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Development of potent ALK inhibitor and its molecular inhibitory mechanism against NSCLC harboring EML4-ALK proteins

Chung Hyo Kang [a, c], Jeong In Yun [a], Kwangho Lee [a, b], Chong Ock Lee [a], Heung Kyoung Lee [a], Chang-Soo Yun [a, b], Jong Yeon Hwang [a, b], Sung Yun Cho [a, b], Heejung Jung [a, b], Pilho Kim [a, b], Jae Du Ha [a], Jeong Hee Jeon [a], Sang Un Choi [a], Hye Gwang Jeong [c], Hyoung Rae Kim [a, **], Chi Hoon Park [a, b, *]

[a] Bio & Drug Discovery Division, Korea Research Institute of Chemical Technology, PO Box 107, Daejeon 305-600, Republic of Korea
[b] Medicinal & Pharmaceutical Chemistry, Korea University of Science and Technology, Daejeon 305-350, Republic of Korea
[c] College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea

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ABSTRACT

Here, we show the newly synthesized and potent ALK inhibitor having similar scaffold to KRCA-0008, which was reported previously, and its molecular mechanism against cancer cells harboring EML4-ALK fusion protein. Through ALK wild type enzyme assay, we selected two compounds, KRCA-0080 and KRCA-0087, which have trifluoromethyl instead of chloride in R2 position. We characterized these newly synthesized compounds by *in vitro* and *in vivo* assays. Enzyme assay shows that KRCA-0080 is more potent against various ALK mutants, including L1196M, G1202R, T1151_L1152insT, and C1156Y, which are seen in crizotinib-resistant patients, than KRCA-0008 is. Cell based assays demonstrate our compounds downregulate the cellular signaling, such as Akt and Erk, by suppressing ALK activity to inhibit the proliferation of the cells harboring EML4-ALK. Interestingly, our compounds induced strong G1/S arrest in H3122 cells leading to the apoptosis, which is proved by PARP-1 cleavage. *In vivo* H3122 xenograft assay, we found that KRCA-0080 shows significant reduction in tumor size compared to crizotinib and KRCA-0008 by 15–20%. Conclusively, we report a potent ALK inhibitor which shows significant *in vivo* efficacy as well as excellent inhibitory activity against various ALK mutants.

1. Introduction

It is quite well known that cancer target therapies produce much superior result to conventional treatments [1–3]. After the success of gleevec, enormous efforts have been performed to find a promising cancer target. Most of targets which gave successful result in clinical test are kinase, such as Bcr-Abl, EGFR, B-Raf, and ALK. Especially, ALK is the recent target which is proved to be an excellent target for the cancer target therapy.

The first paper which describes the ALK chromosomal translocation t(2; 5) (p23:q25), whose production is NPM-ALK, was published in 1994 [4]. After NPM-ALK discovery, multiple ALK fusion forms were found in a variety of cancers, including anaplastic large cell lymphoma (ALCL), inflammatory myofibroblastic tumor (IMT), diffuse large B cell lymphoma (DLBCL), breast cancer, colon cancer, and non-small cell lung cancer (NSCLC) [5]. Strikingly, these ALK fusion forms show a strong ALK autophosphorylation to have a strong transforming activity to tumorigenesis [6,7]. Tumors having ALK fusion gene exhibit 'oncogene addiction' to ALK signal pathway. Preclinical studies show the apoptosis and tumor regression by ALK inhibition in ALK fusion gene expression tumors, which demonstrate ALK as a therapeutic target [8].

EML4-ALK fusion gene is found in 2–5% of NSCLC patients, mainly in adenocarcinoma patients who are young age and never or light smokers [9]. As other ALK fusion genes, EML4-ALK show constitutive activation of ALK independent on ligand dimerization by the coiled-coil domain of EML4 to result in the downstream signaling pathways such as Jak-Stat, Erk, and PI3K-Akt pathway [10,11]. Crizotinib, the first ALK inhibitor introduced clinically,

* Corresponding author. Bio & Drug Discovery Division, Korea Research Institute of Chemical Technology, PO Box 107, Daejeon305-600, Republic of Korea.
** Corresponding author.
E-mail addresses: hyungrk@krict.re.kr (H.R. Kim), chpark@krict.re.kr (C.H. Park).

