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The preclinical profile of crizotinib for the treatment of non-small-cell lung cancer and other neoplastic disorders

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Introduction: The critical role of the activity of the nucleophosmin- anaplastic lymphoma kinase (NPM-ALK) fusion protein in anaplastic large-cell lymphoma prompted drug discovery programs directed against ALK. Drug discovery efforts increased after finding that about 4% of non-small-cell lung cancers (NSCLCs) possess an EML4-ALK fusion protein.

Areas covered: The author provides a review of the development of crizotinib, an orally effective c-Met and ALK protein kinase inhibitor. The article highlights its beginning with the X-ray crystallographic structure of a lead compound (PHA-0665752) bound to the active site of the kinase domain of c-Met.

Expert opinion: Studies of patients with EML4-ALK–positive NSCLC showed that crizotinib was clinically effective and led to its approval in August 2011. The use of lipophilic efficiency played a crucial role in the development of crizotinib from a lead c-Met inhibitor. The use of X-ray crystal structures from lead compounds, bound to their targets, is increasing in the drug discovery process owing to its effectiveness. That the drug also inhibits ALK and ALK-fusion proteins was serendipitous, however. The discovery of the EML4-ALK fusion protein in some NSCLC patients has led to the testing and rapid approval of the compound.

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Keywords: anaplastic lymphoma kinase, c-Met, epidermal growth factor receptor, Hsp90, lung cancer, protein kinase, structure-based drug design, targeted cancer therapy

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1. Introduction

Cancer of the lung is the most prevalent malignancy in the world [1]. In 2008, the estimated global incidence of lung cancer was about 1.6 million with about 1.4 million deaths attributed to this malignancy. In 2012, the estimated incidence of lung cancer in the United States was about 228,000 [2]. Lung cancers are clinically classified into two major groups: non-small-cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers, and small cell lung cancer, which accounts for the remainder [3]. It is important to distinguish between them because their clinical course and treatments vary. The overall 5-year survival rate for lung cancer is about 15%. The mainstay of localized (stages I and II) lung cancer is surgical removal; platinum-based adjuvant therapy (carboplatin or cisplatin) is recommended for stage II NSCLC following complete tumor resection [4]. Unfortunately, only 37% of lung cancers are diagnosed before the tumor has spread from its site of origin [2]. Pre-existing medical conditions in patients with stages I or II lung cancers decrease the number of patients that can undergo surgery to about 25 – 33% of the total (down from 37%). In metastatic disease, combined radiotherapy and chemotherapy improves survival. Commonly used therapeutic regimens include carboplatin and

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Article highlights.

- The EML4-ALK fusion protein occurs in about 4% of NSCLCs amounting to about 9000 new cases per year in the United States and 64,000 worldwide.
- Crizotinib was developed as an inhibitor of c-Met (hepatocyte growth factor receptor) by SBDD.
- Crizotinib is an effective inhibitor of EML4-ALK and other ALK-fusion proteins, which occur in several types of tumors including ALCL, the disease which gave ALK its name.
- Crizotinib is an effective therapy for ALK-positive NSCLC with 90% or more patients experiencing tumor shrinkage.
- Essentially all patients who have a beneficial effect from crizotinib develop drug resistance and relapse.
- Formulating alternative drugs and alternative treatments in patients before and after the development of acquired crizotinib resistance is an important problem that needs further investigation.

This box summarizes key points contained in the article.

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paclitaxel [5]. Other agents that have been used in various combinations include cisplatin, docetaxel, etoposide, gemcitabine, pemetrexed and vinorelbine [5,6]. Bevacizumab, a monoclonal antibody that binds vascular endothelial growth factor and inhibits angiogenesis, improves survival of patients with advanced non-squamous cell NSCLC in combination with carboplatin and paclitaxel [7].

Molecular targeted therapies have been developed for the treatment of advanced lung cancer in people with activating epidermal growth factor receptor (EGFR) mutations or with the occurrence of EML4-ALK fusion proteins [8-10]. Both of these targets possess protein-tyrosine kinase activity, and currently approved small molecule drugs reversibly inhibit this activity. Erlotinib (Tarceva®, OSI Pharmaceuticals and Genentech) was approved by the United States Food and Drug Administration (USFDA) in 2004 for second-line treatment of advanced NSCLC following platinum-based therapy. It is most effective in treating people with activating EGFR mutations, which occur in about 12% of lung cancer cases in the United States [11] or about 27,000 new cases per year. The incidence of EGFR mutations in Asian patients with NSCLC is much higher (about 31%) [11].

Another molecular target in NSCLC is the EML4-ALK fusion protein, which possesses protein-tyrosine kinase activity and was discovered in 2007 [12,13]. Kwak *et al.* reported that the incidence of EML4-ALK fusion proteins in NSCLC was 5.5% (82/1500), which amounts to ~ 82/1800 for all lung cancers assuming that NSCLCs account for 85% of all lung cancers [8]. Dai *et al.* reported that the incidence was about 3.5% in all lung cancers (49/1387) [14]. Adding these two large studies (~ 131/3200) gives an incidence of about 4% or about 9000 new cases in the United States per year.

Although crizotinib (Xalkori®, Pfizer) was initially developed as an inhibitor of the c-Met (hepatocyte growth factor) receptor protein-tyrosine kinase, it also inhibits anaplastic lymphoma kinase (ALK) and the EML4-ALK fusion protein kinase [15]. Cui *et al.* began with a lead compound (PHA-665752) and systematically altered it to better its pharmacological properties using lipophilic efficiency (LipE) as a criterion [15]. The dose-escalation portion of the Phase I study of crizotinib was underway when EML4-ALK was first reported in lung cancer [12,16]. Soon thereafter, patients with ALK-positive lung cancer were enrolled in dose-escalation trials. By 2010, Kwak *et al.* reported that EML4-ALK–positive NSCLC patients exhibited an overall crizotinib response rate of about 55% [8]. This orally effective agent was approved by the USFDA in 2011 for the first-line treatment of ALK-positive NSCLCs that are identified with the USFDA-approved Vysis® ALK Break-apart Fluorescence *in situ* Hybridization (FISH) Probe Kit (Abbott Molecular). Although many patients initially respond to this small molecule ALK protein kinase inhibitor, acquired resistance to crizotinib is a common, if not universal, occurrence [16].

2. ALK and hepatocyte growth factor receptor (c-Met)

The protein kinase family consists of > 500 members [17]. These enzymes catalyze the reaction shown in the following equation:

$$\mathrm{MgATP^{1-} + protein\text{-}OH \rightarrow Protein\text{-}OPO_3^{2-} + MgADP + H^+} \quad (1)$$

based on the nature of the phosphorylated –OH group, these proteins are classified as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90 members) and tyrosine-kinase-like proteins (43 members). Protein phosphorylation is the most widespread class of posttranslational modification used in signal transduction. Families of protein phosphatases catalyze the dephosphorylation of proteins, thus making phosphorylation–dephosphorylation an overall reversible process [18]. Perhaps one-quarter to one-third of all funds supporting drug discovery worldwide are directed toward protein kinases. For a list of USFDA currently approved small molecule protein kinase inhibitors and their indications, see www.brimr.org/PKI/PKIs.htm.

