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Discovery of a Potent Inhibitor of Anaplastic Lymphoma Kinase with in Vivo Antitumor Activity

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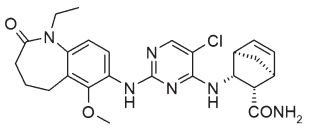
ABSTRACT A series of novel 7-amino-1,3,4,5-tetrahydrobenzo[*b*]azepin-2-one derivatives within the diaminopyrimidine class of kinase inhibitors were identified that target anaplastic lymphoma kinase (ALK). These inhibitors are potent against ALK in an isolated enzyme assay and inhibit autophosphorylation of the oncogenic fusion protein NPM-ALK in anaplastic large cell lymphoma (ALCL) cell lines. The lead inhibitor **15**, which incorporates a bicyclo[2.2.1]hept-5-ene ring system in place of an aryl moiety, activates the pro-apoptotic caspases (3 and 7) and displays selective cytotoxicity against ALK-positive ALCL cells. Furthermore, **15** provides more than 40-fold selectivity against the structurally related insulin receptor, is orally bioavailable in multiple species, and displays in vivo antitumor efficacy when dosed orally in ALK-positive ALCL tumor xenografts in Scid mice.

KEYWORDS Anaplastic lymphoma kinase inhibitor, ALK, anaplastic large cell lymphoma, ALCL



15

Approximately 60% of human anaplastic large cell lymphoma (ALCL) cases are associated with a t(2;5)(p23;q35) chromosome translocation.[1,2] The t(2;5) translocation contains the N-terminal portion of nucleophosmin (*NPM*) gene, a nuclear phosphoprotein, fused to the catalytic domain of anaplastic lymphoma kinase (*ALK*) gene. The *NPM-ALK* fusion gene encodes for an 80 kDa NPM-ALK chimeric oncoprotein with constitutively active ALK tyrosine kinase activity, which plays a key role in lymphomagenesis by aberrant phosphorylation of intracellular substrates.[1,2] This activity is directly implicated in the pathogenesis of ALCL, and inhibition of ALK could markedly impair the growth of ALK-positive lymphoma cells.[1−5] Furthermore, various isoforms of a fusion gene comprised of portions of the echinoderm microtubule-associated protein-like 4 (*EML4*) gene and the *ALK* gene were identified in NSCLC cells.[6] The EML4-ALK fusion transcript was detected in approximately 3−7% of NSCLC patients examined.[6] Experimental evidence from in vitro and in vivo studies demonstrated oncogenic transforming activity of the EML4-ALK fusion proteins and reinforced the pivotal role of EML4-ALK in the pathogenesis of NSCLC in humans.[6] Moreover, recent studies have implicated various mutations of the *ALK* gene in both familial and sporadic cases of neuroblastoma.[7] ALK mutations in neuroblastoma cells resulted in constitutive ALK phosphorylation and attenuation or inhibition of ALK by siRNA, and small-molecule ALK inhibitors resulted in profound growth inhibition in those cell lines.[7]

Because of the strong link of aberrant expression and activation of ALK with the onset and progression of ALK-positive cancers, inhibition of ALK with a selective small molecule represents a potentially viable enhancement to the clinical outcome of patients with well-defined ALK-mediated cancers. As a result, interest in small-molecule ALK inhibitors has surged, and a plethora of unique chemical scaffolds (Figure 1) have been disclosed including indolocarbazole (**1**),[3] tetrahydropyrido[2,3-*b*]pyrazine (**2**),[8] pyridone (**3**),[9] dianilinopyrimidine (**4**),[4] 1*H*-pyrrolo[2,3-*d*]pyrimidine (**5**),[10] and aminopyridine (**6**)[5] pharmacophores.[11−13]

As noted, ALK is a member of the insulin receptor (IR) family, displaying high sequence homology (>90%) to IR within the kinase catalytic domain. The biological implications of inhibiting IR with a small-molecule kinase inhibitor have been sporadically reported, although recent reports have demonstrated compound-dependent fluctuation in glucose homeostasis in preclinical animal studies.[10,14,15] Therefore, obtaining adequate selectivity may provide a more favorable safety profile. Herein, we report the identification of a novel ALK inhibitor that is potent in enzymatic and cellular assays and displays >40-fold selectivity against the structurally related IR. Furthermore, this lead inhibitor induces pro-apoptotic caspase activation and displays selective cytotoxicity against ALK-positive ALCL cells. Acceptable oral bioavailability in multiple species was realized along

