Clinical Cancer Research

The Potent ALK Inhibitor Brigatinib (AP26113) Overcomes Mechanisms of Resistance to First- and Second-Generation ALK Inhibitors in Preclinical Models

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Abstract

Purpose: Non–small cell lung cancers (NSCLCs) harboring *ALK* gene rearrangements (ALK^+) typically become resistant to the first-generation anaplastic lymphoma kinase (ALK) tyrosine kinase inhibitor (TKI) crizotinib through development of secondary resistance mutations in ALK or disease progression in the brain. Mutations that confer resistance to second-generation ALK TKIs ceritinib and alectinib have also been identified. Here, we report the structure and first comprehensive preclinical evaluation of the next-generation ALK TKI brigatinib.

Experimental Design: A kinase screen was performed to evaluate the selectivity profile of brigatinib. The cellular and *in vivo* activities of ALK TKIs were compared using engineered and cancer-derived cell lines. The brigatinib–ALK co-structure was determined.

Results: Brigatinib potently inhibits ALK and ROS1, with a high degree of selectivity over more than 250 kinases. Across a panel of ALK^+ cell lines, brigatinib inhibited native ALK (IC_{50}, 10 nmol/L) with 12-fold greater potency than crizotinib. Superior efficacy of brigatinib was also observed in mice with ALK^+ tumors implanted subcutaneously or intracranially. Brigatinib maintained substantial activity against all 17 secondary ALK mutants tested in cellular assays and exhibited a superior inhibitory profile compared with crizotinib, ceritinib, and alectinib at clinically achievable concentrations. Brigatinib was the only TKI to maintain substantial activity against the most recalcitrant ALK resistance mutation, G1202R. The unique, potent, and pan-ALK mutant activity of brigatinib could be rationalized by structural analyses.

Conclusions: Brigatinib is a highly potent and selective ALK inhibitor. These findings provide the molecular basis for the promising activity being observed in ALK^+, crizotinib-resistant patients with NSCLC being treated with brigatinib in clinical trials. *Clin Cancer Res; 22(22); 5527–38. ©2016 AACR.*

Introduction

Activating gene rearrangements in anaplastic lymphoma kinase (ALK^+), first identified in patients with anaplastic large-cell lymphoma (ALCL), have been shown to be oncogenic drivers in patients with non–small cell lung cancer (NSCLC; 3%–7%) and other cancers (1). The ALK/ROS1/MET tyrosine kinase inhibitor (TKI) crizotinib is highly active in ALK^+ NSCLC, inducing responses in 60% to 74% of patients (2, 3). However, the majority of patients progress within 1 year [e.g., median progression-free survival (PFS) is 8–11 months; refs. 2, 3], with multiple mechanisms of resistance having been identified.

ALK-dependent mechanisms of resistance, observed in approximately 30% of patients, include the acquisition of secondary mutations in ALK that interfere with crizotinib binding and/or amplification of the *ALK* fusion gene. More than 10 secondary mutations in ALK have been associated with crizotinib resistance in patients, with the most common being L1196M and G1269A (4, 5). The central nervous system (CNS) is the first site of progression in approximately 50% of patients (6, 7), suggesting inadequate penetration into the CNS by crizotinib (i.e., pharmacologic failure) as the primary cause of resistance in these patients. Finally, ALK-independent mechanisms, for example, via activation of EGFR-, IGF-1R-, or KIT-mediated signaling pathways, have also been shown to contribute to crizotinib resistance (4).

Recently, two second-generation ALK inhibitors, ceritinib and alectinib, have been approved for use in patients with ALK^+ NSCLC previously treated with crizotinib. In preclinical studies, ceritinib and alectinib have been shown to inhibit ALK more potently than crizotinib and to maintain activity against many of the secondary mutants associated with resistance to crizotinib

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Disease progression in patients with anaplastic lymphoma kinase-positive (ALK^+) non–small cell lung cancer (NSCLC) treated with the first-generation tyrosine kinase inhibitor (TKI) crizotinib, or the second-generation inhibitors ceritinib and alectinib, is often associated with secondary resistance mutations in ALK, or relapse in the brain. Here, we present the development and preclinical characterization of brigatinib, a selective ALK inhibitor with substantially more potent (~12-fold) activity against ALK than crizotinib, *in vitro*. Brigatinib inhibits 17 clinically and preclinically observed crizotinib-, ceritinib-, and/or alectinib-resistant ALK mutants tested *in vitro*, has potent effects on the recalcitrant G1202R mutant *in vivo*, and has activity in an orthotopic brain model. These results suggest that brigatinib may effectively address a broad range of resistance mechanisms identified for other ALK TKIs. Brigatinib is currently being evaluated in a global phase 2 registration trial in patients with locally advanced or metastatic ALK^+ NSCLC who were previously treated with crizotinib (NCT02094573).

(8, 9). Clinically, ceritinib and alectinib induce responses in 49% to 58% of patients previously treated with crizotinib, including in patients with and without secondary ALK mutations (10, 11). Consistent with the ability of these agents to overcome non–ALK-dependent mechanisms of resistance, ceritinib and especially alectinib have been shown to have some activity in patients with untreated CNS disease (10, 11). Nonetheless, patients tend to progress even more rapidly than on first-line crizotinib treatment (median PFS, 6.9–8.9 months; refs. 11, 12), and ALK secondary mutations associated with clinical resistance have been identified, including F1174C/V for ceritinib, I1171N/T/S for alectinib, and G1202R for both agents (4, 8, 13–15).

Brigatinib (AP26113) was developed to be a potent, selective inhibitor of ALK, capable of overcoming mechanisms of resistance associated with crizotinib. Brigatinib has been evaluated in a phase 1/2 trial (NCT01449461), and a pivotal phase 2 trial of brigatinib is ongoing in patients with ALK^+ NSCLC resistant to crizotinib, randomized to one of two dose schedules [90 mg once daily or 90 mg once daily for 7 days followed by 180 mg once daily (90 mg and 180 mg once daily schedules, respectively); NCT02094573]. Here, we provide the first comprehensive characterization of the design and preclinical properties of brigatinib.

Materials and Methods

Compounds

Brigatinib (AP26113), crizotinib, ceritinib, and alectinib were synthesized at ARIAD Pharmaceuticals and purchased [crizotinib (A Chemtek), ceritinib, and alectinib (Ontario Chemicals)]. Chemical structures of all TKIs (Supplementary Fig. S1) were confirmed by standard techniques (LC-MS, NMR). Note that a different molecule has been incorrectly cited as AP26113 in review articles (e.g., see refs. 1, 16). This incorrect molecule has been offered by multiple commercial vendors (e.g., Selleck Chemicals; through May, 2016), and several publications cite such vendors as the source of the material used in their study (e.g., see refs. 17–20).

Kinase assay

In vitro HotSpot^{SM} kinase profiling of 289 kinases was performed at Reaction Biology Corp. The assay was conducted in the presence of 10 μmol/L [^{33}P]-ATP, using brigatinib concentrations ranging from 0.05 nmol/L to 1 μmol/L.

Cell lines

HCC78, H2228, H23, H358, H838, U937, HepG2, and H-4-II-E cells were obtained from the ATCC. Karpas-299, SU-DHL-1, DEL, L-82, SUP-M2, and Ba/F3 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and H3122 cells from the National Cancer Institute (Bethesda, MD). All cell lines were used within 20 passages of the initial thaw; no further authentication was performed.

Expression constructs encoding native EML4-ALK (variant 1), EGFR, ROS1, and FLT3, and mutant variants thereof were synthesized (Genscript), cloned into a pLVX-IRES puro vector (Clontech), and introduced into Ba/F3 cells by lentiviral transduction. After selection with puromycin, cells were grown in the absence of IL3.

In vitro cellular assays

Following TKI treatment for 72 hours, cell growth was assessed using either CellTiter 96 AQueous One solution (Promega) to determine the concentration that causes 50% inhibition of cell viability (IC_{50}) or CyQuant Cell Proliferation assay (Invitrogen) to determine the concentration that causes 50% growth inhibition (GI_{50}). The GI_{50} value was determined by correcting for the cell count at time zero (initiation of treatment). To evaluate effects on levels of phosphorylated (p-) proteins, cells were treated with increasing concentrations of compound for 1 hour and then lysed in RIPA buffer. Lysates were subjected to p-ALK/total ALK ELISA (Cell Signaling Technology) and/or immunoblotted using indicated antibodies purchased from Cell Signaling Technology (p-ALK, total ALK, ROS1, p-ROS1, p-AKT, and pERK1/2). *In vitro* cellular assays related to IGF-1R, INSR, and EGFR are described in the Supplementary Methods.