ALK is a member of the insulin receptor protein-tyrosine kinase superfamily [19]. In 1995, Morris *et al.* characterized an unknown protein-tyrosine kinase in anaplastic large-cell lymphoma (ALCL) cell lines [20]. About two-third of ALCLs possess a balanced chromosomal translocation in which the entire *NPM* (nucleophosmin) gene on chromosome 5 is fused to the 3′ portion of the *ALK* gene on chromosome 2. This chromosomal rearrangement results in the ectopic expression of the nucleophosmin-ALK (NPM-ALK) fusion protein that has a constitutively activated ALK kinase domain; the kinase was named after the disease [20]. Moreover, two groups identified an EML4-ALK oncokinase in

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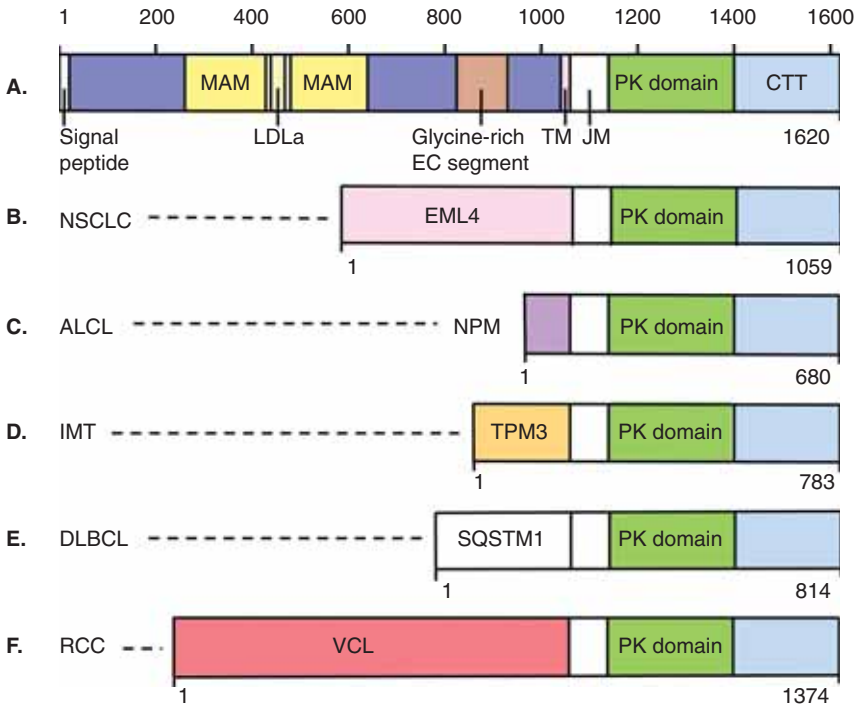

Figure 1. (**A**) General structure of the native human ALK receptor protein-tyrosine kinase. The extracellular segment (residues 19 – 1038) of ALK contains two MAM components (264 – 427 and 480 – 626), an LDLa domain (453 – 471) and a glycine-rich segment (816 – 940). A transmembrane segment (residues 1039 – 1059) connects the extracellular domain with the juxtamembrane segment (1060 – 1115) of the intracellular domain (1060 – 1620). The protein kinase domain consists of residues 1116 – 1392. (**B** – **F**) Structures of selected ALK- fusion proteins that occur in the listed diseases. The numbers denote amino acid residues, and those for ELM4-ALK correspond to variant 1. The entire intracellular domain (excluding the transmembrane segment) occurs in each of the fusion proteins.

ALCL: Anaplastic large-cell lymphoma; CTT: Carboxyterminal tail; DLBCL: Diffuse large B-cell lymphoma; EML4: Echinoderm microtubule-associated protein-like 4; IMT: Inflammatory myofibroblastic tumor; JM: Juxtamembrane; NSCLC: Non-small-cell lung cancer; NPM, nucleophosmin; RCC: Renal cell carcinoma; SQSTM1: Sequestome-1; TM: Transmembrane; TPM3: Tropomyosin α3-chain; VCL: Vinculin.

NSCLCs [12,13]. About 20 variants of this fusion protein depend on the length of EML4 that is attached to ALK. More than two dozen other ALK fusion proteins have been described that occur in a variety of diseases [21].

In 1997, Morris *et al.* reported that physiological ALK consists of an 18 amino-acid-residue signal peptide, a long extracellular domain (1020 amino acid residues in humans), a 21-residue transmembrane segment and a 561 amino acid intracellular domain (UniprotKB: Q9UM73) [22]. The intracellular portion consists of a juxtamembrane segment, a protein kinase domain, and a carboxyterminal tail (**Figure 1**) [23]. The extracellular domain contains two MAM segments, one LDLa domain and a glycine-rich portion. Each MAM (meprin, A5 protein, and receptor protein-tyrosine phosphatase mu) domain consists of about 170 amino acid residues containing four cysteines. Two proteins, pleiotrophin and midkine, have been reported to be the activating ligands for mammalian ALK [24,25]. However, these studies were not confirmed and the nature of the physiological ligand(s) for ALK is unclear [26,27].

c-Met is a receptor protein-tyrosine kinase whose activating ligand is hepatocyte growth factor/scatter factor (HGF/SF) [28]. c-Met is the hepatocyte growth factor receptor (HGFR). The term 'Met' originally referred to the methyl group in the carcinogen (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) used in generating the fusion protein in a human osteogenic sarcoma cell line. In addition to its role in promoting cell division and survival, c-Met also plays a role in the metastasis of cancer cells. c-Met may be thought of as an abbreviation for 'metastasis' or an acronym for 'mesenchymal-epithelial transition' factor [15]. In this review, the cellular proto-oncogene will be referred to as c-Met in order to distinguish it from the abbreviation for the amino acid methionine (Met).

Similar to ALK, c-Met consists of an extracellular ligand-binding domain, a transmembrane segment, a juxtamembrane segment, a protein kinase domain and a carboxyterminal tail [28]. Following HGF/SF binding, protein kinase activity is activated following receptor dimerization and phosphorylation of two tyrosine residues in its activation segment. Following activation, c-Met-mediated phosphorylation of two tyrosine