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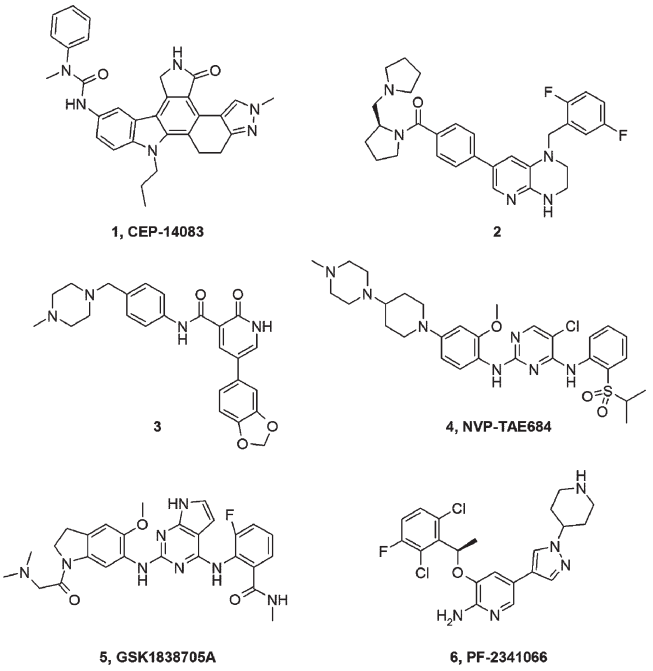
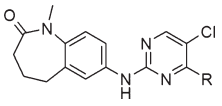


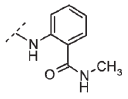
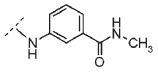
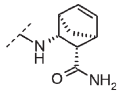
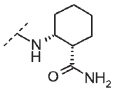
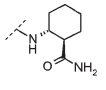
Figure 1. Small-molecule ALK inhibitors.

with in vivo antitumor efficacy when dosed orally in ALK-positive ALCL tumor xenografts in Scid mice.

In our current pursuit of small-molecule ALK inhibitors, we identified the novel 7-amino-1,3,4,5-tetrahydrobenzo-[*b*]azepin-2-one derivative **7** (Table 1) within the dianilinopyrimidine class that displayed potent activity against ALK (IC_{50} = 15 nM) with only a 1.5-fold margin against IR. Importantly, inhibition of NPM-ALK autophosphorylation in ALK-positive ALCL cells (Karpas-299) was quite robust (IC_{50} = 30 nM). Early structure−activity relationships (SAR) identified the benzamide functionality as a key potency determinant for ALK. Shifting the benzamide one carbon to provide **8** resulted in substantial loss in ALK potency, whereas the introduction of a 2-amino-*N*-methylsulfonamide (**9**) provided a boost in ALK activity (IC_{50} = 1 nM), although only a slight improvement to cellular activity (IC_{50} = 20 nM). IR selectivity for **9** increased to 10-fold. Surveying several kinase active saturated/partially saturated 2-amino-carboxamide substructures[16,17] to gauge the impact on potency and selectivity, the aryl ring was replaced with *cis*-3-amino-bicyclo[2.2.1]hept-5-ene-2-carboxamide (cf. **10**), which resulted in a slight drop-off in ALK activity and only 5-fold IR selectivity, although acceptable ALK cell translation was noted. *cis*- and *trans*-2-Aminocyclohexanecarboxamide derivatives **11** and **12** provided no advantage. Early screening for pharmacokinetic (PK) parameters identified

Table 1



Ex.	R	ALK IC_{50}[a] (nM)	Karpas-299 CELL IC_{50}[b] (nM)	IR IC_{50}[a] (nM)	IR/ALK	Rat F%
7		15	30	23	1.5	9
8		568	NT	247	0.4	NT
9		1	20	10	10	11
(±)-10		18	100	91	5	13
(±)-11		180	1000	319	1.8	NT
(±)-12		2139	NT	2012	0.9	NT

[a] IC_{50} values are reported as an average of ≥3 determinations; for values ± SD, see the Supporting Information; NT, not tested. [b] IC_{50} values are reported as a mean of at least two determinations.