In vitro mutagenesis screen

Ba/F3 cells expressing native EML4-ALK were treated overnight with 100 μg/mL *N*-ethyl-*N*-nitrosourea (ENU; Sigma-Aldrich), pelleted, resuspended in fresh medium without IL3, and then distributed into 96-well plates containing various concentrations of brigatinib, crizotinib, ceritinib, or alectinib. Cells were grown in standard growth medium without IL3 for 3 to 5 weeks and monitored for outgrowth of resistant clones. Cells from wells containing substantial outgrowth were expanded under the original selection conditions, gDNA extracted, and the ALK kinase region sequenced by Taq BigDye Deoxy Terminator Cycle Sequencing (Applied Biosystems). Results are presented from two independent screens.

In vivo tumor models

All animal experiments were carried out under a protocol approved by the ARIAD Institutional Animal Care and Use Committee (IACUC). Tumors were established by subcutaneous injection of Karpas-299, H2228, or engineered Ba/F3 cells into the right flank of about 8-week-old CB-17/SCID or SCID beige female mice (Charles River Laboratory). When the average tumor volume reached about 200 mm^3 (Karpas-299 and Ba/F3 models) or 300

mm^3 (H2228 model), mice were randomized to the various treatment groups and administered vehicle or TKI once daily by oral gavage. Mean tumor volumes (length × width^2 × 0.5), tumor growth inhibition, and tumor regression rates were calculated for each treatment group.

In vivo brain tumor studies

Orthotopic brain tumors were generated by intracranial injection of H2228 cells into CB-17/SCID mice. Animals were randomized by body weight on day 7 postsurgery, and TKI treatment was initiated and continued until mice were euthanized, according to IACUC guidelines. Median survival time was calculated, and statistical significance for each survival curve examined using a log-rank Mantel–Cox test.

Pharmacodynamic and pharmacokinetic analyses

Tumor samples were lysed in RIPA buffer and subjected to ELISA or immunoblotting to evaluate pharmacodynamic effects. TKI concentrations in mouse plasma were assessed using LC/MS-MS. Plasma samples were deproteinized before analysis, and TKI levels quantitated using an internal standard LC/MS-MS assay.

Crystallography

A native ALK construct spanning residues 1,093 to 1,407, with His and GST tags at the N-terminus, was expressed in baculovirus and Sf9 cells and copurified using affinity and size exclusion chromatography in the presence of brigatinib. Co-crystals were generated at 4°C using 0.1 mol/L Tris-HCl (pH 8.0), 16% to 18% PEG3350, and 2 mmol/L TCEP. The atomic coordinates and structure factors have been deposited in the RCSB PDB (ID: 5J7H). Additional crystallographic analysis–related methods are described in the Supplementary Methods.

Results

Identification of brigatinib, a potent and selective ALK and ROS1 inhibitor

To identify a potent ALK inhibitor, a focused chemical library was screened using *in vitro* assays, and resulting hits were optimized using structure-based approaches guided by an ALK homology model. We identified a pyrimidine-based molecule, brigatinib (AP26113; Fig. 1A), containing a C4 aniline with an ortho dimethylphosphine oxide (DMPO) substituent. Brigatinib potently inhibited the *in vitro* kinase activity of ALK (IC_{50}, 0.6 nmol/L) and all five mutant variants tested, including G1202R (IC_{50}, 0.6–6.6 nmol/L; Table 1). Brigatinib demonstrated a high degree of selectivity, only inhibiting 11 additional native or mutant kinases (of 289 screened) with IC_{50} <10 nmol/L (Supplementary Table S1). These included ROS1, FLT3, and mutant variants of FLT3 (D835Y) and EGFR (L858R; IC_{50}, 1.5–2.1 nmol/L; Table 1). Brigatinib exhibited more modest activity against EGFR with a T790M resistance mutation (L858R/T790M), native EGFR, IGF1R, and INSR (IC_{50}, 29–160 nmol/L) and did not inhibit MET (IC_{50} >1000 nmol/L).

The activity of brigatinib against native ALK and a number of non-ALK kinases was next examined in cellular assays (Table 1). Consistent with *in vitro* kinase data, brigatinib inhibited ALK and ROS1 with similar potencies (IC_{50}, 14 and 18 nmol/L, respectively). Compared with ALK, brigatinib inhibited FLT3 and IGF-1R with about 11-fold lower potency (IC_{50}, 148–158 nmol/L) and inhibited mutant variants of FLT3 and EGFR with 15- to 35-fold lower potency (IC_{50}, 211–489 nmol/L), despite inhibiting some of these kinases (e.g., FLT3, FLT3 [D835Y], and EGFR [L858R]) with potency similar to ALK in *in vitro* kinase assays. Finally, brigatinib lacked cellular activity against INSR and native EGFR (IC_{50} > 3,000 nmol/L). The activity of brigatinib against non-ALK kinases, including additional mutant variants, is described in further detail in the Supplement (Supplementary Results and Supplementary Figs. S2–S4). Overall, these results suggest that brigatinib is a highly potent and selective ALK and ROS1 inhibitor.

Brigatinib displays superior *in vitro* and *in vivo* potency in ALK$^+$ ALCL and NSCLC models, compared with crizotinib

In a panel of seven ALCL and NSCLC cell lines that express NPM-ALK or EML4-ALK fusions, the concentration of brigatinib that inhibited growth by 50% (GI_{50}) ranged from 4 to 31 nmol/L, and the concentration that inhibited ALK phosphorylation by 50% (IC_{50}) ranged from 1.5 to 12 nmol/L (Fig. 2A and B). Potent inhibition of downstream signaling was also observed in both ALCL and NSCLC cell lines (Supplementary Fig. S5). Across three ALK-negative ALCL and NSCLC cell lines, the GI_{50} values for brigatinib ranged from 503 to 2,387 nmol/L. Thus, overall, brigatinib potently inhibited ALK activity and proliferation in all ALK$^+$ cell lines tested and exhibited more than 100-fold selectivity over ALK-negative lines. In contrast, crizotinib exhibited about 12-fold lower potency compared with brigatinib in ALK$^+$ cell lines (GI_{50}, 62–309 nmol/L; IC_{50}, 23–55 nmol/L), and only about 10-fold selectivity over ALK-negative lines (GI_{50}, 928–1773 nmol/L; Fig. 2A and B; Supplementary Fig. S5).

The *in vivo* activity of brigatinib was examined in ALK$^+$ Karpas-299 (ALCL) and H2228 (NSCLC) xenograft mouse models, with oral administration of brigatinib (10, 25, or 50 mg/kg once daily) leading to a dose-dependent inhibition of tumor growth in both models. In the Karpas-299 model, 25 mg/kg inhibited tumor growth by 87% and 50 mg/kg induced near complete tumor regression that was maintained for at least 13 days after treatment (Fig. 2C). Increased efficacy was associated with increased plasma levels of brigatinib and deeper and more sustained inhibition of ALK signaling in the tumor (Supplementary Fig. S6A and S6B). A 100 mg/kg daily dose of crizotinib inhibited tumor growth (by 90%) and ALK signaling to a similar degree as 25 mg/kg brigatinib, despite about fivefold higher levels of exposure (Fig. 2C; Supplementary Fig. S6A and S6C). Comparison of mouse and human exposure levels (Supplementary Fig. S6C) demonstrated that plasma levels of brigatinib in mice dosed at 25 and 50 mg/kg once daily were similar to those achieved in patients dosed at 90 and 180 mg once daily, respectively. Notably, levels of exposure in mice dosed with 100 mg/kg crizotinib exceeded those achieved in patients dosed on the approved 250 mg twice-daily schedule by about sixfold (Supplementary Fig. S6C). Brigatinib and crizotinib doses used in these studies were well tolerated (Supplementary Fig. S6D).

H2228-derived tumors, possibly because of their slower growth rate, were even more sensitive to ALK inhibition than Karpas-299–derived tumors, with 10, 25, or 50 mg/kg brigatinib inducing substantial tumor regression that was maintained for more than 28 days after treatment (Fig. 2C). In this model, 100 mg/kg crizotinib was required to achieve efficacy similar to that achieved by 10 mg/kg brigatinib (Fig. 2C). Thus, the substantially greater potency of brigatinib over crizotinib observed in Karpas-299 and H2228 cells in cellular assays is also maintained *in vivo*.

