Metabolism-Based Structure Optimization: Discovery of a Potent and Orally Available Tyrosine Kinase ALK Inhibitor Bearing the Tetracyclic Benzo[b]carbazolone Core

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ABSTRACT

A metabolism-based fine-tuning structure-optimization was conducted to address the oxidative metabolism and hERG blockade of our early ALK inhibitor. Compound **8** was identified showing high potency against both ALK wild type and gatekeeper mutant. In addition to the optimal PK properties and significant cell antiproliferative effects, **8** showed complete tumor growth inhibition at doses of 50 or 10 mg/kg once daily in the Karpas299 xenograft model. All these results encouraged the further development of **8** as a potent and orally bioavailable ALK inhibitor.

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The anaplastic lymphoma kinase (ALK) protein, encoded by the ALK gene is a transmembrane tyrosine kinase. Although its normal function has not been fully understood and high expression of ALK only exists in children's nervous systems, gene fusions determining the aberrant expression of ALK proteins have been found widely existing in tumors¹⁻³. Concerning non-small cell lung cancers (NSCLC), the fusion of ALK with the partner gene - echinoderm microtubule-associated protein-like 4 (EML4) represents one of the most frequent genetic alternations, accounting for 3-5% patient populations⁴. Since its recognition as a tumor-driven gene in 2007⁵⁻⁷, EML4-ALK has become an attracting drug targets, and the first-in-class ALK inhibitor crizotinib (**1**) has been approved by the FDA in 2010 for NSCLC patients whose tumors harbor ALK rearrangements.^{8,9} In spite of the remarkable clinical response, most ALK-rearranged tumors eventually acquire resistance within one year since the initiation of crizotinib treatment.^{10,11} Crizotinib (**2**) and alectinib (**3**) are the newly approved second-generation ALK inhibitors (Figure 1) with the capacity of overcoming acquired crizotinib-resistance induced by the mutated ALK, especially the gatekeeper mutation L1196M.¹²⁻¹⁵

Figure 1. Representative ALK inhibitors.

In addition, a number of other small molecules, including brigatinib (**4**)¹⁶⁻¹⁸, lorlatinib (**5**)^{16,19,20}, and entrectinib (**6**)²¹ are also under extensive clinical trials with high potency against both ALK and its active mutants.

Structurally, most of the reported ALK inhibitors belong to aminopyridines (**1** and **5**) and 2,4-aminopyrimidines (**2** and **4**). Benzo[b]carbazolone **3** and indazole **6** represent two different types of structures, especially the benzo[b]carbazolones that are not frequently used as templates of TK inhibitors and only limited structure-activity relationship studies have been reported.²²⁻²⁴

To ultimately explore the structure variations of benzo[b]carbazolones as ALK inhibitors,²⁵⁻²⁸ we recently conducted a structural optimization by introducing a rotatable C8-side chain to the tetracyclic center that led to the potent ALK inhibitor **7**^{27,28} (SOMCL-13-93) (Figure 2), which showed high potency against both wild ALK and L1196M gatekeeper mutant with IC₅₀ values of 3.4 and 3.9 nM, respectively. Compound **7** is also highly potent to inhibit the proliferation of ALK-dependent lung cancer H3122 cells and showed >100 % tumor growth inhibition (TGI) in the NIH-3T3 xenografts at the dose of 20.0 mg/kg.²⁸ Unfortunately, further development of this compound was halted due to its metabolic instability and significant inhibition to the hERG K⁺ channels (2.65 μM), an indication of potential cardiac toxicity. The rat liver microsomal study of **7** indicated that oxidations at C1', C6', and C8'-positions are the major metabolic sites, leading to several oxidative products as the major metabolites (Figure 2). To address these issues related to metabolic instability and hERG inhibition, a fine-tuning structure-optimization was recently conducted by our group that rapidly delivered a potent and metabolically stable ALK inhibitor **8** (SOMCL-13-456). Herein, we report our structural optimization process and further profiling of **8** (Figure 2).

Figure 2. Known ALK inhibitors and our new lead compound 8.

