Alectinib shows potent antitumor activity against RET-rearranged non-small cell lung cancer

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Abstract

Alectinib/CH5424802 is a known inhibitor of anaplastic lymphoma kinase (ALK) and is being evaluated in clinical trials for the treatment of ALK fusion-positive non-small cell lung cancer (NSCLC). Recently, some RET and ROS1 fusion genes have been implicated as driver oncogenes in NSCLC and have become molecular targets for antitumor agents. This study aims to explore additional target indications of alectinib by testing its ability to inhibit the activity of kinases other than ALK. We newly verified that alectinib inhibited RET kinase activity and the growth of RET fusion-positive cells by suppressing RET phosphorylation. In contrast, alectinib hardly inhibited ROS1 kinase activity unlike other ALK/ROS1 inhibitors such as crizotinib and LDK378. It also showed antitumor activity in mouse models of tumors driven by the RET fusion. In addition, alectinib showed kinase inhibitory activity against RET gatekeeper mutations (RET V804L and V804M) and blocked cell growth driven by the KIF5B-RET V804L and V804M. Our results suggest that alectinib is effective against RET fusion-positive tumors. Thus, alectinib might be a therapeutic option for patients with RET fusion-positive NSCLC.
Introduction

Rearranged during transfection (RET) is an oncogene that when genetically altered, is involved in the development of several human cancers. Activation of the RET point mutation is associated with medullary thyroid carcinoma (MTC) (1), and RET rearrangement has been detected in papillary thyroid carcinoma (PTC) (2). In addition, some RET fusion genes such as KIF5B-RET and CCDC6-RET have also recently been identified as driver oncogenes in non-small cell lung cancer (NSCLC), with the associated fusion genes present in approximately 1%–2% of cases (3-5). Vandetanib and cabozantinib, which are multi-kinase inhibitors targeting RET, were approved by the FDA in 2011 and 2012, respectively, for the treatment of patients with MTC (6). In a prospective phase 2 trial that included RET fusion-positive NSCLC patients, 2 patients with RET fusion-positive NSCLC responded to cabozantinib (7). Thus, the genetic alterations of RET are considered to be promising targets for anticancer therapy.

Most of the clinically approved small-molecule inhibitors that are currently available are able to inhibit multiple kinases, and in fact, some inhibitors are approved or are currently in clinical trials for several indications. For example, imatinib, which has inhibitory activity against ABL and KIT, was approved for use in the treatment of Philadelphia chromosome (BCR-ABL fusion)-positive chronic myeloid leukemia and KIT-positive metastatic gastrointestinal stromal tumor (8). Additionally, crizotinib, which was granted accelerated approval by the FDA in 2011 as the first ALK inhibitor for advanced ALK-positive NSCLC patients (9), inhibits not only ALK but several other kinases including MET, ROS1, and RON (10). Clinical trials with crizotinib have recently demonstrated the drug’s efficacy in the treatment of NSCLC with ROS1 translocations.
(11-13). In contrast, off-target toxicities are generally observed when therapeutic agents inhibit kinases that were not intended as targets (14). For example, anticancer therapies involving the use of multi-kinase inhibitors that target KDR are associated with the development of hypertension and proteinuria. These side effects commonly disappear upon drug withdrawal (15, 16).

Alectinib is a potent ALK inhibitor that exhibits antitumor activity against cancers with ALK gene alterations (17). A recent report on a phase 1/2 clinical study shows that alectinib is well tolerated and highly active in patients with advanced ALK-rearranged NSCLC (18). One ongoing clinical study is testing the activity of alectinib in patients in whom crizotinib treatment failed (Trial registration ID: NCT01588028). In this study, we explored indications for alectinib treatment in non-ALK-positive cancers, and provide evidence that alectinib is a potent RET inhibitor for RET fusion-driven NSCLC. Alectinib inhibited RET kinase activity and RET fusion-driven cell growth by suppressing phospho-RET. Alectinib also showed antitumor activity in mouse models of a RET fusion-positive tumor.
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, cabozantinib, and vandetanib were purchased from Selleck Chemicals; gefitinib was purchased from Kemprotec Limited; and LDK378 was purchased from Active Biochemicals.

LC-2/ad cells were obtained from RIKEN in September 2011, NCI-H2228 cells were obtained from American Type Culture Collection (ATCC) in February 2008, NCI-H522 cells were obtained from ATCC in July 2001, and Ba/F3 cells were obtained from RIKEN in July 2008. Each cell line was cultured using the medium recommended by the suppliers. The cell lines have not been authenticated by the authors.