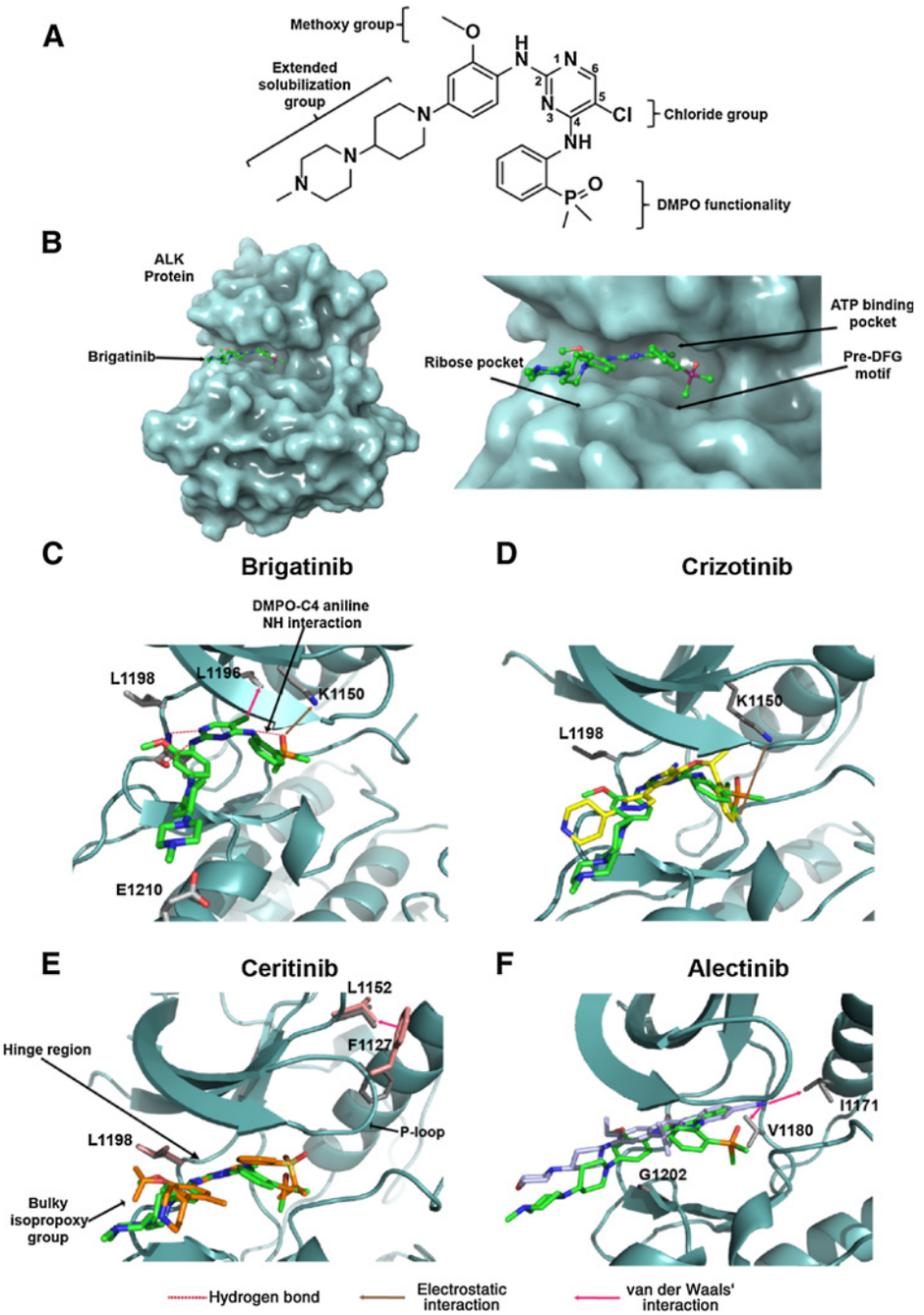


Figure 1.
Brigatinib structure and interactions with ALK, compared with other ALK TKIs. **A,** chemical structure of brigatinib (AP26113), with its unique molecular features attached to the core bisanilinopyrimidine scaffold highlighted. **B,** solid-surface representation of brigatinib (green) bound to ALK. Select binding regions are highlighted. **C–F,** crystal structure of brigatinib (green) bound to ALK, alone (**C**) or overlaid with crizotinib (yellow; **D**), ceritinib (orange; **E**), and alectinib (purple; **F**). In all cases, amino acid residues important for interacting with ALK are mapped for each corresponding inhibitor on the ALK structure in cyan. In **E**, the presentation of F1127, L1152, and L1198 residues on ALK in a ceritinib-bound state are depicted in pink. See Supplementary Fig. S1 for chemical structures of brigatinib, crizotinib, ceritinib, and alectinib.

Brigatinib enhances survival in an orthotopic mouse brain tumor model, compared with crizotinib

Activity of brigatinib in the CNS was assessed using an orthotopic brain tumor model. ALK$^+$ H2228 (NSCLC) cells were injected intracranially to form tumors in the brain, and tumor-bearing mice were treated with vehicle, crizotinib, or brigatinib. Daily oral administration of crizotinib at 100 mg/kg extended median survival to 47.5 days, compared with 28 days observed in vehicle-treated mice (Fig. 3A). Compared with crizotinib-treated mice, daily oral dosing with 25 and 50 mg/kg brigatinib significantly prolonged median survival to 62 and >64 days [i.e., 10 of 10 mice were alive at study termination (day 64)], respectively (Fig. 3A). Histologic evaluation demonstrated a significant reduction in tumor burden in the brains of mice treated with 50 mg/kg brigatinib compared with crizotinib-treated mice (Fig. 3B and C).

Potent *in vitro* activity of brigatinib against ALK mutants that confer resistance to crizotinib, ceritinib, and alectinib

At least 16 unique secondary mutations in ALK, at 11 different amino acid residues (G1123, T1151, L1152, C1156, I1171, F1174, L1196, G1202, D1203, S1206, and G1269), have previously been associated with clinical resistance to crizotinib and/or

Table 1. *In vitro* and cellular activity of brigatinib against select kinases

	Kinase assay	Cellular assay	
Kinase[a]	IC_{50}, nmol/L	IC_{50}, nmol/L	Cellular system[b]
ALK	0.6	14	EML4-ALK
ALK (C1156Y)	0.6	45	EML4-ALK (C1156Y)
ALK (F1174L)	1.4	55	EML4-ALK (F1174L)
ALK (L1196M)	1.7	41	EML4-ALK (L1196M)
ALK (G1202R)	4.9	184	EML4-ALK (G1202R)
ALK (R1275Q)	6.6	ND	N/A
ROS1	1.9	18	CD74-ROS1
FLT3	2.1	158	FLT3-ITD
FLT3 (D835Y)	1.5	211	FLT3-ITD (D835Y)
EGFR	67	>3,000	p-EGFR in EGF-stimulated H358 cells
EGFR (L858R)	1.5	397	EGFR (L858R)
EGFR (L858R/T790M)	29	489	EGFR (L858R/T790M)
IGF-1R	73	148	p-IGF-1R in IGF1-stimulated HepG2 cells
INSR	160	9,331	p-INSR in insulin-stimulated H-4-II-E cells
MET	>1000	ND	N/A

Abbreviations: N/A, not applicable; ND, not determined.

[a]Additional data regarding kinases inhibited with IC_{50} < 10 nmol/L [FER, FES, FAK/PTK2, PTK6, TSSK1, CHEK2, CHEK2 (I157T)] and all other kinases screened are provided in Supplementary Table S1.

[b]Viability assay using Ba/F3 cells whose survival was dependent on activity of the indicated fusion protein, unless otherwise noted; additional details are provided in Fig. 4B and Supplementary Figs. S2 and S7.

the second-generation ALK inhibitors ceritinib and alectinib (Fig. 4A). To search for novel mutations that might be associated with resistance to brigatinib, as well as crizotinib, ceritinib, or alectinib, we performed an *in vitro* mutagenesis screen. To allow direct comparison of TKI resistance profiles, a large batch of Ba/F3 cells expressing EML4-ALK were mutagenized to create a common pool of mutants, and mutagenized cells were plated in the presence of graded concentrations of each inhibitor.

Treatment with 500 nmol/L brigatinib was sufficient to suppress the emergence of any mutant, whereas higher concentrations of all of the other TKIs (≥1,000 nmol/L) were required for pan-ALK inhibitory activity (Fig. 4A). All clones that survived in the presence of the next highest brigatinib concentration tested (200 nmol/L) contained mutations at positions previously observed to confer resistance to other ALK TKIs (F1174, L1196, and S1206), except E1210K. In addition, L1198F and V1180L were identified as mutations that may confer resistance, uniquely, to ceritinib and alectinib, respectively.

Next, we generated a panel of Ba/F3 cell lines containing native EML4-ALK, or 17 variants with mutations at all amino acids (except G1123) that were either previously associated with clinical resistance or identified in the mutagenesis screen (Fig. 4A). All four TKIs were examined side-by-side, and nonspecific activities were assessed using untransformed Ba/F3 cells grown in the presence of IL3 ("parental" cells; Fig. 4B, Supplementary Fig. S7A). TKI potency was also assessed relative to levels of exposure achieved in patients at the approved or recommended phase 2 doses (Fig. 4A).

