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4-Amino-6-piperazin-1-yl-pyrimidine-5-carbaldehyde oximes as potent FLT-3 inhibitors

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Abstract—A series of 4-amino-6-piperazin-1-yl-pyrimidine-5-carbaldehyde oximes has been discovered and developed as potent FLT3 tyrosine kinase inhibitors. The series exhibited potent antiproliferative activity against both an FLT3 ITD-mutated human leukemic cell line as well as a wild-type FLT3 BaF_3 expressed cell line. The structure–activity relationship of this class of compounds is described.

Normal hematopoiesis involves the regulation of proliferation and differentiation in a number of cell types from early stem cells to mature cells derived from erythroid, lymphoid, and myeloid lineages. This is a highly regulated event and when unregulated can lead to a number of myelodysplastic syndromes. The fms-like tyrosine kinase 3 (FLT3), also known as fetal liver kinase-2 (FLK-2) or human stem cell kinase-1 (STK-1), is a receptor tyrosine kinase that is involved in the differentiation of hematopoietic cells.[1] The expression of FLT3 is limited mainly to early hematopoietic stem cells and myeloid and lymphoid progenitor cells.[2] In particular, FLT3 expression is limited to CD34+ fetal liver and bone marrow stem cells,[3] pre-B cells,[4] and peripheral dendritic cells. Activation of FLT3 is due to binding of the endogenous FLT3 ligand (FLT3L) to the extracellular portion of the receptor. FLT3L is widely expressed in most tissues in the body including many hematopoeitic cells.[5]

FLT3 is expressed on blast cells in >90% of patients with Acute Myeloid Leukemia (AML) and activating mutations of FLT3 are present in approximately 30% of these patients. There are currently two known mutations: an internal tandem duplication (ITD)[6] of the juxtamembrane domain of the protein and specific point mutations within the kinase activation loop.[7] The ITD mutation is found in approximately 25% of AML patients and leads to constitutive activation via autophosphorylation of the FLT3 receptor. Patients carrying the FLT3 ITD mutation were found to have an increased level of leukocytosis and a decreased overall survival time as compared to patients without the activating mutation.[8] Taken together the two activating mutations of FLT3 are the most common genetic abnormality found in AML. Therefore identification of a potent, selective FLT3 inhibitor that is potent against both the ITD mutation as well as wild-type FLT3 kinase may provide a means for therapeutic intervention in AML patients.

Recently it was reported that Tandutinib (Fig. 1) is a dual PDGFR/FLT3 inhibitor[9] that is currently under clinical development in AML patients.[10] We envisioned that replacement of the quinazoline found in Tandutinib with our recently reported 4-aminopyrimidine 5-carboxaldehyde oxime scaffold[11] (Fig. 1) could lead to the identification of potent FLT3 tyrosine kinase inhibitors. Herein, we report on the design, synthesis, and biological activity of this new class of pyrimidine carbaldehyde oxime FLT3 inhibitors.

The synthesis of the molecules is outlined in Scheme 1. Treatment of 4-amino-6-chloropyrimidine-5-carboxaldehyde[12] **1** with N-Boc piperazine provided the pyrimidine carboxaldehyde **2**. Oxime formation utilizing

