Discovery of a Highly Potent and Selective Indenoindolone Type 1 Pan-FLT3 Inhibitor

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Supporting Information

ABSTRACT: For a subpopulation of acute myeloid leukemia (AML) patients, the mutationally activated tyrosine kinase FLT3, has emerged as a promising target for therapy. The development of drug resistance due to mutation is a growing concern for mutant FLT3 inhibitors, such as PKC412, Quizartinib, PLX3397, and Crenolanib. Thus, there is a need to develop novel FLT3 inhibitors that overcome these mutations. Here we report the development of a novel type I ATP competitive inhibitor, JH-IX-179, that is extremely potent and selective for FLT3. JH-IX-179 also has the highest affinity for three constitutively active isoforms of FLT3 (FLT3-ITD, FLT3-N841I, and FLT3-D835V) compared to a panel 456 other kinases. The unique and specific kinase inhibition profile suggests that this chemotype may represent an attractive starting point for the development of further improved FLT3 inhibitors with therapeutic potential in tumors harboring deregulated FLT3 activity.

KEYWORDS: Acute myeloid leukemia, FLT3, JH-IX-179, kinase inhibition profile, chemotype

	Crenolanib	IC50 (nM)	JH-IX-179
FLT3-D835Y	4		4
FLT3-ITD	3		10
MOLM-13	7		2
MOLM-14	6		11
Ambit S-Score(10)	0.12		0.03

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by aberrant growth of myeloid precursor cells and a partial block in cellular differentiation. There are approximately 10,000 new cases reported each year in the U.S.¹ Approximately 30% of AML patients harbor a mutant form of the class III receptor tyrosine kinase, FLT3 (Fms-Like Tyrosine kinase-3; STK-1, human Stem Cell Tyrosine Kinase-1; or FLK-2, Fetal Liver Kinase-2).² Constitutively activated FLT3 occurs most often as internal tandem duplications (ITD) within the juxtamembrane domain and is observed in approximately 20–25% of AML patients.^{3,4} The transplantation in mice of murine bone marrow cells infected with a retrovirus expressing a FLT3-ITD mutant leads to the development of a rapidly lethal myeloproliferative disease.⁵ Approximately 7% of AML patients harbor point mutations within the “activation loop” of FLT3, which are believed to predispose the kinase to assume an “activated” conformation.⁶ The majority of patients harbor a missense mutation at position 835. Other less prevalent point mutations in the kinase domain have been identified, including N841I⁷ and Y842C.⁸ FLT3 mutations are associated with a poorer prognosis in both overall and disease-free survival.⁹ Molecular targeting of FLT3 is an attractive therapeutic approach

that has led to the development of numerous small molecule ATP-competitive inhibitors of FLT3 as shown in Table 1.

To date, several potent FLT3 inhibitors have been evaluated in clinical trials, although no FLT3 inhibitor has yet received regulatory approval. Some examples of first generation FLT3 inhibitors include Sunitinib (SU11248 and Sutent; Pfizer), which is equipotent against both FLT3-ITD and D835Y.¹⁰ Sunitinib induced transient responses in early stage clinical trials; however, two fatal cardiotoxicity cases led to its discontinuation.^{11,12} Midostaurin (PKC412; N-benzoyl-staurosporine; Novartis Pharma AG) has demonstrated limited clinical effectiveness against mutant FLT3-positive AML.¹³ However, recent results of Phase II clinical testing yielded favorable results for midostaurin, which in combination with standard chemotherapy significantly prolonged survival in a large number of AML patients as compared to placebo.¹⁴ Lestaurtinib (CEP-701; Cephalon) was tested in clinical trials, but only short duration responses were observed.^{15–17} Combination of lestaurtinib with chemotherapy

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Table 1. Second Generation FLT3 Inhibitors

<p>1 PLX3397 2 Quizartinib 3 Crenolanib 4 JH-IX-179</p>			
compd	IC ₅₀ (nM)		
	FLT3 D835Y	FLT3 ITD	
1, PLX-3397	5	130	
2, Quizartinib	93	2	
3, Crenolanib	4	3	
4, JH-IX-179	4	10	

Table 2. Indenoindolone SAR against FLT3-D835Y and FLT3-ITD Transduced Ba/F3 Cells^a