Brigatinib was the most potent inhibitor of native EML4-ALK (IC_{50}, 14 nmol/L), with crizotinib, ceritinib, and alectinib all having lower potency (IC_{50}, 107, 37, and 25 nmol/L, respectively). Brigatinib also demonstrated substantial activity against all 17 ALK mutants (IC_{50}, 9–184 nmol/L), with potencies substantially greater (range, 2.2- to 77-fold) than those of crizotinib (IC_{50}, 170–1,109 nmol/L) for all mutants except L1198F. Furthermore, brigatinib inhibited five mutants with ≥3-fold greater potency than ceritinib (range, 3.2- to 40-fold; L1152R/P, C1156Y, L1198F, and G1269A) and inhibited six mutants with ≥3-fold greater potency than alectinib (range, 3.2- to 54-fold; L1152R, I1171N, V1180L, L1196M, G1202R, and G1269A). Neither ceritinib nor alectinib had more than twofold greater potency than brigatinib against any of the 17 mutants tested. Similar trends were observed when making comparisons based on IC_{90} values (Supplementary Fig. S7A), with concentrations required to inhibit native and mutant EML4-ALK activity by 90% generally about threefold higher than IC_{50} values for all four TKIs.

Brigatinib also inhibited native EML4-ALK with the greatest selectivity over parental Ba/F3 cells (230-fold; based on IC_{50} values), with crizotinib, ceritinib, and alectinib all having reduced selectivity (12-, 45-, and 83-fold, respectively; Supplementary Fig. S7B). In addition, crizotinib exhibited relatively poor selectivity (≤5-fold vs. parental cells) for 11 mutants, all of which involved residues previously associated with clinical resistance to crizotinib (T1151Tins, L1152R/P, C1156Y, I1171N, F1174L, F1174V, L1196M, G1202R, D1203N, and G1269A). Ceritinib exhibited relatively poor selectivity for four mutants (L1152R/P, L1198F, and G1202R) and alectinib for three (I1171N, V1180L, and G1202R). In contrast, brigatinib exhibited more than 17-fold selectivity for all mutants, with G1202R being the most recalcitrant (IC_{50}, 184 nmol/L).

Finally, brigatinib also exhibited superior preclinical activity compared with crizotinib, ceritinib, and alectinib when *in vitro* potencies were related to steady-state TKI plasma levels (C_{ave}; AUC/dosing interval) achieved in patients (Fig. 4A; Supplementary Fig. S7A). Of the 12 mutants previously associated with clinical resistance to crizotinib, nine were inhibited with IC_{90} values that exceed levels reported to be achieved in patients. In contrast, only two of 12 such mutants were inhibited with IC_{50} values that exceed plasma levels, suggesting that relating IC_{90}, instead of IC_{50}, values to plasma levels may better predict clinical resistance mutations, at least when the potential effects of protein binding are not taken into account. For ceritinib, IC_{90} values for two mutants, L1152R and L1198F, exceeded levels in patients, whereas for alectinib, IC_{90} values for three mutants, I1171N, V1180L, and G1202R, exceeded levels in patients. In contrast, for brigatinib, levels achieved in patients dosed at 180 mg exceeded the IC_{90} for all 17 mutants and levels achieved in patients dosed at 90 mg exceeded the IC_{90} for all mutants except

A

Model	Cell line	ALK fusion status	Brigatinib Cell growth GI_{50} ± SD (nmol/L)	Brigatinib p-ALK IC_{50} ± SD (nmol/L)	Crizotinib Cell growth GI_{50} ± SD (nmol/L)	Crizotinib p-ALK IC_{50} ± SD (nmol/L)
ALCL	Karpas 299	NPM-ALK	10 ± 2	3.2 ± 2.8	119 ± 22	33 ± 15
	SU-DHL-1	NPM-ALK	9 ± 2	1.5 ± 1.3	99 ± 21	23 ± 13
	DEL	NPM-ALK	31 ± 19	6.6 ± 3.2	309 ± 122	ND
	L-82	NPM-ALK	10 ± 3	2.1 ± 0.5	140 ± 49	ND
	SUP-M2	NPM-ALK	15 ± 5	12 ± 12	139 ± 4	ND
	U937	ALK-negative	2387 ± 400	ND	928 ± 155	ND
NSCLC	H3122	EML4-ALK v1	4 ± 1	3.7 ± 1.6	62 ± 18	43 ± 37
	H2228	EML4-ALK v3a/3b	10 ± 7	4.5 ± 2.2	121 ± 61	55 ± 4
	H23	ALK-negative	1337 ± 875	ND	1773 ± 743	ND
	H838	ALK-negative	503 ± 400	ND	1307 ± 270	ND

ND, not determined

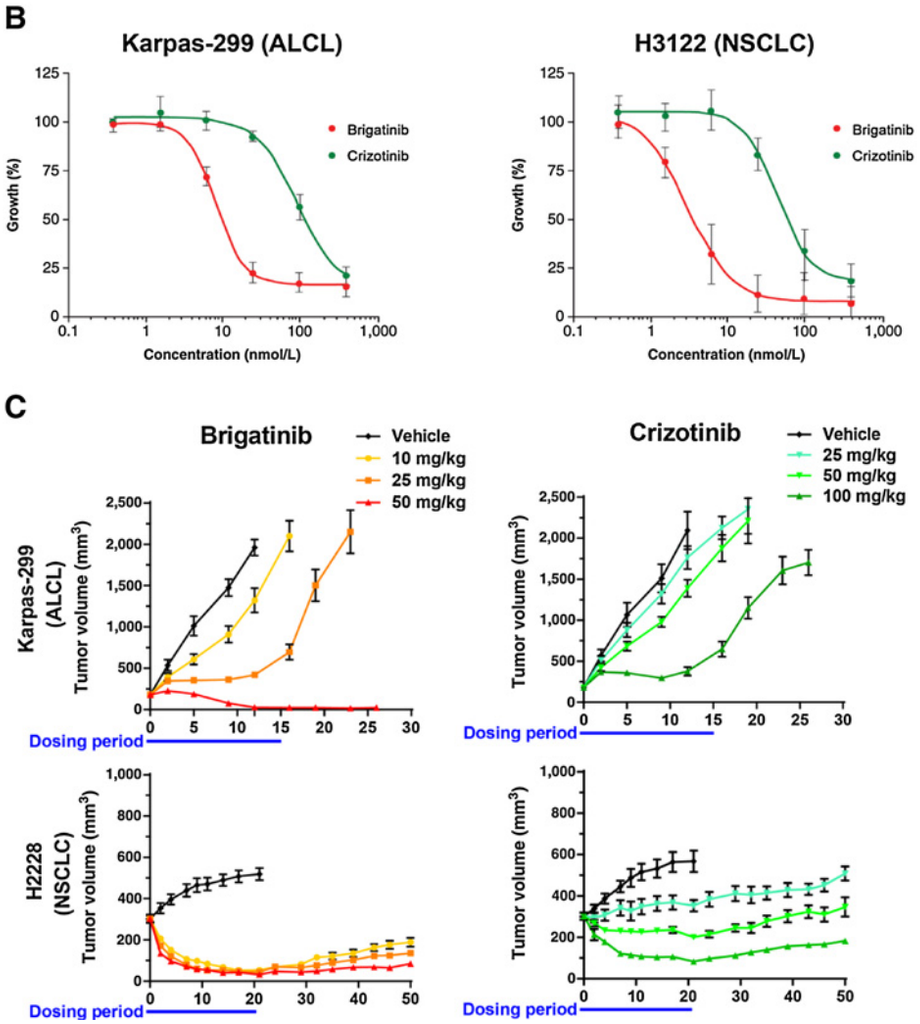


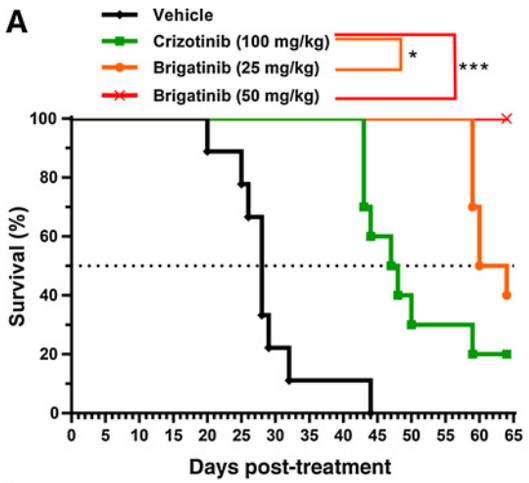
Figure 2.
Cellular and *in vivo* activity of brigatinib, compared with crizotinib, in ALK^+ ALCL and NSCLC models. **A,** TKI-mediated inhibition of cell growth and target phosphorylation in ALK^+ and ALK^- cell lines. **B,** *in vitro* growth inhibition of ALK^+ Karpas-299 (NPM-ALK) and H3122 (EML4-ALK) cells treated with increasing concentrations of brigatinib or crizotinib for 72 hours. Error bars, SD. **C,** efficacy of brigatinib and crizotinib in ALK^+ tumor models *in vivo*. SCID beige mice were implanted subcutaneously with either Karpas-299 (ALCL) or H2228 (NSCLC) cells and dosed orally with brigatinib (10, 25, or 50 mg/kg once daily) or crizotinib (25, 50, or 100 mg/kg once daily) for 14 days (Karpas-299) or 21 days (H2228). Mean tumor volumes shown for each treatment group ($n = 10$ mice/group). Error bars, SE.