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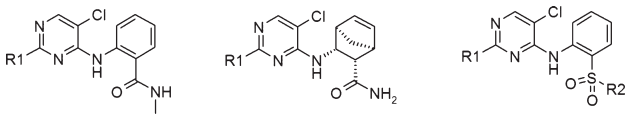
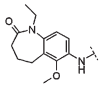
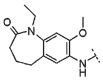
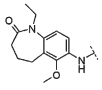
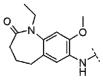
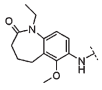
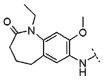
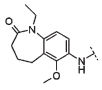
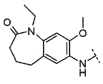
Table 2



Ex	R1	Scaffold	R2	ALK IC_{50}[a] (nM)	Karpas-299 CELL IC_{50}[b] (nM)	IR IC_{50}[a] (nM)	IR/ALK
13		A	--	11	200	215	20
14		A	--	5	40	34	6.8
15		B	--	14	45	597	43
16		B	--	7	40	89	13
17		C	-NMe_2	8	65	71	8.9
18		C	-NMe_2	4	30	37	9.2
19		C	-*i*-Pr	5	50	32	6.4
20		C	-*i*-Pr	2.7	30	12	4.4

[a] IC_{50} values are reported as an average of ≥ determinations; for values ± SD, see the Supporting Information. [b] IC_{50} values are reported as a mean of at least two determinations.

members of this series displaying modest oral bioavailability; **7**, **9**, and **10** displayed 9, 11, and 13% oral bioavailability in rat, respectively.

On the basis of the promising SAR with respect to enzymatic ALK activity, cellular inhibition, and modulation of IR activity, we sought to further optimize IR selectivity and improve upon the modest oral bioavailability on the 2-aminobenzamide, 2-aminobenzenesulfonamide, and the 3-aminobicyclo[2.2.1]hept-5-ene-2-carboxamide scaffolds through modification of the 7-amino-1,3,4,5-tetrahydrobenzo[*b*]azepin-2-one. Toward this end, while maintaining acceptable potency, we identified the ethyl substituent on the azepinone nitrogen for improved PK parameters and *ortho*-methoxy substituents[18] at either the 6-position or the 8-position of the benzazepinone as preferable for selectivity. These structural modifications were combined with the 5-chloro-4-aminopyrimidine scaffolds A, B, and C (Table 2). The 6-methoxy derivative **13** and the 8-methoxy derivative **14** both provided acceptable ALK potency with **14** having about 2−5-fold improved activity relative to **13**, at the expense of IR selectivity. This trend was apparent for all scaffolds tested. Importantly, **13** displayed 20-fold separation of ALK versus IR, an improvement relative to parent **7**. Incorporating these 6-methoxy- and the 8-methoxybenzazepinones on the single isomer (1*S*,2*S*,3*R*,4*R*)-aminobicyclo-[2.2.1]hept-5-ene-2-carboxylic acid amide scaffold B[19] gave **15** and **16**. Although **16** was 2-fold more potent in the isolated

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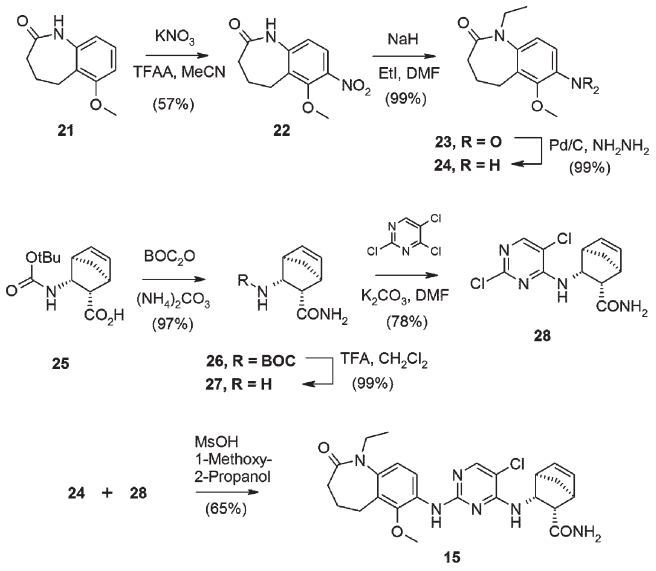
Scheme 1. Synthesis of Compound **15**



enzyme assay, near equivalent results were obtained in the cell assay.[20] Importantly, compound **15** displayed improved IR selectivity to that observed for compound **13**. Surveying a series of sulfonamide derivatives (**17**−**20**), low nanomolar ALK inhibition could be achieved with potent activity in cellular inhibition of NPM-ALK autophosphorylation. Unfortunately, improved IR selectivity was not obtained with these modifications with margins ranging from 4-fold for **20** to 9-fold for **17** and **18**. On the basis of these results, compound **15** displayed the most promising balance between ALK activity (enzyme and cells) and IR activity. Furthermore, when assessed for PK parameters, **15** displayed favorable attributes (vide infra).