Kinase inhibitory assays

Recombinant human RET, mutated RET (G691S, Y791F, V804L, V804M, S891A, and M918T), ROS1, RON, EGFR, KDR, PDGFRβ, FGFR2, KIT, SRC, HER2, MET, RAF1, and MEK1 were purchased from Carna Biosciences or Merck. Inhibitory activity against each kinase was evaluated using the time-resolved fluorescence resonance energy transfer (TR-FRET) assay to examine each compound’s ability to phosphorylate substrate peptide Biotin-EGPWLEEEEEAYGWMDF in the presence of the drug and 30 μM ATP (17). The IC\textsubscript{50} values were calculated using XLfit software (ID Business Solutions).

In silico modeling

The structure models of RET with alectinib, crizotinib, and LDK378 were modeled by
Discovery Studio 3.5 (Accelrys). All figures were drawn using PyMol software (Schrödinger K.K.).

**Generation of Ba/F3 cells expressing KIF5B-RET and mutated KIF5B-RET**

The plasmids that express KIF5B-RET, KIF5B-RET V804L, and KIF5B-RET V804M, respectively named CS-GS104J-M67, CS-GS104J-M67-m1, and CS-GS104J-M67-m2, were purchased from Genecopoeia. To generate Ba/F3 cells that stably expressed KIF5B-RET and mutated KIF5B-RET, Ba/F3 cells were transfected with the appropriate expression plasmids using a Nucleofector device (Amaxa). Stable transfectants were then isolated from the cultured medium without IL-3.

**Cell growth inhibition and caspase-3/7 assay**

Cells were cultured in 96-well spheroid plates (Sumilon Celltight Spheroid 96U; Sumitomo Bakelite Inc.) overnight and incubated with various concentrations of compound for the indicated time. The viable cells were measured by the CellTiter-Glo® luminescent cell viability assay (Promega). The IC₅₀ values were calculated using XLfit software. The caspase-3/7 assay was evaluated using the Caspase-Glo® 3/7 Assay Kit (Promega). Fluorescence was quantified using Envision (PerkinElmer).

**Transcriptome profiling and quantitative RT-PCR**

Total RNA was extracted using the RNeasy mini-kit (Qiagen), and cDNA libraries were generated using the TruSeq RNA sample preparation kit (Illumina). The cDNA libraries were sequenced by an Illumina HiSeq 2000 instrument according to the manufacturer’s
instructions. RSEM software (19) was used to align reads against the RefSeq transcript and to calculate the expression value for each gene. For quantitative RT-PCR, RNA was amplified by QuantiFast Multiplex RT-PCR (Qiagen) using a Universal probe library (Roche Applied Science) and the LightCycler System (Roche). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control.

**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 primary antibody diluted with anti-RET (Santa Cruz Biotechnology, sc-167 or Cell Signaling Technology, #3341), anti-Phospho-RET (Tyr 905) (Cell Signaling Technology, #3221), anti-STAT3 (Cell Signaling Technology, #9132), anti-Phospho-STAT3 (Tyr 705) (Cell Signaling Technology, #9131), anti-AKT (Cell Signaling Technology, #9277), anti-Phospho-AKT (Ser 473) (Cell Signaling Technology, #9271), anti-p44/42 MAP Kinase (ERK1/2; Cell Signaling Technology, #9102), anti-Phospho-ERK1/2 (Thr 202/Tyr 204; Cell Signaling Technology, #9101), anti-Cleaved PARP (Asp214; Cell Signaling Technology, #9546), anti-BIM (Cell Signaling Technology, #2819), anti-MCL-1 (Cell Signaling Technology, #4572), anti-BAX (Cell Signaling Technology, #2774), or anti-β-actin (Sigma, A5441). To detect phosphorylated RET, cell lysates were
immunoprecipitated with anti-phosphotyrosine (PY-20) antibody (BD Biosciences). The membranes were incubated with an anti-rabbit or anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology). The bands were detected using Chemi-Lumi One Super (Nacalai Tesque, Inc.) technology with LAS-4000 (Fujifilm).

