Selective ALK inhibitor alectinib with potent antitumor activity in models of crizotinib resistance
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The clinical efficacy of the ALK inhibitor crizotinib has been demonstrated in ALK fusion-positive NSCLC; however, resistance to crizotinib certainly occurs through ALK secondary mutations in clinical use. Here we examined the efficacy of a selective ALK inhibitor alectinib/CH5424802 in models of crizotinib resistance. Alectinib led to tumor size reduction in EML4-ALK-positive xenograft tumors that failed to regress fully during the treatment with crizotinib. In addition, alectinib inhibited the growth of some EML4-ALK mutant-driven tumors, including the G1269A model. These results demonstrated that alectinib might provide therapeutic opportunities for crizotinib-treated patients with ALK secondary mutations.

Introduction
EML4-ALK is a driver oncogene in non-small cell lung cancer (NSCLC) [1] and the ALK inhibitor crizotinib showed remarkable activity against ALK-positive NSCLC patients with an objective response rate of 60.8% (95% CI 52.3–68.9) and median progression-free survival (PFS) of 9.7 months (95% CI 7.7–12.8) [2]. On the basis of its demonstrated efficacy, crizotinib was granted accelerated approval by FDA in 2011 as the first ALK inhibitor for advanced ALK-positive NSCLC patients. Despite the exciting performance of crizotinib in ALK-positive NSCLC patients, most patients who initially responded to crizotinib have relapsed due to the development of acquired resistance to crizotinib [3,4].

Acquired resistance poses a significant challenge to oncogene-targeted therapy and is caused by various mechanisms, such as gene alterations of target molecules or other gene alterations. About half of NSCLC patients with activating EGFR mutations developed acquired resistance that has been associated with a secondary mutation of EGFR T790M [5]. In addition, a major resistant mechanism of ABL inhibitors in chronic myelogenous leukemia (CML) is caused by secondary mutations of the BCR-ABL kinase domain such as ABL T315I [6,7]. Recently, pan-ABL inhibitor ponatinib was highly active in pretreated CML patients with resistance to ABL inhibitors, including patients with the ABL T315I mutation [8]. Similarly, several different point mutations within the ALK tyrosine kinase domain (L1196M, G1269A, F1174L, L1152R, I1151Tins, S1206Y, C1156Y, and G1202R) have been identified from the biopsy samples of patients who relapsed on crizotinib [3,4,9–14]. Thus ALK inhibitors that retain inhibitory potency against the secondary mutants would be expected to overcome the acquired resistance.

Alectinib is a potent and selective ALK inhibitor, and reveals antitumor activity against cancers with ALK gene alterations [15]. A recent report on phase 1/2 clinical study shows that out of 46 crizotinib-naïve patients treated with alectinib, 43 achieved an objective response of 93.5% (95% CI 82.1–98.6) [16]. Currently, the clinical study of alectinib in patients who are resistant to crizotinib is also ongoing (Trial registration ID: NCT01588028). We have already shown that alectinib could overcome EML4-ALK L1196M and C1156Y in preclinical models [15]. However, the potency of alectinib against crizotinib resistance caused by other newly identified ALK secondary mutations remains unclear. Here, we investigated the efficacy of alectinib in models of crizotinib resistance linked to ALK secondary mutations.

Materials and methods
Compounds and cell lines
Alectinib was synthesized at Chugai Pharmaceutical Co. Ltd. according to the procedure described in patent publication WO2010143664. Crizotinib was purchased from Selleck Chemicals or synthesized according to the procedure described...
In vitro kinase inhibitory assays

Recombinant human ALK and ALK mutants were purchased from Carna Biosciences. The inhibitory activity against each kinase was evaluated by examining their ability to phosphorylate the substrate peptide Biotin-EGPWLEEEEEAYGWMDF (PDB code: 1mmR). Each EML4-ALK mutant gene was generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore). After blocking in Blocking One (Nacalai Tesque, Inc.), the membranes were incubated independently in the primary antibodies diluted with 5% non-fat milk in PBS for 1 h. The membranes were washed three times in 1X PBS containing 0.05% Tween 20 and incubated with secondary antibodies (GOAT–HRP) followed by LAS-4000 (Fujifilm). The bands were detected with Chemi-Lumi One (Nacalai Tesque, Inc.) followed by LAS-4000 (Fujifilm). The bands were detected with Chemi-Lumi One (Nacalai Tesque, Inc.). The IC50 values were calculated using the following formula: IC50 = [D] × [IC50], where [D] is the drug concentration and [IC50] is the IC50 value of the drug.

