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Characterization of a novel series of potent, selective inhibitors of wild type and mutant/fusion anaplastic lymphoma kinase

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Background

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase that is an oncogene in a number of cancers, including non-small cell lung cancer (NSCLC), anaplastic large cell lymphoma (ALCL), neuroblastoma and inflammatory myofibroblastic tumors (IMT). Numerous genetic aberrations at the *ALK* locus are observed in tumors including point mutations, amplifications, translocations and inversions. Inversions are exemplified by inv(2)(p21;p23), seen in ~5% of NSCLC and generates the oncogenic fusion protein, EML4-ALK. Crizotinib, a dual cMet/ALK kinase inhibitor, was recently approved by the FDA to be used to treat ALK-positive NSCLC patients with locally advanced or metastatic disease. Clinical responses were seen in >50% of ALK positive patients, validating ALK as a therapeutic target. Here we describe the pharmacological characterization of a novel series of potent, selective and orally bioavailable ALK kinase inhibitors.

Methods

Kinase inhibition assay: Kinase profiling and K_d values were determined using Kinomescan. The inhibition of protein kinases was assessed through Carna Biosciences using a mobility shift assay (MSA) or internal Amgen assays utilizing time-resolved fluorescence resonance energy transfer (TR-FRET) assays. *(Drew et al, J.Biomol.Med. 2011, 16(2), 164).*

Cell growth: Cells (10,000 per well) were plated in culture medium in 96-well plates. Each well contained 1 µL of test compound at 100X concentration in 100% DMSO, for a final incubation concentration of 1% DMSO. Cells and compound were incubated at 37°C. After 72 hours, 100 µL of CellTiter Glo™ reagent was added to each well according to the manufacturer's instructions, and plates read. IC_{50} values for compounds were calculated from the magnitude of the luminescence signal, and were expressed as the mean of three replicates.

Cellular ALK phosphorylation: Cells (250,000) and compound were incubated at 37°C for 1 hour, washed with BSA-containing stain buffer, fixed with 4% formalin for 30 minutes at room temperature, and then permeabilized overnight with ice-cold 90% MeOH at -20°C. Primary antibodies pY^{1604} ALK rabbit polyclonal antibody, obtained from Cell Signaling Technologies (Danvers, MA), and total ALK (5A4) mouse monoclonal antibody from Abcam (Cambridge, MA) were added for 1 hour at room temperature. Following a wash, fluorescein isothiocyanate- or allophycocyanin-conjugated secondary antibodies were added for 30 minutes at room temperature. Cells were finally washed and suspended in 100 µL of stain buffer. Data from 10,000 cells per well were acquired on an LSRII flow cytometer. IC_{50} values for compounds were calculated from the median fluorescence intensity and expressed as the mean of two or three replicates.

In vivo xenograft: SCID-beige mice were implanted with 0.1 ml of the Karpas-299 cell suspension by subcutaneous injection on the shaved right flank. After tumor growth, mice with well formed tumors of similar size were placed into groups so that the average tumor size was similar in each group (ranging from approximately 200-300 mm^3). The groups of mice (n = 10 per group) were orally dosed with vehicle or ALK inhibitors daily until the end of the study. Tumor volumes (length x width) were measured twice weekly using vernier calipers. Statistics were performed at the end of the study using repeated measures ANOVA and a Dunnett's *post-hoc* test. Body weights were determined twice weekly.

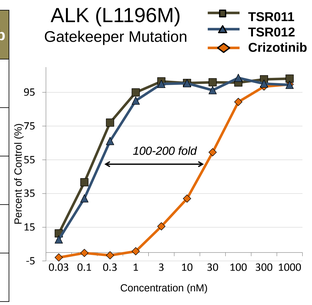
Pharmacokinetics/Pharmacodynamics: Plasma levels of the inhibitors were determined by LC/MS methods specific to each inhibitor. Tumors were collected and snap frozen at specified times points from mice orally dosed with vehicle or ALK inhibitors. Frozen established tumors were pulverized and cell pellets were suspended in a lysis buffer and then homogenized using an acoustic sonicator. The samples were then clarified by centrifugation at 14,000 rpm for 10 minutes at 4° C. The supernatant was then transferred into a new tube and protein was quantified with a BCA kit.

Enzymatic inhibition and selectivity

- TSR-011 and TSR-012 are small molecule ALK inhibitors developed using structure-based drug design.
- TSR-011 and TSR-012 exhibit strong binding to recombinant ALK and potent inhibition against all recombinant ALK mutants tested to date (only selected mutants shown).

