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Design and Discovery of Anaplastic Lymphoma Kinase (ALK) Inhibitors

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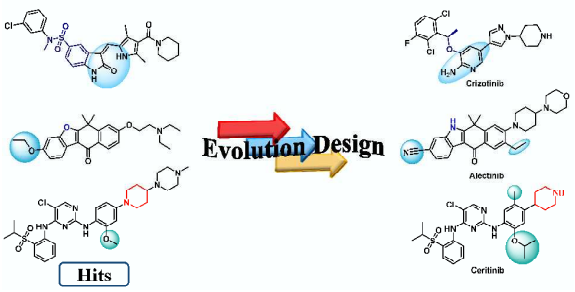
This review descripts the hit-to-drug evolution design of three ALK inhibitors.



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REVIEW

Anaplastic Lymphoma Kinase (ALK) Inhibitors: A Review of Design and Discovery

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Since the launch of Gleevec in 2001, several protein kinases have emerged as attractive therapeutic targets for the treatment of various cancers. One of these, anaplastic lymphoma kinase (ALK), have attracted a great deal of attention due to its oncogenic potential and essential role in the pathogenesis of wide variety of human cancers, such as ALCLs, NSCLC, breast cancer, colorectal cancer, neuroblastoma, and ovarian cancer etc. To date, nine ALK inhibitors have entered clinical investigation for the treatment of cancers, including crizotinib, the first ALK inhibitor to be approved by the US FDA (in 2011) for the treatment of NSCLC patients. As resistance to crizotinib treatment has been reported in both preclinical and clinical settings, several second-generation ALK inhibitors to overcome both the wide-type and mutant ALK are being developed. In 2014, shortly after crizotinib was approved, the second-generation ALK inhibitor ceritinib was approved by the FDA. Biological activities of eight second-generation clinical ALK inhibitors are discussed in this review. Furthermore, the hit-to-drug evolution strategies used in the design of three ALK inhibitors - crizotinib, alectinib and ceritinib - are described in detail, to help the medicinal chemist to understand and devise similar strategies to overcome lead development issues, such as potency, selectivity and metabolism, in the drug discovery projects.

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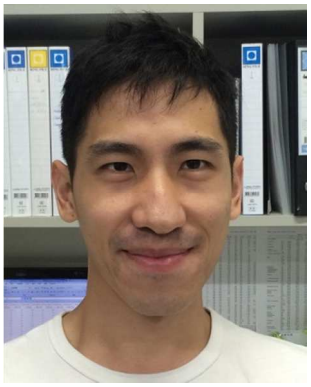

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Introduction

Cancer is the leading cause of mortality worldwide, accounting for 7.6 million deaths in 2008 and an estimated 13.1 million by 2030. Traditional therapies involve surgical removal, radiotherapy, and chemotherapy; but surgery and radiotherapy are only applicable in tumors that have not spread from the tissue of origin; and traditional chemotherapies often cause serious side effects as the non-specific nature of these agents can result in damage to both normal and cancer cells. Therefore, modern cancer treatments have evolved from traditional therapy to the target-based therapy including antibodies and small molecules which are designed to interfere with a specific biochemical pathway that is central to the development, growth and spread of that particular cancer.

Protein kinases regulate most aspects of the cell cycle; their deregulation of protein kinases is both a cause and consequence of diseases such as diabetes, inflammation and cancer; and they have become the second most popular class of drug targets (after G-protein-coupled receptors).[1] Since Gleevec® (imatinib) was first launched in May 2001, a total of 27 small molecule kinase inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for human use. Recently, anaplastic lymphoma kinase (ALK), a receptor tyrosine kinase (RTK) belonging to the insulin receptor (IR) superfamily, has gained much attention as a cancer therapeutic target. ALK was first identified in 1994 as the nucleophosmin (NPM)-ALK fusion protein in 60-80% of anaplastic large cell lymphomas (ALCLs) with a *t*(2;5) chromosomal translocation.[2] Although the physiological function of ALK in cancer remains unclear, ALK fusion proteins have been found in ALCLs as well as in various human cancers, such as breast cancer, colorectal cancer, inflammatory myofibroblastic tumor (IMT), diffuse large B-cell lymphoma (DLBCL) and most notably in non-small cell lung cancer (NSCLC) (Table 1).[3, 4] Therefore, the kinase activity associated with ALK fusion protein is believed to play an essential role in the survival and proliferation of human cancer cells.

The appearance of an ALK translocated gene mutation in NSCLC, an echinoderm microtubule-associated protein like 4 (EML4)-ALK, was first identified in 2007 by Hiroyuki Mano at Jichi Medical University in Japan from a lung cancer patient, and later in five others.[5] The novel EML4-ALK fusion protein

contains an N-terminus of EML4 and C-terminal kinase domain of ALK. Since the discovery of this fusion protein, multiple variants encoding different truncations of EML4 with the same ALK kinase domain have been described.[6] Furthermore, multiple ALK fusion partner genes have been identified in NSCLC, such as TFG, KLC1, KIF5B and PTPN3, which are comparatively rare (Table 1). Clinical studies have shown that EML4-ALK translocation occurs in approximately 5% of all NSCLC patients, and is mutually exclusive with other oncogenes such as epidermal growth factor receptor (EGFR) and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutations.[7] Importantly, xenograft studies showed that EML4-ALK gene fusion strongly contributed to tumor growth. In addition, recent studies have implicated ALK overexpression in the oncogenesis of neuroblastoma,[8-10] inflammatory breast cancer,[11] and ovarian cancer.[12] Therefore, ALK inhibitors targeting the ATP binding site could be a possible treatment option for ALK-driven cancers.

Table 1. Different fused ALK in cancer and their incidence

Tumor type	Translocation	Incidence	Refs.
Breast cancer	EML4-ALK	0-2.4%	13-15
Colorectal cancer	EML4-ALK	0.2.4%	13-15
	C2orff44-ALK	ND	16
NSCLC	EML4-ALK	3-7%	5, 13, 17
	TFG-ALK	Rare	17
	KLC1-ALK	Rare	18
	KIF5B-ALK	Rare	19, 20
	PTPN3-ALK	Rare	21
ALCL	NPM-ALK	60-80%	2
	TMP3-ALK	Rare	22, 23
	TMP4-ALK	12-18%	24
	CLTCL-ALK	Rare	25
	TFG-ALK	Rare	26, 27
	ATIC-ALK	Rare	28-30
	MSN-ALK	Rare	31, 32
	ALO17-ALK	Rare	33
	MYH9-ALK	Rare	34
IMT	TMP3-ALK	50-60%	35
	TMP4-ALK	Rare	35
	CTLC-ALK	Rare	36, 37
	ATIC-ALK	Rare	38
	CARS-ALK	Rare	33, 39
	RANBP2-ALK	Rare	40, 41
	SEC31L1-ALK	Rare	42
DLBCL	NPM-ALK	Rare	43, 44
	CTLC1-ALK	Rare	45
	SQSTM1-ALK	Rare	46
	SEC31A-ALK	Rare	47, 48

NSCLC: non-small cell lung cancer, ALCL: anaplastic large cell lymphoma, IMT: inflammatory myofibroblastic tumor, DLBCL: diffuse large B-cell lymphoma.