**In vivo studies**

Male SCID mice (C.B-17/Icr-scid/scidJc, 5-week-old) were obtained from CLEA Japan, Inc. Cells (LC-2/ad cells at 8.4 × 10⁶ or Ba/F3 cells expressing KIF5B-RET at 5.0 × 10⁶) were grown as subcutaneous tumors in SCID mice. Mice were randomized to treatment groups to receive vehicle or alectinib (oral, qd) for the indicated duration of treatment. The 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 tumor volume (TV) was calculated as: \( TV = \frac{L \times W^2}{2} \). The rate of change in body weight (BW) was calculated using the following formula: \( BW = \frac{W}{W_0} \times 100 \), 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

Inhibition of RET kinase activity mediated by alectinib

The ALK inhibitor crizotinib inhibits several kinases including ALK, MET, ROS1, and RON (10). Several ROS1 fusion genes have recently been identified in NSCLC (4, 11, 12), and crizotinib has recently shown efficacy in the treatment of NSCLC with ROS1 translocations (13). Having previously reported that alectinib potently inhibited ALK (IC$_{50}$ = 1.9 nM) but not inhibit MET kinase (IC$_{50}$, >5000 nM) (17), we further checked the inhibitory activity of alectinib for ROS1 and RON. However, alectinib hardly inhibited ROS1 (IC$_{50}$ = 3700 nM) and did not inhibit RON (IC$_{50}$, >5000 nM; Fig. 1A, Table 1).

To examine the additional kinase inhibitory activity of alectinib, we evaluated 451 biochemical kinases, including several mutated kinases, using KINOMEscan technology (DiscoveRx). Using this technology, we had previously found that alectinib at 10 nM bound to only three kinases: ALK, LTK, and GAK (17). In this study, we found that in addition to ALK and LTK, alectinib at 100 nM bound to CHEK2, FLT3 (D835Y), PHKG2, RET, and RET (M918T) with greater than 95% inhibition (data not shown). Because several RET fusion genes have recently been identified as driver oncogenes in NSCLC (3-5), we focused on the potential of alectinib as a RET inhibitor. The results of a kinase inhibitory assay revealed that alectinib strongly inhibited RET kinase activity (IC$_{50}$ = 4.8 nM; Table 1) and RET kinases with point mutations that were identified in MTC (IC$_{50}$ = 5.7–53 nM, Table 1) (20-22). In contrast, crizotinib hardly inhibited RET (IC$_{50}$ = 3200 nM) and LDK378, a second-generation ALK inhibitor (23, 24), did not inhibit RET kinase activity (IC$_{50}$, >5000 nM). In addition, ATP-competitive binding assay showed that alectinib bound to RET at a dissociation constant (K$_D$) value of 7.6 nM. The kinase domain
of RET shares 36.6% amino acid sequence homology with ALK in the kinase domains, and a structure-based in silico analysis revealed that common hydrophobic interactions of alectinib bound to ALK (25) and RET (Fig. 1B). In addition, the differences among ALK inhibitors show that alectinib has a unique chemical scaffold (it is a benzo[b]carbazole derivative) that is unlike the scaffold used in ALK inhibitors of other chemical classes, such as crizotinib and LDK378 (17, 23, 26, 27). Moreover, in silico analysis suggested that RET would cause steric hindrance, thereby interfering with the action of crizotinib and LDK378 but not with that of alectinib (Fig. 1C).

Alectinib mediated inhibition of RET fusion-positive cell growth

Next, to investigate the sensitivity of RET fusion-positive cells to alectinib, we evaluated alectinib’s capacity to inhibit cell growth in LC-2/ad NSCLC cells harboring the CCDC6-RET fusion gene (28). Alectinib, similarly to well-known RET inhibitors cabozantinib and vandetanib, inhibited the growth of LC-2/ad cells (Fig. 2A). However, other ALK inhibitors (crizotinib and LDK378) and the EGFR inhibitor gefitinib did not inhibit cell growth. To investigate the sensitivity of another line of RET fusion-positive cells to alectinib, we generated Ba/F3 cells expressing KIF5B-RET. An established Ba/F3 transfectant expressing KIF5B-RET shows IL-3 independent growth and the cells depend on KIF5B-RET. Alectinib, cabozantinib, and vandetanib were also effective against Ba/F3 cells expressing KIF5B-RET (Fig. 2B). From public information, ponatinib also inhibited Ba/F3 cells expressing KIF5B-RET (IC$_{50}$ = 11 nM) (29), consistent with the potency of enzyme inhibition on RET (Supplementary Fig. S1). In contrast, gefitinib and crizotinib did not inhibit these cells and LDK378 hardly inhibited these cells (Fig. 2B, Supplementary Fig.
Moreover, alectinib induced caspase-3/7 activation in LC-2/ad cells as well as in NCI-H2228 cells harboring *EML4-ALK* (Fig. 2C), indicating that apoptosis is involved in the antitumor activity of alectinib. To understand the effects of phospho-RET suppression and the contribution of factors downstream to RET, we conducted a cellular phosphorylation assay using LC-2/ad cells that had been treated with alectinib. Alectinib suppressed the auto-phosphorylation of RET in a concentration-dependent manner (Fig. 2D). Alectinib also suppressed the phosphorylation of ERK1/2, but not of STAT3 and AKT (Fig. 2D), suggesting that the proliferation of LC-2/ad cells requires MAPK signaling pathways. Taken together, these results indicate that alectinib exhibits potent inhibitory activity against RET fusion-positive cells by suppressing RET phosphorylation.