The synthesis of compound **15** followed the sequence outlined in Scheme 1. Nitration of benzo[*b*]azepinone **21**[21] controlling the stoichiometry of the nitrating reagent[22] readily gave the desired product as a mixture of nitro regiomers separable by silica gel chromatography. Alkylation of the azepinone nitrogen gave **23**. Reduction provided 7-aminobenzo[*b*]azepinone **24**. The β-aminoamide derivative was prepared from (1*S*,2*S*,3*R*,4*R*)-3-methoxycarbonylaminobicyclo[2.2.1]hept-5-ene-2-carboxylic acid **25**.[23] Formation of a mixed anhydride and displacement with ammonia provided the amide **26**. Deprotection of the amine to give **27** was followed by a highly convergent assembly of the target. The addition of **27** to 2,4,5-trichloropyrimidine provided **28**, which was then treated with **24** using an acid-mediated addition protocol to provide **15** in 65% yield.

The effects of **15** on the proliferation and survival of ALK-positive ALCL cells (Karpas-299 and Sup-M2) and ALK-negative cells (K562, a leukemia cell line) in culture were evaluated by MTS and caspase 3/7 activation assays. Compound **15** displayed concentration-dependent growth inhibition of Karpas-299 and Sup-M2 cells in culture, with the extent of growth inhibition consistent with cellular inhibition of NPM-ALK phosphorylation in these cells (Supporting Information, Figure 1A). Because of extremely high levels of NPM-ALK expressed in ALCL cells, at least 80−90% inhibition of NPM-ALK phosphorylation in cells is required to induce measurable cytotoxicity and caspase activation.[3] Therefore, the calculated cytotoxicity EC_{50} values (~200−267 nM) are close to the cellular IC_{80-90} values. In contrast, **15** had minimal growth inhibition on ALK-negative K562 cells at concentrations up to 1 μM (Supporting Information, Figure 1A), suggesting that at the concentrations studied, **15** exerts growth inhibition on ALK-positive ALCL cells primarily through inhibiting NPM-ALK activity. The in vitro cytotoxicity of **15** in cells was also assessed via measurement of caspase 3/7 activity. Treatment of ALK-positive cell lines with **15** led to concentration-related caspase 3/7 activation (Supporting Information, Figure 1B). In contrast, no caspase 3/7 activation was detected in K562 cells treated with **15**. These data indicate that **15** exerts in vitro cytotoxicity on ALK-positive ALCL cells mainly through inhibiting NPM-ALK activity. As a further assessment of selectivity, compound **15** was profiled in the Ambit KINOMEscan against 402 kinases.[24] At screening concentrations of 1 and 0.1 μM (roughly 100- and 10-fold above the ALK IC_{50}), selectivity scores for 90% inhibition [S(90) values][24,25] of 0.090 and 0.015, respectively, were observed suggesting that off-target kinase activity is minimal and that the induction of pro-apoptotic proteins and antiproliferative effects of **15** is primarily driven by inhibition of ALK.

Table 3. PK Parameters of **15**[a]

PK parameters	Scid mouse	rat	dog
	iv		
dose (mg/kg)	1.0	1.0	1.0
$t_{1/2}$ (h)	0.3	0.8	1.1
Cl (mL/min/kg)	37	13	22
% Cl/hepatic blood flow	41	20	58
V_d (L/kg)	0.9	0.9	2.1
AUC (ng h/mL)	446	1276	773
	po		
dose (mg/kg)	10.0	5.0	10
t_{max} (h)	0.25	0.8	1.1
$t_{1/2}$ (h)	2.4	2.2	1.6
AUC (ng h/mL)	1406	4088	2482
F (%)	32	64	31

[a] Determinants of three for each dosing group, average value; for values ± SEM, see the Supporting Information.

Compound **15** was profiled in three species for PK parameters (Table 3). Following iv dosing, clearance in Scid mouse (37 mL/min/kg) was 41% of hepatic blood flow,[26] while the clearance observed in rat was more moderate at 20% of liver blood flow. Clearance in dog was the highest as a percentage of hepatic blood flow (58%). The volume of distribution was near unity for rodents with a higher value observed in dogs. Following oral dosing, rapid absorption was observed in Scid mouse with a more extended t_{max} for rat and dog. Oral bioavailability was 2-fold higher in rats than dog or Scid mice. On the basis of PK, potency, and selectivity, **15** advanced to in vivo PK/pharmacodynamic (PD) and antitumor xenograft efficacy studies.