IC ₅₀ (nM)										
Compd	R ₁	R ₂	R ₃	FLT3 D835Y	FLT3 ITD	Compd	R ₁	R ₂	R ₃	FLT3 D835Y FLT3 ITD
5	H		CN	96	128	15		H	CN	365 280
6		H	CN	40	74	16	H		CN	5815 1577
7	H		CN	62	146	17		H	CN	692 937
8		H	CN	83	97	18	H		CN	502 356
9	H		CN	26	14	19		H	CN	946 2244
10		H	CN	430	424	20		H		126 182
11	H		CN	31	33	21	H			34 55
12		H	CN	96	143	22		H		1526 2485
13		H	CN	106	135	23	H			3166 5707
14	H		CN	114	124	24		H		6 18
						4	H			4 10

^aFLT3-D835Y and FLT3-ITD transduced Ba/F3 cells were treated with compound for 3 days. Results are average of triplicate experiments.

did not result in clinical benefit versus chemotherapy alone, and its further clinical development was discontinued.¹⁸ Some examples of “second generation” FLT3 inhibitors include, sorafenib, a so-called “type II” inhibitor, which binds kinases in the “DFG-out” conformation. Sorafenib is a more potent inhibitor of FLT3-ITD versus D835Y.¹⁰ Sorafenib showed some clinical efficacy as a monotherapy before or after allogeneic stem cell transplantation in relapsed or refractory FLT3-ITD-positive AML.¹⁹ However, the combination of sorafenib and standard chemotherapy was not found to be superior to chemotherapy alone in a clinical study involving elderly patients.²⁰ Quizartinib (AC220) exhibits higher potency and selectivity against FLT3-ITD when compared to first generation FLT3 inhibitors, although it is associated with emergence of D835 residue mutations and the gatekeeper residue mutation, F691L.²¹ In Phase I and Phase II clinical trials, quizartinib displayed superior efficacy to other clinically evaluated FLT3 inhibitors; however, severe bone marrow suppression and QTc prolongation are significant toxicity concerns. In addition,

patients treated with quizartinib failed to achieve complete remission.²² PLX3397 (Plexxikon) is a novel FLT3 inhibitor that overrides F691L; however, in the context of D835 FLT3, several mutations are known to confer drug resistance.^{23,24} The investigational type I inhibitor, crenolanib, is active against TKD mutations that are resistant to quizartinib; however, it shows a loss of potency against the gatekeeper mutation F691L.²⁴

Despite the numerous FLT3 inhibitors under clinical investigation, issues such as transient single agent clinical responses, toxicity, bioavailability, low potency with respect to FLT3 TKD inhibition drug resistance, warrant the development of novel agents conferring higher potency and selectivity toward the FLT3 TKD with less toxicity. Such agents would be expected to be effective as single agents or when used in combination with other agents to suppress disease progression and prolong the lifespan of patients. Moreover, a recent report highlighted the need for FLT3-specific inhibitors for patients with more advanced disease as opposed to newly diagnosed FLT3 mutant

Scheme 1^a

^aReagents and conditions: (a) K_2CO_3 , 90 °C, 4 h, 91%; (b) Fe, AcOH, 100 °C 2 h, 94%; (c) Pd(dppf)Cl₂, *t*-BuXPhos, Na₂CO₃, 31, H₂O/dioxane, 60 °C, 1 h, then 32, 100 °C 2 h, 47%.

AML patients since the more advanced disease stage has a higher mutant allelic burden and is more responsive to cytotoxicity from selective FLT3 inhibition.²⁵