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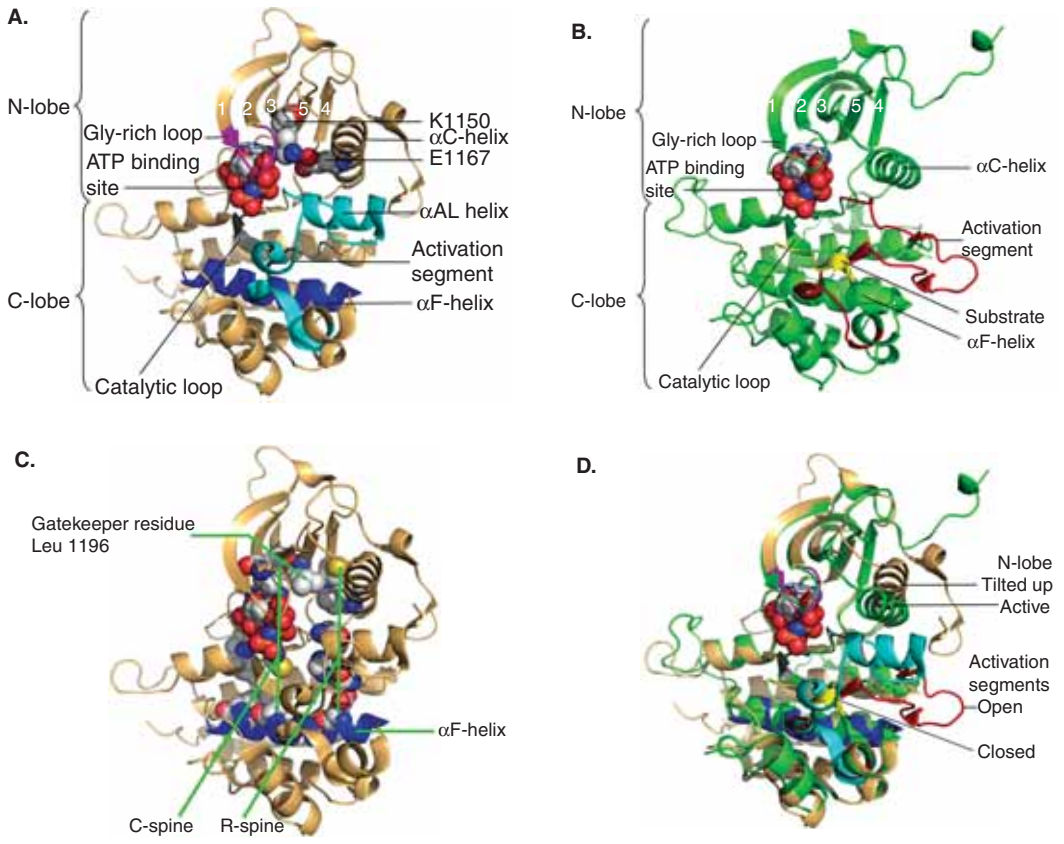

Figure 2. (**A**) Ribbon diagram of the dormant human ALK kinase domain. β-strands 1 – 5 are labeled. β3 Lys1150 and αC Glu1167, which form a salt bridge, are shown as space-filling models. (**B**) Ribbon diagram of the activated insulin receptor protein kinase domain. (**C**) Catalytic and regulatory spines and the gatekeeper residue of ALK are shown as space-filling models. The view is the same as above. (**D**) The activated insulin receptor kinase domain is superposed on the dormant ALK domain. The activation segment of the ALK kinase domain is in an inactive closed conformation, and its N-lobe is tilted upward from the C-lobe when compared with that of the activated insulin receptor. The structures of dormant ALK were prepared from PDB 3LCS and those of the activated insulin receptor were prepared from PDB 1IR3 using the PyMOL Molecular Graphics System Version 1.5.0.4 Schrödinger, LLC.

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residues in the carboxyterminal tail provides binding sites for signal-transduction docking proteins including Gab1, Grb2, phospholipase C (PLC) and Src.

A large number of malignancies that exhibit sustained c-Met activation owing to its stimulation, overexpression or mutation are potential c-Met inhibitor drug targets, which include lymphomas, rhabdomyosarcomas and carcinomas of the kidney, liver, ovary, stomach and thyroid [29-33]. Moreover, activating mutations of c-Met are clonally selected during the metastasis of human head and neck cancers [34] as their frequency increased from 2% in primary tumors to 50% in the metastatic tumors. Activated c-Met also promotes migration, invasion and metastasis, thereby increasing the number of c-Met inhibitor targets. Furthermore, c-Met promotes angiogenesis [35], which participates in tumor progression [36], suggesting that c-Met inhibition may have additional beneficial effects.

3. ALK and c-Met protein kinase structure and function

3.1 Catalytic protein kinase signature residues

All protein kinases including ALK and c-Met have a small amino-terminal lobe and large carboxyterminal lobe that contain several conserved α-helices and β-strands. The small lobe is dominated by a five-stranded antiparallel β-sheet (β1 – β5) [37]. It also contains an important regulatory αC-helix that occurs in active or inactive positions, the orientation of which is important in the drug discovery process. The small lobe contains a conserved glycine-rich (GxGxxG) ATP-phosphate-binding loop (**Figure 2A**). The glycine-rich loop helps position the β- and γ-phosphates of ATP for catalysis. A conserved glutamate occurs near the center of the αC-helix in protein kinases. The presence of a salt bridge between the conserved β3-lysine (K1150) and the αC-glutamate (E1167)

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in ALK is a prerequisite for the formation of the active state and corresponds to the 'αC-in' conformation (**Figure 2A**). The αC-in conformation is necessary but not sufficient for the expression of full kinase activity. However, the absence of this salt bridge in the αC-out conformation indicates that the kinase is inactive.

The large lobe of protein kinase domains including those of ALK and c-Met is mainly α-helical with six conserved segments (αD – αI) [37]. The large lobe also contains short conserved β-strands that contain most of the catalytic residues associated with the phosphoryl transfer from ATP to kinase substrates. Hanks *et al.* identified 12 subdomains (I – VIa, VIb – XI) with conserved amino acid residue signatures that constitute the catalytic core of protein kinases [38]. The catalytic loops surrounding the actual site of phosphoryl transfer are different between the protein-serine/threonine and protein-tyrosine kinases. This loop is made up of YRDLKPEN canonical sequence in protein serine/threonine kinases and an HRDLAARN sequence in protein-tyrosine kinases. The occurrence of HRDIAARN in NPM-ALK, which was initially determined by Morris *et al.* [20], allowed them to identify ALK as a receptor protein-tyrosine kinase. The AAR sequence in the catalytic loop is a receptor protein-tyrosine kinase signature and RAA is a non-receptor protein-tyrosine kinase signature.

The following four amino acids, which define a K/E/D/D (Lys/Glu/Asp/Asp) motif, illustrate the catalytic properties of protein kinases. The invariant β3-strand K (Lys1150), which binds to the αC-helix E (Glu1167), forms salt bridges with the α- and β-phosphates of ATP. An aspartate (D1247) occurring within the catalytic loop HRD (His–Arg–Asp) sequence accepts a proton from the protein substrate –OH group. The second aspartate (D1270) of the K/E/D/D signature is the first residue of the activation segment. The activation segment of nearly all protein kinases begins with DFG (Asp–Phe–Gly) and ends with APE (Ala–Pro–Glu). The ALK activation segment begins with DFG but it ends with PPE (Pro–Pro–Glu). The DFG-aspartate binds Mg^{2+} that in turn coordinates the α-, β- and γ-phosphates of ATP. The large lobe characteristically binds the peptide/protein substrates as depicted for the insulin receptor kinase domain (**Figure 2B**). The activation segment is an important regulatory element in protein kinases, which influences both substrate binding and catalytic efficiency [39].

3.2 Hydrophobic spines

Taylor and Kornev [37] and Kornev *et al.* [40] analyzed the structures of active and inactive conformations of some two dozen protein kinases and determined functionally important residues by a local spatial pattern alignment algorithm. This analysis revealed a skeleton of four nonconsecutive hydrophobic residues that constitute a regulatory or R-spine and eight hydrophobic residues that constitute a catalytic or C-spine. Each spine consists of residues derived from both the small and large lobes (**Figure 2C**). The regulatory spine contains residues from the αC-helix and the activation loop (AL), whose conformations are important in defining active or dormant enzyme states. The catalytic spine governs catalysis by directing ATP binding. The two spines dictate the positioning of the protein substrate (R-spine) and ATP (C-spine) that results in catalysis. The proper alignment of the spines is necessary for the assembly of an active kinase.