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showed a dramatic effect on the ALK rearrangement-positive patients in NSCLC. The objective response rate (ORR) is 61%, and the median progression free survival (PFS) is 9.7 months in Phase I [12]. Because of its impressive outcome, crizotinib was approved by the US FDA in August 2011. After crizotinib, it is very active to develop excellent ALK inhibitors in global pharmaceutical company.

Previously, we reported KRCA-0008 to have a tumor suppression activity in mouse xenograft model [13]. Here, we report that a new compound having similar scaffold shows potent activity *in vivo* xenograft assay and excellent inhibition effect against ALK mutants which are found in crizotinib-resistant patients compared to KRCA-0008. We also suggest its inhibitory molecular mechanism against EML4-ALK positive cancer cells.

2. Materials and methods

2.1. Compounds and reagent

KRCA-0080 and KRCA-0087 were synthesized according to procedures published in PCT/KR2013/004767. All compounds including crizotinib were dissolved in DMSO. Compounds were formulated in 20% PEG-400, 3% Tween-80, 77% DI water for all *in vivo* studies. Kinase domain of ALK is purchased from CarnaBio Science (JAPAN).

2.2. ALK in vitro enzyme assay

Experimental procedure was followed by the manufactured instruction (Cisbio, France). The reaction was initiated by ATP addition to a mixture containing the ALK enzyme, peptide substrates, and inhibitors. After 30 min, EDTA containing solution was added to stop the reaction. EDTA containing solution has Europium conjugated anti-phosphoresidue antibody and streptavidine-XL665 (SA-XL665) for the detection of the phosphorylated peptide product. After 1 h incubation, fluorescence was measured with 337 nm excitation and dual 665 and 620 nm emission of the Envision reader. IC_{50} was calculated using GraphPad Prism version 5 for Windows. The curves were fit using a nonlinear regression model with a log (inhibitor) versus response formula.

2.3. Cell culture

H3122 and Ba/F3 cells were maintained in RPMI 1640 medium supplemented with 10% FBS (HyClone, US) using a humidified incubator with 5% CO_2 at 37 °C.

2.4. Antibodies and immunoblotting

The following antibodies were obtained from Cell Signaling Technology: phospho ALK tyrosine 1604 (Catalog No. 3341), phospho-Erk threonine 202/204 (Catalog No. 4370), phospho-Akt serine 473 (Catalog No. 4060). Tubulin antibody (Catalog No. T6199) was purchased from Sigma–Aldrich. HRP-conjugated anti-mouse (Catalog No. NCI1430KR), and HRP-conjugated anti-rabbit (Catalog No. NCI1460KR) antibodies were obtained from Thermo Scientific. For immunoblotting, cells were washed in PBS, lysed in 1× sample buffer (50 mmol/L Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 3% β-mercaptoethanol), and boiled for 10 min. Lysates were subjected to SDS-PAGE followed by blotting with the indicated antibodies and detection by Western blotting substrate ECL reagent (Thermo Scientific). Images were quantified using a LAS3000 and Image Lab software.

2.5. Cell cytotoxicity assay

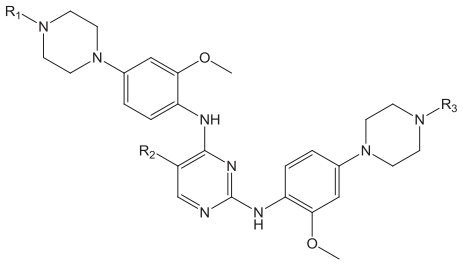
For viability experiments, cells were seeded in 96-well plates at 30% confluency and exposed to chemicals the next day. After 72 h, WST-1 reagent was added and absorbance at 450 nm was measured on a Spectramax spectrophotometer (Molecular Devices, US) according to the manufacturer's instructions. IC_{50}s were calculated using GraphPad Prism version 5 for Windows. The curves were fit using a nonlinear regression model with a log (inhibitor) versus response formula.