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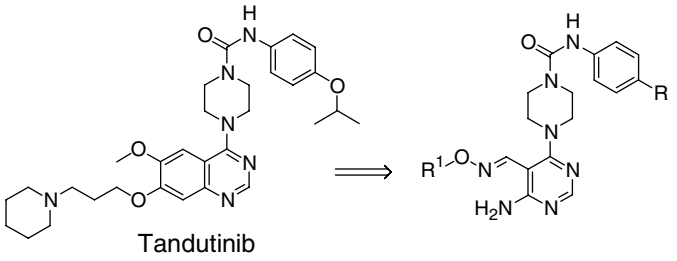
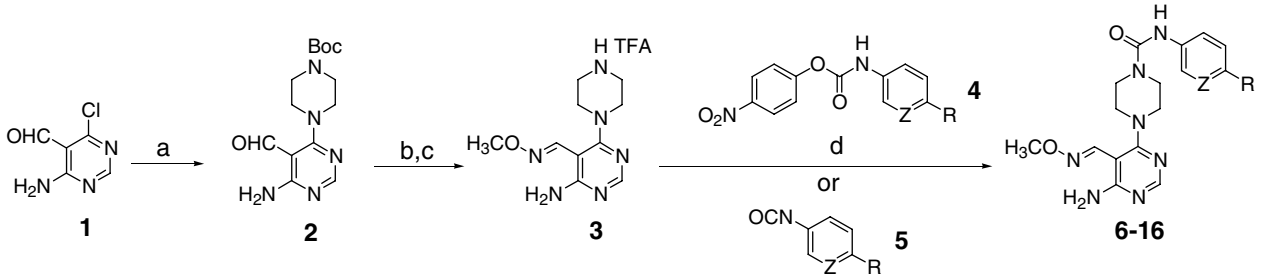


Figure 1. Inhibitor design derived from reported FLT3 inhibitor.



Scheme 1. Reagents and conditions: (a) N-Boc piperidine, diisopropylethylamine, CH_3CN, 100 °C, 94%; (b) CH_3ONH_2 HCl, MeOH, 75 °C, 75%; (c) TFA/CH_2Cl_2 (1:1, v/v), rt, >95%; (d) **4** or **5**, diisopropylethylamine, CH_3CN, reflux, 47–70%.

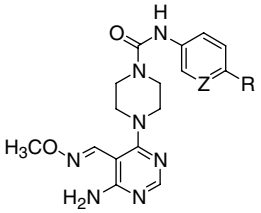
O-methyl hydroxylamine to the *E*-isomer oxime followed by subsequent removal of the N-Boc protecting group provided the piperazinopyrimidine **3**. It should be pointed out that the *E*-isomer oxime[13] was either the major or exclusive product obtained. Treatment of piperazine **3** with either the *o*-nitrophenyl ester carbamate[14] **4** or isocyanate reagent **5** under similar conditions provided the desired final pyrimidines **6**–**16**.

The oxime pyrimidines were evaluated for FLT3 tyrosine kinase inhibition[15] as well as for antiproliferative activity against two FLT3-driven cell lines: an MV4-11[16] line that is a human AML cell line expressing the ITD activating mutation and a wild-type FLT3 expressed BaF_3 cell line.[17] Our initial efforts focused on developing the SAR of the *para*-substituted aryl urea and the data are summarized in Table 1. Generally we found that small, branched lipophilic substituents such as isopropoxy, isopropyl, and pyrrolidin-1-yl provided potent FLT3 enzyme inhibition as well as good antiproliferative activity in the FLT3-driven cell lines. Specifically, compounds **6**, **10**, and **12** provided potent antiproliferative activity against the ITD mutation MV4-11 cell line below 100 nM while maintaining excellent activity against the wild-type FLT3-driven BaF_3 cell line. Not all substitutions were favorable; the use of morpholin-4-yl or chlorine (compounds **8** and **14**) decreased activity against both the FLT3 kinase and MV4-11 cells. It should be noted that each of the molecules was tested against a parental BaF_3 cell line to determine general cytotoxic activity and all of the compounds displayed <50% antiproliferative activity when tested at 10 μM (data not shown), inferring that the antiproliferative activity observed in the FLT3-driven cell line was a result of FLT3 kinase inhibition.

