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Selective ALK inhibitor alectinib with potent antitumor activity in models of crizotinib resistance

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ABSTRACT

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, 1151Tins, 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

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in WO2006021884. NCI-H2228 and Ba/F3 cell lines were obtained from American Type Culture Collection (ATCC) or RIKEN. Each cell line was cultured using the medium recommended by the suppliers.

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 in the presence of drug and 30 μM ATP using time-resolved fluorescence resonance energy transfer (TR-FRET) assay. The IC_{50} values were calculated using XLfit software (ID Business Solutions).

Generation of Ba/F3 cell lines expressing EML4-ALK mutant

The EML4-ALK mutant genes (1151Tins, C1156Y, F1174L, L1196M, G1202R, S1206Y, and G1269A) were inserted into pcDNA3.1/hygro vector (Life Technologies). Each EML4-ALK mutant gene was generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). Each Ba/F3 cell line stably expressing the EML4-ALK mutant was generated by transfecting with pcDNA3.1/hygro-EML4-ALK mutant genes using a Nucleofector device (Amaxa); stable transfectants were then isolated from the culture medium without IL-3.

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 IC_{50} values were calculated using XLfit software.

Immunoblotting

Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology) containing 1 mM PMSF, 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). After blocking in Blocking One (Nacalai Tesque, Inc.), the membranes were incubated independently in the primary antibodies diluted with anti-ALK (Life Technologies, #51-3900), anti-Phospho-ALK (Tyr 1604) (Cell Signaling Technology, #3341), anti-STAT3 (Cell Signaling Technology, #9132), anti-Phospho-STAT3 (Tyr 705) (Cell Signaling Technology, #9131), anti-PARP (Cell Signaling Technology, #9542), or anti-β-actin (Sigma, A5441). The membranes were incubated with an anti-rabbit or anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology). The bands were detected with Chemi-Lumi One Super (Nacalai Tesque, Inc) followed by LAS-4000 (Fujifilm).

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: $TV = (L \times W^2)/2$. Tumor growth inhibition was calculated using the following formula: Tumor growth inhibition = $[1-(T-T_0)/(C-C_0)] \times 100$, where T and T_0 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 C_0 are the mean tumor volumes for the control group. The rate of change in body weight (BW) was calculated using the following formula: $BW = W/W_0 \times 100$, where W and W_0 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 regrowth 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,

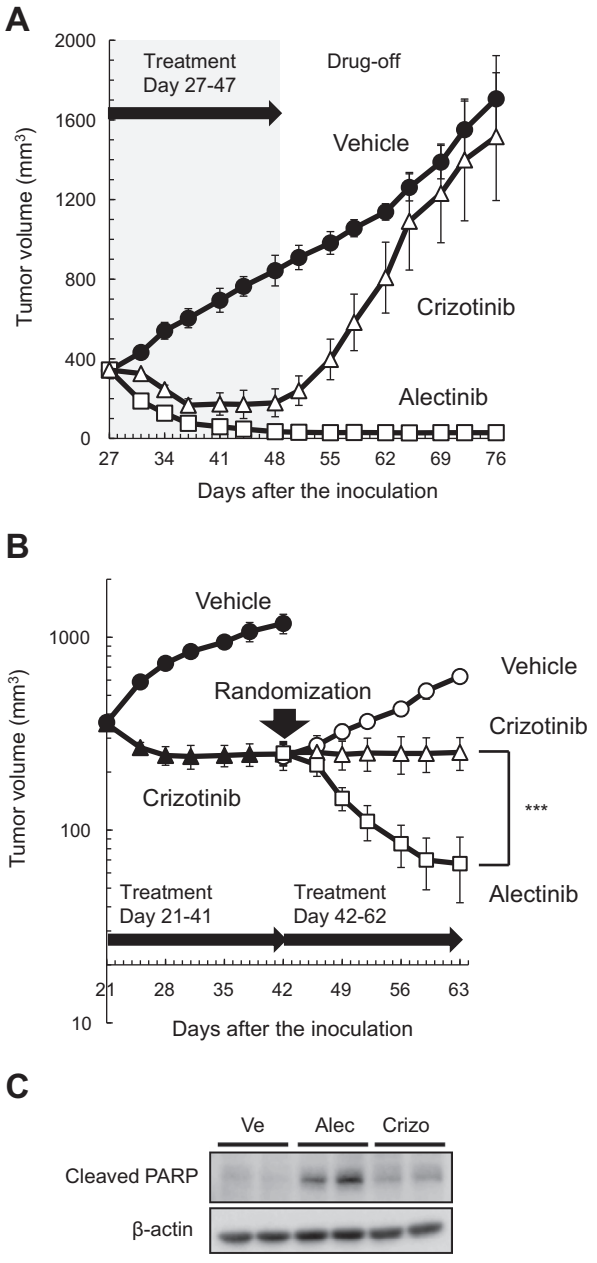

Fig. 1. Efficacy of alectinib against tumors remaining after treatment with crizotinib. (A) Mice bearing NCI-H2228 cells orally treated with alectinib at 60 mg/kg or crizotinib at 100 mg/kg on a daily basis for 21 days (days 27–47). Then tumor volume (mean ± SD) was continuously measured during the treatment and the 4-week drug-free period that followed. Data are shown as mean ± SD ($n = 5$ per group). (B) Mice bearing NCI-H2228 cells were orally treated with crizotinib at 100 mg/kg on a daily basis for 21 days and then randomized. After randomization, the mice were orally treated with vehicle, alectinib at 60 mg/kg, or crizotinib at 100 mg/kg on a daily basis for 21 days. Parametric Dunnett's test: ***, $p < 0.001$, treatment with alectinib versus crizotinib at final day ($n = 5$ per group). (C) Apoptosis-inducing ability of alectinib. Mice bearing alectinib cells were orally treated with a single dose of vehicle, alectinib at 60 mg/kg, or crizotinib at 100 mg/kg and the tumors were collected and lysated at 6 h post-dosing. Cleavage of PARP and β-actin were detected by immunoblot analysis using antibodies against each of them ($n = 2$ per group).