Cell growth inhibition

Cells were cultured in 96-well plates (Corning), and incubated with various concentrations of compound for 48 h. The viable cells were measured by the CellTiter-Glo luminescent cell viability assay (Promega). Luminescence was quantified by Envision (PerkinElmer). The IC50 values were calculated using XLfit software.

Immunoblotting

Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM PMFS, 1% (v/v) phosphate inhibitor cocktail 2 (Sigma), 1% (v/v) phosphate inhibitor cocktail 3 (Sigma), and Complete Mini, EDTA-Free (Roche). Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore). The membranes were incubated independently in the primary antibodies diluted with 5% non-fat milk in PBS for 1 h. The membranes were washed three times in 1X PBS containing 0.05% Tween 20 and incubated with secondary antibodies (GOAT–HRP) followed by LAS-4000 (Fujifilm). The bands were quantified by Envision (PerkinElmer). The IC50 values were calculated using XLfit software.

Subcutaneous xenograft models

To evaluate the antitumor activity against EML4-ALK mutant-driven tumors in vivo, cell lines were grown as subcutaneous tumors in SCID mice (CLEA Japan, Inc.). Mice were randomized to treatment groups to receive vehicle, alectinib or crizotinib (oral, qd) for the indicated duration. Final concentration of vehicle was 0.02N HCl, 10% DMSO, 10% Cremophor EL, 15% PEG400, and 15% HPCD (2-hydroxypropyl-β-cyclodextrin). The length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated as: TV = (L × W2) / 2. Tumor growth inhibition was calculated using the following formula: Tumor growth inhibition = 1−[TV (Vehicle) / TV (Drug)] × 100, where L and W are the mean tumor volumes on a specific experimental day and on the first day of treatment, respectively, for the experimental groups and likewise, where C and G are the mean tumor volumes for the control group. The rate of change in body weight (BW) was calculated using the following formula: BW = W × W0 / 100, where W and W0 are the body weight on a specific experimental day and on the first day of treatment, respectively. All animal experiments in this study were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Chugai Pharmaceutical Co., Ltd.

Results

Alectinib is effective against tumor remaining after treatment with crizotinib

To compare the maximum efficacy of alectinib and crizotinib, we conducted an efficacy study in a mouse model of EML4-ALK-positive NCI-H2228 cells during long-term observation. Alectinib at 60 mg/kg caused tumor regression and after administration of the drug for 21 days, tumor growth did not occur for 4 weeks (Fig. 1A) [15]. We have already shown that the levels of phosphorylated ALK are decreased in NCI-H2228 xenograft tumor after a single dose of alectinib [15]. In addition, in mice at dose levels up to 60 mg/kg of alectinib, there was no body weight loss, no significant change in peripheral blood cell count.
no elevations of aspartate aminotransferase or alanine aminotransferase, and no substantial change in electrolytes [15]. In contrast, crizotinib at the maximum tolerated dose (MTD: 100 mg/kg) in mice [17] also caused tumor regression for a period of up to 10 days after treatment, but subsequently led to tumor stasis rather than shrinkage, and during drug-free periods, tumors started regrowing (Fig. 1A). In another study, we tried to investi-

Table 1  
The total number of ALK secondary mutations in patients with crizotinib resistance.

<table>
<thead>
<tr>
<th>ALK mutation</th>
<th>Total number of patients</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1196M</td>
<td>9</td>
<td>[3,4,9,10,13,14]</td>
</tr>
<tr>
<td>G1269A</td>
<td>7</td>
<td>[4,9,13,14]</td>
</tr>
<tr>
<td>C1156Y</td>
<td>2</td>
<td>[10,14]</td>
</tr>
<tr>
<td>F1174L</td>
<td>1</td>
<td>[12]</td>
</tr>
<tr>
<td>L1151Tins</td>
<td>1</td>
<td>[3]</td>
</tr>
<tr>
<td>L1152R</td>
<td>1</td>
<td>[11]</td>
</tr>
<tr>
<td>G1202R</td>
<td>1</td>
<td>[3]</td>
</tr>
<tr>
<td>S1206Y</td>
<td>1</td>
<td>[3]</td>
</tr>
</tbody>
</table>

Fig. 3. Kinase inhibitory activity of alectinib or crizotinib against ALK mutants. The in vitro kinase inhibitory assays of purified native ALK (amino acids 1081–1410), 1151Tins, L1152R, C1156Y, F1174L, L1196M, G1202R, and G1269A fused to GST in the presence of alectinib or crizotinib were carried out as described in “Materials and Methods”.

