ABSTRACT

Non–small cell lung cancers (NSCLC) harboring anaplastic lymphoma kinase (ALK) gene rearrangements invariably develop resistance to the ALK tyrosine kinase inhibitor (TKI) crizotinib. Herein, we report the first preclinical evaluation of the next-generation ALK TKI, ceritinib (LDK378), in the setting of crizotinib resistance. An interrogation of in vitro and in vivo models of acquired resistance to crizotinib, including cell lines established from biopsies of patients with crizotinib-resistant NSCLC, revealed that ceritinib potently overcomes crizotinib-resistant mutations. In particular, ceritinib effectively inhibits ALK harboring L1196M, G1269A, I1171T, and S1206Y mutations, and a cocrystal structure of ceritinib bound to ALK provides structural bases for this increased potency. However, we observed that ceritinib did not overcome two crizotinib-resistant ALK mutations, G1202R and F1174C, and one of these mutations was identified in 5 of 11 biopsies from patients with acquired resistance to ceritinib. Altogether, our results demonstrate that ceritinib can overcome crizotinib resistance, consistent with clinical data showing marked efficacy of ceritinib in patients with crizotinib-resistant disease.

SIGNIFICANCE: The second-generation ALK inhibitor ceritinib can overcome several crizotinib-resistant mutations and is potent against several in vitro and in vivo laboratory models of acquired resistance to crizotinib. These findings provide the molecular basis for the marked clinical activity of ceritinib in patients with ALK-positive NSCLC with crizotinib-resistant disease. Cancer Discov; 4(6); 662–73. © 2014 AACR.

See related commentary by Ramalingam and Khuri, p. 634.
INTRODUCTION

Chromosomal rearrangements of anaplastic lymphoma kinase (ALK) are detected in 3% to 7% of non–small cell lung cancers (NSCLC; refs. 1, 2). These rearrangements result in constitutively active ALK fusion proteins with potent transforming activity (2, 3). Lung cancers with ALK rearrangements are highly sensitive to ALK tyrosine kinase inhibition, underscoring the notion that such cancers are addicted to ALK kinase activity. On the basis of early-phase studies, the multitargeted tyrosine kinase inhibitor (TKI) crizotinib was approved by the FDA in 2011 to treat patients with advanced NSCLC harboring ALK rearrangements (1). However, despite a high response rate of 60% in ALK-rearranged NSCLC, most patients develop resistance to crizotinib, typically within 1 to 2 years.

Studies of ALK-rearranged lung cancers with acquired resistance to crizotinib have identified ALK fusion gene amplification and secondary ALK tyrosine kinase (TK) domain mutations in about one third of cases (4–6). To date, seven different acquired resistance mutations have been identified among crizotinib-resistant patients. The most frequently identified secondary mutations are L1196M and G1269A. In addition to these mutations, the 1151T-ins, L1152R, C1156Y, G1202R, and S1206Y mutations have also been detected in crizotinib-resistant cancers (4, 6–10). In approximately one third of crizotinib-resistant tumors, there is evidence of activation of bypass signaling tracts such as EGFR or c-KIT (6, 9). In the remaining one third of crizotinib-resistant tumors, the resistance mechanisms remain to be identified.

Next-generation ALK inhibitors with improved potency and selectivity compared with crizotinib have been developed to overcome crizotinib resistance in the clinic. We previously evaluated the ability of several ALK TKIs (TAE684, AP26113, ASP3026, and CH5424802) to inhibit ALK activity in models harboring different ALK secondary mutations (6, 11). These studies revealed variable sensitivity to these ALK inhibitors depending on the specific resistance mutation present. For example, the gatekeeper L1196M mutation was sensitive to TAE684, AP26113, and ASP3026, whereas 1151T-ins conferred resistance to all next-generation ALK TKIs. Ceritinib is an ATP-competitive, potent, and selective next-generation ALK inhibitor (12). The kinase selectivity has been tested in a cellular proliferation assay against 16 different kinases, and aside from ALK, no inhibition below 100 nmol/L was observed (12). In the phase I study of ceritinib in ALK-positive NSCLC, marked antitumor activity has been observed in both crizotinib-relapsed and crizotinib-naïve patients (13, 14). On the basis of this impressive clinical activity, ceritinib received FDA approval on April 29, 2014.

Herein, we present the first report examining the activity of ceritinib in preclinical models of ALK-positive lung cancer with acquired resistance to crizotinib, as well as an early biologic insight into mechanisms of resistance to ceritinib arising in patients.

