatekeeper mutation, L1196M, while optimizing bioavailability. Designs that mimic the crizotinib–ALK binding elements are popular, while alternative compounds have been identified from high-throughput screens. Of particular note are the Novartis agent ceritinib, now FDA approved, and the Ariad agent AP26113, which has been granted FDA breakthrough therapy designation, for their success in treating both crizotinib-naïve and crizotinib-resistant patients with NSCLC (21, 22). However, interrogation of crizotinib-resistant mutations in NSCLC has found that a small proportion of acquired mutations, including those at the F1174 residue, also exhibits resistance to ceritinib (23).

The first clinical trial of crizotinib in pediatric patients, although successful in demonstrating responses in a number of patients with tumors harboring ALK aberrations, suggested that ALK F1174L defined relative resistance to this inhibitor (13). The sensitivity of ALK F1174L to crizotinib inhibition is complex, as cocystalization of this inhibitor within the ALK F1174L catalytic domain predicts an adequate fit to impinge upon access to the ATP-binding site (24), and in vitro studies demonstrate that ceritinib can inhibit ALK F1174L, albeit at greater doses than required for the other neuroblastoma ALK mutants, most likely due to the increased affinity of ALK F1174L for ATP (25, 26).

A mutation at F1174 is particularly intriguing, as it results in a subtle change to the chemical structure of ALK, yet has a significant biological effect. The F1174 residue lies at the C terminus of the γC helix within the hydrophobic spine of the kinase domain, but distinct from the ATP-binding domain (Fig. 1B,ii). This is in contrast to other multi-inhibitor–resistant mutations, such as G1202R, which is found at the ATP-binding site (Fig. 1B,i; ref. 23). The F1174L mutation, which results in the replacement of the large phenylalanine side chain with a smaller leucine residue, changes the geometry of the γC helix, and overall the kinase domain of ALK adopts a catalytically active conformation (27). Moreover, the switch to a smaller residue at F1174 is likely to have an impact on the ability of the neighboring DFG motif to coordinate a magnesium ion, which is essential for the kinase activity (26).

Steady-state kinetic parameters of wild-type ALK and ALK F1174L were compared by Bresler and colleagues (26). They found that phosphorylation of the wild-type protein leads to a 45-fold activation, and that unphosphorylated F1174L had 86% of the activity of the phosphorylated wild-type enzyme. This group went further to investigate the ability of neuroblastoma ALK mutants within the kinase domain to phosphorylate a peptide corresponding to the ALK activation loop, in order to predict the transforming ability of individual mutants (4). Mutations at the F1174 and the F1245 residues, both within the hydrophobic core, promoted the strongest activation of ALK, coupled with the greatest transforming ability. Sensitivity or resistance to ceritinib correlated with IC50 values generated using transformation assays, rather than biochemical indices in several cases, suggesting that a thorough biological evaluation of next-generation inhibitors to target individual crizotinib-resistant ALK mutations will be required. Indeed, further experimental interrogation of the second-generation ALK inhibitor ceritinib has revealed that it was unable to inhibit ALK harboring the crizotinib-resistant F1174C mutation, which would structurally have a similar impact upon the kinase domain of ALK to F1174L (22).

The resistance of an F1174 mutation to two structurally diverse ALK inhibitors emphasizes the urgent need to evaluate alternative

<table>
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<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>IC50 of compound in cells (nmol/L)</th>
<th>Stage of clinical development</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Adults</td>
<td>Children</td>
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<tr>
<td>Crizotinib</td>
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<td>0.6 vs. 19</td>
<td>7.5 vs. 319</td>
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<tr>
<td>PF06463922</td>
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<td>Phase I/II</td>
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NOTE: The eight selective ALK inhibitors currently in clinical trials are shown alongside the data available to demonstrate their potency versus crizotinib in cells expressing wild-type ALK and the most frequent ALK mutations found in NSCLC and neuroblastoma, L1196 and F1174, respectively.
compounds, in order to treat patients with neuroblastoma who are resistant to currently available ALK inhibitors. There are seven ALK inhibitors in clinical use available to adult patients, including those with crizotinib-resistant NSCLC (Table 1). This is in stark contrast to only two that are in clinical trials for pediatric patients. However, the recently reported potency of the Pfizer compound, PF6463922, against F1174L in vitro clearly suggests that it could show efficacy against F1174L in vivo and warrants further investigation (28). It is clear that tackling ALK resistance will require very careful molecularly personalized medicine, with transparent collaborations between pharmaceutical companies, academia, and clinicians to select the appropriate agent, or combinations, for the specific resistant mutation or mutations acquired.

Preclinical Models of Neuroblastoma: Predicting the Most Potent Compounds for Clinical Use

An evaluation of next-generation ALK inhibitors to treat neuroblastoma using a rational methodology will allow selection of the most appropriate compounds to be taken forward into the clinic. To achieve this aim, we have developed a transgenic model of neuroblastoma where tyrosine hydroxylase drives expression of ALK F1174L and MYCN (Th-ALKF1174L/MYCN), serving as an invaluable platform for the evaluation of novel therapeutics (15). In this transgenic model, ALK F1174L expression upregulates the transcription of native Mycn, which further increases the relevance of the model to clinical cases of neuroblastoma. Transgenic models are superior to human xenograft models for translational pharmacokinetic and pharmacodynamic modeling due to spontaneous tumor onset in an immunologically competent animal, which allows the study of a comparable tumor microenvironment to the human disease equivalent. Indeed, through Th-ALKF1174L/MYCN, we were able to predict that neuroblastomas harboring ALK F1174L would not respond to crizotinib treatment in the clinic (13).

Despite the ongoing efforts to bring next-generation ALK inhibitors into clinical practice, it may be that some crizotinib-resistance mutants will require a combinational therapeutic approach to achieve tumor response. We have already demonstrated tumor regression in the Th-ALKF1174L/MYCN model when crizotinib is combined with the mTOR inhibitor Torin2 (15). As a direct result of this preclinical study, the combination is now being pursued clinically in pediatrics, with a trial planned to include crizotinib combined with temsirolimus, an agent known to be well tolerated in the combinational therapeutic setting. Further potential for combinational drug therapy to overcome crizotinib resistance in dual ALK-positive, MYCN-amplified neuroblastoma might include crizotinib with an ERK5 inhibitor, as ALK-induced transcription of MYCN and subsequent stimulation of cell proliferation requires signaling through this pathway (19).

Conclusion

Resistance to crizotinib, whether acquired, as in NSCLC, or intrinsic, as in neuroblastoma, requires a complex clinical strategy due to the wide range of resistance mechanisms. Preclinical models of neuroblastoma in which there is targeted expression of a resistant ALK mutation have a crucial role to play in the development of next-generation ALK inhibitors. Their relevance is not only toward the pediatric population, as results generated with novel compounds could provide a rationale for the treatment of adult patients with multidrug-resistant ALK mutations.

Traditionally, successful drug development requires that a large number of patients be treated to see a small effect. As genomically personalized medicine becomes a reality, developing drugs to treat specific molecular aberrations, a small population is targeted, but the benefit will be of greater magnitude (29). This is especially poignant for pediatric drug development, as the comparative rarity of childhood cancer to adult malignancies suggests that pharmaceutical companies will not be incentivized to concentrate funding in this area. However, we believe in this case that a deeper understanding of the biology of ALK mutants seen in neuroblastoma will aid the development of ALK inhibitors to treat both adult and pediatric disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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