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Running Title: Crizotinib in NSCLC

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**ABSTRACT**

Crizotinib (PF02341066, Xalkori, Pfizer) was recently approved by the Food and Drug Administration for treatment of ALK-positive non-small cell lung cancer (NSCLC) as defined by a jointly approved diagnostic test using a break-apart fluorescence in-situ hybridization (FISH) assay. The approval was made on dramatic response rates in ALK-positive NSCLC patients of 54-61% in phase I and II trials. Overall disease control rates on these trials to date are close to 90%. Progression-free survival approaches 10 months. This review focuses on the ALK inhibitory activity of crizotinib in preclinical and clinical trials that led to approval as well as the diagnostic methodologies to classify patients with ALK-positive NSCLC. Although these patients represent a small subset of all NSCLC, the rapid time course from identification of this unique target to an approved targeted therapy with striking benefit serves as a paradigm for the development of targeted therapeutics in an era of personalized medicine.
Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related deaths in both men and women in the U.S. Most patients present with advanced disease and standard chemotherapeutic agents have marginal effects on survival. However, select populations of patients with non-small cell carcinoma carry somatic genomic activating mutations, that when present, appear to singularly drive the growth of the cancer. The most frequent examples of this are mutations in the epidermal growth factor receptor (EGFR), present in approximately 15% of all NSCLC in the U.S. EGFR-targeted tyrosine kinase inhibitors show markedly greater response rates and progression-free survival than 1st-line chemotherapy in EGFR mutant patients (1-3).

**ALK rearrangement in NSCLC**

ALK (anaplastic lymphoma kinase) rearrangements were first identified as a fusion to a portion of the nucleophosmin (NPM) gene in 60% of anaplastic large cell lymphoma, a small subset of non-Hodgkin’s lymphomas.

**ALK** rearrangements in NSCLC were identified in late 2007, primarily as fusions to EML4 (echinoderm microtubule-like protein 4) (4, 5). These fusion proteins are found in approximately 3-7% of NSCLC patients overall, but have been identified primarily in lung adenocarcinomas and more frequently in younger patients and in never or light smokers (4, 6, 7). Like EGFR, KRAS, ERBB2, and BRAF, they are mostly found exclusive of other known activating mutations(8, 9).

Although the most frequent fusion partner is EML4, several different breakpoints of EML4 have been described ((4, 10), reviewed in (11)). In addition, rare other fusion partners
have also been described, including \textit{TFG} and \textit{KIF5B} (12). Most fusions link to \textit{ALK} in frame with exon 20 and the resulting protein carries a coiled-coil basic domain from the upstream fusion partner, which may facilitate activation of the \textit{ALK} tyrosine kinase by promoting dimerization (4).

Most of the identified EML4-ALK fusion proteins have been demonstrated to be oncogenic both \textit{in vitro} and \textit{in vivo} (4, 10). EML4-ALK variant 1 expressed in mouse fibroblasts generates tumors in nude mice and results in multiple adenocarcinomas when expressed in the lungs of transgenic mice (4, 13, 14). While the detailed mechanism of oncogenic activation by \textit{ALK} is not fully characterized, overexpression in cell lines results in both STAT3 and ERK activation (15). Studies of crizotinib and TAE684, another small molecule \textit{ALK} inhibitor, have demonstrated downregulation of both AKT and ERK phosphorylation (16, 17); one study demonstrated that TAE684 results in apoptosis mediated by BIM upregulation that is ERK-dependent and survivin downregulation that is STAT3-dependent (15).

\textit{Crizotinib}

Crizotinib is a potent and specific small molecule inhibitor of both \textit{ALK} and \textit{c-MET} tyrosine kinases. It is a 3-benzyloxy-2-aminopyridine series inhibitor derived from a first generation \textit{c-MET} inhibitor PHA-66752 using the co-crystal structure of this inhibitor with \textit{c-MET} to further optimize active site binding (18). In a screen for kinase selectivity, crizotinib inhibited autophosphorylation of both \textit{c-MET} and \textit{ALK} with high potency and specificity across a panel of > 120 kinases (18). Crizotinib binds in a bidentate manner at the hinge region of \textit{c-MET} and competes with ATP binding in both kinases (a crystal structure of crizotinib binding in the inactive conformation of \textit{ALK} has been reported as well (PDB ID: 2XP2)).
**Pharmacokinetics and pharmacodynamics**

Crizotinib blocks c-MET phosphorylation and c-MET dependent cell growth and invasion in a variety of cell lines at an IC$_{50}$ of 5-20 nM (18, 19). The IC$_{50}$ of crizotinib in blocking cell proliferation and apoptosis in ALK-rearranged lymphoma cell lines is 25-50 nM (18, 20). The IC$_{50}$ of crizotinib in NSCLC lines carrying an *EML4-ALK* fusion is significantly less, ranging from 250-340 nM (16, 21). This is notably less potent than other ALK inhibitors in development (17).