Currently, there are 9 ALK inhibitors either approved or under clinical development, which are summarized in Table 2.[4, 49] In this review, we will describe the design strategies of three ALK inhibitors: crizotinib (PF-02341066), alectinib (AF802, CH5424802, RO5424802), and ceritinib (LDK378). Crizotinib is the first FDA approved ALK inhibitor, whereas alectinib is currently undergoing intensive clinical investigations for ALK-positive patients, particularly for crizotinib-resistant patients. Furthermore, ceritinib is the second FDA approved ALK inhibitor as a treatment for patients with ALK-positive metastatic NSCLC that progressed on crizotinib. In addition, the most recent preclinical and clinical trial data of 6 other second-generation ALK inhibitors are also discussed in brief.

In modern drug development, chemists play an essential role in several stages of drug discovery. When a molecular target associated with a disease pathology is discovered and potential hits for this target identified using screening technologies, chemists need to obtain structure-activity relationship (SAR) information of the hit in order to develop efficacious leads for the target. However, lead optimization may encounter setbacks due to inadequate physicochemical properties; and therefore ADME profiles (absorption, distribution, metabolism, and elimination), oral bioavailability, and toxicity data will also be sought out as part of the structure-property relationships (SPR) analysis (Fig. 1).

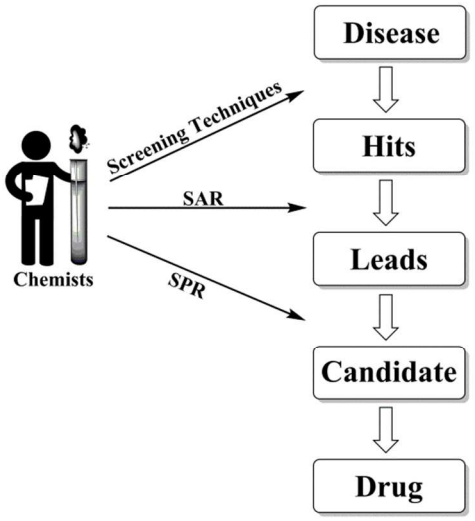


Fig. 1 The role of chemists in drug discovery.

Particularly, the most crucial role for medicinal chemists in drug discovery and development is to focus on SAR, as well as SPR studies to help in developing a suitable drug candidate from the initial hits identified for the target. Medicinal chemists employ several design strategies, such as variation of substituents, extension of the structure, (bio)isosteric replacement, ring variation, ring expansion/contraction, chain extension/contraction, simplification, rigidification, conformational blocking etc., to achieve the goal of designing a new drug. This review highlights the design strategy used in the development of three ALK drug candidates from hits, and could act as a catalyst for other drug candidate development.

Crizotinib

Crizotinib is the first-in-class FDA approved treatment for ALK-positive NSCLC. It emerged from a drug discovery project at Pfizer which initially focused on the development of c-Met kinase inhibitors. Crizotinib's "off-target" anti-ALK activity was recognized only at later stages of the project. It entered into clinical trials for ALK-positive NSCLC in 2006,

Table 2. List of ALK inhibitors under clinical development.

Compound	Company	ALK activity		Other targets	Clinical states	Refs
		WT	Mutations			
Crizotinib PF-02341066 Xalkori®	Pfizer	24 nM	--	c-Met ROS1	FDA Approved	50-56
Ceritinib LDK378 Zykadia®	Novatis	0.15 nM	L1196M G1269A S1206Y I1171T	ROS1	FDA Approved	57-60
Alectinib AF802 CH5424802 RO5424802	Roche/Chugai	1.9 nM	L1196M F1174L R1275Q C1156Y	GAK, LTK	Phase II/III	61-66
AP26113	Ariad	0.62 nM	L1196M F1174C I1171T F1245C E1210K S1206R G1269A L1196Q I1171N	Multiple kinases ROS1	Phase I/II	67-72
X-396	Xcovery	<0.4 nM	L1196M C1156Y F1174L	--	Phase I	73
CEP-37440	Cephalon/Teva	3.5 nM	--	FAK	Phase I	74
TSR-011	Tesaro	0.7 nM	L1196M R1275Q		Phase I/II	75, 76
NMS-E628	Nerviano	55 nM	L1196M C1156Y	Aurora B, IGF-1R, ROS1	Phase I/II	77, 78
PF-06463922	Pfizer	2 nM	G1269A L1196M C1156Y L1152R F1174L S1206Y 1151Tins G1202R	ROS1	Phase I/II	79, 80

c-Met: hepatocyte growth factor receptor, GAK: cyclin G-associated kinase, LTK: leukocyte tyrosine kinase, FAK: focal adhesion kinase, IGF-1R: insulin-like growth factor 1 receptor

and was approved by FDA in 2011. This section describes the strategic development of crizotinib from the early identification of hit **1** to FDA approval (Scheme 1).