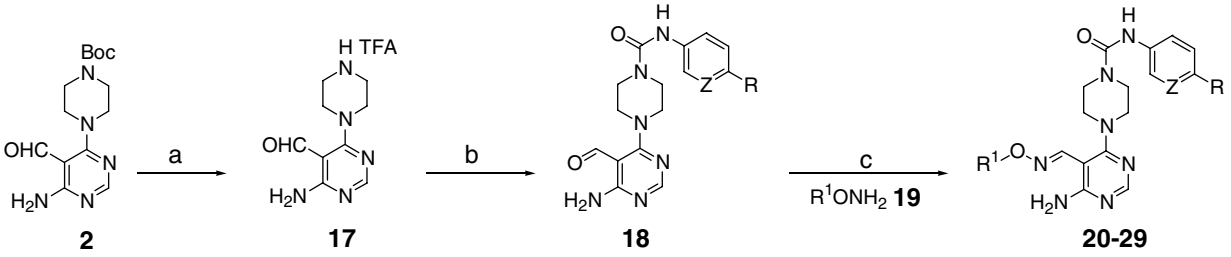
Our next objective was to study the effects of using a variety of substituents off the oxime moiety. The synthesis of the molecules is outlined in Scheme 2. Beginning with the previously disclosed **2**, deprotection of the N-Boc group and subsequent acylation of the resulting piperazine **17** with an appropriate *o*-nitrophenyl ester carbamate[14] **4** provided the pyrimidine carboxaldehyde **18**. Treatment of the aldehyde **18** with an appropriate substituted hydroxylamine[18] **19** provided the desired oxime pyrimidines **20**–**29**. Table 2 summarizes the FLT3 kinase and antiproliferative activities of this set of compounds. Excellent activity was maintained when the oxime substituent was increased in size to ethyl substitution as compared to the methyl oxime analogue (compounds **27**–**29**). The use of 2-(morpholin-1-yl)ethyl side chain with an isopropoxy group as the R substituent **25** provided similar kinase and antiproliferative activity as the corresponding methyl oxime **6**. However changing the oxime substituent to a more solubilizing group diminished the activity in some compounds. Specifically changing the oxime substituent to 2-(morpholin-1-yl)ethyl, 2-(methylsulfonamido)ethyl or 3-hydroxypropyl led to decreased activity in both FLT3 kinase inhibition as well as antiproliferative activity as compared to the methyl oximes when R is either an isopropoxy group or pyrrolidin-1-yl.

In order to determine kinase selectivity for this series of FLT3 inhibitors a representative example was tested against other Class III receptor tyrosine kinase class comprising of PDGFRβ, c-kit, and c-fms. Compound **6** was found to be a potent c-kit inhibitor with an IC_{50} of 10.5 nM but was much less potent versus PDGFRβ (IC_{50} = 10.7 μM) and c-fms (IC_{50} = 1.28 μM). Compound **6** was also screened against a diverse panel of 20 kinases at a fixed concentration

Table 1. FLT3 kinase- and FLT3-mediated cellular antiproliferative activity of compounds **6**–**16**

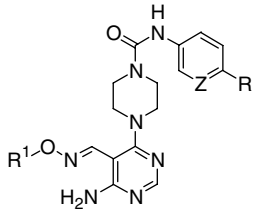


Compound	Z	R	FLT3 IC_{50} (μM)	MV4-11IC_{50} (μM)	BaF_3 FLT3 IC_{50} (μM)
6	CH	–$OCH(CH_3)_2$	0.05	0.079	0.017
7	CH	Piperidin-1-yl	0.05	0.089	0.153
8	CH	Morpholin-4-yl	0.34	0.515	0.105
9	N	–*O*(cyclobutyl)	0.075	0.111	0.104
10	CH	–$CH(CH_3)_2$	0.015	0.078	0.008
11	N	–*O*(cyclopentyl)	0.018	0.112	0.068
12	CH	Pyrrolidin-1-yl	0.003	0.099	0.312
13	CH	Cyclohexyl	0.099	0.052	0.012
14	CH	–Cl	1.37	>1	0.11
15	CH	–OPh	0.001	0.086	0.102
16	CH	–$N(CH_3)_2$	0.014	0.472	0.058



Scheme 2. Reagents and conditions: (a) TFA/CH_2Cl_2 (1:1, v/v), rt, >95%; (b) **4**, diisopropylethylamine, CH_3CN, reflux, 32–70%; (c) **19**, MeOH, 75 °C, 50–60%.