G1202R. Importantly, the improved potency margin for brigatinib is even greater if its relatively low degree of protein binding is taken into account. The percentage of "free" brigatinib (34.3%), that is not bound to human plasma proteins *in vitro*, is four- to 100-fold greater than that of crizotinib (9.3%), ceritinib (2.8%), and alectinib (0.3%; Fig. 4A).

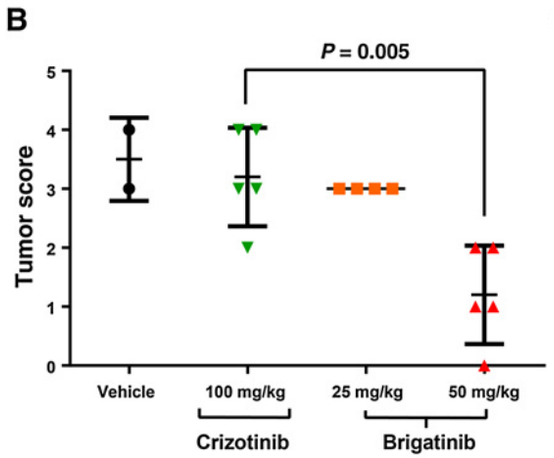
Taken together, these results demonstrate that brigatinib is a more potent ALK inhibitor than crizotinib, ceritinib, and alectinib *in vitro* and is the only one that exhibits substantial activity against all 17 secondary ALK mutants tested.

Brigatinib demonstrates antitumor activity against L1196M and the highly resistant G1202R mutant *in vivo*

Engineered Ba/F3 cells were next used to explore the *in vivo* inhibitory profile of brigatinib and other ALK TKIs against native EML4-ALK and two ALK mutants of particular interest: L1196M,



Treatment	Median survival (days)
Vehicle	28
Crizotinib (100 mg/kg)	47.5
Brigatinib (25 mg/kg)	62
Brigatinib (50 mg/kg)	>64



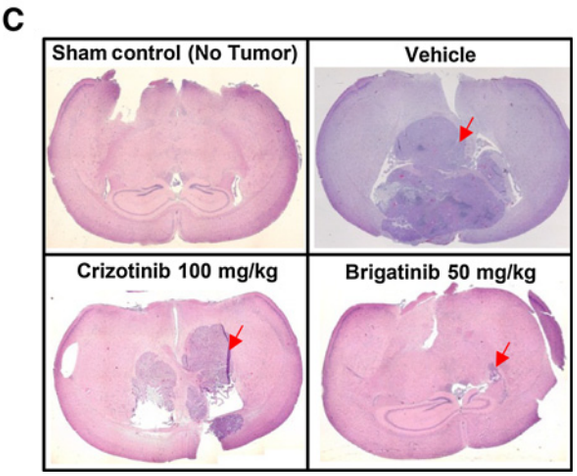


Figure 3.

Brigatinib markedly enhances survival of mice bearing ALK$^+$ brain tumors compared with crizotinib. **A,** Kaplan–Meier survival curves of CB17 SCID mice with intracranial ALK$^+$ H2228 (NSCLC) tumors following treatment with vehicle, crizotinib (100 mg/kg once daily), or brigatinib (25 or 50 mg/kg once daily). Median survival time and statistical significance (examined using a log-rank Mantel–Cox test) were calculated for each survival curve. *, $P < 0.05$; ***, $P = 0.0002$; median survival was significantly longer in all TKI treatment groups compared with vehicle ($P < 0.0001$). Injected tumor cells were localized in the brains of all mice examined; no metastases to other parts of body were observed. Therefore, the survival benefit conferred by TKI treatment was likely due to a reduction of tumor burden in the brain and not due to a reduction in systemic tumor burden. **B,** brain tumor pathology score. Tumor score was evaluated on the basis of visual quantitation of tumor area in hematoxylin and eosin (H&E)-stained sections of brain samples (collected at the time of euthanasia, see Materials and Methods) by 2 independent reviewers in a blinded manner; an unpaired *t* test was performed to determine statistical significance. Error bars, SD. Brains analyzed were harvested on days 32 ($n = 1$) and 44 ($n = 1$) for vehicle, 43 ($n = 2$), 50 ($n = 1$), 59 ($n = 1$), and 64 ($n = 1$) for crizotinib 100 mg/kg, 59 ($n = 2$) and 64 ($n = 2$) for brigatinib 25 mg/kg, and 64 ($n = 5$) for brigatinib 50 mg/kg. Note 3 of 5 mouse brains analyzed in the crizotinib-treated group and all of the mouse brains in the vehicle group were collected between days 32 and 50, whereas all of the mouse brains analyzed in the brigatinib-treated groups were collected on days 59 or 64. Longer times until collection could potentially lead to a bias for higher tumor scores for brigatinib-treated mice compared with vehicle- and crizotinib-treated mice. **C,** histopathology of brain tumors. Representative images of H&E-stained sections from samples collected on treatment days 44, 59, and 64 from mice treated with vehicle, 100 mg/kg crizotinib, and 50 mg/kg brigatinib, respectively. Red arrows indicate tumor location.

the most common mutant associated with resistance to crizotinib (4), and G1202R, the mutant brigatinib inhibited least potently and the only mutant thus far associated with clinical resistance to crizotinib, ceritinib, and alectinib (Fig. 4A).

Mice bearing subcutaneous tumors expressing native EML4-ALK or L1196M-mutant EML4-ALK were administered vehicle, crizotinib, or brigatinib orally once daily. High-dose crizotinib (200, but not 100 mg/kg) induced regression of tumors expressing native EML4-ALK but did not show antitumor activity against L1196M (Fig. 5A; Supplementary Fig. S8A). In contrast, 25 mg/kg brigatinib induced regression and substantial growth inhibition of tumors expressing native EML4-ALK and L1196M, respectively, and 50 mg/kg brigatinib induced tumor regression in both models. The increased efficacy of brigatinib against L1196M was associated with deeper and more sustained inhibition of ALK signaling in the tumor (Fig. 5B; Supplementary Fig. S8A). Ceritinib (25 mg/kg) and alectinib (60 mg/kg) have previously been shown to inhibit growth of tumors expressing L1196M mutant EML4-ALK (8, 9).

In mice bearing tumors expressing G1202R-mutant EML4-ALK, 25 and 50 mg/kg brigatinib inhibited tumor growth by 55% and 88%, respectively, whereas 100 and 200 mg/kg crizotinib only

A

TKI	Clinical trial data				*In vitro* mutagenesis screen results	
	Dose[a]	C_{ave}[b] (nmol/L)	PPB (%)	ALK Secondary mutations previously associated with clinical resistance[c,e]	TKI Conc. (nmol/L)	ALK Secondary mutations that emerged[e]
Crizotinib	250 mg bid	718	90.7	T1151Tins, L1152R, C1156Y, I1171N/T, F1174L/V, L1196M, G1202R, D1203N, S1206F/Y, G1269A	500	T1151K, I1171T, F1174L/I, S1206A, F1245C, G1269A
					750	C1156Y, I1171N/T, F1174C/V, L1196M
					1000	L1196M
Brigatinib	90 mg qd	582	65.7	N/A[d]	200	F1174C/V/I, L1196M, S1206A, **E1210K**
	180 mg qd	1447			500	None
Ceritinib	750 mg qd	1232	97.2	G1123S, F1174C/V, G1202R	200	F1174C/V/I, **L1198F**, S1206A
					500	**L1198F**
					1000	None
Alectinib	600 mg bid	1120	99.7	I1171N/T/S, G1202R	200	I1171N/S, **V1180L**, L1196M
					500	I1171N
					1000	I1171N

[a]Approved or recommended phase 2 doses. [b]C_{ave}, Steady-state plasma levels ($AUC_{0\text{-}24}$ corrected for 24 h exposure); crizotinib (32), brigatinib (30, 33), ceritinib (11), alectinib (34). [c]Crizotinib (5), ceritinib (5), alectinib (5). [d]Mutations associated with resistance to brigatinib in the phase I/II trial have not yet been reported. [e]Underlined mutations were included in the panel of 17 mutants analyzed further (Fig. 4B); bolded mutations are at amino acids not previously associated with clinical resistance. PPB, Plasma protein binding (30, 32, 33, 35, 36); Conc., concentration. $AUC_{0\text{-}24}$, area under the concentration-time curves from time 0 to 24 h.