Although the tertiary structure of catalytically active protein kinase domains are strikingly similar, Huse and Kuriyan reported that the crystal structures of inactive enzymes reveal a multitude of distinct protein kinase conformations [41]. The practical consequence of this is that drugs targeting specific inactive conformations may be more selective than those targeting the active conformation [42]. Huse and Kuriyan noted that protein kinases usually assume their less active conformation in the basal or non-stimulated state, and the acquisition of their activity may involve several layers of regulatory control [41]. As mentioned above, the three main regulatory elements within the kinase domain include the small lobe αC-helix (αC-in, active; αC-out, inactive), the large lobe DFG-Asp (DFG-Asp in, active; DGF-Asp out, inactive), and the large lobe AL (AL open, active; AL closed, inactive).

3.3 ALK and insulin receptor protein kinase X-ray crystal structures

X-ray crystallographic structures of unphosphorylated and dormant ALK indicate that it does not possess all of the negative regulatory elements observed in other protein kinases. First, dormant ALK assumes the DFG-Asp in conformation [43,44], which corresponds to the active state, rather than the inactive DFG-Asp out conformation. In dormant ALK, the αC-helix is rotated into the active site properly positioning Glu1167 of the αC-helix with Lys1150 of the β3-strand. This structurally important Glu-Lys salt bridge, which occurs in active protein kinase conformations, is observed in unphosphorylated dormant ALK (**Figure 2A**). However, the ALK AL precludes peptide/protein substrate binding.

The ALK αC-helix is restricted in its mobility by an α-AL helix in the activation segment (**Figure 2A**), which plays an important role in maintaining ALK kinase in its dormant state [43,44]. Middle residues of the AL block peptide/protein substrate binding, thereby contributing to the dormant status of the enzyme. The relative interlobe closure between the small and large lobes of ALK is intermediate between that of the less active open and more active closed conformations exhibited by the insulin receptor protein kinase domain. This difference in interlobe closure is depicted by the positions of the αC-helices where the more active insulin receptor αC-helix is closer to the C-lobe than that of ALK (**Figure 2D**). The restricted mobility of the αC-helix, the obstruction of the peptide-binding site by the AL and the lesser degree of interlobe closure all contribute to ALK enzyme dormancy.

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3.4 ALK and c-Met protein kinase activation

Ligand binding to the extracellular domain of receptor protein-tyrosine kinases including ALK and c-Met usually activates them by inducing receptor dimerization [19]. The AL of receptor protein-tyrosine kinases contains one or more tyrosines that, on phosphorylation, lead to enzyme activation. Both ALK and c-Met contain three such tyrosine residues. The probable mechanism for the ligand and dimer-induced activation of ALK and c-Met involves the transphosphorylation of AL tyrosines by the partner protein kinase. The trisphosphorylated kinase may then catalyze the transphosphorylation of the partner AL tyrosines. In the case of EML4-ALK, the EML4 at the amino terminus of the fusion protein induces ligand-independent dimer formation, which is followed by AL phosphorylation and kinase activation.

4. ALK activation following the formation of fusion proteins

Since the discovery of the NPM-ALK fusion protein in human anaplastic lymphoma cell lines [20], more than two dozen different ALK fusion proteins have been described in several malignancies including NSCLC [45]. NPM refers to nucleophosmin, which participates in ribosome biogenesis and serves as a protein chaperone. ELM4 refers to echinoderm microtubule-associated protein-like 4, which may participate in microtubule assembly. The prevalence of the ALK fusion proteins in many of these malignancies is generally low, which thereby limits their usefulness as drug targets. Despite the occurrence of the EML4-ALK fusion protein in only 4% of NSCLCs, the large number of patients with this disease makes it the most prevalent disease target for ALK kinase inhibitors.

The transcription of the fusion protein is driven by the promoter of the ALK partner's protein [46]. This accounts for the ectopic expression of the ALK kinase domain in various cell types; full-length ALK is ordinarily expressed in significant amounts only in the developing nervous system [46]. The fusion partner mediates the ligand-independent dimerization of the ALK-fusion protein, which leads to activation of the protein kinase domain by transphosphorylation as described above. Dimerization, thus, produces kinase activation.

5. Discovery strategy and preclinical development of crizotinib

5.1 Development of crizotinib

Although crizotinib is approved by the USFDA for the treatment of NSCLCs that possess the EML4-ALK fusion protein, the initial inhibitory drug target was c-Met [15]. Cui *et al.* published a comprehensive paper describing their development of crizotinib using structure-based drug design (SBDD) [15]. These investigators monitored the progress of optimization using LipE, which is given by the following equations:

$$\text{LipE} = \text{p}K_\text{i}\text{-cLogD} \quad (2)$$

$$\text{LipE} = \text{pIC}_{50}\text{-cLogD} \quad (3)$$

Equation 2 corresponds to the inhibition of the purified protein kinase domain (K_i), and Equation 3 corresponds to the inhibition protein kinase activity in intact cells (IC_{50}). The first term on the right side of each equation is a measure of drug potency. The p of pK_i or of pIC_{50} is the negative of the Log_{10} of K_i or IC_{50}. The second term (cLogD) represents the lipophilicity of a drug where c indicates that the value is calculated using algorithms based on the behavior of thousands of organic compounds. cLogD is the calculated logarithm of the distribution coefficient, which is the ratio of drug solubility in octanol/water at a specified pH, usually 7.4. The more soluble the compound is in octanol, the greater is its lipophilicity and the greater is the value of cLogD. A related term, cLogP, is the calculated log of the solubility in octanol/water ignoring the ionizations that change as a function of pH. Leeson and Springthorpe suggest that compound lipophilicity, as estimated by cLogP, is the most important chemical property to consider during drug development [47].

Optimal values of LipE range from 5 to 10 [48]. Increasing the potency (larger pK_i or pIC_{50}) and decreasing the lipophilicity (less negative cLogP) during drug development, in general, lead to better pharmacological properties. Lipophilicity plays a dominant role in promoting binding to unwanted drug targets. The goal for drug optimization in the developmental stage is to increase potency without increasing lipophilicity.

Their search for a c-Met inhibitor began with 3-substituted indolin-2-ones using the structure of PHA-665752 (**Figure 3A**), a competitive inhibitor of molecular weight 642, bound to the protein kinase domain of c-Met (PDB: 2WKM) [15]. This lead compound has poor solubility and permeation properties. Cui *et al.* found that the activation segment of dormant unphosphorylated c-Met forms a wedge between the small lobe five-stranded β-sheet and αC-helix that displaces the helix from its catalytically competent position, and they used this unique inactive conformation as a basis for drug design [15]. In the initial series of experiments, Cui *et al.* modified the indolinone group and sulfone linker and arrived at a novel 5-aryl-3-benzyloxy-2-aminopyridine scaffold (**Figure 3B**) [15]. ATP, the indolinone group of PHA-665752, and 2-aminopyridine of the scaffold bind to backbone residues of the hinge region that links the small and large lobes.