2.6. Cell cycle analysis

We followed the manufacturer's instruction to NucleoCounter NC-250 (chemometec, Denmark) to analyze the cell cycle distribution. Briefly, cells treated with vehicle or compounds for 24hr were suspended by lysis buffer supplemented with 10ug/ml DAPI. After 5 min incubation at 37 °C, cells were suspended by stabilization buffer. Cells were loaded into the chambers of the slide and were analyzed by NucleoCounter NC-250.

2.7. Xenograft studies

Female athymic BALB/c(nu/nu) mice (6 weeks old) were obtained from Charles River of Japan. Animals were maintained under clean room conditions in sterile filter top cages and housed on high efficiency particulate air-filtered ventilated racks. Animals received sterile rodent chow and water ad libitum. All of the procedures were conducted in accordance with guidelines approved by the Laboratory Animal Care and Use Committee of Korea Research Institute of Chemical Technology. H3122 cells (5×10^6 in 100ul) were implanted s.c. into the right flank region of each mouse and allowed to grow to the designated size. Once tumors reached an average volume of 200 mm^3, mice were randomized and dosed via oral gavage daily with 80 mpk of compounds for 35 days. Mice were observed daily throughout the treatment period for signs of morbidity/mortality. Tumors were measured twice weekly using calipers, and volume was calculated using the formula: length × $width^2$ × 0.5. Body weight was also assessed twice weekly. Statistical significance were evaluated by using Mann–Whitney U-test (a = 0.01).



	R1	R2	R3
8	COMe	Cl	COMe
80	COMe	CF_3	H
87	Me	CF_3	COMe

Fig. 1. Chemical structures of synthesized ALK inhibitors.

Table 1
IC_{50} (uM) in enzyme assays.

KRCA-	ALK WT	T1151_L1152 insT	G1202R	G1269A	F1174L	C1156Y	L1196M
8	0.0039	0.0036	0.038	0.012	0.0044	0.0036	0.018
80	0.0020	0.00058	0.014	0.0012	0.0017	0.0021	0.0092
87	0.0035	0.0019	0.012	0.0013	0.0044	0.0027	0.0089
Crizotinib	0.0063	0.030	0.052	0.074	0.0046	0.012	0.28

Table 2
IC_{50} (uM) cell cytotoxic assays.

KRCA-	H3122	Ba/F3 WT	Ba/F3 L1196M	A549	Ba/F3
8	0.037	0.015	0.089	3.0	3.5
80	0.045	0.046	0.19	>5	2.1
87	0.052	0.024	0.11	1.7	1.7
Crizotinib	0.28	0.058	0.72	1.3	1.6

3. Results

3.1. Identification of new ALK inhibitors

To develop an excellent ALK inhibitor, we synthesized the derivatives of KRCA-0008, which was proven to have anti-ALK kinase activity [13]. To improve the efficacy, we substituted trifluoromethyl (CF_3) for chloride (Cl) in R2 position (Fig. 1). Among the synthesized ones, two compounds, KRCA-0080 and KRCA-0087, show the potent inhibition against ALK WT in biochemical assay (Table 1, Supplementary Figure 1). With these compounds, we performed the ALK mutants biochemical assay, such as T1151-L1152insT, G1202R, G1269A, F1174L, C1156Y, and L1196M. These mutations have been found in crizotinib-resistant patients [14] [15]. All three compounds show superior inhibitory activity against ALK mutants to crizotinib. Regarding the L1196M, which is the most popular mutation found in crizotinib-resistant patients, the IC_{50} ratio of L1196M/WT is 3–5 in our synthesized compounds, however, the IC_{50} ratio of L1196M/WT is over 40 in crizotinib. It means that our compounds inhibit the kinase activity of ALK L1196M mutant very efficiently. Our compounds also show highly inhibitory effect compared to crizotinib against other ALK mutants as well as L1196M. In addition, the inhibitory effect against ALK mutants by KRCA-0080 is better than KRCA-0008 by 2–10 fold. We anticipate that substituted CF_3 in R2 position enhances the inhibitory activities against ALK mutants. Next, we checked the cell cytotoxic activities of these compounds. Like biochemical assay, cell cytotoxicities of our synthesized compounds are much better than crizotinib (Table 2, Supplementary Figure 2). They suppress the cell growth of H3122 selectively, which is addicted to ALK signaling, not of A549. H3122 has fusion form of ALK, EML4-ALK, whereas A549 has ALK WT. This means that our ALK inhibitors have no side effect on normal cells harboring ALK WT proteins. We also have done the cytotoxic assay with Ba/F3 cells transduced by EML4-ALK wt gene (Ba/F3 WT) or EML4-ALK L1196M mutant gene (Ba/F3 L1196M). Parental Ba/F3 cells grow dependent on IL-3 [16]. However, Ba/F3 cells transduced by EML4-ALK wt gene or EML4-ALK L1196M mutant gene grow independent on the IL-3 [17]. ALK inhibitors show low suppressive activity on the cell growth of Ba/F3 parental cells. However, Ba/F3 WT cell and Ba/F3 L1196M cell are very sensitive to ALK inhibitors as H3122 cells are. Table 2 shows that our compounds have much higher cytotoxic effect against Ba/F3 L1196M than crizotinib. Cytotoxic data show that our ALK inhibitors target the cells having ALK fusion protein specifically and have excellent effect on ALK mutants.