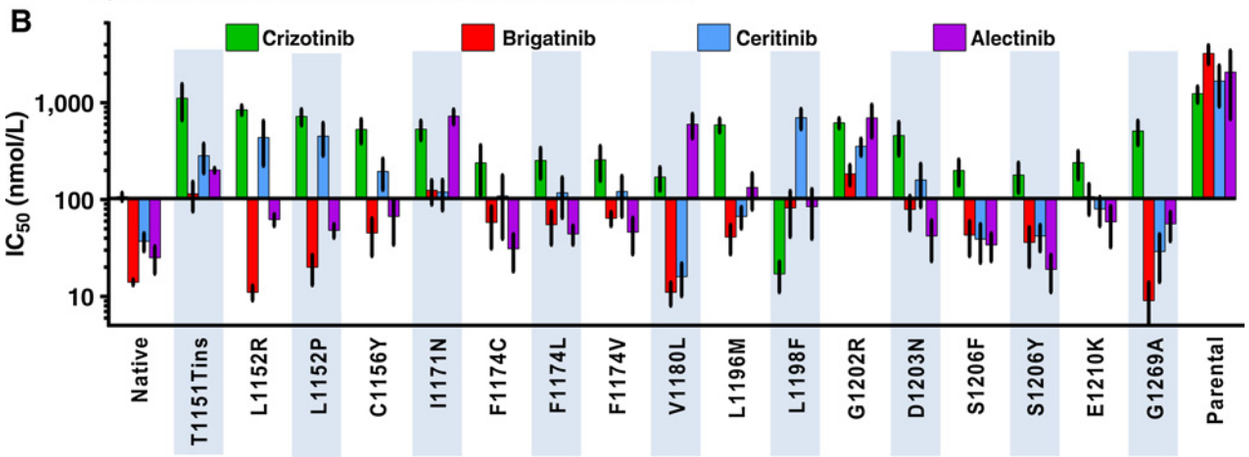


Figure 4.

Brigatinib has reduced susceptibility to ALK mutations compared with other first- and/or second-generation ALK TKIs *in vitro*. **A,** left, clinical trial data for ALK TKIs: steady-state plasma levels (C_{ave}; AUC/dosing interval) at the recommended phase 2 dose, *in vitro* plasma protein binding (PPB), and ALK mutations previously associated with clinical resistance. Right, ALK resistance mutations identified in an ENU mutagenesis screen as performed in this study. **B,** IC_{50} values (nmol/L) of crizotinib, brigatinib, ceritinib, and alectinib in Ba/F3 cells harboring native EML4-ALK or 17 mutant variants, and in ALK^- (parental) cells. Data for each cell line are derived from at least 4 independent experiments. Error bars, SD. See Supplementary Fig. S7A for IC_{50} and IC_{90} values.

inhibited tumor growth by 23% and 46%, respectively (Fig. 5A; Supplementary Fig. S8A). Doses of ceritinib (50 mg/kg) and alectinib (60 mg/kg) that strongly inhibited growth of native EML4-ALK tumors (by >95%) had little to no impact on growth of G1202R tumors (<15% inhibition). The increased efficacy of brigatinib against G1202R was also associated with deeper and more sustained inhibition of ALK signaling in the tumor (Fig. 5B). Pharmacokinetic analysis demonstrated that superior efficacy of 25 and 50 mg/kg brigatinib was associated with plasma levels that were similar (within twofold) to those of ceritinib and alectinib and similar to or below those of crizotinib (Supplementary Fig. S8A). All dosing regimens used in these studies were well-tolerated (Supplementary Fig. S8B).

Structural basis for the improved potency of brigatinib

To examine the structural basis for the potent activity of brigatinib against native ALK and its reduced susceptibility to ALK mutations (Fig. 4B; Supplementary Fig. S7A), a crystal structure of the ALK kinase domain in complex with brigatinib was determined and compared to those of crizotinib, ceritinib, and alectinib (chemical structures: Supplementary Fig. S1).

Brigatinib is constructed around a bisanilinopyrimidine core that occupies the ATP-binding site of ALK in a U-shaped conformation (Fig. 1A–C). Brigatinib contains several chemical features that increase its affinity for ALK that are lacking in crizotinib (Fig. 1D), including a methoxy substituent, and an extended solubilization group on the C2 aniline, a chlorine atom at C5, and a unique DMPO functionality on the C4 aniline. The methoxy group binds in a pocket located under the hinge residue L1198 and the extended solubilization group fills part of the ribose-binding pocket and interacts with additional residues from alpha helix D (e.g., E1210). The chlorine atom at C5 interacts with the gatekeeper residue L1196. The DMPO moiety interacts with the small pre-DFG pocket and, to some extent, with the catalytic lysine (K1150). In addition, because of an intramolecular H-bond between the DMPO and the C4 aniline NH, brigatinib is more conformationally preorganized than crizotinib and therefore pays a reduced entropic penalty during complexation with ALK.

ALK mutations that reduce ceritinib activity to a much greater degree than brigatinib include L1198F and L1152R/P. While both compounds share the same bisanilinopyrimidine core and a chlorine atom at C5, ceritinib has an isopropoxy substituent on

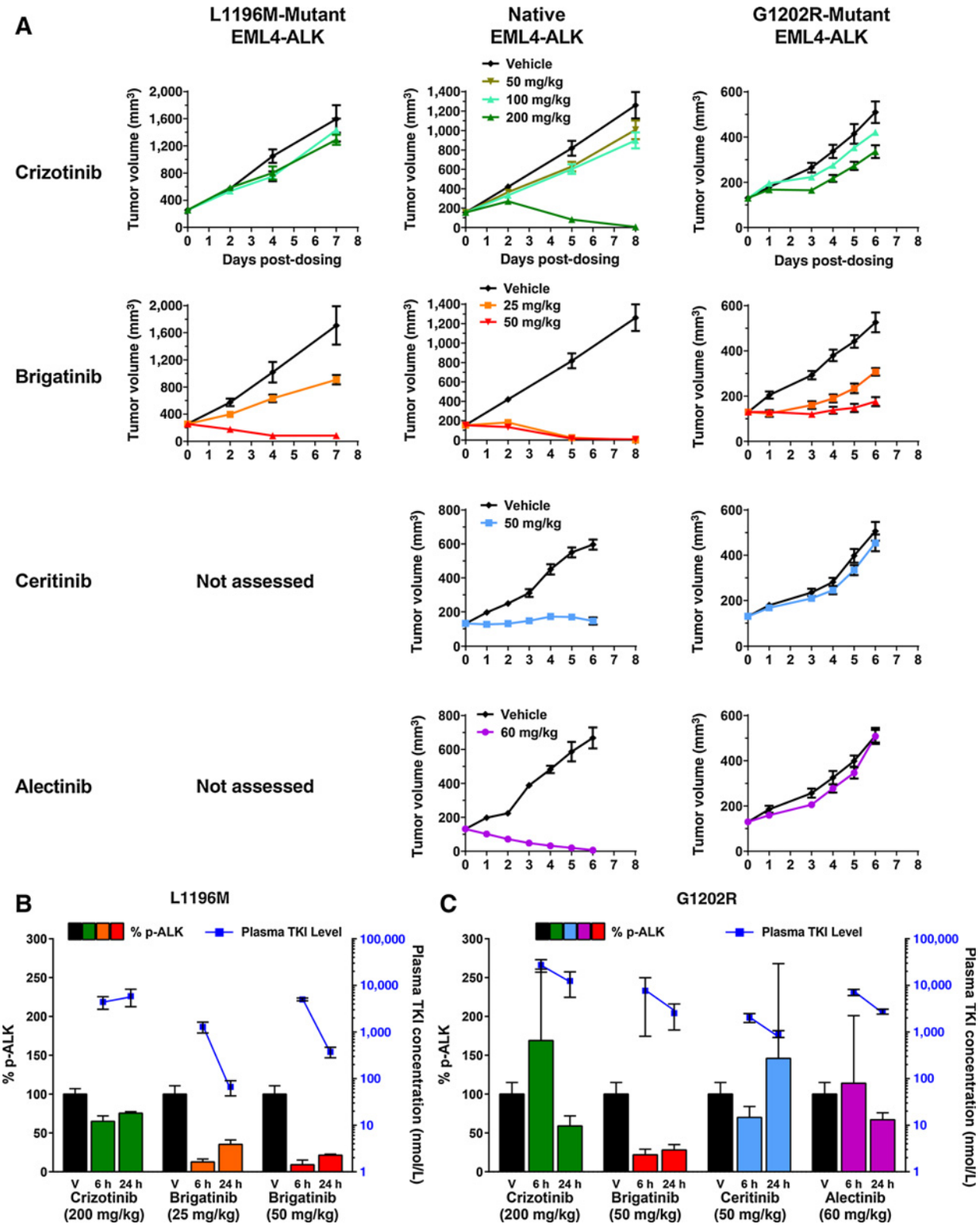


Figure 5.