Next, Cui *et al.* optimized the 3-benzyloxy group that binds to the hydrophobic pocket and interacts with Tyr1230 of the c-Met activation segment [15]. After characterizing more than a dozen compounds, they found that the attachment of an α-methyl-2, 6-dichloro-3-fluoro-benzyloxy group to the scaffold yielded a drug with better properties

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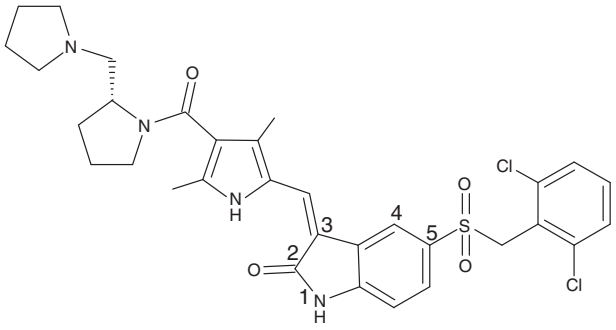
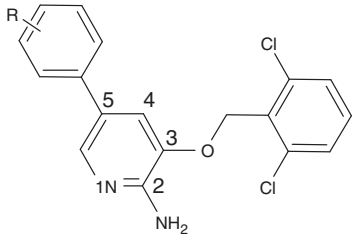
A. PHA-665752: $C_{32}H_{34}Cl_2N_4O_4S$; MW = 642



B. 5-Aryl-3-benzyloxo-2-aminopyridine



C. Crizotinib: $C_{21}H_{22}Cl_2FN_5O$; MW = 450

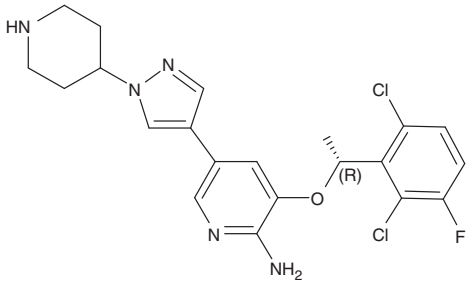


Figure 3. Structures of crizotinib and its lead compounds.

and a cellular IC_{50} for c-Met of 20 nM. After studying several dozen compounds with 5-aryl substitutions of the ethoxypyridin-2-amine core, they synthesized and characterized the racemic version of crizotinib. The R-form of the compound (**Figure 3C**) with a molecular weight of 450 proved to be the most effective. Although several purified protein kinases exhibited nanomolar IC_{50} values, they found that only c-Met (8.0 nM) and ALK (20 nM) exhibited cell-based IC_{50} values of < 75 nM [15].

Cui *et al.* determined the X-ray crystal structure of crizotinib bound to the kinase domain of ALK, which was not their original target [15]. Similar to many protein kinase inhibitors [49], the amino group of crizotinib forms a hydrogen bond with the carbonyl group of a hinge residue (Asp1197) (**Figure 4**). Crizotinib also interacts with the G-rich loop Leu1122, the gatekeeper Leu1196 and the C-spine Val1130 and Ala1148. It also forms a hydrophobic bond with Leu1256 on the floor of the ATP-binding site. Similar to ALK, crizotinib forms hydrogen bonds with the carbonyl group of Pro1158 and the NH group of Met1160 of the hinge region and residues in the G-rich loop and the C- and R-spines of c-Met [15].

5.2 Inhibition of tumor growth

The initial crizotinib studies were performed on human ALCL Karpas-299 and SU-DHL-1 cells that express NPM-ALK [50]. Christensen *et al.* reported that crizotinib inhibits the growth of these cells with IC_{50} values of 32 and 43 nM, respectively [50]. The drug also increases the number of apoptotic cells after 24 and 48 h of treatment. In mice bearing Karpas-299 tumor xenografts (200 mm^3 initial tumor volume), oral crizotinib produced complete tumor regression in 15 days [50]. Zou *et al.* reported that crizotinib inhibited the growth of NCI-H441 NSCLC cell growth in xenografts in athymic mice [51]. These cells lack the EML4-ALK fusion protein and inhibition was thought to occur because of c-Met inhibition.

Analysis of the tumors revealed that crizotinib reduced the levels of phosphorylated NPM-ALK, ERK, Akt, PLC-γ1 and STAT3 [50]. Decreased phosphorylation of NPM-ALK indicates that crizotinib decreases ALK protein kinase activity. Decreased phosphorylation of ERK, which is part of the mitogenic Ras/Raf/MEK/ERK pathway, leads to inhibition of cell division. Akt and STAT3 phosphorylation activates cell survival pathways and their decreased phosphorylation leads to apoptosis. Phosphorylation of PLC-γ1 leads to the activation of protein kinase C, which in turn activates Raf/MEK/ERK-mediated cell survival [49] and crizotinib inhibits PLC-γ1 activation.

Sun *et al.* studied the effects of crizotinib on two NSCLC cell lines *in vitro* and *in vivo*: H3122, which harbors the EML4-ALK fusion protein and H460, which does not [52]. They reported that crizotinib inhibits ALK downstream signaling to ERK, Akt and STAT3 in H3122 cells but not in H460 cells in culture. They found that 400 nM crizotinib (a rather high concentration) decreases ALK autophosphorylation as early as 15 min following treatment. It also inhibits cell proliferation of H3122 cells. They found that radiation and crizotinib treatment increased apoptosis in H3122 cells in culture and more effectively inhibited tumor growth in lung cancer xenografts than either agent alone. In contrast to this study, Tumati *et al.* reported that crizotinib failed to increase radiosensitivity in five NSCLC cell lines (A549, H460, H3122, H2228 and H1993) in culture or in xenograft studies *in vivo* [53]. The reason for the discrepancy between these two studies is unclear and deserves follow up.

6. Clinical data

Camidge *et al.* [54] updated the results from the first Phase I study by Kwak *et al.* [8] on the activity and safety of crizotinib.

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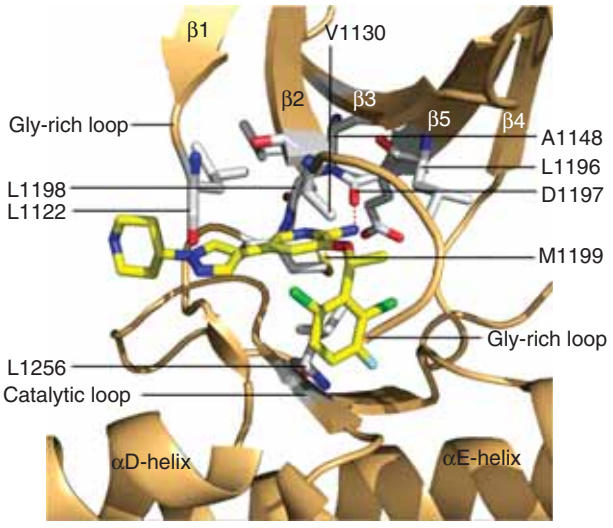

Figure 4. Close-up view of crizotinib bound to the ALK active site. Leu1122, Val1130 and Ala1148 of the N-lobe and Leu 1256 from the C-lobe form hydrophobic contacts. Met1199 is the gatekeeper, and residues 1197 – 1199 are part of the hinge region. The amino group of crizotinib, which is shown in the stick representation with yellow carbon atoms, forms a hydrogen-bond with the carbonyl oxygen of Asp1197, indicated by the red dots. The Gly-rich loop, which is the most flexible part of the protein kinase domain, cannot be shown in its entirety owing to its disorder. The figure was generated from PDB 2XP2 using the PyMOL Molecular Graphics System.