3.2. ALK inhibitors suppress the cellular ALK activity as to downregulate Akt and Erk signaling

To see the inhibitory effect on cellular ALK activity, western blot was done after H3122 cells were treated with inhibitors for 6hr. Fig. 2 shows that ALK inhibitors suppress the cellular ALK activity in a dose dependent manner. Our ALK inhibitors suppress the ALK auto-phosphorylation effectively compared to crizotinib. The downstream signals such as Akt and Erk, which are important for cell survival and proliferation, are also inhibited by ALK inhibitors. This data is consistent with the previous reports demonstrating that cells having ALK fusion protein are addicted to ALK activity. However, due to the poor inhibitory activity of crizotinib against ALK, crizotinib does not suppress the downstream signal efficiently. Taken together, our data show that newly synthesized compounds inhibit ALK activity efficiently to downregulate the cellular signaling such as Akt and Erk.

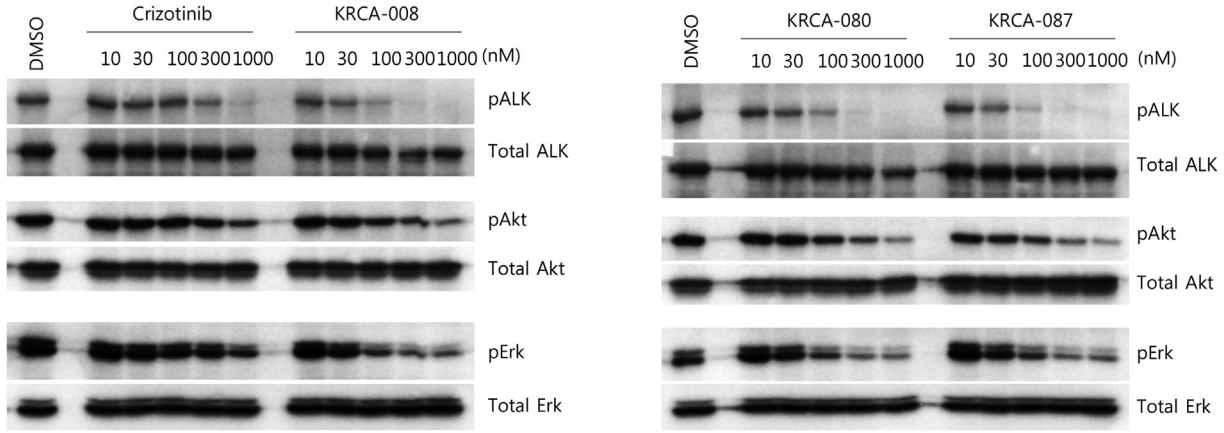


Fig. 2. ALK inhibitors suppress the downstream signals as well as ALK auto-phosphorylation. H3122 cells were treated with ALK inhibitors including crizotinib, KRCA-0008, KRCA-0080, and KRCA-0087 for 6hr. Cell lysates were subjected to western blot using antibodies against phospho-ALK, phospho-Akt, and phospho-Erk. Total ALK, Akt, and Erk was also measured by western blot.