Table 2. FLT3 kinase- and FLT3-mediated cellular antiproliferative activity of compounds **20**–**29**



Compound	Z	R	R^1	FLT3 IC_{50} (μM)	MV4-11 IC_{50} (μM)	BaF_3 FLT3 IC_{50} (μM)
20	CH	Pyrrolidin-1-yl	–$CH_2CH_2NHSO_2CH_3$	0.15	0.554	0.025
21	CH	Pyrrolidin-1-yl	–CH_2CH_2(morpholin-1-yl)	0.165	0.362	0.53
22	CH	–$OCH(CH_3)_2$	–CH_2CO(morpholin-1-yl)	0.062	0.268	0.187
23	CH	Pyrrolidin-1-yl	–CH_2CO(morpholin-1-yl)	0.041	0.458	0.066
24	CH	–$OCH(CH_3)_2$	–$CH_2CH_2NHSO_2CH_3$	0.146	0.415	0.028
25	CH	–$OCH(CH_3)_2$	–CH_2CH_2(morpholin-1-yl)	0.036	0.177	0.081
26	CH	–$OCH(CH_3)_2$	–$CH_2CH_2CH_2OH$	0.26	0.283	0.072
27[21]	CH	–$OCH(CH_3)_2$	–CH_2CH_3	0.018	0.077	0.022
28[21]	CH	Piperidin-1-yl	–CH_2CH_3	0.049	0.058	0.026
29[21]	N	–*O*(cyclobutyl)	–CH_2CH_3	0.058	0.065	0.063

of 3 μM in the presence of 100 μM ATP.[19] It was observed that the compound was highly selective versus the kinase panel with the highest activity obtained for FES and ALK (34% and 31% inhibition, respectively). The series also displayed good in vitro microsomal stability when incubated in the presence of human liver microsomes as demonstrated by examples **6**, **7**, **25**, and **27** (78.5%, 69.2%, 82.9%, and 78.9% parent compound remaining after 10 min of incubation, respectively).

In summary, we have described a new class of oxime pyrimidines[20] as potent inhibitors of FLT3 kinase. In addition the compounds show excellent antiproliferative activity against both a FLT3 ITD-mutated human leukemic cell line as well as a wild-type FLT3 cell line. A variety of small lipophilic substitutions off the phenyl urea head group such as isopropoxy, pyrrolidin-yl, and isopropyl were preferred substituents. Substitution off the oxime side chain exhibited either the same or less activity as compared to the parent methyl oxime. Further studies with this series of molecules will be reported in due course.

Acknowledgment

We thank Malini Dasgupta for conducting the human liver microsomal stability studies.