**Fig. 2.** X-ray structure of alectinib with ALK (PDB ID: 3AOX), crizotinib with ALK (PDB ID: 2XP2), and NVP-TAE684 with ALK (PDB ID: 2XB7). Alectinib (C in yellow), crizotinib (C in pink), and NVP-TAE684 (C in aqua) are shown in stick form. Figures were drawn using PyMol software (Schrödinger K.K.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
gate whether alectinib is effective against the tumor that remains after administration of crizotinib for 21 days. Switching from crizotinib to alectinib led to a significant reduction in tumor size (Fig. 1B). There were no mutations in the ALK kinase domain of the remaining tumor after 21-day-treatment with crizotinib (data not shown).

In order to understand this difference in antitumor activity between alectinib and crizotinib, the apoptosis-inducing potency of these drugs in NCI-H2228 tumors was tested. Both alectinib and crizotinib induced cleavage of PARP as an apoptotic marker in the tumor samples after initial drug treatment but the apoptosis induced by alectinib in these xenograft tumors was higher (Fig. 1C). Accordingly, the potent tumor regression associated with alectinib in a crizotinib resistant model and increased apoptosis, indicate that alectinib might be a therapeutic agent either for crizotinib-treated patients or in the first-line setting where it could lead to more durable antitumor activity.

Alectinib inhibited kinase activity of ALK mutations related to crizotinib resistance

Secondary acquired mutations of ALK have been identified from patients who have relapsed after initially responding to crizotinib. So far, there are a total of eight ALK mutations including L1196M,
G1269A, C1156Y, F1174L, 1151Tins, L1152R, S1206Y, and G1202R in crizotinib-relapsed NSCLC and inflammatory myofibroblastic tumor patients (Table 1).

Alectinib comprises a unique chemical scaffold (a benzo[b]carbazole derivative) that is unlike the scaffold used in ALK inhibitors of another chemical class, such as crizotinib and NVP-TAE684 [15,18,19]. They all bind into the kinase domain of ALK in different ways (Fig. 2), suggesting that alectinib might overcome resistance to other chemical classes caused by mutational mechanism. To evaluate the inhibitory effect of alectinib on each ALK secondary mutation, we conducted kinase inhibition assays of the ALK mutants. Alectinib has substantial inhibitory potency against native ALK as well as ALK L1196M, G1269A, C1156Y, F1174L, 1151Tins, and L1152R. In contrast, alectinib was less potent against only ALK G1202R (Fig. 3).

Alectinib is active in tumors driven by ALK mutations related to crizotinib resistance

Next, to investigate the sensitivity of these mutant-driven cells to alectinib, we generated Ba/F3 cell lines expressing EML4-ALK and the mutated EML4-ALK. Parent Ba/F3 cells are dependent on IL-3 for growth, but each established Ba/F3 transfectant expressing EML4-ALK or mutated EML4-ALK shows IL-3-independent growth. Alectinib was effective against each mutant-driven cell except for G1202R mutant, and the IC50 ratio of each Ba/F3 cell line expressing EML4-ALK mutations other than ALK G1202R to the parent Ba/F3 cells with alectinib (8.3- to 57-fold) was higher than that with crizotinib (1.3- to 5.1-fold; Fig. 4A). The cell sensitivity to drugs was consistent with the suppression of phospho-ALK (Fig. 4B).