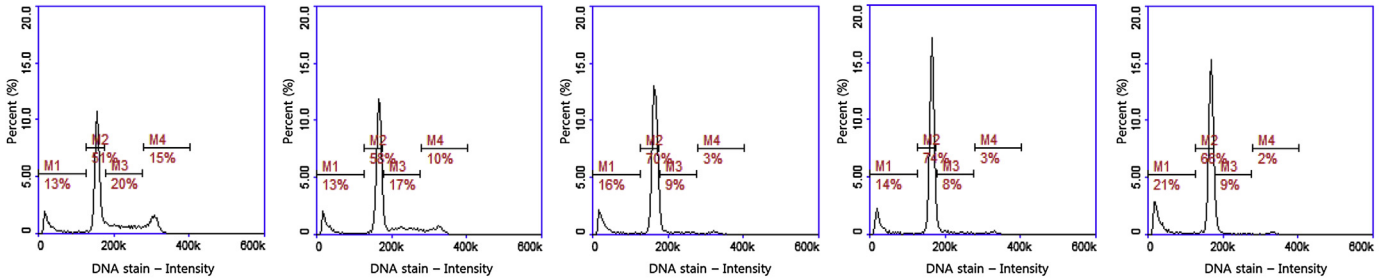
3.3. ALK inhibitors show G1/S arrest, which leads to the apoptosis

We checked the cell cycle change after ALK inhibitors treatment. H3122 cells were treated with ALK inhibitors for 18hr. As shown in Fig. 3A, ALK inhibitors induce G1/S arrest significantly in H3122 cells. Our ALK compounds are more efficient for G1/S arrest than crizotinib (Fig. 3A and C). 70% of the cells show G1/S arrest by 100 nM of our compounds, whereas 300 nM of crizotinib is required for 70% of the cells to be arrested at G1/S boundary. However, ALK inhibition doesn't induce the G1/S arrest in A549 at all (Fig. 3B and C). It means that ALK inhibitors have influence only on ALK-addicted cell lines. In addition, to see whether ALK inhibition causes apoptosis in H3122, we treated cells with ALK inhibitors for 1 day, 2 days or 3days. H3122 cells treated with ALK inhibitors show PARP-1 cleavage, which means that cells undergo apoptosis (Fig. 3D). Consistent with this data, Supplementary Figure 3 shows that 30 hr inhibitor treatment elevates the sub G1 population in H3122 cells, whereas G1 population is decreased. It means that the cells arrested at G1/S phase undergo apoptosis.

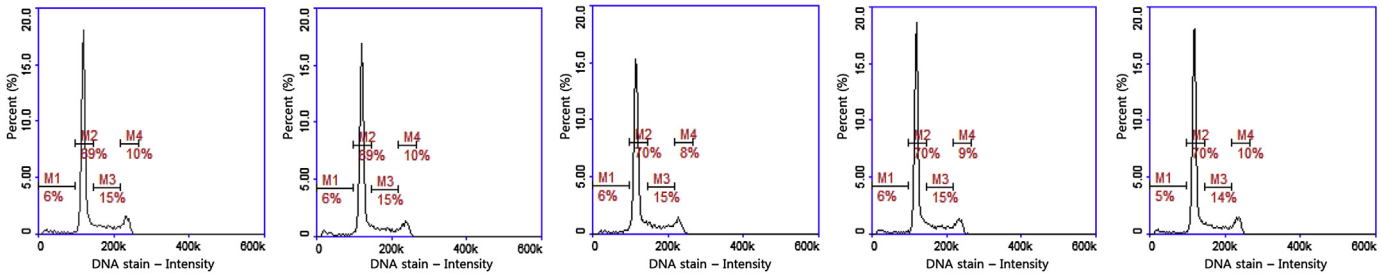
3.4. KRCA-0080 shows an excellent effect in tumor growth inhibition in mouse H3122 xenograft assay

To see the anti-tumor effect of our compounds *in vivo*, we used H3122 xenograft model. After H3122 cells were implanted on nude mouse, ALK inhibitors were started to be administered orally at

(A)



(B)



(C)