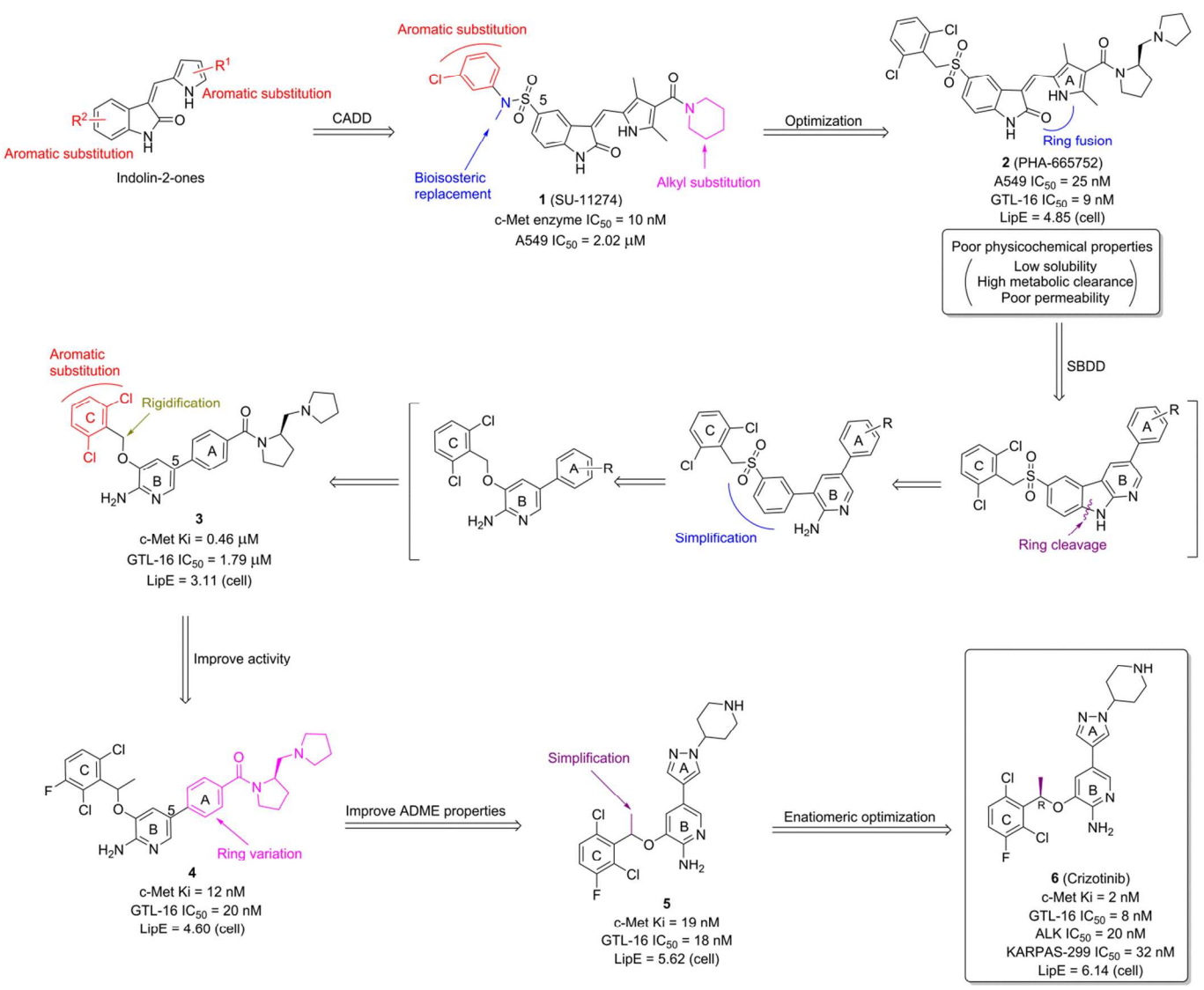
Sugen (now Pfizer) screened approximately 7,500 compounds from their indolin-2-one library against phosphorylation of c-Met kinase;[81] but no potent inhibitors were identified. In order to enhance the activity of existing indolinone series of compounds, a homology model of c-Met kinase was constructed. Based on the information from homology modeling, modifications were carried out by introducing different amine substituents on the pyrrole ring and C-5 sulfonamide substituents on the indolinone ring. This led to the identification of compound **1** (SU-11274), which displayed potent activity against c-Met enzyme with an IC_{50} value of 10 nM. Furthermore, compound **1** inhibited HGF-induced tyrosine autophosphorylation of c-Met in A549 cell line with an IC_{50} value of 2.02 μM.[82] To further improve the cellular activity, SAR studies were carried out by replacing the piperidine ring on the amide moiety with substituted pyrrolidine ring, followed by incorporation of 2,6-dichloro substituent on the *N*-phenylsulfonamide moiety, and subsequent bioisosteric replacement to furnish the sulfone derivative **2** (PHA-665752). PHA-665752 demonstrated an improved cellular potency (IC_{50} = 25 nM in A549 and 9 nM in GTL-16) with >50-fold selectivity for c-Met kinase against various tyrosine and serine-threonine kinases.[83, 84]

Although compound **2** exhibited good *in vitro* activities, further development was limited due to poor physicochemical properties such as low solubility at pH 7.4 (0.9 μg/ml), high metabolic clearance (CL = 77 mL/min/kg in rat), and poor permeability.[53] To improve the drug-like properties of compound **2**, lipophilic efficiency (LipE) was used to guide further optimization in conjunction with structure-based drug design strategy.[85, 86] LipE [p*K*i (or pIC_{50}) – cLog D] is an important parameter, which is used to evaluate the effect of structure modification on both potency and lipophilicity, simultaneously. Use of the LipE parameter instead of binding affinity or IC_{50} during ligand optimization will facilitate the

identification of potent compounds with the desirable physiochemical properties required for a clinical candidate.

First, modification of the pyrrole indolin-2-one scaffold was carried out by ring fusion to generate the carbazole; ring cleavage of the resultant carbozole ring and simplification of the sulfone linker led to the design of novel 5-aryl-3-benzoxy-2-aminopyridine scaffold. After synthesizing a few analogues bearing different substitution at the amide position and an *O*-methylene-2,6-dichlorophenyl group at the 3-position, compound **3** (with the same amide group as **2**) was identified with better lipophilic efficiency value (LipE = 3.11) compared to the other analogues, while displaying moderate inhibition against c-Met with enzymatic Ki of 0.46 μM. Despite the fact that compound **3** displayed a lower potency and LipE value than compound **2**, the co-crystal structure showed that 2-aminopyrimidine core (B-ring) participated in H-bonding with the hinge region residues Pro-1158 and Met-1160 of c-Met, and was consistent with the binding mode of indolin-2-one (compound **2**) to the hinge residues. The *O*-methylene-2,6-dichlorophenyl (C-ring) extended outward to form an efficient π-π stacking interaction with Tyr-1230 in the activation loop. These structural biology findings validate the design of inhibitor **3** as a potential lead for further optimization by changing the scaffold from indolin-2-one to 2-amino-pyrimidine.[53]

The strategy to further improve the potency of **3** was to replace the 3-benzyloxy group (C-ring) with various aryl analogues. Subsequent aromatic substitution by 2,6-dichloro-3-fluoro group and rigidification of the 3-benzyloxy ring by addition of an α-methyl group to give compound **4** resulted in an increase in both the enzymatic and cellular activities. Racemate **4** bearing an α-methyl-2,6-dichloro-3-fluorobenzyl-oxy group improved enzymatic (Ki = 12 nM) and cellular activity (IC_{50} = 20 nM) with a cell based LipE value of 4.60.



Scheme 1 The strategic development of crizotinib from early identification of hit 1 to FDA approval.