In a pharmacokinetic/pharmacodynamic modeling study from Pfizer, crizotinib was projected to reach adequate plasma levels to adequately inhibit ALK phosphorylation to achieve an anti-tumor effect. Specifically, in xenograft models using either H3122 cells (NSCLC line with EML4-ALK fusion) or Karpas299 cells (ALCL line with NPM-ALK fusion), ALK inhibition was seen at an EC$_{50}$ of 233 and 660 nM, respectively, and tumor growth inhibition at 255 and 875 nM, suggesting that ≥50% ALK inhibition is required for anti-tumor efficacy (22). Using clinical pharmacokinetic data, >70% ALK inhibition was projected at the now FDA-approved dose of 250 mg twice daily.

On a phase I clinical study of crizotinib, patients treated at the recommended phase 2 dose (RP2D) were noted to reach a C$_{\text{max}}$ of crizotinib at a median T$_{\text{max}}$ of 4 hours with a terminal half-life of 42 hours after a single dose (23). Steady-state levels were reached within 15 days, with non-linear pharmacokinetics (decreasing CL/F with multiple dosing). This is felt to be due to autoinhibition of CYP3A after reaching steady state. Because of these observations, an amendment to the phase I study is now evaluating once daily dosing with safety observed at doses up to 650 mg daily to date (Pfizer, pers. comm.). Notably, crizotinib C$_{\text{max}}$ and AUC in
Asian patients were approximated 1.5X that of non-Asian patients, but without increased toxicities.

**Safety**

Crizotinib was well-tolerated overall, with rare grade 3/4 toxicities that included pneumonitis (1.6% across phase I and II studies) which usually occurred within 2 months of initiating drug (24). Two deaths attributed to treatment-related pneumonitis were reported in the phase II study (25). Grade 3/4 ALT elevation (4% on phase I study and 7% on stage II) was also noted resulting in one treatment discontinuation. Overall, elevated transaminases were attributable to drug in 19% of patients.

More common treatment-related toxicities included visual disturbances, which occurred in 62% of patients, but all were grade 1 or 2 effects. These were primarily described as light trails (following a moving lighted object) or light flashes or brief image persistence (i.e. following camera flashbulb use). Flipped dark-light registration in high-contrast images was also reported. No ophthalmologic abnormalities were noted in the limited exams that were performed.

Other frequent treatment-related toxicities that were primarily grade 1 or 2 included nausea (53%), diarrhea (43%), vomiting (40%), constipation (27%), edema (28%), fatigue (20%), anorexia (19%), dizziness (16%), neuropathy (13%), and dysguesia (12%). Rash was noted in 10% of patients and reflux or other esophageal symptoms in 11%. After a food effect evaluation was done with the findings of only 14% reduction in Cmax and AUC after a high-fat meal, patients were allowed to take the drug in a non-fasting state, which resulted in substantial reduction of nausea and vomiting.

**Clinical Efficacy**
Preclinical data suggested potential efficacy of crizotinib against both MET and ALK; however, it was the dramatic results seen in ALK-positive NSCLC that served as the impetus for accelerated FDA approval. Following responses seen in two ALK-positive patients during dose-escalation, the phase I study of crizotinib in solid tumors was amended to include an expanded cohort for ALK-positive lung cancer, onto which 119 patients have been enrolled. The overall response rate amongst these patients is 61% (26) with an overall clinical benefit rate (complete response (CR) + partial response (PR) + stable disease (SD)) of 88%. In patients who were chemotherapy-naïve (24 patients), the response rate was 68%. Preliminary median survival was 10 months. These striking data served as the basis for rapid initiation of both a phase II trial of crizotinib for patients who have relapsed following 1st line chemotherapy as well as phase III randomized trial of crizotinib vs. 2nd-line therapy. As reported to date, the response rate on the phase II trial in 76 patients evaluable for response was 54% with a 91% disease control rate (PR + SD) (25).