Given the enhanced potency and selectivity of the type 1 FLT3 inhibitor crenolanib compared to type 2 FLT3 inhibitors, we decided to explore alternative type 1 scaffolds. The indenoindolones 5 and 6 were developed during a previous medicinal chemistry effort to diversify the tetracyclic core of alectinib, a reported inhibitor of anaplastic lymphoma kinase (ALK).²⁶ Compounds 5 and 6 were identified as FLT3 inhibitors during a chemical screen testing for small molecule inhibitors that exhibited selective antiproliferative activity against FLT3-D835Y-dependent Ba/F3 cells. Compound 5 showed an IC₅₀ of 96 and 128 nM against FLT3-D835Y and FLT3-ITD respectively, while the regioisomer compound 6 had an IC₅₀ of 40 and 74 nM against FLT3-D835Y and FLT3-ITD, respectively. Based on these promising results, we decided to further develop this scaffold. We began by making the methylated pyrazole regioisomers 7 and 8, which shared similar potencies as their nonmethylated counterparts. We then decided to employ substituted pyrazoles, compounds 9–15. The dimethylamino propyl (9) and dimethylamino ethyl compound (11) substituted at the 3 position showed enhanced activity with IC₅₀s of 26 and 31 nM, respectively, against FLT3-D835Y and IC₅₀s of 14 and 33 nM, respectively, against FLT3-ITD. Interestingly, the dimethylamino propyl (10) and dimethylamino ethyl compound (12) substituted at the 2 position showed less potency with IC₅₀s of 430 and 96 nM, respectively, against FLT3-D835Y and IC₅₀s of 424 and 143 nM, respectively, against FLT3-ITD. We then made the dimethylacetamide compound (13), which resulted in an inseparable mixture of regioisomers. However, the mixture showed less potency than compound 9, so we decided not to pursue it. We then synthesized the methylacetamide compounds 14 and 15, which resulted in a loss of activity for both regioisomers compared to compound 9. At this point, we replaced the pyrazole substituent with both 3-pyridine (18 and 19) and 4-pyridine (16 and 17). However, all of the pyridine substituted compounds showed much less potency. We modified the left side of the molecule by replacing the cyano group with pyrazole and methyl pyrazole. We hypothesized that the pyrazole could form a stronger interaction with E661 as predicted by molecular modeling and discussed further below. We began by making the dipyrazole substituted compounds 20 and 21, which showed similar potency compared to compound 9. Interestingly, the methyl-pyrazole substituted compounds 22 and 23 showed a dramatic loss in potency. We then made the dimethylamino-propyl compounds 4 and 24, which gratifyingly showed enhanced activity compared to compound 9. Compound 24

showed IC₅₀s of 6 and 18 nM against FLT3-D835Y and FLT3-ITD, respectively, while compound 4 showed IC₅₀s of 4 and 10 nM against FLT3-D835Y and FLT3-ITD, respectively, which is similar to Crenolanib (Table 2). A complete listing of IC₅₀s with standard deviations can be found in Table 1 in the Supporting Information section.

Compound 4 was prepared from commercially available 5-chloro-indenedione and 1-fluoro-4-iodo-2-nitrobenzene (Scheme 1). 5-Chloro-indenedione 25 underwent an addition reaction with 1-fluoro-4-iodo-2-nitrobenzene 26 under basic conditions to give the corresponding adduct 27, which then underwent reduction and cyclization using a solution of iron in acetic acid to give the corresponding indenoindolone as a 1:1 mixture of regioisomers 28 and 29. The mixture of regioisomers was then subjected to sequential Suzuki coupling reactions with two boronic acids 31 then 32 to yield the desired compounds as a 1:1 mixture of regioisomers 4 and 24, which were separated by HPLC. Other analogues were prepared in a similar manner starting from 4-fluoro-3-nitrobenzonitrile 26.

A molecular docking study was performed based on the known cocrystal structure of FLT3 with Quizartinib (PDB code: 4XUF),²⁷ which suggested a type 1 binding mode, and revealed a single hydrogen bond to the hinge region between backbone Y693 and C694. Additionally, the docking study shows a hydrogen bond interaction between the pyrazole NH and E661 as shown in Figure 1.

To extend our cellular profiling to human-derived cell lines we tested compound 4 against commonly used AML lines: MOLM-13 and MOLM-14, which are known to be “addicted” to mutant FLT3 kinase activity. Our most potent compound, JH-IX-179, exhibited an IC₅₀ of 2 and 11 nM against MOLM-13 and

Figure 1. Molecular model of JH-IX-179 with FLT3.