Brigatinib demonstrates superior antitumor activity against L1196M- and G1202R-mutant ALK *in vivo*. **A,** CB-17/SCID or SCID beige mice bearing Ba/F3 cells expressing L1196M-mutant, native, or G1202R-mutant EML4-ALK were administered TKIs orally, once daily, at the indicated dose for the number of days shown; mean tumor volume (±SE) is shown for each group. Evaluation of levels of TKI in mouse plasma and TKI-mediated inhibition of ALK phosphorylation (p-ALK) in L1196M- (**B**) or G1202R- (**C**) mutant EML4-ALK Ba/F3 tumors *in vivo*, following a single oral dose of the TKI. p-ALK levels, evaluated using ELISA, are shown as the percentage relative to vehicle-treated animals ± SD.

the C2 aniline and an isopropyl sulfone functionality on the C4 aniline, both of which create unique susceptibilities for ceritinib (Fig. 1E). For example, while the smaller methoxy substituent of brigatinib remains largely compatible with mutation of the hinge residue L1198 to a more bulky phenylalanine, the isopropoxy substituent of ceritinib does not. In addition, the isopropyl sulfone functionality on the C4 aniline of ceritinib promotes a P-loop conformation stabilized via a hydrophobic interaction between L1152 and F1127, making ceritinib, but not brigatinib, highly susceptible to mutations that disrupt this interaction.

ALK mutations I1171N and V1180L significantly attenuate alectinib potency but have more modest (I1171N) or no (V1180L) effects on brigatinib potency. Comparison of co-crystal structures reveals that alectinib binds more deeply into the ALK-binding pocket, in part, through I1171 and V1180 interactions with a pendant nitrile functionality, whereas brigatinib interacts minimally with these residues (Fig. 1F). Finally, the G1202R mutation has a more pronounced effect on alectinib, versus brigatinib, binding, which is principally due to their different chemical scaffolds. While this mutation sterically clashes with the extended solublization groups present on both inhibitors, alectinib, with its rigid tetracyclic core and reduced rotational flexibility, experiences greater potency loss.

Discussion

Targeted therapies have reshaped the treatment of oncogene-driven malignancies, including chronic myeloid leukemia (CML), gastrointestinal stromal tumors, and most recently ALK^+ NSCLC. In most cases, including imatinib for CML and crizotinib for ALK^+ NSCLC, the first TKI approved for these indications had not been optimized to inhibit the oncogenic driver. Suboptimal target inhibition likely explains the high susceptibility to both target-based resistance mechanisms and pharmacologic failure, as next-generation TKIs have been able to overcome these limitations: for example, nilotinib, dasatinib, and ponatinib achieve high response rates in patients with CML who progressed on earlier lines of therapy, with or without BCR-ABL resistance mutations (21–23). This TKI evolution is now being recapitulated for ALK^+ NSCLC, with the development of ceritinib and alectinib. An ideal TKI for a given target would act as a pan-inhibitor, inhibiting all potential secondary resistance mutations, at clinically achievable levels (e.g., ponatinib for BCR-ABL; ref. 24), and would also have a high degree of selectivity for the target, allowing it to have a favorable safety profile at high levels of exposure necessary to limit pharmacologic failure.

Here we describe the development and preclinical characterization of brigatinib, a potent ALK/ROS1 inhibitor designed to overcome the limitations of crizotinib. Across a panel of eight ALK^+ tumor-derived and engineered cell lines, brigatinib (median IC_{50}, 10 nmol/L) inhibits ALK with 12-fold greater potency than crizotinib. This superior potency is also observed *in vivo* in multiple xenograft models, including in an orthotopic mouse brain tumor model in which brigatinib significantly enhanced survival compared to crizotinib. This could potentially reflect enhanced CNS penetration by brigatinib or the greater potency of brigatinib over crizotinib making it less susceptible to pharmacologic failure in mice.

When evaluating the *in vitro* potency of TKIs and the potential therapeutic implications, it is critical to consider these data in the context of TKI levels achieved in patients at the recommended dose (25). Possibly due to its high selectivity for ALK (and ROS1), two kinases with limited expression in adults (26), brigatinib can achieve levels of exposure in patients that substantially exceed those required to inhibit native ALK. Total steady-state plasma levels (C_{ave}) of brigatinib in patients dosed at 90 (582 nmol/L) and 180 mg (1,447 nmol/L), the two regimens being studied in a pivotal phase 2 trial, exceed the IC_{90} for native ALK inhibition by 15- to 38-fold (relating *in vitro* IC_{90} values, vs. IC_{50} values, to total plasma TKI levels was a better predictor of efficacy). In contrast, the crizotinib C_{ave} only exceeds the native ALK IC_{90} by twofold, suggesting that brigatinib may be much less susceptible to pharmacologic failure than crizotinib in patients with ALK-driven cancer.

Perhaps most notably, brigatinib is less susceptible to secondary resistance mutations in ALK than ceritinib and alectinib, as well as crizotinib. In an *in vitro* mutagenesis screen in which all four TKIs were compared side-by-side, only brigatinib was able to suppress emergence of any ALK secondary mutant at 500 nmol/L. Using a panel of cell lines containing 17 different secondary ALK mutations, the inhibitory profile of brigatinib was found to be superior to that of all three other TKIs. For example, when comparing absolute IC_{50} (and IC_{90}) values, brigatinib had similar or substantially greater potency than all three TKIs against all 17 mutants (except L1198F compared with crizotinib). Notably, brigatinib inhibited nine different mutants with three- to 54-fold greater potency than ceritinib and/or alectinib. Moreover, the C_{ave} achieved in patients dosed with 180-mg brigatinib in the phase 1/2 clinical trial exceeds the IC_{90} for all 17 mutants (levels at the 90-mg dose exceed all mutants except G1202R). In contrast, for ceritinib, IC_{90} values for two mutants (L1152R and L1198F) exceeded the C_{ave} observed in patients, whereas for alectinib, IC_{90} values for three mutants (I1171N, V1180L, and G1202R) exceeded the C_{ave}.

Importantly, our preclinical studies predict that the higher potency of brigatinib may be even greater in the clinical setting, as the percentage of brigatinib not bound to human plasma proteins *in vitro* is four- to 100-fold greater than that of crizotinib, ceritinib, and alectinib. Consistent with this, performing cellular assays in the presence of physiologic levels of human plasma proteins leads to a smaller reduction in brigatinib potency (2.0-fold) compared with crizotinib, ceritinib, and alectinib (2.7- to 4.0-fold; ref. 27). In addition, in an *in vivo* assay (in which effects of protein binding are operative), ceritinib and alectinib had no effect on growth of a G1202R-mutant tumor, whereas brigatinib substantially inhibited tumor growth.

Thus, on the basis of a variety of analyses, brigatinib is predicted to have the broadest spectrum of activity against ALK mutants, including activity against secondary mutants predicted to confer a high degree of resistance to ceritinib and/or alectinib, including L1152R, L1198F, I1171N, V1180L, and G1202R. Thus, while we, and others (28, 29), have identified mutants that brigatinib inhibits less potently than native ALK, we predict that when levels of exposure achieved in patients are taken into account, brigatinib has the potential to have pan-ALK inhibitory activity such that no single ALK mutant will be associated with a high degree of resistance, especially at the higher 180-mg dose level.

Comparison of the brigatinib–ALK costructure to that of crizotinib, ceritinib, and alectinib reveals the molecular interactions that drive the superior potency of brigatinib and its reduced susceptibility to secondary mutations. In particular, major unique mutational liabilities of ceritinib (L1152R/P and L1198F) and

alectinib (I1171N and V1180L), are shown to result from their differing molecular structures and interactions with ALK compared with brigatinib. Notably, the dimethylphosphine oxide moiety present on the C4 aniline of brigatinib is critical for balancing ALK potency and selectivity over the highly homologous IGF-1R and INSR kinases (30).

In cellular assays, brigatinib also inhibits the activity of FLT3-ITD, and variants with certain clinically observed resistance mutations (D835Y and F691L), with potencies similar to those against the most recalcitrant ALK mutants. Brigatinib also has substantial activity against one of the two most common EGFR-activating mutants (exon 19 deletion; IC_{90}, 314 nmol/L) but lower potency against EGFR L858R (IC_{90}, 1.2 μmol/L) and against T790M-containing mutants (IC_{90} >2 μmol/L). Finally, brigatinib inhibits ROS1 with potency similar to that of ALK; therefore, a high degree of ROS1 inhibition and low susceptibility to pharmacologic failure in patients is predicted. Brigatinib was shown to maintain substantial activity against ROS1 with a secondary mutation at the gatekeeper residue (L2026M), although not against the G2032R mutant that has been associated with clinical resistance to crizotinib. A phase 2 trial to evaluate the activity of brigatinib in patients with NSCLC with ROS1 rearrangements is planned.

The superior potency of brigatinib versus crizotinib observed in our preclinical studies supports the ongoing testing of brigatinib in patients with ALK^+ patients who have progressed on crizotinib and suggests several clinically testable hypotheses. Indeed preliminary results from a phase 1/2 trial have already shown high objective response rates in this setting, including in the CNS (31). Our preclinical studies also predict brigatinib may have activity against most or all mutants that confer resistance not only to crizotinib, but also to ceritinib and alectinib; a clinical trial to confirm the preclinical findings of the ability of brigatinib to overcome ceritinib and alectinib resistance is planned. These data also suggest that higher doses (such as 180 mg once daily) should help overcome certain mutants and may give better CNS penetration. Finally, our data support testing brigatinib as initial TKI therapy, to see whether the substantially greater *in vitro* potency of brigatinib also manifests as deeper and more durable responses than those seen with crizotinib and whether emergence of ALK resistance mutations can be delayed or even circumvented. A phase 3 trial to compare brigatinib and crizotinib in patients with ALK^+ NSCLC who have not received prior ALK inhibitors is ongoing (NCT02737501).