**Downstream signaling pathway in LC-2/ad cells harboring CCDC6-RET**

To understand the downstream signal pathway of CCDC6-RET in NSCLC, we performed comprehensive gene expression analysis of alectinib-treated LC-2/ad cells based on Illumina HiSeq 2000 next-generation sequencing. The majority of genes down-regulated by alectinib were negative feedback regulators of ERK such as *DUSP6* (30) and *DUSP2* (31) and MAPK pathway downstream genes such as *ETV1*, *ETV4*, *ETV5*, *FOS* (32), and *EREG* (33). A previous report has demonstrated that certain MAPK-related genes, such as *DUSP6* and *EREG*, are suppressed by a knockdown of RET using siRNA in LC-2/ad cells (34). To validate our data, we conducted quantitative RT-PCR and confirmed significant decreases in the expression of these genes (e.g., *DUSP2*, *EREG*, *ETV5*, and *FOS*) (Fig. 3A). We also examined the effects of alectinib on the expression of apoptosis-related proteins in LC-2/ad cells. Alectinib induced PARP cleavage in LC-2/ad cells and increased the abundance of
BIM, a key proapoptotic member of the BCL-2 family of proteins, whereas the levels of BCL-2 family members MCL-1 and BAX remained unaffected (Fig. 3B), suggesting that alectinib induces apoptosis through the up-regulation of BIM via inhibition of the MAPK signaling pathway. In Ba/F3 cells expressing KIF5B-RET, alectinib also suppressed phosphorylation of ERK and increased the abundance of BIM (Supplementary Fig. S3). These data were consistent with a previous report that inhibition of the MAPK signaling pathway contributes to EGFR inhibitor-induced BIM up-regulation in EGFR mutated NSCLC cells (35). However, we recognize that the full downstream signal pathway of RET fusion protein in NSCLC, including that in LC-2/ad cells, remains unknown. Further detailed studies are needed to elucidate the downstream signal pathway of RET fusion protein in NSCLC to explore options for combination therapy.

Antitumor activity of alectinib against RET fusion-positive tumors

To evaluate the in vivo antitumor activity of alectinib against RET fusion-positive tumors, we used a mouse xenograft model of LC-2/ad cells expressing CCDC6-RET. Although the engraftment of LC-2/ad cells using SCID mice was slow, RET break-apart FISH analysis using the RET Split Dual Color FISH Probe (ver. 1 SP018, GSP Lab., Inc.) (3) showed that LC-2/ad xenograft tumors continued to harbor the RET rearrangement up to 141 days after the inoculation (data not shown). Using this xenograft model, we confirmed that once-daily oral administration of alectinib resulted in remarkable tumor regression at all doses without significant weight loss (Fig. 4A). Similar efficacy was also observed in the case of treatment with vandetanib at 50 mg/kg, but significant body weight loss was seen at this dose level (Fig. 4A).
We next tested a subcutaneous mouse model of Ba/F3 cells expressing KIF5B-RET. We found that treatment with alectinib at 60 mg/kg significantly inhibited the growth of KIF5B-RET-driven tumors (Fig. 4B). In a phosphorylation assay of the Ba/F3 cells expressing KIF5B-RET tumors, alectinib suppressed auto-phosphorylation of RET in vivo (Fig. 4C). These in vitro and in vivo findings confirmed that alectinib is effective against RET fusion-positive tumors.