These results led to the accelerated FDA approval of crizotinib for ALK-positive NSCLC in record time—the time frame from discovery of the target in late 2007 to FDA approval of a targeted therapy in August 2011 was less than 4 years (Figure 1). The approval comes while both the phase I and phase II trials are still ongoing. In addition, a phase III trial comparing crizotinib against standard 2nd-line therapy is still accruing and a more recent phase III trial comparing crizotinib to first-line platinum doublet chemotherapy has been launched as well. Importantly, the FDA approval was not linked to any line of therapy and given the commercial availability of crizotinib, accrual to these large phase III trials, especially the first line trial, may be slowed.
The approval of the drug also makes a prospective evaluation of the overall survival benefit of crizotinib challenging. Although the use of EGFR-directed TKIs is markedly better than 1st-line chemotherapy in terms of progression-free survival for EGFR mutation patients, the overall survival is not statistically different because most patients are likely to get an EGFR TKI during some point of their therapy (1-3) and the same is likely to be true of crizotinib. A retrospective study examining the outcomes of 36 ALK-positive patients NOT treated with crizotinib compared with 56 patients treated on the phase I expanded cohort of crizotinib demonstrated that amongst patients treated in the second or third line, there was improved overall survival compared with those treated with other therapies (HR 0.36 (95% CI 0.17-0.75; p=0.004)(27).

Future Clinical Direction

Although much of the attention on crizotinib has focused on its activity against ALK-positive NSCLC, equally dramatic responses have been seen against ALK positive lymphoma (28) as well as MET-amplified gastroesophageal cancers and NSCLC (29, 30). An independent study for crizotinib in ALK-positive lymphoma is now ongoing (NCT00939770). In addition, in vitro studies identified a ROS1-rearranged cell line as sensitive to TAE684, another ALK inhibitor, (16) and this alteration has been identified in clinical specimens as well (5). An effort to look for ROS1 translocations (using a break-apart FISH probe similar to that for ALK) demonstrated ROS1 rearrangements in 18 of 1073 of NSCLC patients screened (31). One of these was treated on the expanded cohort of the phase I study of crizotinib and achieved a dramatic and sustained response similar to those of ALK-positive patients. These observations
suggest a broader role for crizotinib as an effective therapy against multiple targets in multiple tumor types.

**ALK Diagnostics**

Although the phase I trial of crizotinib allowed several different local lab determinations of *ALK* rearrangement for approval of eligibility, the phase II trial mandated central testing for *ALK* rearrangement using the now FDA-approved Vysis ALK Break Apart FISH Probe Kit (Abbott Molecular). The test, which utilizes a red and a green probe flanking the *ALK* translocation breakpoint, is considered positive if 15% or more at minimum 50 nuclei evaluated have a split or isolated red signal that results from the intervening fusion of a translocated protein fragment such as *EML4*. Although “clinically validated” in the sense that it was the test used in crizotinib trials, when compared to other methodologies, it is associated with several practical challenges. FISH requires specialized equipment and expertise and can cost > $1000 per test. In addition, the interpretation is sometimes not clear-cut depending on how close the signals are to each other. Multiple alternative methods of evaluation have been and are being studied, but each has potential drawbacks (Table 1).

Chromogenic in-situ hybridization (CISH) offers a lower-cost alternative to FISH and allows simultaneous histologic evaluation. A study utilizing CISH for detection of *ALK* rearrangement demonstrated high concordance with FISH (95%) (32). However, CISH does not get around the problem of sometimes subtle or difficult to interpret hybridization pattern.

RT-PCR is an alternative diagnostic methodology that utilizes multiplexed primer pairs to amplify known possible *ALK* fusions. This test has been shown to be sensitive and specific for detecting and *identifying* particular *ALK* fusions, but will by definition miss as yet
uncharacterized fusion partners (10). In addition, the success of the test depends on the quality of the sample and whether the RNA is degraded. The test has been optimized on formalin-fixed paraffin embedded specimens (FFPE) in addition to fresh tissue, but the quality of the former is more variable.

Immunohistochemistry for ALK protein is another alternative test as there is essentially no ALK expression in normal lung epithelia. However, even within ALK-positive lung tumors, the expression of ALK is relatively low (compared to ALK expression in ALK-rearranged lymphoma) and antibodies that are used routines for detection of ALK-positive lymphoma, such as the DAKO clone ALK1, demonstrated specificity but relatively low sensitivity for detecting ALK in NSCLC (33). Increased concentration of antibody and the use of novel methods to amplify the secondary signal did result in improved sensitivity and higher concordance with FISH (34).

Other antibodies also have improved sensitivity and higher concordance with FISH, including one developed by Cell Signaling Technology, DF3, which demonstrated a sensitivity of 100% and specificity of 99% and showed much improved staining compared to the ALK1 antibody (35). Another promising antibody for routine use may be the 5A4 antibody from Novocastra, which demonstrated a sensitivity of 100% and specificity of 96% (36). However, moderate staining (+2) is more variable in determining ALK-rearrangement as detected by other methodologies (36, 37).