In this study, 149 patients with ALK-positive stages III or IV NSCLC received 250 mg b.i.d. in 28-day cycles between 27 August 2008 and 1 June 2011 (ClinicalTrials.gov number NCT00585195). People with ALK-positive NSCLC were identified using the USFDA-approved FISH Probe Kit. Although the age range was large, the median age range was 52 years compared with a median of 71 years for unselected lung cancer patients. Most of the patients were never smokers bearing adenocarcinomas that develop independently of *EGFR* and *KRAS* mutations. These data raise the question of how does smoking inhibit the chromosomal translocation that produces EML4-ALK? More than 90% of patients exhibited tumor shrinkage with crizotinib and 61% achieved an objective response. Median progression-free survival (time from the initiation of therapy until the tumor objectively increased in size as determined by a CT scan) was about 10 months. The estimated overall survival at 12 months was 75%.

Alk$^{-/-}$ deficient mice are viable and fertile without any known abnormalities [46,55]. Thus, inhibition of ALK activity *per se* should be associated with little toxicity owing to the nonessential nature of physiological ALK. Camidge *et al.* reported that crizotinib was well tolerated [54] but 97% experienced treatment-related effects. Most of these adverse effects were mild (grade 1 or grade 2) and consisted of gastrointestinal (diarrhea, constipation, nausea and vomiting) and visual disturbances (light trails, flashes and brief image persistence). Grade 3 and grade 4 events occurred in 24% (36/149) of the patients and included neutropenia (n = 9), elevated alanine aminotransferase (n = 6), hypophosphatemia (n = 6), lymphopenia (n = 6), elevated aspartate aminotransferase (n = 5), pneumonitis (n = 3) and fatigue (n = 2). About 7% (10/149) required dose reduction owing to treatment-related adverse effects.

Camidge and Doebele reported that the rapid onset of low testosterone in men is a commonly occurring adverse event attributed to crizotinib [56]. Of interest is the possibility that the gastrointestinal symptoms and low testosterone may be related to the inhibition of physiological ALK, the mRNA of which is expressed in small amounts in the human small intestine and significantly higher amounts in the testes [20]. Since c-Met, a crizotinib target, is expressed in adults, it is possible that some of its toxicities result from c-Met inhibition.

Based on the initial findings of Kwak *et al.* [8], the USFDA approved crizotinib for the treatment of locally advanced or metastatic non-small-cell ALK-positive lung cancer. This approval does not specify a requirement for prior therapies, and crizotinib can be used in the first-line setting. Preliminary data reported by Camidge *et al.* indicate that crizotinib is effective in this first-line setting [54].

7. Acquired resistance to crizotinib

As noted by Winer *et al.*, 'Biologically, the cancer cell is notoriously wily; each time we throw an obstacle in its path, it finds an alternate route that must then be blocked' [57]. Not surprisingly, crizotinib resistance occurs in people who initially benefited from treatment, and this resistance develops rapidly. For example, Katayama *et al.* reported that crizotinib resistance occurred in ALK-positive NSCLC patients (n = 18) in a range of 4 – 34 months with a median of 10.5 months [58]. Katayama *et al.* [58] and Doebele *et al.* [59] identified mutations in the ALK protein-tyrosine kinase domain in eight and gene amplification in 3 of 29 patients amounting to 38% (**Table 1**).

Both groups found that bypass signaling pathways including those of the EGFR and Kit protein-tyrosine kinases were upregulated in patient samples [58,59]. For example, Katayama *et al.* reported that four of nine samples and Doebele *et al.* reported that four of ten samples exhibited an increase in EGFR activation in the resistant samples when compared with the corresponding pretreatment sensitive samples supporting a possible role of EGFR in mediating crizotinib resistance [58,59]. One of these cases had a secondary ALK mutation indicating that more than one mechanism for resistance occurs within the same tumor [58].

To add further complications, Choi *et al.* reported that a Cys1156Tyr and a Leu1196Met mutation occurred in two different clones in one person who had undergone treatment for ALK-positive NSCLC with crizotinib [60]. Leu1196 is the gatekeeper residue that occurs next to the ATP and

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Table 1. Molecular mechanisms of acquired crizotinib resistance in EML4-ALK-positive NSCLC.

Mechanism	n found/N tested (%)	Refs.
ALK mutation	8/29 (28)	[58,59]
Leu1196Met	3/29 (10)	[58,59]
Gly1269Ala	2/29 (6.9)	[59]
1151ThrIns	1/29 (3)	[58]
Gly1210Arg	1/29 (3)	[58]
Ser1206Tyr	1/29 (3)	[58]
ALK amplification	3/29 (10)	[58,59]
↑ EGFR activity post-crizotinib	8/19 (42)	[58,59]
EGFR activating mutation	1/10 (10)	[59]
Kit amplification	2/26 (8)	[58,59]
KRAS activating mutation	2/11 (18)	[59]
Unknown	9/29 (31)	[58,59]

crizotinib-binding site (**Figure 2C**), and the gatekeeper mutation appears to be the most common mutation in crizotinib-resistant patients (**Table 1**). Gatekeeper mutations often influence type II drug binding, where type II inhibitors bind to the dormant DFG-Asp out conformation [49]. Moreover, these mutations involve the substitution of a larger residue such as methionine for a smaller residue such as threonine, and the larger residue blocks access to a hydrophobic pocket next to the ATP-binding site [61]. This accounts for the resistance of the BCR-Abl Thr315Ile mutant to imatinib and the resistance of the EGFR Thr790Met mutant to erlotinib [62,63]. In the case of ALK, we have the substitution of a residue of about equal size (methionine for leucine) and the basal ALK enzyme has a DFG-Asp in conformation that is the target of type I, and not type II, kinase inhibitors [43,44,49].

The presence of the DFG-Asp in conformation and the substitution of a similar-sized residue indicate that the ALK Leu1196Met gatekeeper mutation is a unique case that does not result in crizotinib resistance by blocking access to a hydrophobic pocket [61]. Azam *et al.* reported that the substitution of isoleucine or methionine for the threonine gatekeeper in the Src, Abl, platelet-derived growth factor receptors, and EGFR protein kinases results in the activation of enzyme activity [64]. They reported that methionine increases activity more than isoleucine (they did not compare it with leucine). The gatekeeper residue occurs near the tip of the hydrophobic spines (**Figure 2C**), and these investigators ascribed enzyme activation to the ability of the mutant hydrophobic gatekeeper (methionine or isoleucine) to strengthen the R-spine and promote formation of the active conformation of the protein.

Lovly *et al.* reported that the EML4-ALK Leu1196Met mutant exhibits greater cellular baseline levels of phosphorylation, thereby suggesting that this gatekeeper mutation leads to increased protein kinase activity [65]. Moreover, direct protein kinase activity measurements indicated that the ALK Leu1196Met mutant kinase domain is catalytically more active than the wild-type enzyme. This raises the likelihood that the substitution of methionine for leucine destabilizes the wild-type autoinhibitory conformation to which crizotinib binds, and it is the change from the dormant conformation to a more active conformation that confers drug resistance. Thus, crizotinib resistance is due to a conformational change and not to the blockade of an adjacent hydrophobic pocket by a larger gatekeeper residue. Additional mutations in samples from NSCLC have been identified in the ALK kinase domain in people resistant to crizotinib (**Table 2**).