With the potent inhibitor **4** in hand, the next strategy was focused on the A ring variation, in order to increase the LipE. Modeling of 2-aminopyridine suggested that extending a polar basic amino group into the solvent exposed part of the protein could improve both the potency and property. On the other hand, smaller and less lipophilic 5-heteroaryl analogues could reduce the torsion strain associated with planar H-bonding. With the application of these design concepts, the analogue bearing pyrazol-4-yl group at the 5-position showed the most potent activity against c-Met (Ki = 81.5 nM) and higher LipE value (2.52) compared to other heteroaryl and phenyl group (LipE = -0.47 to 2.04). In addition, *N*-methyl substitution on the pyrazol-4-yl group demonstrated the potency (Ki = 46 nM) with improved LipE value of 2.83. This indicates that the *N*-substitution on the pyrazol-4-yl group plays a critical role on the potency and LipE. Based on the above study, the analogues with polar basic *N*-substituents on the pyrazol-4-yl group were synthesized, as shown in racemate **5** bearing piperidine ring attached to *N*1-position of pyrazol-4-yl group. Racemate **5** showed potent cellular activity with an IC_{50} value of 18 nM and good LipE of 5.62. In order to identify the potency and LipE of each enantiomer, typical simplification strategy was then employed. Each enantiomer was separately synthesized, and the *R*-enantiomer **6** (crizotinib) was found to show higher potency against c-Met kinase (K_i = 2 nM and GTL-16 cell IC_{50} = 8 nM) than the *S*-enantiomer (K_i = 161 nM). Crizotinib inhibited the migration and invasion of HGF-induced human NCI-H441 lung carcinoma cell with IC_{50} values of 11 and 6.1 nM, respectively, as well as MDCK inhibition with an IC_{50} value of 16 nM. Moreover, *in vivo* study revealed that crizotinib exhibited potent efficacy in suppressing GTL-16 tumor growth for >3 months at a dose of 50 mg/kg/day. These results suggest that crizotinib is a potent c-Met inhibitor suitable for the treatment of various cancers.[50]

Crizotinib was tested against >120 human kinases and found to display >100-fold selectivity for c-Met while showing "off-target" anti-ALK (20 nM) and anti-ROS1 (80 nM) activities.[50] Although ROS1 shares only 49% amino acid homology with the kinase domains of ALK and 77% homology of the ATP-binding site, several ALK inhibitors showed inhibitory activity against ROS1. In different ALK-related cellular assays, crizotinib inhibited the tyrosine phosphorylation of NPM-ALK in KARPAS-299 and SU-DHL-1 ALCL cells with IC_{50} values of 32 nM and 43 nM, respectively. *In vivo* study following oral administration of crizotinib in a mouse model bearing the KARPAS-299 tumor xenograft at 10 mg/kg/day dose for 15 days resulted in complete tumor regression.[51] These promising results suggest that crizotinib is a potent ALK inhibitor and more suitable for the treatment of ALK-related cancers.

In 2006, Pfizer commenced clinical studies of crizotinib in patients with ALCL and neuroblastoma. A phase I dose-escalation study was already in progress at the time when EML4-ALK fusion gene was first identified in NSCLC patients in 2007; which strongly contributes to tumor growth, and found in approximately 6.7% of NSCLC patients.[5, 87, 88] Based on this new breakthrough finding, Pfizer and Massachusetts General Hospital immediately initiated a new phase I study for the treatment of EML4-ALK-positive NSCLC patients with crizotinib. At the same time, a fluorescence *in situ* hybridization (FISH) assay was quickly developed by Dr. John Iafrate at Massachusetts General Hospital and used as diagnostic method for identifying patients with advanced ALK-positive NSCLC. The phase I study of crizotinib in 82 ALK-FISH-positive NSCLC patients showed a 57% overall response rate (ORR), 87% disease control rate (DCR) for 8 weeks and 72% progression-free survival (PFS) for 6 months.[52] In a global phase II study of crizotinib, the ORR was 51.1% among 133 NSCLC patients and the median duration of response was 41.9 weeks. The efficacy and safety of crizotinib were further investigated in two centers, single-arm studies. The ORR was 50% and 61%, and the median duration of response was 41.9 weeks and 48.1 weeks, respectively.[54, 55] Based on the phase I and II studies, crizotinib (Xalkori®, Pfizer) was granted an accelerated approval by FDA in August 2011 for the treatment of patients with ALK-positive NSCLC. Only 6 years elapsed between initial discovery and market availability, a new record in the history of drug development.

Resistance to crizotinib was first observed in a young patient with ALK-positive NSCLC in 2007.[89] Two secondary mutations (Cys1156Tyr and Leu1196Met) were documented in the patient. The mutation information provided the basis for the development of second-generation ALK inhibitors in order to overcome ALK secondary mutations. Several second-generation ALK inhibitors, such as alectinib, ceritinib, AP26113, X-396, TSR-011 etc., are currently under evaluation in clinical trials.

Alectinib

Alectinib **11** bearing a unique benzo[*b*]carbozole scaffold, is an orally active, ALK-selective second-generation inhibitor developed by Chugai/Roche. Importantly, alectinib showed potent antitumor activity by overcoming cirzotinib-resistant L1196M and C1156Y mutants, as well as inhibiting EML4-ALK^{L1196M}-driven cell growth. Currently, alectinib is undergoing clinical investigations. Herein, we describe the design strategy used from the initial screening hit **7** to the clinical candidate **11** (alectinib) (Scheme 2).

Hit **7** was identified as a weak ALK inhibitor (IC_{50} = 1.3 μM) without c-Met inhibitory activity (IC_{50} >50 μM) through HTS of an in-house library.[90] From the lessons learned from crizotinib (a dual c-Met/ALK inhibitor) that a highly selective inhibitor may increase the therapeutic window of drug safety, selective ALK inhibitor **7** was further developed.

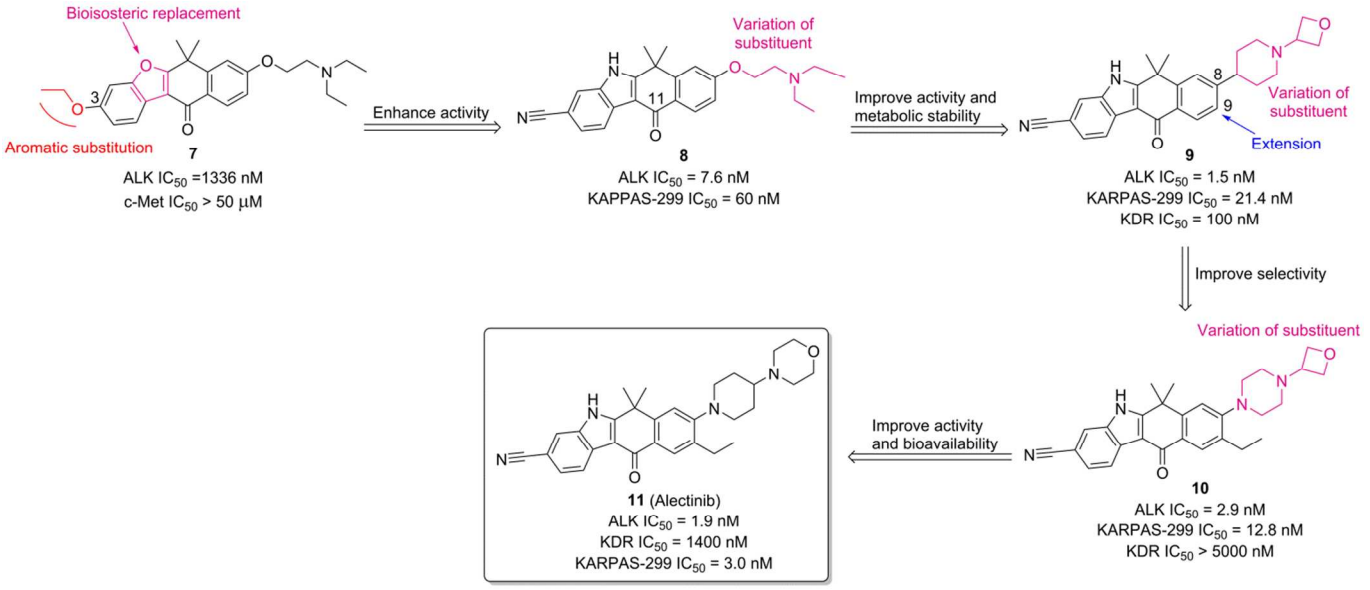
Initially, variation of the benzofuran scaffold by bioisosteric replacement with an indole ring in order to increase the H-bonding interaction between the carbonyl group at the 11-position and the hinge region Met-1199 of ALK was attempted. The replacement of ethoxy group at the 3-position with a cyano group also showed a favorable interaction with the protein. The cyano group was thought to form a critical interaction with the ALK protein, and played an important role in the development