**Alectinib is active in cancers driven by RET gatekeeper mutations**

Point mutations in the kinase domain are known as a mechanism of acquired resistance to small-molecule kinase inhibitors. In particular, gatekeeper mutations, such as T790M in EGFR, T315I in ABL, and L1196M in ALK, are one of the most frequent causes of resistance (36-38). To evaluate the inhibitory effect of alectinib on the most predictable resistant RET mutations (V804L and V804M), which are known as gatekeeper mutations (20), we measured the kinase inhibitory activity using recombinant glutathione S-transferase (GST)-fused RET V804L and V804M. Alectinib had substantial inhibitory potency against both RET gatekeeper mutants and the IC$_{50}$ values on RET V804L and V804M were 32 nM and 53 nM, respectively (Table 1). Next, to investigate the sensitivity of these mutant-driven cells to alectinib, we generated Ba/F3 cells expressing KIF5B-RET V804L and V804M. Alectinib was effective against each mutant-driven cell, and the IC$_{50}$ ratio of alectinib against the mutated KIF5B-RET as compared to the non-mutated KIF5B-RET (6.5- to 8.1-fold) was higher than that of cabozantinib (15- to 25-fold) and vandetanib (50- to 57-fold; Fig. 5A). The degree of cell sensitivity to these drugs was consistent with the suppression of phospho-RET (Fig. 5B).
In order to understand the difference in the inhibitory effects on RET V804L and V804M as exhibited by alectinib versus vandetanib, we examined an *in silico* model based on the crystal structures of alectinib with ALK (PDB ID: 3AOX). This model was then superimposed on the crystal structure of RET bound to vandetanib. Both V804L and V804M mutation would cause steric hindrance that interfered with the action of vandetanib but not with that of alectinib (Supplementary Fig. S4). These results indicated potential antitumor activity of alectinib against tumors harboring gatekeeper-mutated RET fusion genes.
Discussion

Discovery of the RET fusion gene in NSCLC has led to the development of RET inhibitors for NSCLC patients with RET fusion-positive tumors. Clinical trials have been designed to investigate the therapeutic effects of multi-kinase inhibitors targeting RET, such as vandetanib (Trial registration ID: NCT01823068), cabozantinib (NCT01639508), sunitinib (NCT01829217), lenvatinib (NCT01877083), dovitinib (NCT01831726), and ponatinib (NCT01813734). In a recent study of cabozantinib in three NSCLC patients with the RET fusion gene, cabozantinib achieved partial responses in 2 patients and stable disease in 1 patient (7). Of those patients, Grade 3 toxicities of fatigue, proteinuria, and hypertension were observed, and two patients treated with cabozantinib required dose reduction of cabozantinib. In order to understand the differences between alectinib and other RET inhibitors, we confirmed the associated kinase selectivity profiles. Cabozantinib, vandetanib, sunitinib, sorafenib, ponatinib, lenvatinib, and dovitinib inhibited not only RET kinase but also other kinases such as KDR, which is a receptor for vascular endothelial growth factor (VEGF) (Supplementary Fig. S1) (39, 40). In contrast, alectinib only slightly inhibited KDR kinase activity (Supplementary Fig. S1). The major toxicities of KDR tyrosine kinase inhibitors are associated with the development of hypertension and proteinuria and they are commonly regressive on drug withdrawal (15, 16). In a clinical trial of alectinib in patients with ALK-rearranged NSCLC, alectinib was generally tolerable with no dose limiting-toxicities observed and KDR-related toxicities such as hypertension observed rarely (18). Therefore, potent RET inhibitors with weak KDR inhibition may offer a clinical advantage in the treatment of RET fusion-positive NSCLC.

Although alectinib inhibited the growth of RET C634W mutation-positive MTC
TT cells (data not shown) (41), this effect was weak in a mouse xenograft model. Nevertheless, alectinib inhibited RET phosphorylation in these tumor samples (data not shown). In contrast, a multi-kinase inhibitor, sorafenib, showed tumor growth inhibition against this mouse model and inhibited RET phosphorylation in these tumor samples (data not shown). In a previous report, cabozantinib also inhibited tumor growth in a mouse xenograft model of TT cells (42). Thus, the growth of TT xenograft tumors might depend on not only RET but also other kinases such as KDR. Further investigation is needed to confirm the mechanisms underlying the effects of several RET inhibitors in RET-mutated MTC xenograft models.