	Concentration	Sub G1 (%)	G1 (%)	S (%)	G2/M (%)	Cell
DMSO		13.35	50.9	19.81	15.17	H3122
Crizotinib	30nM	11.48	59.01	15.24	13.69	
	100nM	13.49	57.71	17.35	10.07	
	300nM	18.58	71.16	5.71	4.1	
KRCA-8	30nM	11.26	63.51	15.74	8.97	
	100nM	15.93	70.39	9.03	3.35	
	300nM	19.49	76.62	1.84	1.86	
KRCA-80	30nM	12.27	62.55	15.02	9.53	
	100nM	14.32	73.66	7.84	3.32	
	300nM	19.15	77	2.02	1.48	
KRCA-87	30nM	12.69	69.43	10.15	7.41	
	100nM	21.05	66.24	8.64	2.24	
	300nM	22.83	73.2	2.17	1.46	
DMSO		5.61	68.59	16.05	9.36	A549
Crizotinib	300nM	5.95	68.5	15.85	9.48	
KRCA-8		5.87	69.61	17.03	7.31	
KRCA-80		5.69	69.16	16.33	8.61	
KRCA-87		5.19	69.69	15.48	9.36	

(D)

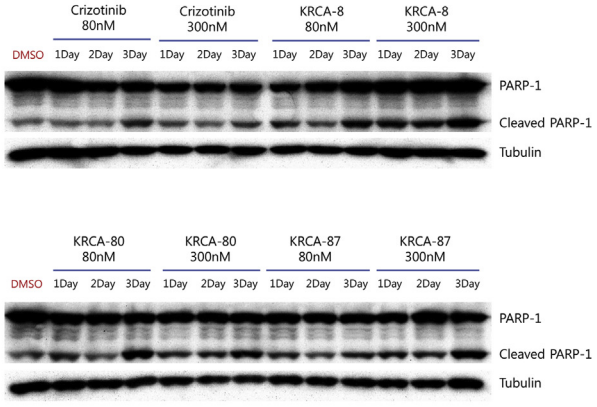


Fig. 3. ALK inhibitors induce G1/S arrest and induce the PARP-1 cleavage in ALK addicted cells. H3122 (A) and A549 (B) cells were treated with DMSO or ALK inhibitors for 18hr and 30hr, respectively. Cells were collected to be analyzed for cell cycle by NucleoCounter NC-250 instrument according to the manufacture's instruction. (C) The populations of each phase are shown. (D) H3122 cells were treated with the ALK inhibitors for 1 day, 2 day or 3 days. Cell lysates were prepared for the western blot against PARP-1 antibody. Tubulin blot indicates the equal loading.

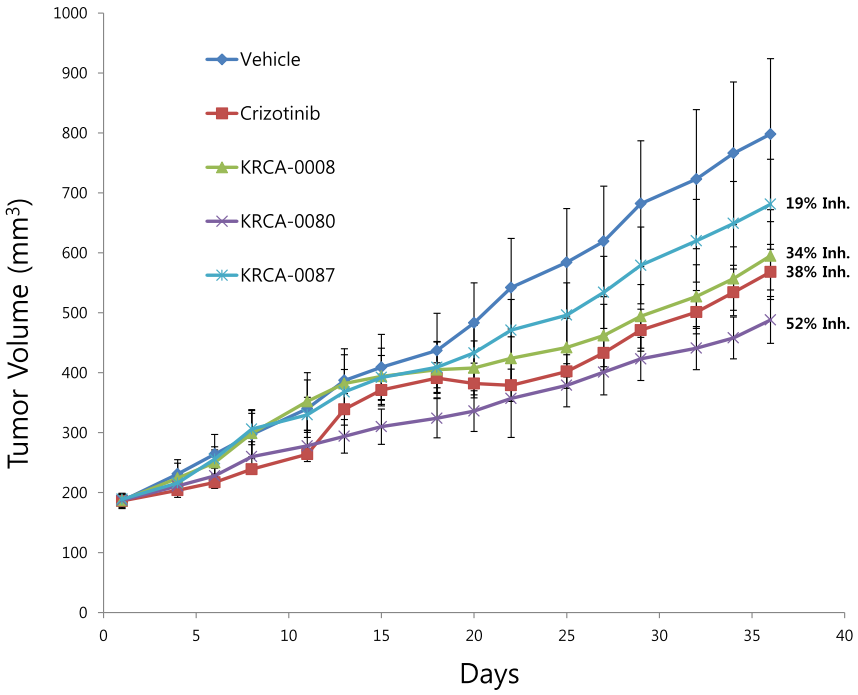


Fig. 4. *In vivo* H3122 xenograft assay. H3122 cells were implanted into the mouse and allowed to grow to the designated size. Vehicle and ALK inhibitors were orally administered to mouse daily at doses of 80 mpk. Tumor sizes were measured using calipers daily throughout the treatment period.