of ALK inhibitor. The above two modifications led to the identification of compound **8** which showed a 100-fold ALK activity increase compared to compound **7** and also possessed good cell growth inhibition against NPM-ALK driven cell line KARPAS-299, with an IC_{50} value of 60 nM.[90]

Unfortunately, compound **8** was found to have high *in vitro* clearance in both mouse (CL = 51.4 μL/min/mg) and human (CL = 54.5 μL/min/mg) liver microsomes, perhaps due to oxidative dealkylation of the diethylamino group. The next strategy used was to modify the 8-position substituent of 3-cyanobenzo[*b*]carbozole scaffold in order to block or slow down metabolism and clearance. It was found that substituents with a cationic nitrogen atom at the 8-position would interact with the solvent-accessible region of the ALK protein, as seen in the *N*-oxetan-3-yl-piperidin-4-yl derivative **9**. Improved metabolic stability (CL = 10.2 μL/min/mg in human live microsome) was the result. Derivative **9** showed inhibitory activity against enzymatic ALK and cellular KARPAS-299 with IC_{50} values of 1.5 nM and 21.4 nM, respectively. Compound **9** further showed significant tumor regression in both KARPAS-299 (20 mg/kg, po, qd) and NCI-H2228 (6 mg/kg, po, qd) mouse xenograft models for 11 days without significant body weight loss.[90]

Even though ALK inhibitor **9** had weak inhibitory activity against c-Met (IC_{50} = 7200 nM), it was much more active against KDR (IC_{50} = 100 nM), which is associated with hypertensive side effects. Hence, further optimization focused on improving the selectivity towards KDR activity. Docking studies of compound **9** to an ALK homology modeled structure showed that the hydrophobic substituents at the 9-position play an essential role in kinase selectivity.[91] Among several synthesized analogs bearing various alkyl and alkynyl groups at the 9-position and an *N*-(oxetan-3-yl)piperazine moiety at the 8-position, compound **10** bearing a 9-ethyl substituent showed an excellent selectivity against enzymatic ALK (IC_{50} = 2.9 nM) with no KDR inhibition (IC_{50} >5000 nM). The greatly improved target selectivity of compound **10** compared to that of compound **9**, as well as strong anti-proliferative activity against KARPAS-299 (IC_{50} = 12.8 nM) suggested further testing of this compound would be worthwhile. *In vivo* study of compound **10** revealed significant tumor regression at 20 mg/kg oral dosage without significant body weight loss in NPM-ALK positive ALCL mouse xenograft models.[91]

The final design focused on varying the substituents at the 8-position to achieve better ADME properties, since compound **10** only had 28.2% oral bioavailability. Compound **11** (alectinib) bearing the 4-morpholinopiperidine group, displayed superior bioavailability (F = 50.4% in monkey) compared to compound **10**, and showed potent activity against enzymatic ALK^{WT} (1.9 nM) as well as the mutated ALK enzymes, such as ALK^{L1196M} (K_i = 1.56 nM), ALK^{F1174L} (IC_{50} = 1.0 nM), and ALK^{R1275Q} (IC_{50} = 3.5 nM). According to the co-crystal structure of the alectinib-ALK complex (PDB ID: 3AOX), the cyano group was found to form a CH/π hydrophobic interaction with the gatekeeper residue (Leu1196), which is reflected by gatekeeper mutant inhibition. Furthermore, alectinib displayed strong anti-proliferative activity in different ALK-driven cell lines, such as NCI-H2228 (IC_{50} = 53 nM), KARPAS-299 (IC_{50} = 3.0 nM), SR-786 (IC_{50} = 6.9 nM), NB-1 (IC_{50} = 4.5 nM) and KELLY (IC_{50} = 62 nM); as well as cell growth inhibition against Ba/F3 EML4-ALK cell lines harboring L1196M and C1156Y mutation that are resistant to crizotinib. Regarding PK properties, alectinib possesses good oral bioavailability (71%) in mice with plasma half-life of 8.6 h. Oral administration of alectinib resulted in significant tumor regression at 60 mg/kg QD for 8 days in mice bearing Ba/F3-EML4-ALK^{L1196M} and in



Scheme 2 The design strategy used from the initial screening hit 7 to the clinical candidate 11 (alectinib).

other xenograft mouse models, such as two ALK-related ALCL cell lines (KARPAS-299 and SR-786) and ALK-amplified neuroblastoma cells (NB-1).[61, 62] Based on the above promising results, alectinib was chosen as a promising second-generation ALK inhibitor and is currently undergoing phase II/III clinical trials.