Inhibition of ALK kinase and mutants

In vitro assay	TSR-011	TSR-012	Crizotinib
ALK IC50 nM	0.7	0.7	2.3
ALK Kd nM	0.36	0.24	3.3[a]
EML4-ALK IC50 nM	0.6	0.6	3.1
ALK (L1196M) IC50 nM	0.1	0.2	19.2
ALK (R1275Q) IC50 nM	0.5	0.6	3.7

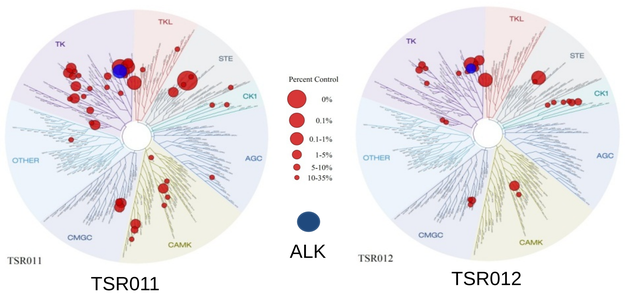


a. Davis et al, Nature Biotech, 2011, 29 (11), 1046

Selectivity Scores and Treespot Diagram of TSR-011 and TSR-012

Analysis	TSR-011	TSR-012
S(35)	0.137	0.08
total kinases tested	386	386

Selectivity (S35) =
bound kinases POC<35%
kinases tested POC<35%



TSR011 TSR012

Cellular and *in vivo* efficacy

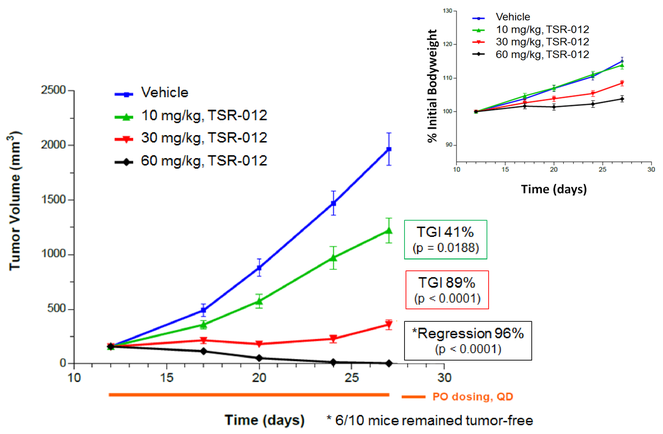
- TSR-011 and TSR-012 potently inhibit cell proliferation and ALK autophosphorylation in ALK-driven cell lines.
- A representative Karpas 299 *in vivo* xenograft model shows potent tumor growth inhibition and regression after daily oral dosing of TSR-012.

Comparison of ALK inhibition in cell lines

ALK Status	Cell Type (model)	Assay[a]	TSR-011 IC50 (nM)	TSR-012 IC50 (nM)	Crizotinib IC50 (nM)
NPM-ALK Translocation	Karpas299 (ALCL)	pY-ALK	2	2	69[b]
		proliferation	1	3	16[b]
	Sup-M2 (ALCL)	pY-ALK	2	ND	ND
		proliferation	4	ND	ND
EML4-ALK translocation	H3122 (NSCLC)	pY-ALK	1	6	108
		proliferation	1	2	25
ALK amplification	NB-1 (neuroblastoma)	pY-ALK	11	ND	ND
		proliferation	10	ND	ND
ALK negative	HT	proliferation	> 1000	> 1000	>1000

a. ALK autophosphorylation (pY) was determined by measuring pY^{1604}
b. Similar to data published in Christensen et. al , Mol. Cancer. Ther., 2007, 6, 3314
ND = not determined

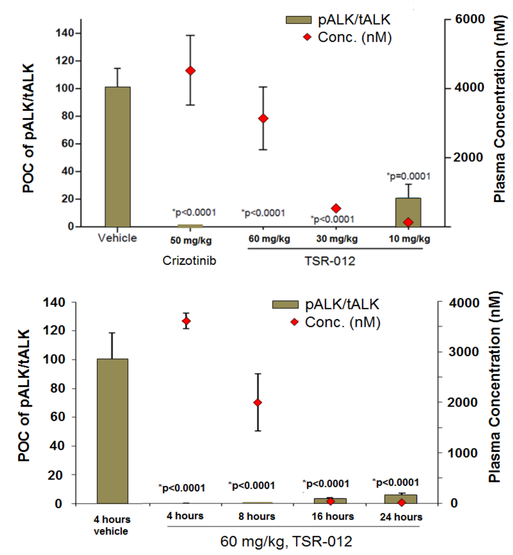
Representative *in vivo* efficacy of TSR-012 in Karpas 299 xenografts



PK/PD

- Complete inhibition of pY^{1604} ALK in Karpas 299 tumors (8 hrs post dose) by TSR-012 was seen at doses of 30 mg/kg.
- A single 60 mg/kg dose of TSR-012 was able to maintain >90% ALK inhibition in Karpas 299 tumors up to 24 hours post-dose, indicating that regression is associated with long term ALK inhibition.

Inhibition of ALK in tumors vs plasma concentration



POC= percent in control
a. Plasma concentrations and ALK inhibition similar to previously published data (Yamazaki et al, J. Pharmacol. Exp. Ther. 2012, 340(3), 549)

Conclusions

- The described compounds are very potent inhibitors of ALK kinase and retain potency across the range of mutant variants described to date, including clinically observed resistance mutations.
- These inhibitors show excellent cellular and *in vivo* activity against ALK-driven tumors.
- Significant ALK inhibition in tumors is maintained well beyond the compound's clearance from plasma.
- These features indicate the potential for significant advantages, including a longer duration of response and the retention of potency against mutant variants with resistance.

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