Figure 2. Inhibition of phosphorylation of FLT3-D835Y by JH-IX-179 following of treatment of Ba/F3-FLT3-D835Y cells for 2 h (A). Inhibition of phosphorylation of signaling molecules downstream of FLT3-D835Y, including phospho-S6 (B), phospho-AKT (C), and phospho-MAPK (D) by JH-IX-179 following treatment of Ba/F3-FLT3-D835Y cells for 2 h. Inhibition of phosphorylation of signaling molecules downstream of FLT3-ITD, including phospho-AKT (E) and phospho-S6 (F) by JH-IX-179 following treatment of Ba/F3-FLT3-ITD cells for 2 h. Inhibition of phosphorylation of FLT3-ITD by JH-IX-179 following treatment of Ba/F3-FLT3-ITD cells for 2 h (G).

Figure 3. KinomeScan results for JH-IX-179 and Crenolanib at 1 μ M concentration with a cutoff of 90% inhibition.

MOLM-14 cell lines, respectively (Supplementary Figure 1). Interestingly, JH-IX-179 showed much less affinity to human serum protein than the FLT3 inhibitor PKC412 (Supplementary Figure 2). Furthermore, JH-IX-179 effectively inhibited autophosphorylation of FLT3 and the activity of key effector signaling molecules downstream of FLT3-D835Y and FLT3-ITD (Figure 2). JH-IX-179 demonstrated targeted inhibition of the growth and viability of FLT3-ITD-driven human acute leukemia cell lines with little-to-no inhibition of human acute leukemia lines not driven by mutant FLT3 (Supplementary Figure 1a). Treatment of FLT3-ITD-expressing cells by JH-IX-179 led to inhibition of cell cycle progression (G1 arrest) as well as induction of apoptosis (Supplementary Figure 3A, 3C, 5, and 7), which suggests that both contribute to the mechanism of

action of JH-IX-179 against cells carrying the FLT3-ITD mutation. In contrast, treatment of D835Y-expressing cells with JH-IX-179 did not lead to cell cycle arrest; however, it did lead to a strong induction of apoptosis (Supplementary Figure 3B, 3D, 6, and 8), which suggests that induction of apoptosis is the primary mechanism of action of JH-IX-179 against cells expressing the D835Y mutant. JH-IX-179 was then tested against a patient-derived FLT3-D835Y and FLT3-ITD positive AML primagraft obtained from mouse spleen and showed an IC_{50} of 70 nM (Supplementary Figure 4).

To evaluate the kinase selectivity we performed KinomeScan binding analysis against a near comprehensive panel of 456 kinases at a concentration of 1 μ M. The profiling demonstrated that JH-IX-179 resulted in no interactions other than FLT3 and

FLT3 mutants with greater than 95% inhibition, with the exception of CIT, ROCK2, PHKG1, and CLK1 demonstrating the outstanding selectivity of this inhibitor. JH-IX-179 inhibited ROCK2 kinase activity with IC_{50} of 936 nM, PHKG1 with an IC_{50} of 78 and CLK4 with an IC_{50} of 142 nM.²⁸ Interestingly, JH-IX-179 displayed a higher level of selectivity ($S(10)$ score of 0.03) toward FLT3 and FLT3 mutants than crenolanib ($S(10)$ score of 0.12), as shown in Figure 3. Complete profiling results provided in the Supporting Information.²⁹

In mouse microsomes, JH-IX-179 showed a half-life of 15 min. In mouse plasma, JH-IX-179 showed no degradation after 6 h of exposure.

In summary, we have discovered that JH-IX-179 is a potent type 1 inhibitor of FLT3. In addition, JH-IX-179 displays similar potency as crenolanib against FLT3-D835Y and FLT3-ITD transduced Ba/F3 cells as well as two human leukemia cell lines MOLM-13 and MOLM-14. However, JH-IX-179 is considerably more selective for FLT3 and FLT3 mutants than crenolanib. Furthermore, JH-IX-179 displayed very little human serum protein binding and effectively inhibited autophosphorylation of FLT3 and the activity of signaling molecules downstream of FLT3-D835Y and FLT3-ITD. Further elaboration of this scaffold and characterization of adsorption, distribution, metabolism, and excretion (ADME) will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00498.

IC_{50} curves, Western blots, full Ambit profiling data for JH-IX-179 and crenolanib, and experimental details (PDF)

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Notes

The authors declare no competing financial interest.

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