RESULTS

Ceritinib Exhibits Potent Activity in Crizotinib-Naïve ALK-Positive NSCLC Models

In vitro enzymatic studies revealed that ceritinib was approximately 20-fold more potent against ALK than crizotinib (Table 1). Similarly, ceritinib was more potent than crizotinib against two ALK-rearranged lung cancer cell lines, H3122 and H2228 (Fig. 1A and B, Table 1). Accordingly, ceritinib led to suppression of ALK phosphorylation as well as the downstream PI3K–AKT, MEK–ERK, and mTOR signaling pathways at lower doses than crizotinib (Fig. 1C and D).

To further assess the cellular specificity of ceritinib, we determined the GI50 (concentration needed to reduce the growth of treated cells to half that of untreated cells) of ceritinib against two lung cancer cell lines with ALK rearrangements, it was not potent against NSCLC or breast cancer cell lines driven by KRAS, EGFR, PI3K, or HER2, with GI50 >1 μmol/L (Supplementary Fig. S1A).

We next compared the efficacy of ceritinib and crizotinib in vivo using treatment-naïve H2228 xenograft models (Fig. 1E). Tumor-bearing animals were treated with either high-dose crizotinib (100 mg/kg) or ceritinib (25 mg/kg or 50 mg/kg) once daily for 14 days. Both crizotinib (100 mg/kg) and ceritinib (25 and 50 mg/kg) were well tolerated in this study (Supplementary Fig. S1B). As expected, marked tumor regression was observed in all groups during the treatment. After treatment was stopped, the animals were monitored for tumor progression. Although recurrent tumors were detected within 11 days of drug withdrawal in mice treated with crizotinib, mice treated with ceritinib at 50 mg/kg remained in complete remission with no discernible tumor growth for 4 months. In the mice treated with ceritinib at 25 mg/kg, tumor regrowth was observed in 4 of 8 animals after 1 month, whereas complete remission was maintained in the other 4 animals for 4 months. Thus, ceritinib had more durable antitumor activity than crizotinib, even after the drugs were discontinued. It is also worth noting that the exposure of crizotinib at 100 mg/kg is approximately 3-fold to 5-fold greater than the exposures achieved at the human maximum tolerated dose (MTD; 250 mg, twice a day; ref. 15) and that ceritinib at 25 to 50 mg/kg is predicted to be achievable at

Table 1. Ceritinib is a potent ALK inhibitor

<table>
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<th>AKT enzymatic assay</th>
<th>Crizotinib</th>
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<tr>
<td>H2228</td>
<td>107</td>
<td>3.8</td>
<td>28</td>
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<td>H3122</td>
<td>245</td>
<td>6.3</td>
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NOTE: GI50 values for in vitro ALK enzymatic assay or H3122 and H2228 cell survival assay for crizotinib and ceritinib are shown.
We also evaluated the efficacy of ceritinib in a primary explant model derived from a crizotinib-naïve NSCLC tumor MGH006 (6). Treatment of these mice with 25 mg/kg ceritinib also led to tumor regressions (Supplementary Fig. S1C). Altogether, these data demonstrate that ceritinib is potent against crizotinib-naïve ALK-rearranged cell lines and tumor models in vivo and in vitro.

Figure 1. Ceritinib is a potent ALK inhibitor in crizotinib-naïve models. A and B, cell survival assay of H3122 (A) and H2228 (B) cells treated with the indicated doses of crizotinib or ceritinib for 72 hours. Cell survival was assayed by CellTiter-Glo. C and D, H3122 (C) and H2228 (D) cells were treated with the indicated concentrations of crizotinib or ceritinib for 6 hours. Lysates were probed with antibodies directed against the specified proteins. E, SCID beige bearing H2228 cells were administered crizotinib or ceritinib orally once daily for 14 days. The arrow indicates when treatments were stopped, and tumor growth was monitored in animals up to 4 months. Tumor volumes, mean ± SD (n = 8). p, phosphorylated.