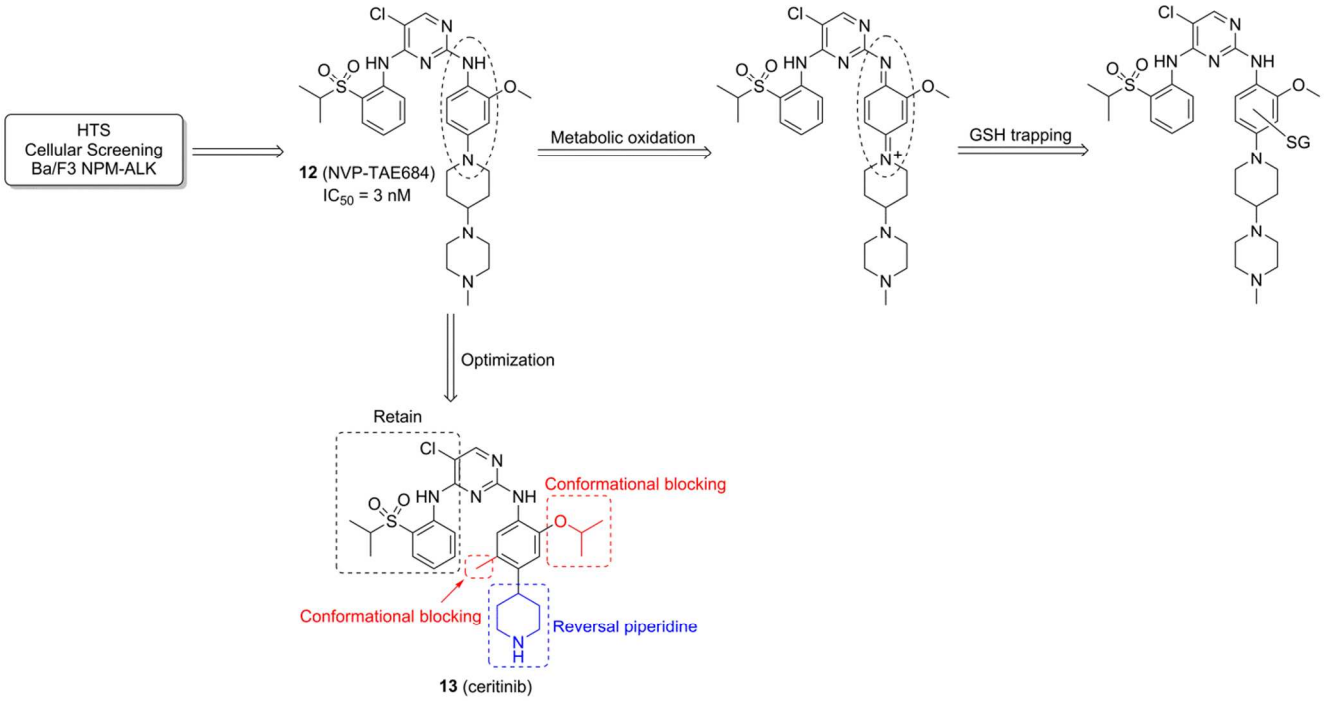
In the phase II study of 46 patients receiving alectinib at a dose of 300 mg BID showed an ORR of 93.5% and treatment continuation beyond 10 months. All the patients had tumor shrinkage of 30% or more.[64, 65] In addition, a phase I dose escalation study of alectinib in crzitoinib-refractory ALK-positive NSCLC patients demonstrated ORR was 59% among 37 evaluable patients.[66] In September 2013, alectinib was awarded FDA breakthrough therapy designation for the treatment of patients with ALK-positive NSCLC.

Ceritinib

Ceritinib is an orally available second-generation ALK inhibitor developed by Novartis, which received accelerated approval by the FDA in April 2014 as a treatment for patients with ALK-positive metastatic NSCLC, who were previously treated with crizotinib. Hence, we will describe the evolution of ceritinib from the HTS hit **12** (NVP-TAE-684) to FDA approval (Scheme 3).

Compound **12** with diaminopyrimidine scaffold as a potent and highly selective ALK inhibitor identified in a cellular screening designed to discover compounds with activities against NPM-ALK activated Ba/F3 cells. Hit **12** showed good activity against Ba/F3-NPM-ALK with an IC_{50} value of 3 nM,[92] and displayed higher potency against the two EML4-ALK positive NSCLC cell lines, NCI-H2228 and NCI-H3122 (IC_{50} values of 16 nM and 44 nM) than crizotinib (871 nM and 1551 nM, respectively).[93]

Although compound **12** showed potent *in vivo* efficacy, clinical development was not possible due to the toxicity associated with the generated 1,4-diiminoquinone as a result of oxidative metabolism. Formation of the 1,4-diiminoquinone intermediate metabolite was confirmed by incubating compound **12** with liver microsome, and trapping the formed 1,4-diiminoquinone by GSH. Approximately 20% conversion of metabolites was detected by LC-MS. Three approaches to slow the metabolism rate were designed: an increase in steric bulk by replacing the methoxy moiety by an *iso*-propoxy group; blockage of the *para* position of the alkyoxy group by adding a methyl group; and reversal of piperidine attachment to avoid the formation of 1,4-diiminoquinone. After synthesizing several analogues using the above design strategy, compound **11** (ceritinib) was found to be the most effective against Ba/F3-NPM-ALK and KARPAS-299 with IC_{50} values of 26 nM and 22.8 nM, respectively. Profiling against a panel of 30 kinases, ceritinib showed ALK inhibition with an IC_{50} value of 200 pM, while displaying 70-230 fold selectivity against three kinases, IGF-1R, InsR, and STK22D with $IC_{50} < 100$ nM. In Ba/F3 cells engineered to express 18 kinases, ceritinib displayed strong inhibitory activity against Ba/F3-ALK and Ba/F3-EML4-ALK with IC_{50} values of 40.7 nM and 2.2 nM, respectively. Furthermore, ceritinib exhibited excellent oral bioavailability in four different species including mouse, rat, dog, and monkey



Scheme 3 The evolution of ceritinib from the HTS hit 12 (NVP-TAE684).

(55%, 66%, >100%, and 56%, respectively). It induced dose-dependent growth inhibition and tumor regression in both KARPAS-299 (25 mg/kg or 50 mg/kg, po, qd) and NCI-H2228 (25 mg/kg, po, qd) mouse xenograft models for 2 weeks with no body weight loss.[57] More importantly, ceritinib exhibited potent inhibitory activity against Ba/F3 cells expressing four crizotinib-resistant ALK mutations, such as L1196M, G1269M, I1171T, and S1206Y mutations. However, Ceritinib is not able to overcome the following five mutations: C1156Y, G1202R, 1151Tins, L1152R and F1174C; even though it is a more potent inhibitor than crizotinib.[59]

In a phase I single-arm study of 144 patients with ALK-FISH-positive NSCLC, 78 patients receiving ceritinib at a dose of 750 mg per day showed an ORR of 59% in crizotinib-resistant patients and 62% in crizotinib naïve patients.[58] These results indicated that ceritinib is a potential second-generation ALK inhibitor for conventional ALK-positive patients, as well as for crizotinib-relapsed patients. In March 2013, ceritinib received FDA breakthrough therapy designation, and FDA approval in April 2014 for the treatment of patients with ALK-positive metastatic NSCLC patients who were previously treated with-, or intolerant to crizotinib. The approval was based on the results from the analysis of 163 patients with metastatic ALK-positive NSCLC, who were treated with ceritinib at a dose of 750 mg QD and had progressed on-, or intolerant to crizotinib. The trials shown an overall response rate of 54.6% and a median duration of response of 7.4 months.[60] These promising results show ceritinib to constitute a new treatment opportunity for patients with ALK-positive metastatic NSCLC.