doses of 80 mpk daily when tumor size reaches to 200 mm^3. Tumor volumes were measured about for 35 days. Data shows that tumor volumes were significantly reduced by KRCA-0080 (Fig. 4). At 35th day, the average tumor size of KRCA-0080 administered mouse is 488 mm^3, whereas those of KRCA-0008 or crizotinib administered mouse are 595 mm^3 and 568 mm^3. It means that KRCA-0008 and crizotinib suppress the tumor growth by 34% and 38% respectively, however, KRCA-0080 suppresses the tumor growth by 52%. However, the effect of KRCA-0087 *in vivo* is worse than KRCA-0008. We checked the body weight of mouse regularly, but the loss of weight was not seen in any mouse (Data not shown). Conclusively, *in vivo* mouse model demonstrates that KRCA-0080 has superior activity against ALK to KRCA-0008 and crizotinib.

4. Discussion

Previously, our novel compound, KRCA-0008, was shown to have ALK inhibitory activity [13]. To find out better one, we synthesized dozens of compounds similar to KRCA-0008 in structure. Especially we focused on R2 position in pyrimidine, therefore we replaced chloride with trifluoromethyl group (Fig. 1). By *in vitro* enzyme assay, we selected 2 compounds among the synthesized ones, and we attempted to see their inhibitory mechanism against H3122 cell survival (Table 1, Table 2). Our compounds show highly inhibitory activity against both ALK WT and ALK mutants, including T1151-L1152insT, G1202R, G1269A, F1174L, C1156Y, and L1196M, which are found in crizotinib-resistant patients, whereas crizotinib shows poor inhibition against ALK mutants. In addition, our newly synthesized compounds, KRCA-0080 and KRCA-0087, have better activity against ALK mutants than KRCA-0008 by 2–10 fold. Because both KRCA-0080 and KRCA-0087 has CF_3 at R2 position, whereas KRCA-0008 has Cl at R2 position, we anticipate that CF_3 confers the potent inhibitory activity against ALK mutants on newly synthesized compounds (Table 1).

Not only cellular ALK activity but also cellular Akt and Erk activities are suppressed dramatically by our compounds (Fig. 2). The inhibitory activities of crizotinib against cellular ALK, Akt and Erk are very weak compared to our compounds. That's why we see poor cytotoxic effect in crizotinib treated cells unlike in our compounds treated cells. Interestingly, ALK inhibitors induced G1/S arrest in H3122 cells to result in apoptosis, which is confirmed by PARP-1 cleavage (Fig. 3D). However, there is neither cytotoxic effect nor cell cycle arrest by our compounds in A549 which has ALK wt gene (Table 2, Fig. 3). This means that our compounds are selective to ALK and have no side effect on normal cells which has ALK wt gene. Therefore, our compounds can be used safely for target therapy for ALK addicted patients. After finishing *in vitro* efficacy test, we performed *in vivo* assay to confirm if the newly synthesized compounds are better than KRCA-0008 in H3122 xenograft model. Xenograft assay shows that KRCA-0008 and crizotinib suppress the tumor growth by 34% and 38% respectively, whereas KRCA-0080 suppresses the tumor growth by 52%. However, KRCA-0087 has less tumor suppressive activity than crizotinib (Fig. 4). Although cellular phosphorylation assay and cytotoxic assay show that the cellular activities of our compounds are similar, *in vivo* efficacies of each compound are quite different.

Conclusively, we report an optimized ALK inhibitor which shows excellent *in vivo* efficacy as well as potent inhibitory activity against ALK mutants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.027.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.027.

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