Fig. 5. Antitumor activity of alectinib against EML4-ALK mutant-driven tumors in a subcutaneous mouse model. (A) Mice bearing Ba/F3-EML4-ALK G1269A, or -EML4-ALK G1202R were treated with vehicle, alectinib, or crizotinib orally once daily for 7 days. Tumor volume for each dose group was measured. Data are shown as mean ±SD (n = 5 per group). Parametric Dunnett’s test: “,” p < 0.001; N.S., not significant, versus vehicle treatment at final day. (B) Pharmacodynamic response of EML4-ALK mutant-driven tumors to alectinib. Mice bearing Ba/F3-EML4-ALK G1269A, or -EML4-ALK G1202R were orally treated with a single dose of vehicle, alectinib, or crizotinib, and the tumors were collected and lysed at 4 h post-dosing. Phosphorylated STAT3 (Tyr 705), STAT3, and β-actin were detected by immunoblot analysis using antibodies against each of them (n = 2 per group). (C) Mice bearing Ba/F3-EML4-ALK 1151Tins, -EML4-ALK F1174L, and -EML4-ALK S1206Y were treated with vehicle, alectinib, or crizotinib orally once daily for 7 days. Tumor volume for each dose group was measured. Data are shown as mean ±SD (n = 5 per group).
To further evaluate the in vivo antitumor activity of aleckitinib against EML4-ALK mutant-driven tumors, we used subcutaneous mouse models of Ba/F3 cell lines expressing EML4-ALK mutations. We have already shown that treatment with crizotinib at 100 mg/kg led to effective antitumor activity against native EML4-ALK-driven tumor, and that aleckitinib at 60 mg/kg resulted in significant tumor regression against both native EML4-ALK and the gatekeeper mutant L1196M [15]. In this study, we first examined the in vivo efficacy against tumors harboring EML4-ALK G1269A, which is the second most common resistant mutation (Table 1), and EML4-ALK G1202R, which is less sensitive to aleckitinib in vitro (Figs. 3 and 4A). We revealed that treatment with aleckitinib at 60 mg/kg led to significant tumor regression against EML4-ALK G1269A-driven tumors, but did not inhibit tumor growth against EML4-ALK G1202R-driven tumors. On the other hand, crizotinib at 100 mg/kg had no tumor growth inhibition against either of these EML4-ALK mutant-driven tumors (Fig. 5A). Consistent with the results of tumor growth inhibition, aleckitinib could block phosphorylation of STAT3, which is one of the ALK downstream components [15,20], in the G1269A model, but not in the G1202R model (Fig. 5B). Additionally, we also confirmed aleckitinib was effective against three other EML4-ALK mutations, 1151Tins, F1174L, and C1156Y-driven tumors (Fig. 5C). These results indicated the potential antitumor activity of aleckitinib against tumors harboring most of the EML4-ALK mutations related to crizotinib–resistance.

Discussion

ALK secondary mutations associated with crizotinib resistance have been identified from crizotinib–relapsed patients. In this study, we also confirmed that aleckitinib is effective against a broad spectrum of EML4-ALK mutants in a Ba/F3 transfectant model. In clinic, the trough plasma concentration of aleckitinib administered at the clinical dose (at 300 mg bid) is reported to be 463 ng/mL (959 nM) [16]. In most of the Ba/F3 transfectants except for G1202R, a concentration of aleckitinib lower than 959 nM resulted in nearly complete inhibition of cell proliferation (Fig. 4A). These results were consistent with the antitumor effects of each drug against mouse models.

We have already shown that aleckitinib could sustain inhibitory activity against ALK L1196M and C1156Y mutants. In the in silico modeling study of L1196M, aleckitinib could also maintain the hydrogen-bonding network [15]. To further understand the difference in the inhibitory effects on G1269A, which is another major crizotinib–resistant mutant, we also examined the in silico model based on the crystal structures of crizotinib with ALK (PDB ID: 2XP2). The G1269A mutation would cause steric hindrance interfering with the action of crizotinib but not with that of aleckitinib (Fig. S1).

A minor mutation, G1202R, showed less sensitivity to either aleckitinib or crizotinib (Figs. 3, 4A, and 5A) and would cause steric hindrance to any ALK inhibitors, including NVP-TAE684 (Fig. S2), which is a finding that is consistent with the results of another report [3]. In our study, the relative phospho-ALK level in the Ba/F3 cell line expressing EML4-ALK G1202R was much lower than in expressing either native EML4-ALK mutants or other EML4-ALK mutants (Fig. S3), and this remained the case even when the expression level of phospho-ALK in all EML4-ALK mutants was assessed across several clones (data not shown). In the report mentioned above, the patient with G1202R mutation also had focal KIT amplification [3]. When taken together, new ALK inhibitors that are effective against ALK G1202R and combination therapies would be expected to overcome the resistance caused by ALK G1202R.

A report on a phase 1/2 clinical study shows that aleckitinib is well tolerated and highly active in crizotinib naive ALK-positive NSCLC patients who received previous chemotherapy regimens [16]. In addition, consistent with the result of our preclinical study, recent clinical study showed that aleckitinib was also highly active against ALK-positive NSCLC patients after treatment with crizotinib [21]. Because it is unclear whether the crizotinib–relapsed patients have ALK secondary mutations to examine at this moment, we recognize that its is important to examine the mechanisms of crizotinib resistance including ALK secondary mutations in patients. Of note, aleckitinib has activity against the crizotinib–relapsed patients with CNS metastases, which is known as the primary site of initial treatment failure in 46% of patients treated with crizotinib [21,22]. Thus, aleckitinib has shown promising antitumor activity in second- and subsequent-line treatment of ALK–positive NSCLC patients. Further investigation of aleckitinib in the first-line setting is needed to confirm the durability of response in ALK–positive NSCLC patients.

Conflict of Interest Statement

All of the authors are employees of Chugai Pharmaceutical Co., Ltd.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2014.05.020.

References