Other Second-Generation ALK inhibitors

Besides alecitinib and ceritinib, currently there are six second-generation ALK inhibitors undergoing clinical trials for the treatment of ALK-positive patients, particularly for crizotinib-resistant patients. Five inhibitors with publicly disclosed structures are shown in Fig. 2. The following section will briefly outline the *in vitro* and *in vivo* activities of these inhibitors.

AP26113

AP26113 is a 2,6-diaminopyrimidine derivative TKI developed by Ariad Pharmaceuticals, orally active against ALK, ROS1 and EGFR. It shows strong inhibition against ALK (IC_{50} of 0.62 nM), inhibited cell growth and phosphorylation of ALK, and induced apoptosis in both wild-type and crizotinib-resistant NCI-H3122 cells. It also exhibited potent activity against Ba/F3 cells engineered to express either naïve EML4-ALK or EML4-ALK^{L1196M} mutant with IC_{50} values of 10 nM and 24 nM, respectively. More importantly, AP26113 overcame pan-ALK inhibitory activity against nine clinical crizotinib-resistant mutations with IC_{50} values in the range of 17-379 nM, and two clinical alectinib-resistant mutations with IC_{50} values of 19 and 123 nM, respectively. *In vivo* study revealed that AP26113 showed complete tumor regression in both KARPAS-299 and NCI-H3122 (50 mg/kg, po, qd) mouse xenograft models for 2-3 weeks, and is currently undergoing evaluation in phase I/II clinical trial.[67, 68] In a clinical study of 34 patients with either naïve or crizotinib-resistant ALK-positive NSCLC receiving AP26113 at a dose of 60 mg QD displayed an ORR of 65% (22 patients) including one complete response and 21 partial response. Of the three TKI-naïve NSCLC patients receiving AP26113 showed objective response, including one complete response. In particular, eight out of ten ALK-positive NSCLC patients with brain metastasis exhibited radiographic improvement.[70] These promising results demonstrate that AP26113 has the potential to be a new treatment option for ALK-positive NSCLC patients.

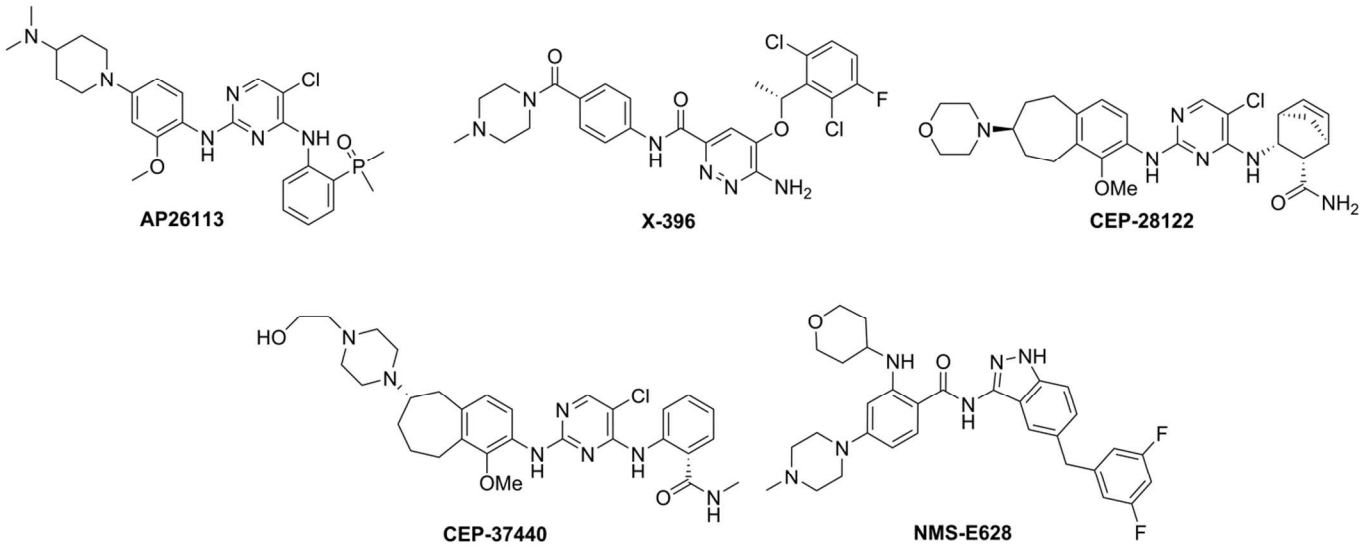


Fig. 2 Structures of second-generation ALK inhibitors.

X-396

Xcovery reported X-396 as a novel ALK inhibitor bearing an aminopyrazine scaffold. It showed comparatively superior ALK inhibition (IC_{50} < 0.4 nM) than crizotinib (IC_{50} = 4.5 nM), and displayed inhibitory activity against NCI-H3122 (IC_{50} = 15 nM), NCI-H2228 (IC_{50} = 45 nM), SUDHL-1 (IC_{50} = 9 nM), and SY5Y neuroblastoma cells (ALK^{F1174L}, IC_{50} = 68 nM). In Ba/F3-NPM-ALK cells, X-396 was approximately 10-fold more potent than crizotinib (IC_{50} of 22 nM and 250 nM, respectively). In two crizotinib-resistant mutations, EML4-ALK^{L1196M} and EML4-ALK^{C1156Y}, it exhibited IC_{50} values of 106 nM and 48 nM, respectively. *In vivo* study of X-396 showed significant tumor inhibition in NCI-H3122 (25 mg/kg, po, bid) mouse xenograft models without any signs of body weight loss or toxicity.[73] Based on these promising preclinical results, X-396 was entered into phase I clinical trial testing.