Ceritinib Is Active against Patient-Derived Cell Lines from Crizotinib-Resistant Cancers with and without Resistant Mutations

To investigate the activity of ceritinib against crizotinib-resistant mutations, we used crizotinib-resistant cell line models harboring the two most common EML4-ALK...
mutations, L1196M and G1269A. We have previously described the H3122 CR1 crizotinib-resistant cell line, which developed resistance in vitro by chronic exposure to crizotinib. This cell line harbors both the L1196M EML4–ALK gatekeeper mutation and amplification of the EML4–ALK allele (11). In addition, we also examined two novel cell lines established from biopsies of patients whose ALK-rearranged lung cancers had become resistant to crizotinib in the clinic. These two patient-derived resistant lines, MGH045 and MGH021-4, harbor the L1196M and G1269A mutations, respectively. The MGH021-4 line is a clonal cell line established from MGH021, a tumor harboring both 1151T-ins and G1269A mutations; MGH021-4 cells harbor only the G1269A mutation (5). This clone, therefore, represents an early generation of the patient-derived cell line. The GI50 values of ceritinib against all of these resistant cell lines were decreased 6-fold to 36-fold compared with crizotinib (Fig. 2A and Supplementary Fig. S2A–S2C). Accordingly, phosphorylation of ALK and downstream ERK and AKT were more effectively suppressed by lower doses of ceritinib compared with crizotinib (Fig. 2B–D).

To further assess the activity of ceritinib against crizotinib-resistant ALK-positive tumors in vivo, we examined the efficacy of ceritinib against xenografts derived from MGH045 cells that harbor the L1196M resistance mutation. As shown in Fig. 2E, treatment of MGH045 tumor-bearing mice with
low-dose ceritinib (25 mg/kg) was more effective than with high-dose crizotinib in controlling tumor growth. These data demonstrate that ceritinib is active against cancers derived from patients with acquired resistance to crizotinib and is more potent than crizotinib against ALK-rearranged cancers harboring the L1196M and G1269A resistance mutations.

The ongoing clinical trial of ceritinib demonstrates that crizotinib-resistant ALK-positive tumors, including tumors without ALK mutation or gene amplification, are responsive to ceritinib treatment (13). This raises the possibility that many of these resistant tumors may develop because of inadequate target suppression. We investigated the efficacy of crizotinib and ceritinib against a ceritinib-resistant ALK-positive cell line without ALK resistance mutations, MGH051. As shown in Fig. 3A, this cell line was derived from a biopsy of a liver lesion that developed in a patient on crizotinib. Assessment of the biopsy sample revealed no ALK mutations or gene amplification. The cell line derived from the biopsy also did not harbor any ALK resistance mutations. This resistant cell line was highly sensitive to ceritinib in vitro, and, surprisingly, the MGH051 cell line was also sensitive to crizotinib (Fig. 3B). Accordingly, phosphorylation of ALK and downstream AKT and ERK was efficiently suppressed by ceritinib and ceritinib (Fig. 3C). These data suggest that cancers with acquired resistance to crizotinib without ALK-resistant mutations may remain sensitive to ALK inhibition (please see “Discussion”).

Assessment of Ceritinib Activity against a Panel of ALK Mutations

To systematically assess the potency of ceritinib against ALK resistance mutations, we used Ba/F3 cells engineered
to express wild-type EML4–ALK or one of the nine different resistance mutations. In this system, ceritinib was approximately 10-fold more potent against wild-type EML4–ALK than crizotinib. Whereas all these secondary mutations induced crizotinib resistance, ceritinib was potent in inhibiting the growth of Ba/F3 cells expressing four of the resistance mutations, including L1196M, G1269A, S1206Y, and I1171T (Fig. 4A; Supplementary Fig. S3; Supplementary Table S1). However, C1156Y, G1202R, 1151T-ins, L1152R, and F1174C mutations also conferred resistance to ceritinib, although ceritinib was still more potent than crizotinib against these mutations. Thus, the most common crizotinib-resistant mutations were substantially more sensitive to ceritinib than crizotinib, whereas less common resistance mutations conferred resistance to both crizotinib and ceritinib.