As shown in Figure 3, we first prepared and evaluated the major metabolite of **7**, the C6'-oxidized product **9** (SOMCL-13-323). Interestingly, compared to the parent compound **7**, compound **9** showed nearly 5- and 25-fold lower potency against ALK and L1196M mutant. However, its blockade on hERG was much reduced (12.15 μM), which may be partially due to the reduced lipophilicity (cLogP = 4.38). Further blocking the C1'-

oxidation by replacement of the piperidine ring with the piperazine ring and introducing C9-ethyl both to spatially block the metabolic oxidation of phenylpiperazine (forming 1,4-diiminoquinone) component and to increase kinase selectivity. Meanwhile, morpholinyl was also replaced by either hexyl or piperidinyl. The resulting compounds **10** and **11** both retained high potency against wild ALK, but the potency to inhibit the proliferation of H3122 cells was reduced (> 50 nM). Introducing two methyls to block the C8'-oxidation was unsuccessful as well and the corresponding compound **12** showed reduced cellular potency and much higher lipophilicity (CLogP = 5.96). Finally, compound **8** bearing a flexible 4-((4-hydroxytetrahydro-2H-pyran-4-yl)methyl)piperazine-1-yl side chain turned out to be the optimal compound. It retained high potency both in the ALK biochemical and cellular antiproliferative assays. Compared to our early lead **7**, compound **8** showed lower lipophilicity (CLogP = 4.92) and much reduced hERG inhibition (33.4 μM). A similar rat liver microsomal assay indicated that compound **8** was much stable than compound **7** remaining as the major product after 3 h incubation. The major metabolite was acid **13** (<2%), a double oxidized product. We then prepared metabolite **13** and found that it only retained good potency against ALK but not in the cell growth inhibition (> 50 nM). Therefore, compound **8** was selected as a new lead for further profiling.

Figure 3. Metabolism-based structure optimization on lead compound 2.

Since overcoming ALK clinical resistant mutants is the feature of second- or third-generation ALK inhibitors, we tested our lead compound **8** on the kinase activity of well-established gatekeeper L1196M ALK mutant^{10,11}. As shown in Table 1, **8** showed high potency against the gatekeeper L1196M with an IC₅₀ of 4.2 nM. Then, the inhibitory activity of lead **8** against a panel of our in-house tyrosine kinases was investigated (Table 1). In contrast to its high potency against ALK (1.5 nM), **8** barely inhibited the kinase activity of 11 other tested TKs, indicating compound **8** is a relatively selective second-generation ALK inhibitor with the capacity to block both wild and the gatekeeper mutated ALK.

Table 1. Kinase Selectivity Profile of Compound **8**

Kinase	8 (IC ₅₀ , nM)
Wild-type ALK	1.5±0.1
ALK L1196M	4.2±1.7
KDR	> 1000
PDGFR-α	> 1000
PDGFR-β	> 1000
IGFIR	> 1000
EGFR	> 1000
ErbB2	> 1000
ErbB4	> 1000
ABL	> 1000
EPH-A2	> 1000
c-Src	> 1000
c-Kit	> 1000

The IC₅₀ values are shown as the mean ± SD (nM) or estimated values from two separate experiments.

To further evaluate the cellular activity of compound **8**, the inhibitory effects on the ALK phosphorylation and its downstream signaling were tested in ALK-addicted NCI-H3122 cell lines. As shown in Figure 4, **8** dose-dependently inhibited the phosphorylation of ALK and the ALK pathway downstream effectors, including p-STAT3, p-AKT and p-ERK, confirming that **8** could suppress ALK signaling.

Figure 4. Compound **8** suppressed ALK phosphorylation and downstream signaling in ALK-addicted cancer cells. NCI-H3122 cells were treated for 1 h with **8** at the indicated concentrations, and then were lysed and subjected to Western blot analysis.

Since activation of ALK triggers cancer cell proliferation, we then set to investigate the inhibitory effects of **8** on cell proliferation in several ALK-addicted human cancer cell lines. As shown in Table 2, **8** significantly inhibited cell proliferation of ALK constitutively activated SU-DHL-1, Karpas299 and NCI-H3122 cells that cover the frequently occurring oncogenic forms of ALK (EML4-ALK or NPM-ALK fusion protein). The IC₅₀ values were 16.7, 22.6 and 24.4 nM, respectively, which were more potent than crizotinib (Table 2).

Table 2. Inhibitory Effect of Compound **8** on Cell Proliferation

Cell Line	IC ₅₀ (nM)	
	8	crizotinib
SU-DHL-1	16.7±1.1	92.3±14.9
Karpas299	22.6±1.3	104.9±3.9
H3122	24.4±6.9	100.9±22.5

The IC₅₀ values are shown as the mean ± SD (nM) or estimated values from two separate experiments.

Since **8** possessed high ALK enzymatic and cellular potencies and was also effective in blocking the ALK resistant mutants, we proceeded to evaluate its antitumor efficacy *in vivo*. The Karpas299 xenograft model, which was specifically driven by constitutively active ALK, was used^{25,28} (Figure 5). Compound **8** was administered orally as its hydrochloride salt with 0.5% CMC-Na (sodium carboxymethyl cellulose) at doses of 50 or 10 mg/kg once daily for 14 consecutive days. Compared with the vehicle treatment, **8** significantly inhibited tumor growth at both doses with tumor growth inhibitory rates more than 100% ($p < 0.001$). Moreover, complete tumor regression was also observed in the 8-treated group. At all doses tested, **8** were well tolerated with no observatory weight loss.