CEP-28122 and CEP-37440

CEP-28122 bearing a diaminopyrimidine scaffold was identified by Cephalon (a subsidiary of Teva) as a highly potent and selective ALK inhibitor (>600-fold selectivity with respect to the insulin receptor), which showed inhibitory activity against enzymatic ALK and KARPAS-299 with IC_{50} values of 1.9 nM and 20 nM, respectively. It also inhibited the growth of neuroblastoma cell lines NB-1643 and SHSY5Y, harboring ALK activating mutants F1174L and R1275Q. Moreover, CEP-28112 led to tumor regression in two ALK-positive ALCL mouse tumor xenografts, KARPAS-299 (30 mg/kg, po, bid) and Sup-M2 (55 or 100 mg/kg, po, bid for 4 weeks). In EML4-ALK-positive NSCLC mouse xenograft models (NCI-H2228, NCI-3122, and NB-1), CEP-28112 also demonstrated good antitumor activities at 50 mg/kg, po, bid for 12 days.[71,72] However, the development of CEP-28122 was terminated due to the occurrence of severe lung toxicity in the 4- and 13-week monkey studies. Cephalon have turned their attention on a new analogue, CEP-37440, which shown potent inhibitory activity against enzymatic ALK and FAK with IC_{50} values of 3.5 and 2.3 nM, respectively. CEP-37440 possessed more favorable properties than CEP-28112, such as superior solubility and metabolic stability, improved oral bioavailability, as well as lower clearance and toxicity. *In vivo* study of CEP-37440 in both Sup-M2 (30 mg/kg, po, bid or 50 mg/kg, po, qd) and KARPAS-299 (30 mg/kg, po, bid or 50 mg/kg, po, qd) mouse xenograft models for 12 days displayed complete tumor regression without overt toxicity and significant body weight loss. Moreover, oral administration of CEP-37440 (HCl salt) caused tumor stasis and partial regression in NCI-H3122 (30 mg/kg, po, bid and 55 mg/kg, po, bid) mouse xenograft models for 12 days, but it exhibited tumor regression in NCI-H2228 (30 mg/kg, po, qd and bid or 55 mg/kg, po, bid) mouse xenograft models with no overt toxicity and body weight loss (except at 30 mg/kg, bid). Based on these results, CEP-37440 underwent a phase I clinical trial in August 2013.[74]

TSR-011

TSR-011 (structure undisclosed) is an orally active dual ALK and TRK (tropomyosin-related kinases) inhibitor developed by Tesaro. It was reported to strongly inhibit ALK, ALK^{L1196M} and ALK^{R1275Q} with IC_{50} values of 0.7 nM, 0.1 nM and 0.5 nM, respectively. It also showed potent anti-proliferative activity against KARPAS-299 (IC_{50} = 1 nM), Sup-M2 (IC_{50} = 4 nM), NCI-H3122 (IC_{50} = 1 nM), and NB-1 (IC_{50} = 10 nM). In addition, it exhibited potent inhibition of TRK with IC_{50} values of 0.5-2.4 nM, and inhibited the proliferation of either TRKA-rearranged (IC_{50} = 25 nM) or NGF-stimulated TRKA (IC_{50} = 42 nM) cell lines. TSR-011 further exhibited promising activity in mouse models and is currently undergoing phase I/II clinical trials. According to the preliminary clinical results in 17 patients with treatment of TSR-011 for 8 weeks, 11 patients (65%) showed disease control. Three patients with ALK-positive NSCLC were progressed on prior treatment of crizotinib, and two patients showed response and one patient has stable disease. In addition, one patient with papillary thyroid carcinoma and one patient with pancreatic cancer exhibited stable disease on treatment with TSR-011.[75, 76]

NMS-E628

NMS-E628 is an orally available ALK inhibitor developed by Nerviano Medical Science. It showed good activity against NPM-ALK with an IC_{50} value of 55 nM. *In vivo* study of NMS-E628 showed complete tumor regression in both KARPAS-299 and SR-768 mouse xenograft models. When NMS-E628 was evaluated in Ba/F3-ALK^{L1196M} and Ba/F3-ALK^{C1156Y} cells that were identified in crizotinib resistant patients, it was able to inhibit them at lower dose than crizotinib, in both *in vitro* and *in vivo* conditions. NMS-E628 also showed ATP competitive inhibition of ROS1 with an IC_{50} value of 7 nM and induced complete tumor regression in Ba/F3-ROS1 mouse xenograft models for 10 days.[77, 78] It is currently being evaluated in phase I/II clinical trials.

PF-06463922

PF-06463922 (structure undisclosed) is a novel, orally active dual ALK and ROS1 inhibitor developed by Pfizer. According to 2013 AACR meeting report, it showed potent inhibitory activity against ALK (Ki < 0.2 nM and Cellular IC_{50} ~ 2 nM) and ROS1 (Ki < 0.005 nM and Cellular IC_{50} ~ 0.2 nM) with >100-fold selectivity against a panel of 207 kinases. More importantly, PF-06463922 demonstrated potent inhibition of 8 ALK mutants (ALK^{G1269A}, ALK^{L1196M}, ALK^{C1156Y}, ALK^{L1152R}, ALK^{F1174L}, ALK^{S1206Y}, $ALK^{1152Tins}$ and ALK^{G1202R}) that have been identified in crizotinib-resistant patients (IC_{50} = 1-65 nM), especially $ALK^{1152Tins}$ and ALK^{G1202R} that are resistant to all second-generation ALK inhibitors. It also induced apoptosis in the NSCLC cells harboring either wide-type or mutant ALK with IC_{50} values of 1-30 nM. Regarding *in vivo* studies, PF-06463922 showed significant tumor regression in mouse tumor xenograft models expressing activated NPM-ALK, EML4-ALK, EML4-ALK^{L1196M}, EML4-ALK^{G1269A} and EML4-

ALK^{G1202R} at low nanomolar plasma concentrations. The promising results indicated that PF-06463922 is the most potent ALK inhibitor against all known crizotinib-resistant mutants. It is also able to cross the blood brain barrier and is currently undergoing evaluation in Phase I/II clinical trials.[79, 80] Clinical trial outcome will decide if PF-06463922 has the potential to replace crizotinib as front-line therapy in ALK mediated NSCLC.

Conclusions

Modern drug discovery is target driven. In particularly, drugs for the treatment of cancer are now developed based on the molecular level alterations in the cancer. ALK has been recognized as an oncology therapeutic target since 1994, when fusion proteins of ALK were found in various human cancers. In 2007, the EML4-ALK driver gene was identified in NSCLC patients, resulting in tremendous interest in the development of ALK inhibitors for the treatment of ALK driven cancers. Crizotinib, originally designed as a c-Met inhibitor, was later found to have off-target ALK activity and has now become the first FDA-approved ALK inhibitor for the treatment of EML4-ALK-positive NSCLC patients. To overcome drug resistance, several second-generation ALK inhibitors are being actively pursued. Drugs with the ability to penetrate into the central nervous system are also being developed to treat brain tumors, since lung cancer is the most common type of cancer to spread to the brain. In 2014, the second-generation ALK inhibitor ceritinib was approved by the FDA for the treatment of patients with ALK-positive metastatic NSCLC, who were previously treated with-, or intolerant to crizotinib.

The successful development of these drugs emphasizes that the lead optimization process is not only focused on improving the potency, but also entails improving target selectivity, ADME, and physicochemical properties. Besides traditional medicinal chemistry efforts, several computer-assisted technologies such as homology modeling, structure-based drug design, and lipophilic efficiency parameters also played critical roles in hit-to-lead optimization of these candidates. Such strategies could be followed by researchers to overcome development issues, such as potency, selectivity and metabolism in drug discovery projects.

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