**Structural Basis for Increased Potency of Ceritinib against ALK Crizotinib-Resistant Mutations**

To glean insights into the structural basis for the ability of ceritinib to maintain activity toward select crizotinib-resistant mutants, the structure of the ALK catalytic domain complexed with ceritinib was determined (Fig. 4B; PDB 4MKC) and compared with the structure of the ALK catalytic domain bound to crizotinib (Fig. 4C; PDB 2X2P; ref. 16). As shown in Fig. 4A, ceritinib retains potency toward the most common G1269A and L1196M crizotinib-resistant mutants. The co-crystal structure reveals that G1269 is situated just proximal to D1270 of the activation loop DFG-motif. Although mutation to Ala in the G1269 mutant would not be predicted to present any steric obstruction to ceritinib binding, it would be predicted to introduce a steric clash to ceritinib binding due to the proximity of the phenyl ring of ceritinib. The Cl moiety of the pyrimidine hinge-binding core of ceritinib is juxtaposed with the L1196 side chain and participates in a hydrophobic interaction with the Leu side chain. In the L1196M mutant, the Cl moiety of ceritinib can interact with Met, which may compensate for the loss of interaction between CI and the Leu side chain in wild-type ALK. In contrast, introduction of a Met at the gatekeeper position 1196 likely adversely affects ceritinib binding through both steric interference and unfavorable interactions with the 2-amino substituent of the pyridinyl hinge-binding core and the methyl substituent of the alkoxy moiety of ceritinib. These structural findings are in agreement with the increased potency of ceritinib versus crizotinib against these resistance mutations.

In contrast with G1269A and L1196M mutations, ceritinib is not potent against the G1202R crizotinib-resistant mutation (Fig. 4A). The crystal structure reveals that mutation of G1202 to a larger, bulky, and charged side chain would be incompatible with ceritinib or crizotinib ALK binding due to steric hindrance (6). This steric obstruction leads to a loss in potency as reflected by the shift in IC₅₀ values observed for ceritinib and crizotinib. In contrast with the G1202R mutation, the T1151 insertion, L1152P, C1156Y, and F1174C inhibitor-resistant mutants all map to the N-terminal lobe of the ALK catalytic domain and flank opposing ends of the αC-helix. The locations of these mutants do not directly contribute to inhibitor binding in co-crystal structures. Interestingly, positions T1151 and F1174 in ALK have been previously identified as sites of activating gain-of-function mutations in neuroblastoma (17). Although difficult to predict without structural and biochemical analyses of these mutants, T1151 is adjacent to the catalytically important K1150, and insertion at this position, along with the F1174C, L1152P, and C1156Y inhibitor-resistant mutants, likely influences αC-helix mobility and conformational dynamics of the catalytic domain. Previously reported structures of nonphosphorylated ALK in the apo, ADP, and inhibitor-bound forms suggest the ALK catalytic domain structure possesses a “DFG-in” conformation (18) with a unique activation loop conformation. It is conceivable that these mutations destabilize the ALK conformation and shift the conformational equilibrium toward those that are no longer able to bind the inhibitor. It is also possible that these mutations could decrease the Kᵦ for ATP, rendering ceritinib/crizotinib a less effective ATP competitive inhibitor.

**Ceritinib-Resistant Tumors Harboring EML4–ALK Wild-Type, I1171T, or C1156Y Mutations Are Sensitive to Ceritinib In Vivo**

To evaluate the activity of ceritinib against crizotinib-resistant tumors in vivo, crizotinib-resistant H2228 xenograft tumors were generated by treatment with escalating doses of crizotinib (from 50 to 100 mg/kg). Tumors that progressed during treatment with 100 mg/kg crizotinib were analyzed for resistance mechanisms. Typical tumor responses and resistance are shown for 3 animals in Supplementary Fig. S4, and are representative of the 80 animals used in this study. To determine mechanisms of resistance to crizotinib, we sequenced the ALK kinase domain of all 80 tumors and identified three distinct resistance mutations in six tumors. The G1202R, C1156Y, and I1171T mutations were detected in three, two, and one resistant tumors, respectively. Of these three mutations, G1202R and C1156Y have been previously reported in patients with NSCLC who relapsed on crizotinib (6, 7). Interestingly, I1171T has not yet been reported from crizotinib-resistant patients but was identified in an in vitro mutagenesis screen for resistance mutations (19).