The acquired resistance of ALK-positive NSCLC to crizotinib can be divided into three groups. The first group, which constitutes about 38% of the cases of acquired drug resistance, is exemplified by kinase domain mutations and *ALK*-gene amplification (**Table 1**). The second group, which constitutes about 40% of cases, is independent of ALK and is related to activation of bypass pathways including those of EGFR, Kit and K-Ras. The third groups, which constitute the remainder, are those for which the mechanism is unknown. Next-generation ALK inhibitors may overcome resistance to mutations or gene amplification, but they are unlikely to overcome resistance to the second or third groups of drug resistance. In the case of increased signaling by EGFR or Kit, treatment with specific inhibitors such as erlotinib or imatinib, respectively, represents a potential therapeutic strategy.

8. Other diseases with ALK activation

8.1 Oncogenic ALK activation as a result of chromosomal rearrangements

Since the initial discovery of the NPM-ALK fusion protein in human anaplastic lymphoma cell lines [20], more than two dozen different ALK fusion proteins have been described in various malignancies [45]. The prevalence of the ALK fusion proteins in these malignancies is generally low (**Table 3**), which thereby limits their usefulness as drug targets. In the case of ALCLs, eight other fusion proteins have been described (see Ref. [45], for a full description of known ALK-fusion partners).

Several ALK-fusion proteins have been described in inflammatory fibroblastic tumors. These rare mesenchymal neoplasms usually occur during the first two decades of life [66]. These tumors are treated by surgical excision, but local recurrence may occur after surgery with the risk of metastasis. About half of these tumors possess the TPM3/4-ALK fusion proteins [45].

Diffuse large B-cell lymphoma is a malignancy that occurs primarily in older individuals with a median age at diagnosis of about 70 years [67]. These aggressive tumors can arise virtually anywhere and a small portion of these lymphomas possesses the NPM-ALK or other fusion protein [67]. Traditional therapy includes cytotoxic drugs and rituximab. Rituximab is a chimeric monoclonal antibody against CD20,

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Table 2. Crizotinib resistance mutations in NSCLC.

Mutation	Inferred mechanisms of resistance	Refs.
Leu1196Met	Activating gatekeeper mutation destabilizes the dormant autoinhibitory conformation to which crizotinib binds	[58,59,60,65,89]
1151ThrIns	The inserted residue occupies position 1152. See Leu1152Arg	[58]
Leu1152Arg	This residue occurs in the β3-strand and interacts with the αC-helix; this mutation may alter the dormant conformation to which crizotinib binds	[90]
Cys1156Tyr	Cys1156 is too far from crizotinib to directly block its binding, but it interacts directly with Leu1152. The tyrosine mutant may perturb Leu1152 (see above entry) and thereby alter the dormant enzyme conformation to which crizotinib binds	[65]
Leu1198Pro	Leu1198 is in the hinge region close to the crizotinib-binding site. Mutation to proline will introduce a kink and alter the structure of the segment	[91]
Gly1202Arg	Gly1202 is in contact with the pyrazolyl ring of crizotinib; mutation to a larger residue may block crizotinib-binding directly	[58]
Asp1203Asn	Aspartate is near the crizotinib-binding site but it is unclear how its mutation to asparagine would decrease the inhibitory potency of crizotinib	[91]
Ser1206Tyr	Ser1206 is about 6 Å from crizotinib; the Tyr1206 mutant may be large enough to block crizotinib-binding directly	[58]
Gly1269Ala	Gly1269 interacts directly with crizotinib; mutation to the larger Ala may block crizotinib-binding directly	[59]

The possible mechanisms of resistance are inferred from the X-ray structure of crizotinib bound to the ALK kinase domain (PDB 2XP2).

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Table 3. Constitutive ALK signaling in human cancers owing to translocations, mutations or overexpression.

Disease	Cohort (mean age)	Estimated number of new cases in the US per year	Comments
NSCLC	Adults (52 years)	4% of 228,000, ~ 9000/year	Most commonly EML4-ALK fusion protein; 99% in never or former smokers; almost all are adenocarcinomas
ALCL	Adults	50% of all adult ALCL are ALK-positive, ~ 160/year	NPM-ALK fusion protein (80%) or other fusion protein (20%)
Breast cancer	Adults	2.4% of 232,000, ~ 5600/yearr	EML4-ALK
Colorectal cancer	Adults	2.4% of 143,000, ~ 3400/year	EML4-ALK and C2orf44-ALK
ALCL	Children	90% of all pediatric ALCL are ALK-positive, ~ 120/year	NPM-ALK fusion protein (80%) or other fusion protein (20%)
Inflammatory myofibroblastic tumor (IMT)	Teenagers (13 years)	~ 75/year	TMP3/4-ALK (50%) or other fusion protein (50%)
Sporadic neuroblastoma	Children (17 months)	10% of 600/year, ~ 60/year	ALK somatic point mutations or ALK amplification
Hereditary neuroblastoma	Children (17 months)	~ 15/year	ALK germline point mutations
Anaplastic thyroid cancer	Adults	~ 100/year	ALK somatic point mutations

Based on the data from [2,69,92].

which is a protein found primarily on the surface of B cells [68]. Remission occurs in 60 – 80% of cases and about 50% of all patients remain free from disease for several years. Whether crizotinib is effective in the cohort of patients that fail to respond to treatment or whether it can constitute a first-line therapy remains to be established.

Lin *et al.* reported that the incidence of the EML4-ALK fusion protein is 2.4% in breast and colorectal cancers [69]. Although the occurrence of the EML4-ALK fusion protein is low, the large incidence of these disorders suggests that many patients may benefit from crizotinib treatment. If these results are confirmed, the number of patients that receive crizotinib may significantly increase. VCL-ALK (**Figure 1**) occurs in renal cell cancers and TPM4-ALK occurs in esophageal cancers [70].

8.2 Oncogenic activation of ALK by mutations in neuroblastoma and anaplastic thyroid cancer

The activation of ALK protein kinase activity in neuroblastoma is unrelated to the generation of fusion proteins but rather results from mutations within the *ALK* gene itself. At

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least 20 mutations have been documented [71-74], and the rationale for kinase activation has been described in most cases [43-45]. Many of these mutations occur in important kinase regulatory positions such as the αC-helix, the activation segment, the regulatory spine or the catalytic loop. Two mutations have been described in samples of anaplastic thyroid cancer [75]. Both of these mutations (Leu1198Phe and Gly1201Glu) occur in the hinge region between the small and large lobes. Anaplastic thyroid carcinomas are one of the most aggressive solid tumors, with a median survival of about 3 – 5 months after diagnosis [76]. However, the incidence of these two malignancies is small (**Table 3**).

8.3 Clinical trials

Crizotinib has undergone > 40 clinical trials for a variety of diseases including locally advanced and/or metastatic i) ALCL, ii) alveolar rhabdomyosarcoma, iii) brain and central nervous system tumors including gliomas, iv) inflammatory myofibroblastic tumor, v) lung cancer, vi) neuroblastoma and vii) papillary renal cell carcinoma (www.ClinicalTrials.gov). Owing to the problem of the near universal development of resistance to crizotinib, alternative therapies that target resistance pathways are under investigation. Increased signaling by EGFR in NSCLC is one such pathway. One ongoing trial is testing the efficacy of erlotinib (an EGFR inhibitor) with crizotinib (NCT00965731). Two other trials will examine the efficacy of combining crizotinib with dacomitinib (an irreversible inhibitor of the EGFR family including EGFR (HER1), HER2, HER3 and HER4 [77]) (NCT01121575 and NCT01441128).