In addition to the RET fusion gene, the ROS1 fusion gene has been identified as an oncogenic driver that is found in approximately 1%–2% of NSCLC (11, 12). Alectinib hardly inhibited ROS1 kinase activity (Table 1) and did not inhibit the growth of HCC78 cells harboring the SLC34A2-ROS1 fusion gene (IC₅₀, >1000 nM) (11, 12, 43), although crizotinib strongly inhibited growth, and LDK378 weakly inhibited the growth of these cells under three-dimensional (3D) spheroid culture conditions. In addition, in silico analysis suggested that ROS1 would cause steric hindrance, thus interfering with the action of alectinib but not with that of crizotinib or LDK378 (Supplementary Fig. S5). In contrast, alectinib inhibited the growth of RET fusion-driven cells but crizotinib and LDK378 did not (Fig. 2A, B). Alectinib also showed antitumor activity in mouse models of RET fusion-driven tumors by suppressing phospho-RET (Fig. 4A, B). On the basis of our studies, in contrast to other ALK inhibitors, alectinib would not be useful for the treatment of ROS1 fusion-positive NSCLC but would be useful for the treatment of RET fusion-positive NSCLC.
Acknowledgements

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Table 1. Kinase inhibitory activity of alectinib against RET, ROS1 and RON.

<table>
<thead>
<tr>
<th>Kinase</th>
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NOTE: The *in vitro* kinase inhibitory assays of purified native RET and mutated RET (amino acids 658–1114), ROS1 (amino acids 1883–2347) and RON (amino acids 979–1400) fused to GST in the presence of alectinib were carried out as described in Materials and Methods.
Figure legends

Figure 1. Structural model of alectinib with RET. A, chemical structure of alectinib. B, x-ray structure of alectinib with ALK at the ATP binding site and 3D model of alectinib with RET. Amino acid residues, which form hydrogen bonds to the ligand directly or via water molecules, are depicted. Alectinib and amino acid residues are shown in stick form (C in yellow, white, and green, O in red, and N in blue). C, x-ray structure of alectinib with ALK (PDB ID: 3AOX), crizotinib with ALK (PDB ID: 2XP2), and LDK378 with ALK (PDB ID: 4MKC) and structural models of RET (PDB ID: 2K2K) with them. Alectinib (C in yellow), crizotinib (C in pink), and LDK378 (C in aqua) are shown in stick form.

Figure 2. Inhibition of RET-driven tumors by alectinib. A, growth inhibition of LC-2/ad cells by alectinib. Cells were seeded in a spheroid culture plate and incubated overnight, and then treated with the indicated concentrations of alectinib, cabozantinib, vandetanib, crizotinib, LDK378, and gefitinib. The viable cells were measured using a CellTiter-Glo® luminescent cell viability assay after 5 days of treatment. Data are shown as mean ± SD (n = 3 per group). B, effect of alectinib against Ba/F3 cells expressing KIF5B-RET. Cells were seeded and then treated with various concentrations of alectinib, cabozantinib, vandetanib, and gefitinib for 2 days. The viable cells were measured using the CellTiter-Glo® luminescent cell viability assay. Data are shown as mean ± SD (n = 3). C, alectinib-mediated increase in caspase 3/7 activity. Cells were seeded in spheroid culture plate and incubated overnight, then treated with alectinib at the indicated concentrations. The viable cells were measured using a Caspase-Glo® 3/7 assay kit after 2 days of treatment. Data are shown as mean ± SD (n = 3 per group). D, effects of alectinib on the phosphorylation of...
RET, STAT3, AKT and ERK1/2 in LC-2/ad cells. LC-2/ad cells were treated with alectinib for 2 hours at the indicated concentrations. Cell lysates were subjected to immunoprecipitation with anti-phosphorylated tyrosine antibodies, and the level of RET phosphorylation was detected by immunoblot analysis using anti-RET antibody. The expression level of RET, phospho-STAT3 (Tyr 705), STAT3, phospho-AKT (Ser 473), AKT, phospho-ERK1/2 (Thr 202/Tyr 204), and ERK1/2 were detected by immunoblot analysis with the appropriate antibodies.

Figure 3. Downstream signaling pathway in LC-2/ad cells harboring CCDC6-RET. A, the levels of each transcript were measured by quantitative RT-PCR. Data are shown as mean ± SD (n = 2). Student’s t test: *, p < 0.05, versus DMSO treatment. B, effects of alectinib on the expression levels of cleaved PARP, BIM, MCL-1, and BAX in LC-2/ad cells. LC-2/ad cells were treated with alectinib for 24 hours at the indicated concentrations. The expression level of cleaved PARP, BIM, MCL-1, BAX, and β-actin were detected by immunoblot analysis using the appropriate antibodies.