Figure 5. The inhibitory effect of **8** on tumor growth in the Karpas299 xenograft model. **8** was administered orally once daily for 14 days after the tumor volume reached 150 to 180 mm³. The results are expressed as the mean±SE (n = 6 in treated group, n = 12 in vehicle group). *** $P < 0.001$ vs vehicle group, determined using Student's t test.**Table 3.** Pharmacokinetic Parameters of **8** in Sprague-Dawley (SD) Rats.^a

iv	Dose (mg/kg)	5
	t _{1/2} (h)	5.5 ± 0.5
	AUC _{0-∞} (ng.h/mL)	6640 ± 1132
	Vd (L/Kg)	5.0 ± 1.1
	CL (mL.min ⁻¹ kg ⁻¹)	0.73 ± 0.11
po	Dose (mg/kg)	10
	C _{max} (ng/mL)	867 ± 92
	T _{max} (h)	4.0 ± 1.4
	AUC _{0-∞} (ng.h/mL)	11737 ± 1675
	F (%)	105.0

^aValues ± SEM are the mean from three animals.

Meanwhile, the pharmacokinetic (PK) parameters of **8** in Sprague-Dawley (SD) rats were also tested. As shown in Table 3, **8** showed a relatively long half-life time (5.5h), high plasma exposure (11737 ng.h/mL, po at 10 mg/kg), and low clearance (0.73 mL.min-1kg-1), confirming its optimal PK profile. Moreover, **8** has an excellent oral bioavailability of > 100%.

The synthesis of compounds **8-13** was outlined in Scheme 1. Commercially available **14a-d** were used as the starting materials to access intermediates **15a-d** by following literature procedures.²⁹ Treating **16** with appropriate oxaspiro intermediates **15a-d** in the presence of DIPEA with or without *N*-deprotection with TFA gave the compounds **8-12** in 53-70% yields. In addition, intermediate **17** was prepared from but-2-yne-1,4-diol in four steps in 5% overall yield. Treating **17** with **16** in the presence of K₂CO₃/KI provided precursor **18**, which was reacted with CsF in DMF followed by reaction with KOH to afford the desired product **13** in 4% overall yield.

Scheme 1. Synthesis of compounds **8-13**.

In conclusion, we have conducted a fine-tuning structure-optimization on our early ALK inhibitor to address its oxidative metabolism and hERG blockade. By synthesis of the metabolites and further blocking the oxidative sites, together with reducing the lipophilicity, compound **8**³⁰ was identified showing high potency against both ALK wild type and gatekeeper mutant. Compound **8** potentially inhibited the proliferation of several ALK-addicted h_uMan cancer cell lines, and showed complete t_uMor growth inhibition at doses of 50 or 10 mg/kg once daily in the Karpas299 xenograft model. Optimal PK properties and greater than 100% bioavailability were also observed. The well balanced *in vitro* and *in vivo* profiles of **8** encouraged its further developability and it is now under early preclinical trials as a potent and orally bioavailable ALK inhibitor.

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30. Representative analytic data. For compound **8**: ^1H NMR (300 MHz, DMSO- d_6) δ 12.69 (s, 1H), 8.32 (d, J = 8.2 Hz, 1H), 8.04 (s, 1H), 7.99 (s, 1H), 7.60 (d, J = 8.1 Hz, 1H), 7.36 (s, 1H), 4.21 (s, 1H), 3.69 – 3.52 (m, 4H), 3.03 – 2.92 (m, 4H), 2.70 (q, J = 7.4, 6.7 Hz, 6H), 2.35 (s, 2H), 1.75 (s, 6H), 1.64 (td, J = 11.8, 10.2, 4.9 Hz, 2H), 1.41 (d, J = 12.7 Hz, 2H), 1.27 (t, J = 7.5 Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 179.62, 160.51, 155.65, 147.27, 136.72, 136.09, 128.16, 126.60, 126.32, 125.22, 122.07, 120.57, 116.98, 116.82, 109.84, 105.00, 69.15, 68.20, 63.55, 55.78, 52.50, 36.92, 36.26, 30.48, 23.08, 14.80. MS (EI, $[\text{M}]^+$) m/z 512. HRMS (ESI) calcd for $\text{C}_{31}\text{H}_{36}\text{N}_4\text{O}_3$, 512.2787; found, 512.2794.

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ALK^{WT}: 1.5 nM
ALK^{L1196M}: 4.2 nM
Cell (H3122): 24.4 nM
TGI (KARPAS-299 xenografts):
> 100% @ 20.0 mg/kg q.d.
hERG: > 30 uM; cLogP: 4.92

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