EML4-ALK variants are stabilized during synthesis and maturation by Hsp90, which is a heat shock protein and chaperone [78]. Two clinical trials will examine the efficacy of treating EML4-ALK–positive NSCLC with crizotinib with or without AT13387, an Hsp90 inhibitor (NCT01712217). Another will compare crizotinib with or without ganetespib, another Hsp90 inhibitor (NCT01579994). As noted above, there seems to be a role for enhanced EGFR signaling in crizotinib-naive as well as crizotinib-resistant NSCLC (**Table 1**) [58]. EGFR is also an Hsp90 client [79], and inhibition of Hsp90 may play an additional therapeutic role by downregulating EGFR signaling. Another trial is planned to determine whether crizotinib and pazopanib (which is a vascular endothelial growth factor receptor and angiogenesis inhibitor [80]) are more efficacious than crizotinib alone (NCT01548144).

Clinical trials for other ALK inhibitors for the treatment of NSCLC are planned or underway. These include i) AP26113 (NCT01449461, ARIAD Pharmaceuticals), ii) CH5424802 (NCT01588028, Hoffmann-La Roche), iii) ASP3026 (NCT01401504, NCT01284192, Astellas Pharma), iv) LDK378 (NCT01283516, Novartis) and v) RO5424802 (NCT01801111, Hoffmann-La Roche). PubMed searches are negative for the latter three drugs and their structures are not in the public domain, hence we know very little about them.

9. Conclusion

Cui *et al.* developed crizotinib as a c-Met inhibitor using SBDD methodologies [15]. The drug is also an effective inhibitor of ALK and ALK fusion proteins. While the preclinical work on crizotinib was directed at the NPM-ALK fusion protein in ALCLs, the EML4-fusion protein was discovered in about 4% of NSCLCs. This finding changed the focus of clinical studies, and later work has centered on NSCLC with a much larger patient cohort. However, the development of crizotinib resistance has been problematic.

10. Expert opinion

Although crizotinib is approved by the USFDA for the treatment of NSCLCs that possess the EML4-ALK fusion protein, the initial inhibitory drug target was c-Met [15]. Cui *et al.* began with PHA-665752, an ATP-competitive inhibitor of c-Met [15]. They systematically altered its structure to better its pharmacological properties, while increasing its c-Met inhibitory properties. These investigators monitored their progress by using LipE, which was a strategy that aided in the development of crizotinib. Modifications were designed to increase the solubility of the derivatives in aqueous solution, while permitting permeation through membranes.

Pharmacologists and medicinal chemists have searched for drug-like chemical properties that result in compounds with therapeutic efficacy in a predictable fashion. Lipinski's 'rule of five' represents an initial experimental and computational approach to estimate solubility, permeability and efficacy in the drug discovery and development setting [81]. This rule predicts that poor absorption or permeation is more likely when there are more than five hydrogen-bond donors, 10 (5 × 2) hydrogen-bond acceptors, a molecular weight > 500 (5 × 100) and a calculated Log P (cLogP) > 5, where P is the partition coefficient that reflects the ratio of the solubility of a compound in octanol/water. The number of hydrogen-bond donors is expressed as the sum of OH and NH groups, and the number of hydrogen-bond acceptors is expressed as the sum of nitrogen (N) and oxygen (O) atoms. Crizotinib fulfills these criteria and possesses only three hydrogen-bond donors, six hydrogen-bond acceptors, a molecular weight of 450 and a calculated LogP of 4.5. Moreover, crizotinib has a lipophilic index of 6.14 [15], which is within the optimal range of 5 – 10 [82].

One of the drawbacks of nearly all of the small molecule protein kinase inhibitors is the development of acquired resistance. Although the data on the mechanisms of crizotinib resistance is too limited to be definitive, about one-third of the cases of resistance in ALK-positive NSCLC are due to mutations or gene amplification of EML4-ALK, one-third are due to upregulation of bypass signaling pathways including EGFR and Kit and one-third are due to unknown mechanisms. The development of second-generation ALK

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inhibitors represents a feasible approach to address the first mechanism. Owing to the role of EGFR signaling in acquired resistance to crizotinib in lung cancers, the coadministration of erlotinib (an EGFR inhibitor) and crizotinib may delay the onset of acquired resistance. In cases where Kit signaling is increased, treatment with dasatinib or sunitinib and crizotinib represents a feasible approach.

Preclinical and clinical experiments will be required to determine whether coadministration, alternating administration or intercalated administration of two inhibitors such as crizotinib and erlotinib is more effective in preventing the acquisition of drug resistance. Unfortunately, clinical trials that compare combinations of inhibitors are time-consuming and expensive. Moreover, deciding which combinations to use is problematic. As Knight *et al.* have pointed out, there is a need 'for companies to collaborate to test new combinations of investigational drugs in oncology, where there is arguably the greatest need and opportunity' [83].

Although several cancers have a major oncogenic driver, all cancers exhibit dysregulation of many signaling pathways that promote proliferation, evade growth suppressors, sustain invasion and metastasis, enable replicative immortality, induce angiogenesis and inhibit apoptosis [84]. Targeting oncogenic drivers often results in short-term benefits, but more must be done to increase long-term benefits. The administration of crizotinib with other agents that inhibit angiogenesis, EGFR family kinases, Akt-mediated apoptosis or Ras/Raf/MEK/ERK-mediated cell proliferation should be tested before resistance to crizotinib occurs. Similarly, coadministration or sequential administration of crizotinib and traditional cytotoxic therapies should be explored in preclinical and clinical settings. The preclinical report that crizotinib may increase radiosensitivity [52] provides a rationale for exploring this possibility in animal models and perhaps in the clinic.

There was a strong rationale for developing a c-Met inhibitor, which can function as a driver mutation in some cancers and may play a secondary role in the development of metastasis. So far these aspects of crizotinib have not been thoroughly explored. Amplification of c-Met is one mechanism of resistance that occurs in NSCLC patients receiving gefitinib [85] so that crizotinib may prove effective in this setting. Owing to the importance of metastasis in malignancies, the use of crizotinib as a potential anti-metastatic agent coadministered with other targeted or standard cytotoxic drugs should be explored preclinically and in humans.

Serendipity has played an important role in developing therapies targeted against protein kinases. Imatinib was developed as a platelet-derived growth factor receptor inhibitor, but its initially approved therapeutic target was BCR-Abl in chronic myelogenous leukemia [49,86]. Further work showed that imatinib also inhibits Kit, which is an oncogenic driver of gastrointestinal stromal tumors [87]. Sorafenib was initially developed as a Raf inhibitor, but it inhibits several protein kinases including the vascular endothelial growth factor receptor, Kit, and Flt-3 [49]. Sorafenib is approved for the treatment of renal cell and hepatocellular carcinomas. Bergethon *et al.* reported that *ROS1* gene rearrangements occur in 1.7% (31/1073) of NSCLCs and crizotinib is a weak inhibitor of ROS1 [88]. This represents another patient cohort that may benefit from crizotinib treatment.

The approval of crizotinib for the treatment of NSCLC bearing the EML4-ALK fusion protein as determined by the Vysis® FISH Probe Kit represents an important therapeutic advance. This approval was based only on response rate and planned or ongoing clinical trials will determine whether the drug increases progression-free survival or overall survival.

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Declaration of interest

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