Figure 4. Antitumor activity of alectinib against RET-fusion driven tumors. A, mice bearing LC-2/ad cells were administered alectinib or vandetanib orally at the indicated doses, once daily for 14 days. Tumor volume and changes in body weight were measured for each dose group. Data are shown as mean ± SD (n = 5 per group). Parametric Dunnett’s test: ***, p < 0.001; *, p < 0.05; N.S., not significant, versus treatment with vehicle of alectinib on the final day of the experiment. B, mice bearing Ba/F3 cells expressing KIF5B-RET were treated with vehicle, 60 mg/kg of alectinib orally once daily for 10 days. Tumor volume
was measured for each dose group. Data are shown as mean ± SD (n = 5 per group). Parametric Dunnett’s test: ***, p < 0.001, versus vehicle treatment on the final day of the experiment. C, mice bearing Ba/F3 cells expressing KIF5B-RET were orally administered a dose of 0 (vehicle) or 60 mg/kg alectinib, and the tumors were collected and lysed at 4 hours post-dosing. The expression levels of phospho-RET and RET were detected by immunoblot analysis using the appropriate antibodies.

Figure 5. Inhibition of RET gatekeeper mutants by alectinib, vandetanib, or cabozantinib. A, effect of alectinib, vandetanib, or cabozantinib against Ba/F3 cells expressing KIF5B-RET and gatekeeper mutations (V804L and V804M). Cells were seeded and then treated with various concentrations of alectinib, vandetanib or cabozantinib for 2 days. The viable cells were measured using a CellTiter-Glo® luminescent cell viability assay. IC_{50} values were determined by plotting the drug concentration versus the percentage of cell growth inhibition. Data are shown as mean ± SD (n = 3). B, effects of alectinib on the phosphorylation levels of RET in Ba/F3 cells expressing KIF5B-RET and gatekeeper mutations (V804L and V804M). Ba/F3 cells expressing KIF5B-RET were treated with alectinib, vandetanib, or cabozantinib for 2 hours at the indicated concentrations. The expression level of phospho-RET, phospho-AKT, AKT, phospho-ERK1/2, ERK1/2, and β-actin were detected by immunoblot analysis using the corresponding antibodies.
Figure 1. Kodama et al

A

![Chemical structure](image)

B

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C

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</table>
Figure 2. Kodama et al

A. LC-2/ad (CCDC6-RET)

B. Ba/F3 KIF5B-RET

Cell viability (%) vs. Drug conc. (nM)

C. Caspase 3/7 activity (fold)

D. Western blot analysis of phosphorylation levels:
   - pRET
   - RET
   - pSTAT3
   - STAT3
   - pAKT
   - AKT
   - pERK1/2
   - ERK1/2

Author Manuscript Published OnlineFirst on October 27, 2014; DOI: 10.1158/1535-7163.MCT-14-0274
Figure 3. Kodama et al

A

**DUSP2**

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**ETV5**

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**EREG**

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**FOS**

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B

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* indicates statistical significance.
Figure 4. Kodama et al

A

Tumor volume (mm$^3$)

Days after tumor implantation

Vehicle

Vandetanib 50 mg/kg

Vandetanib 60 mg/kg

Alectinib 20 mg/kg

Alectinib 60 mg/kg

B

Tumor volume (mm$^3$)

Days after tumor implantation

Vehicle

Alectinib 60 mg/kg

C

Body weight change rate (%)

Days after tumor implantation

Vehicle

Alectinib

pRET

RET

N.S.

*N.*
Figure 5. Kodama et al

A

IC_{50} (nM)

>1500 nM

KIF5B-RET
KIF5B-RET V804L
KIF5B-RET V804M

Alectinib Cabozantinib Vandetanib

B

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<td>1500</td>
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pRET

β-actin

pAKT

AKT

pERK

ERK

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Molecular Cancer Therapeutics

Alectinib shows potent antitumor activity against RET-rearranged non-small cell lung cancer

Tatsushi Kodama, Toshiyuki Tsukaguchi, Yasuko Satoh, et al.

Mol Cancer Ther Published OnlineFirst October 27, 2014.

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Author Manuscript Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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