Disclosure of Potential Conflicts of Interest

S. Zhang, S. Nadworny, T. Zhou, Y. Ning, S. Wardwell, L. Eichinger, W.S. Huang, S. Liu, E. Ye, N. Narasimhan, X. Zhu, T. Clackson, D. Dalgarno, W.C. Shakespeare, and V. Rivera have ownership interest in ARIAD Pharmaceuticals. No other potential conflicts of interest were disclosed.

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Other (proposed/designed brigatinib as a potent ALK inhibitor): W.-S. Huang

Other (pioneered and validated this chemistry project targeting TK and discovered all three lead compounds for ALK includin gbrigatinib): S. Liu

Other (bioanalytical method development and analysis and pharmacokinetics interpretation): N.T. Narasimhan

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References

1. Grande E, Bolós M-V, Arriola E. Targeting oncogenic ALK: a promising strategy for cancer treatment. Mol Cancer Ther 2011;10:569–79.
2. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med 2013;368:2385–94.
3. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. N Engl J Med 2014;371:2167–77.
4. Katayama R, Lovly CM, Shaw AT. Therapeutic targeting of anaplastic lymphoma kinase in lung cancer: a paradigm for precision cancer medicine. Clin Cancer Res 2015;21:2227–35.
5. Toyokawa G, Seto T. Updated evidence on the mechanisms of resistance to ALK inhibitors and strategies to overcome such resistance: clinical and preclinical data. Oncol Res Treat 2015;38:291–8.
6. Weickhardt AJ, Scheier B, Burke JM, Gan G, Lu X, Bunn PAJr, et al. Local ablative therapy of oligoprogressive disease prolongs disease control by tyrosine kinase inhibitors in oncogene-addicted non-small-cell lung cancer. J Thorac Oncol 2012;7:1807–14.
7. Costa DB, Shaw AT, Ou SH, Solomon BJ, Riely GJ, Ahn MJ, et al. Clinical experience with crizotinib in patients with advanced ALK-rearranged non-small-cell lung cancer and brain metastases. J Clin Oncol 2015; 33:1881–8.
8. Friboulet L, Li N, Katayama R, Lee CC, Gainor JF, Crystal AS, et al. The ALK inhibitor ceritinib overcomes crizotinib resistance in non-small cell lung cancer. Cancer Discov 2014;4:662–73.
9. Sakamoto H, Tsukaguchi T, Hiroshima S, Kodama T, Kobayashi T, Fukami TA, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. Cancer Cell 2011;19:679–90.
10. Gadgeel SM, Gandhi L, Riely GJ, Chiappori AA, West HL, Azada MC, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. Lancet Oncol 2014;15:1119–28.
11. Shaw AT, Kim DW, Mehra R, Tan DS, Felip E, Chow LQ, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. N Engl J Med 2014; 370:1189–97.

12. Ou SI, Ahn JS, De Petris L, Govindan R, Yang JC, Hughes B, et al. Alectinib in crizotinib-refractory ALK-rearranged non-small-cell lung cancer: a phase II global study. J Clin Oncol 2016;34:661–8.
13. Ou SH, Greenbowe J, Khan ZU, Azada MC, Ross JS, Stevens PJ, et al. I1171 missense mutation (particularly I1171N) is a common resistance mutation in ALK-positive NSCLC patients who have progressive disease while on alectinib and is sensitive to ceritinib. Lung Cancer 2015;88:231–4.
14. Ou SH, Klempner SJ, Greenbowe JR, Azada M, Schrock AB, Ali SM, et al. Identification of a novel HIP1-ALK fusion variant in Non-Small-Cell Lung Cancer (NSCLC) and discovery of ALK I1171 (I1171N/S) mutations in two ALK-rearranged NSCLC patients with resistance to Alectinib. J Thorac Oncol 2014;9:1821–5.
15. Ou SH, Milliken JC, Azada MC, Miller VA, Ali SM, Klempner SJ. ALK F1174V mutation confers sensitivity while ALK I1171 mutation confers resistance to alectinib. The importance of serial biopsy post progression. Lung Cancer 2016;91:70–2.
16. Iwama E, Okamoto I, Harada T, Takayama K, Nakanishi Y. Development of anaplastic lymphoma kinase (ALK) inhibitors and molecular diagnosis in ALK rearrangement-positive lung cancer. Onco Targets Ther 2014;7:375–85.
17. Hatcher JM, Bahcall M, Choi HG, Gao Y, Sim T, George R, et al. Discovery of inhibitors that overcome the G1202R anaplastic lymphoma kinase resistance mutation. J Med Chem 2015;58:9296–308.
18. Katayama R, Kobayashi Y, Friboulet L, Lockerman EL, Koike S, Shaw AT, et al. Cabozantinib overcomes crizotinib resistance in ROS1 fusion-positive cancer. Clin Cancer Res 2015;21:166–74.
19. Shaw AT, Friboulet L, Leshchiner I, Gainor JF, Bergqvist S, Brooun A, et al. Resensitization to crizotinib by the lorlatinib ALK resistance mutation L1198F. N Engl J Med 2016;374:54–61.
20. Katayama R, Friboulet L, Koike S, Lockerman EL, Khan TM, Gainor JF, et al. Two novel ALK mutations mediate acquired resistance to the next-generation ALK inhibitor alectinib. Clin Cancer Res 2014;20:5686–96.
21. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–41.
22. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006;354:2542–51.
23. Cortes JE, Kim DW, Pinilla-Ibarz J, le Coutre P, Paquette R, Chuah C, et al. A phase 2 trial of ponatinib in Philadelphia chromosome-positive leukemias. N Engl J Med 2013;369:1783–96.
24. Deininger MW, Hodgson JG, Shah NP, Cortes JE, Kim DW, Nicolini FE, et al. Compound mutations in BCR-ABL1 are not major drivers of primary or secondary resistance to ponatinib in CP-CML patients. Blood 2016;127:703–12.
25. Smith MA, Houghton P. A proposal regarding reporting of in vitro testing results. Clin Cancer Res 2013;19:2828–33.
26. Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, et al. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. Oncogene 1997;14:439–49.
27. Zhang S, Nadworny S, Wardwell SD, Eichinger L, Das B, Emily YY, et al. The potent ALK inhibitor AP26113 can overcome mechanisms of resistance to first-and second-generation ALK TKIs in preclinical models. Cancer Res 2015;75:781.
28. Fontana D, Ceccon M, Gambacorti-Passerini C, Mologni L. Activity of second-generation ALK inhibitors against crizotinib-resistant mutants in an NPM-ALK model compared to EML4-ALK. Cancer Med 2015;4:953–65.
29. Ceccon M, Mologni L, Giudici G, Piazza R, Pirola A, Fontana D, et al. Treatment efficacy and resistance mechanisms using the second-generation ALK inhibitor AP26113 in human NPM-ALK-positive anaplastic large cell lymphoma. Mol Cancer Res 2015;13:775–83.
30. Huang WS, Liu S, Zou D, Thomas M, Wang Y, Zhou T, et al. Discovery of brigatinib (AP26113), a phosphine oxide-containing, potent, orally active inhibitor of anaplastic lymphoma kinase. J Med Chem 2016;59:4948–64.
31. Gettinger SN, Bazhenova L, Salgia R, Langer C, Gold K, Rosell R, et al. Brigatinib (AP26113) efficacy and safety in ALK+ NSCLC: phase 1/2 trial results. J Thorac Oncol;10:S238.
32. Gehrke BJ. Clinical pharmacology review. Xalkori, crizotinib. Center for Drug Evaluation and Research: U.S. Food and Drug Administration; 2011. Report No.: 202570.
33. Brigatinib (data on file). ARIAD Pharmaceuticals, Inc.
34. Ou S, Gadgeel S, Chiappori A, Riely G, Lee R, Garcia L, et al. Safety and efficacy analysis of RO5424802/CH5424802 in anaplastic lymphoma kinase (ALK)-positive non-small cell lung cancer (NSCLC) patients who have failed crizotinib in a dose-finding phase I study (AF-002JG, NCT01588028). Eur J Cancer 2013;49:S19.
35. Brower M, Fox E. Clinical pharmacology review. Zykadia, ceritinib. Center for Drug Evaluation and Research: U.S. Food and Drug Administration; 2014. Report No.: 205755.
36. Zahalka E, Ringgold K. Clinical pharmacology review. Alecensa, alectinib. Center for Drug Evaluation and Research: U.S. Food and Drug Administration; 2015. Report No.: 208434.