Following oral administration of **15** (30 mg/kg) in vehicle (PEG400), inhibition of NPM-ALK autophosphorylation in tumor lysates from ALK-positive ALCL tumor xenografts

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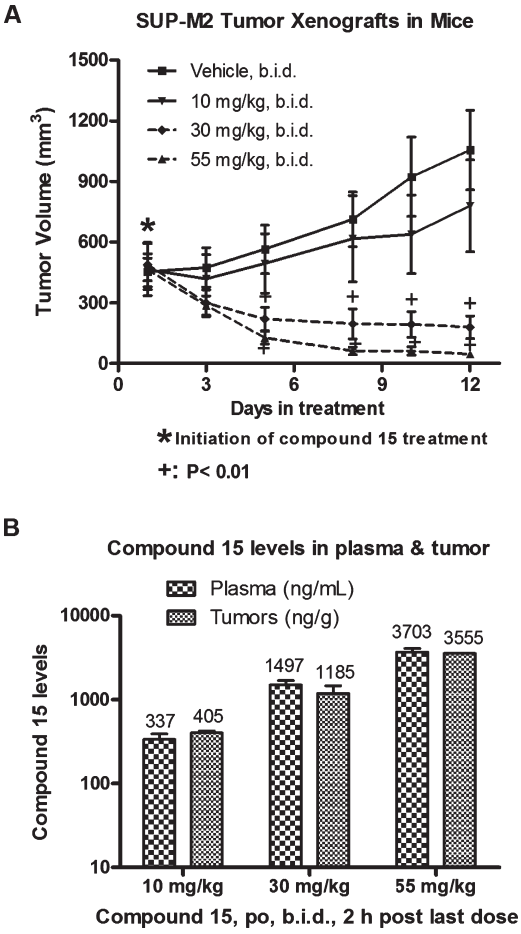

Figure 2. (A) In vivo antitumor efficacy of **15** following oral dosing in SUP-M2 tumor xenografts. (B) Compound levels in plasma and tumor lysates at 2 h postfinal dose.

(SUP-M2) in Scid mouse was detected. Significant (>80%) target inhibition in tumor xenografts up to 12 h was observed postsingle oral administration; compound levels in tumor roughly paralleled levels in plasma albeit about 1.3−2.3-fold lower (Supporting Information, Figure 2). The calculated cellular IC_{50} of **15** in the presence of murine plasma is shifted ~5-fold [IC_{50} = 225 nM (112 ng/mL)], attributed to the effect of plasma protein binding (92%). This value is an approximation of the in vivo IC_{50} and consistent with the extent of target inhibition/compound levels detected in tumors. These results suggested that with daily b.i.d. dosing at 30 mg/kg or higher, sustained target inhibition would be obtained in tumor xenografts, which is necessary to achieve in vivo efficacy (i.e., tumor regressions).[5]

Thus, antitumor efficacy studies were conducted with **15** at 10, 30, and 55 mg/kg, po, b.i.d. (Figure 2A). Dose-dependent antitumor activity was observed with no significant antitumor efficacy following 12 days of treatment at the 10 mg/kg dosing regimen, with 100% partial tumor regressions at 30 mg/kg po, b.i.d., and complete/near complete tumor regressions at 55 mg/kg, po. The compound was well tolerated with no overt toxicity and no significant body weight loss at all doses (Table 4 in the Supporting Information). Dose-related levels of **15** were found in plasma and tumor lysates collected at 2 h post final dose (Figure 2B). Relative to the single dose PK/PD study, an approximately 2-fold increase in compound levels was observed in both plasma and tumors with 12 days of b.i.d. dosing. The result is consistent with the observation that the antitumor efficacy in this model is an area under the curve (AUC) driven event, in which sustained target inhibition is required to achieve significant tumor growth inhibition and tumor regression.

In summary, novel small-molecule inhibitors of ALK within the diaminopyrimidine class have been identified. Optimization of potency, off-target selectivity, and PK parameters led to the identification of compound **15**, which displayed favorable potency in an ALK enzymatic assay as well as a cellular assay measuring oncogenic NPM-ALK autophosphorylation. Furthermore, oral administration of compound **15** to mice bearing ALK-positive ALCL sc xenografts resulted in complete/near-complete regressions. Compound **15** advanced to late preclinical candidate evaluation, benchmarking the program with a defining tool compound. Further optimization of this structural class will be reported in future publications.

SUPPORTING INFORMATION AVAILABLE

Procedures for the preparation of **15**, analytical data for compounds **7**−**20**, and procedures for in vitro and in vivo assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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