Studies comparing all three methodologies (excluding CISH) do not demonstrate clear superiority in all aspects of one methodology (reviewed in (38)). Rather, they suggest that ALK testing may one day mirror HER2 testing in breast cancer, with an initial screen by IHC (using an as yet to be agreed upon antibody and IHC procedure) with grading of intensity on a +1 to +3
range, followed by FISH confirmation of moderate staining. RT-PCR may be used to identify specific fusion partners when indicated.

**Crizotinib resistance**

Additional methodologies for ALK evaluation will also be required to streamline the detection of resistance mutations. Several resistance mutations have already been identified in patients treated with crizotinib (12, 17, 39); the first report of two acquired point mutations, C1156Y and L1196M, was published concurrently with the first report of activity of crizotinib (12). While some of the identified resistance mutations, such as L1196M and G1269A, are found within the active site and likely interfere with crizotinib binding (L1196M is analogous to resistance mutations involving a “gatekeeper” residue found in EGFR and ABL), other mutations (i.e. L1152 and C1156Y) do not have a clear mechanism for causing resistance to crizotinib (40). Studies of acquired resistance in vitro (41, 42) have identified these clinically identified mutations as well as other resistance mutations—all have variable resistance to crizotinib in vitro. Of the 7 clinically identified mutations to date, the degree of resistance to crizotinib is variable. Some are still sensitive to structurally different ALK inhibitors with greater potency than crizotinib as well as to Hsp90 inhibitors, which are active clinically against ALK positive non-small cell lung cancers (17).

Unlike EGFR resistance to erlotinib, which occurs by a common mechanism in over 50% of cases (T790M gatekeeper mutation), ALK resistance appears more variable. Not only are there more varied mutations identified, but many cases have no identified ALK secondary mutation. EGFR activation and ALK copy number gain have been identified as mechanisms of acquired resistance in both patients and in vitro models (17);(31, 39, 40). Clinically, KIT amplification (with concomitant increased expression
of the KIT ligand, SCF) and the emergence of independent, KRAS mutant clones have also been identified as mechanisms of resistance to crizotinib (17, 39). The largest series published to date suggests that less than 25% of resistance occurs via acquisition of a secondary point mutation and greater than 50% of resistance may occur via alternate tyrosine kinase activation. With the use of more potent ALK inhibitors, alternate pathway activation may become an even more important issue with which to grapple.

ALK-targeted therapy, as well as the identification of ALK-positive NSCLC tumors, is a rapidly evolving field, which has been put on the map by crizotinib. Although future refinements in diagnostic tests are likely and more potent inhibitors are currently in clinical development, crizotinib and the break-apart FISH test have clearly separated a group of NSCLC patients with dramatically improved outcomes than the majority of patients. This example of a molecularly –defined target and an effective therapeutic strategy mirrors the experience with EGFR mutant NSCLC and EGFR-directed TKIs and hopefully serves as a model for more examples to come.
Figure legend

**Figure 1.** Time line for approval of kinase inhibitors for molecular subsets of NSCLC. EGFR inhibitors were approved initially for a broader population and subsequently for *EGFR* mutant NSCLC patients. In contrast crizotinib clinical development and approval has been limited to *ALK*-positive NSCLC patients.
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<th>TEST</th>
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| Break-apart FISH | Can detect any rearrangement  
|              | Performed on FFPE tissue  
|              | Used clinically in crizotinib trials | Cost  
|              |                           | Expertise required  
|              |                           | Often difficult to interpret      |
| CISH        | Cost  
|             | Performed on FFPE tissue  
|             | Simultaneous histology evaluation | Expertise required  
|             |                           | Often difficult to interpret      |
| RT-PCR      | Needs minimal material  
|             | Can identify fusion partners | Will not identify new fusions  
|             |                           | RNA degradation  
|             |                           | Possibility of contamination      |
| IHC         | Cost  
|             | Ability to perform in any lab  
|             | Performed on FFPE tissues | Low-level ALK expression  
|             |                           | No preferred antibody as yet      |
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EGFR mutations identified in NSCLC

Quinazoline EGFR inhibitors discovered

Erlotinib approved for NSCLC

Gefitinib approved for EGFR mutant NSCLC

1990s 2004 2005 2009

ALK Timeline

Crizotinib developed

Responses seen in ALK+ NSCLC treated on phase I study of crizotinib

2005 2007 2009 2011

EML4-ALK discovered in NSCLC

Crizotinib approved for ALK+ NSCLC

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