The efficacy of ceritinib was tested against these crizotinib-resistant H2228 xenograft tumor models as well as one of the resistance models that did not harbor a resistance mutation nor ALK amplification (data not shown). Although each was resistant to crizotinib at 100 mg/kg, ceritinib suppressed tumor growth in multiple resistance models (Fig. 5A–D). In the wild-type and I1171T resistant models, ceritinib demonstrated impressive antitumor activity, whereas it was less active in the C1156Y-resistant model and was inactive against the G1202R-resistant model. These data are consistent with the Ba/F3 models in which ceritinib was more potent against I1171T than the C1156Y and G1202R mutants (Fig. 4A). The studies shown herein provide evidence that ceritinib can overcome resistance in vivo, especially in tumors harboring wild-type, L1196M, or I1171T ALK fusions at a dose that is predicted to be achievable in humans. Of note, it is rather interesting that ceritinib overcame crizotinib resistance in the
**Figure 4.** Ba/F3 models of ALK-crizotinib-resistant mutations. **A,** IC\textsubscript{50} values of ceritinib across different Ba/F3 cell lines expressing wild-type or mutated ALK TK and including parental, IL3-dependent Ba/F3 cells are shown. **B** and **C,** ALK-resistant mutations mapped onto ALK/ceritinib (PDB 4MKC, **B**) and ALK/crizotinib (PDB 2XP2, **C**) cocrystal structures. \( \beta \)-Strand secondary structural elements of the N-terminal lobe and the \( \alpha \)C-helix of the N-terminal lobe are shown in orange and purple, respectively. Helical structural elements of the C-terminal lobe are shown in blue. Residues involved in resistant mutations are depicted as green spheres. Inhibitor molecules are depicted as stick representations with carbons colored yellow and cyan for crizotinib and ceritinib, respectively. Nitrogen is colored dark blue, oxygen is colored red, and chlorine green for both inhibitors. Fluoride is colored white (crizotinib) and sulfur atoms are colored yellow (ceritinib). Transparent surfaces for the inhibitors are displayed. Zoomed-in view boxes for G1269 and L1196 residues are shown. Figures were rendered with MacPymol (The PyMOL Molecular Graphics System, Version 1.4 Schrödinger, LLC).
tumor that did not harbor an ALK resistance mutation, as this recapitulates observations in the clinic and with the patient-derived cell line shown in Fig. 3 (please see “Discussion”).

**Acquired Resistance to Ceritinib in Patients**

Ceritinib has demonstrated impressive activity in the clinic in crizotinib-resistant patients (13). However, similar to other targeted therapy successes, despite initial and durable responses, tumors do develop resistance. We have now biopsied 11 cancers with acquired resistance to ceritinib (two of which were from different sites from the same patient). As shown in Fig. 6A, five of these biopsies revealed the development of mutations at either G1202 or F1174 in the ceritinib-resistant cancers. In the patient JFCR021, who had two sites of disease biopsied, two different ceritinib-resistant mutations were identified, underscoring the heterogeneity of resistance mechanisms that can be identified in a single patient (6). Of

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**Figure 5.** EML4–ALK C1156Y, I1171T, G1202R mutations’ sensitivity to ceritinib. A to D, SCID beige mice bearing H2228 crizotinib-resistant tumors EML4–ALK wild-type (WT; A), I1171T (B), C1156Y (C), or G1202R (D) were treated with 100 mg/kg crizotinib or 50 mg/kg ceritinib once daily for 12 to 22 days. Tumor volumes, mean ± SD (n = 5–8).
note, 2 of the patients had crizotinib-resistant mutations before enrolling on ceritinib (MGH011; Fig. 6B; and JFCR021) that our laboratory studies suggested would be sensitive to ceritinib. In the ceritinib-resistant cancers, those mutations were no longer detected, but the G1202R mutation emerged (Fig. 6B). These findings are consistent with preclinical studies presented in this article demonstrating the activity of ceritinib against G1269A and S1206Y crizotinib-resistant mutations, and its lack of potency against the G1202R mutation.

**DISCUSSION**

Since its approval in the United States in 2011, the ALK inhibitor crizotinib has emerged as a standard of care for patients with advanced NSCLC harboring the ALK fusion oncogene. Unfortunately, as has been observed with other targeted therapies, the emergence of resistance has ultimately limited the benefit of this therapy. Next-generation ALK inhibitors (ceritinib, CH5424802, ASP3026, AP26113, and X-396) have been developed with the hope that they may overcome acquired resistance to crizotinib. We previously reported differential activity of some of these ALK inhibitors depending on the resistance mutations present within the ALK TK domain (6, 11). In an ongoing early-phase clinical study, ceritinib has exhibited dramatic activity in patients with ALK-rearranged NSCLC (13).

In these studies, we find that ceritinib is a more potent ALK inhibitor than crizotinib, and has marked activity in crizotinib-naïve models of ALK-positive NSCLC, including H2228, H3122, and Ba/F3 cell lines in vitro and MGH006 primary explants in vivo. To better characterize the activity of ceritinib in crizotinib resistance, we developed a variety of crizotinib-resistant models, including cell lines derived from biopsies from patients whose cancers had developed resistance to crizotinib in the clinic. These models harbored different ALK resistance mechanisms, including various ALK resistance mutations. The activity of ceritinib varied depending on the specific ALK resistance mutation. For example, in Ba/F3 models, ceritinib was highly active against L1196M, G1269A, S1206Y, and I1171T EML4–ALK mutants, and less active against the less common mutations C1156Y, G1202R, L1152P, and F1174C. It is notable that in the phase I
study of ceritinib, five of 19 crizotinib-resistant tumors harbored resistance mutations at residues 1196, 1269, and 1206, with one tumor harboring both G1269A and 1151T-ins. The patients harboring these resistance mutations all exhibited significant tumor shrinkage (13). Importantly, as has been observed in the clinic, ceritinib showed potent efficacy in vitro and in vivo against a crizotinib-resistant tumor that did not harbor an ALK resistance mutation or gene amplification (Fig. 3B). Interestingly, the patient-derived cell line also retained sensitivity to crizotinib in vitro, demonstrating that these cells are still sensitive to ALK inhibition. One potential explanation for this finding is that, in the clinic, crizotinib fails to achieve tumor levels that completely inhibit ALK, and that tumor cells can survive through modest input from activation of bypass tracks such as EGFR. However, these cells remain sensitive to complete ALK inhibition. In the setting of a more potent ALK inhibitor, ALK is inhibited fully, abrogating the functional role of bypass tracks and leading to the elimination of tumor cells. It is also possible that this patient relapsed on crizotinib because of poor adherence to therapy or due to tumor cells. It is noteworthy that in two cases, the crizotinib-resistant mutations at residues 1196, 1206, and 1269, were no longer observed in the ceritinib-resistant biopsies in which the G1202R mutations were observed (Fig. 6A). This suggests that predominant populations of resistant clones whose emergence is dependent on ceritinib are able to suppress many crizotinib-resistant mutations, but that the G1202R and F1174V/C mutants are resistant to ceritinib. It is noteworthy that in two cases, the crizotinib-resistant mutations, S1206Y and G1269A, were no longer observed in the ceritinib-resistant biopsies in which the G1202R mutations were observed (Fig. 6A). This suggests that predominant clones with the S1206Y and G1269A mutations were suppressed by ceritinib, whereas much more rare clones with G1202R mutations were selected by ceritinib. These findings give further support to the notion that there are multiple populations of resistant clones whose emergence is dependent on the selective pressure applied. Altogether, our in vitro and in vivo data, including cell line models established from crizotinib-resistant patient samples, demonstrate that the next-generation ALK inhibitor ceritinib is active against most crizotinib-resistant tumors. This is consistent with the marked clinical activity of ceritinib in patients with ALK-positive NSCLC who progressed on crizotinib. As resistance to ceritinib has already been observed in the clinic, future studies will need to identify mechanisms of resistance to ceritinib other than mutations in the G1202 and F1174 residues to maximize the clinical benefit afforded by next-generation ALK-targeted therapies.

METHODS

Cell Lines and Reagents

All human lung cancer samples were obtained from patients with informed consent at the Massachusetts General Hospital (MGH) and the Japanese foundation for Cancer Research (JFCR), and all procedures were conducted under an Institutional Review Board (IRB)-approved protocol. Cells in pleural effusion were collected by centrifugation at 440 x g for 10 minutes. After red blood cells were lysed with the Red Blood Cell Lysis Solution (BioLegend), cells were grown in ALC (Invitrogen) supplemented with 1% FBS or RPMI-1640 supplemented with 10% FBS and 1x Antibiotic-Antimycotic. After the cells started growing stably, clonal cell lines were also established.

H3122, H2228, A549, H460, H1299, HCC827, and H522 cell lines were provided by the Center for Molecular Therapeutics (CMT) at Massachusetts General Hospital (Boston, MA), which performs routine cell line authentication testing by single-nucleotide polymorphism and short-tandem repeat analysis. BT-474, SKBR3, and the ALK-positive patient-derived cell lines used in this study are from the Engelman laboratory (Boston, MA) and have been previously tested for mutation status to confirm their authenticity. A549, H460, H1299, HCC827, H522, SKBR3, H2228, H3122, H3122 CR1, and MGH021-4 cell lines were cultured in RPMI-1640 supplemented with 10% FBS. For survival assays, H2228 were cultured in 1% FBS. The MGH045 cell line was cultured in ALC supplemented with 1% FBS, and MGH051 and BT-474 were cultured in DMEM supplemented with 10% FBS.

Mouse myeloma Ba/F3 cells were cultured in DMEM supplemented with 10% FBS with (parental) or without (EML4–ALK) IL3 (0.5 ng/mL). cDNAs encoding EML4–ALK variant1 or EML4–ALK variant3 containing different point mutations were cloned into retroviral expression vectors, and virus was produced as previously described (11). After retroviral infection, Ba/F3 cells were selected in puromycin (0.5 µg/mL) for 2 weeks. IL3 was withdrawn from the culture medium for more than 2 weeks before experiments.

Ceritinib was purchased from ChemieTek, and crizotinib was provided by Novartis. Both were dissolved in DMSO for in vitro experiments. Ceritinib was formulated in 0.5% methyl cellulose/0.5% Tween 80 and crizotinib in 0.1 N HCl or 0.5% methyl cellulose/0.5% Tween 80 for in vivo studies.

Western Blot Analysis

A total of 5 x 10⁶ cells were treated in 6-well plates for 6 hours with the indicated drugs. Cell protein lysates were prepared as previously described (6, 11). Phospho-ERK (T202/Y204), ERK, S6, phospho-S6, phospho-AKT (S473 and T308), AKT, phospho-ALK (Y1282/1283), and AKT antibodies were obtained from Cell Signaling Technology. GAPDH was purchased from Millipore.

Survival Assays

Cells (2,000 or 5,000) were plated in triplicate into 96-well plates. Seventy-two hours (48 hours for Ba/F3 cells and 7 days for MGH051) after drug treatments, cells were incubated with a CellTiter-Glo assay reagent (Promega) for 15 minutes, and luminescence was measured with a Centro LB 960 Microplate Luminometer (Berthold Technologies).

In Vivo Efficacy Study of Ceritinib

SCID beige mice for crizotinib-resistant H2228 xenograft tumor models, nude mice for MGH006 primary explants and MGH045 cells were randomized into groups of 5, 6, or 8 mice with an average tumor volume of approximately 150 mm³ and received ceritinib or crizotinib daily treatments by oral gavage as indicated in each study. Tumor volumes were determined by using caliper measurements and calculated with the formula (length x width x height)/2.

In Vitro Enzymatic Assay

An enzymatic assay for the recombinant ALK kinase domain (1066–1459) was conducted using the Caliper mobility shift methodology, using fluorescently labeled peptides as kinase substrates. The
Caliper assay was performed at 30 °C for 60 minutes in a total volume of 9 μL. The reaction was terminated by the addition of 16 μL of stop solution [100 mmol/L HEPES, 5% (v/v) DMSO, 0.1% (v/v) coating reagent, 10 mmol/L EDTA, 0.015% (v/v) Brij 35]. After termination of the reactions, the plates were transferred into the Caliper LabChip 3000 workstation for analysis.

Analysis of ALK/Ceritinib and ALK/Crizotinib Costructures

The ALK/ceritinib costructure was determined by the soaking of 2 mmol/L ceritinib into apo crystals grown in 0.2 mol/L sodium acetate trihydrate/20% PEG3350 using protein expressed and purified as previously described (18). The ALK/ceritinib final model determined to 2.0 Å (PDB 4MKC on hold) was superimposed with the coordinates of the ALK/crizotinib costructure (PDB 2XP2) for analyses.

Patient Sample Analyses

The patients with ALK-positive NSCLC with acquired ceritinib resistance underwent biopsy of their resistant tumors between January 2011 and September 2013. Standard histopathology was performed to confirm the diagnosis of malignancy as previously described (6). The electronic medical record was reviewed retrospectively to obtain clinical information under an IRB-approved protocol. This study was approved by the IRB of MGH or the Cancer Institute Hospital of JFCR.

Disclosure of Potential Conflicts of Interest

M. Nishio has received honoraria from the speakers’ bureaus of Pfizer and Chugia Pharmaceutical Co., Ltd. A.T. Shaw is a consultant/advisory board member of Novartis, Pfizer, and ARIAD. J.A. Engelman has received commercial research grants from Novartis and Sanofi-Aventis, and is a consultant/advisory board member of Novartis, Sanofi-Aventis, Chugia Pharmaceutical Co., Ltd., and Ventana Medical Systems, Inc. No potential conflicts of interest were disclosed by the other authors.

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