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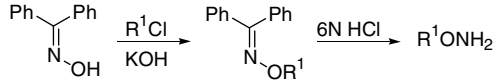
14. *p*-Nitrophenyl ester carbamates were prepared from the corresponding anilines by treating with 1 equiv *p*-nitrophenylchloroformate and 1 equiv diisopropylethylamine.
15. *FLT3 kinase assay protocol*. To determine the activity of the compounds in an in vitro kinase assay, inhibition of the isolated kinase domain of the human FLT3 receptor (aa 571–993) was performed using the following fluorescence polarization (FP) protocol. The FLT3 fluorescence polarization assay utilizes the fluorescein-labeled phosphopeptide and the anti-phosphotyrosine antibody included in the Panvera Phospho-Tyrosine Kinase Kit (Green) supplied by Invitrogen. The FLT3 kinase reaction is incubated at room temperature for 30 min under the following conditions: 10 nM FLT3 571–993, 20 μg/mL poly Glu4Tyr, 150 μM ATP, 5 mM $MgCl_2$, 1% compound in DMSO. The kinase reaction is stopped with the addition of EDTA. The fluorescein-labeled phosphopeptide and the anti-phosphotyrosine antibody are added and incubated for 30 min at room temperature and polarization was read.
16. MV4-11 cell-based assay: (a) Quentmeier, H.; Reinhardt, J.; Zaborski, M.; Drexler, H. G. *Leukemia* **2003**, *17*, 120; (b) MV4-11 (ATCC No. CRL-9591) cells were plated at 10,000 cells per well in 100 μl of RPMI media containing penn/strep, 10% FBS, and 0.2 ng/ml GM-CSF. Compound dilutions or 0.1% DMSO (vehicle control) is added to cells and the cells are allowed to grow for 72 h at standard cell growth conditions (37 °C, 5% CO_2). To measure total cell growth an equal volume of CellTiterGlo reagent (Promega Madison, WI) was added to each well, according to the manufacturer's instructions, and luminescence was quantified. Total cell growth was quantified as the difference in luminescent counts (relative light units, RLU) of cell number at Day 0 compared to total cell number at Day 3 (72 h of growth and/or compound treatment). All IC_{50} values are calculated in GraphPadPrism using non-linear regression analysis with a multiparameter (variable slope) equation.
17. *wt FLT3 BaF$_3$ cell-based assay*. BaF_3 FLT3 cells were maintained in RPMI 1640 with 10% FBS, penn/strep, and 10 ng/ml of FLT ligand at 37 °C, 5% CO_2. BaF_3 FLT3 cells (2×10^5) were plated in 96-well dishes in RPMI 1640 with 0.5% serum and 0.01 ng/mL IL-3 for 16 h prior to 1 h compound or DMSO vehicle incubation. Cells were treated with 100 ng/mL Flt ligand (R&D Systems Cat# 308-FK) for 10 min at 37 °C. Cells were pelleted, washed, and lysed in 100 μl lysis buffer (50 mM Hepes, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 10 mM NaF, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM Na-pyrophosphate) supplemented with phosphatase (Sigma Cat # P2850) and protease inhibitors (Sigma Cat # P8340). Lysates were cleared by centrifugation at 1000*g* for 5 min at 4 °C. Cell lysates were transferred to white wall 96-well microtiter (Costar # 9018) plates coated with 50 ng/well anti-FLT3 antibody (Santa Cruz Cat # sc-480) and blocked with SeaBlock reagent (Pierce Cat # 37527). Lysates were incubated at 4 °C for 2 h. Plates were washed 3× with 200 μl/well PBS/0.1% Triton X-100. Plates were then incubated with 1:8000 dilution of HRP-conjugated anti-phosphotyrosine antibody (Clone 4G10, Upstate Biotechnology Cat # 16-105) for 1 h at room temperature. Plates were washed 3× with 200 μl/well PBS/0.1% Triton X-100. Signal detection with Super Signal Pico reagent (Pierce Cat # 37070) was done according to manufacturer's instruction with a Berthold microplate luminometer. All data points are an average of triplicate samples. The total relative light units (RLU) of Flt ligand stimulated FLT3 phosphorylation in the presence of 0.1% DMSO control was defined as 0% inhibition and 100% inhibition was the

total RLU of lysate in the basal state. All IC_{50} values are calculated in GraphPadPrism using non-linear regression analysis with a multiparameter (variable slope) equation.

18. Prepared by the following 2-step protocol:



Gaul, M.D.; Xu, G.; Baumann, C.A. PCT Int. Appl. WO 2006/135719 A1 20061221, 2006.

19. Upstate Cell Signaling Solutions, Charlottesville, VA.
20. Compounds **6**–**16** and **20**–**29** were characterized by ^{1}H NMR and LC/MS. Representative experimental procedures.

Compound 6. To a mixture of 4-amino-6-piperazin-1-yl-pyrimidine-5-carbaldehyde *O*-methyl-oxime trifluoroacetic acid salt **3** (Ref. 18) (23 mg, 0.066 mmol) and (4-isopropoxy-phenyl)-carbamic acid 4-nitro-phenyl ester **4** (22.8 mg, 0.072 mmol) in CH_3CN (1.5 mL) was added DIEA (17 mg, 0.13 mmol). The mixture was heated at reflux with stirring for 3 h and the solvents were evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (EtOAc as eluent) to afford Compound **6**. ^{1}H NMR ($CDCl_3$)δ 8.19 (s, 1H), 8.12 (s, 1H), 7.21 (d, J = 8.93 Hz, 2H), 6.81 (d, J = 8.94 Hz, 2H), 6.45 (br, 1H), 4.46 (m, 1H), 3.96 (s, 3H), 3.58 (m, 4H), 3.42 (m, 4H), 1.30 (d, J = 6.06 Hz, 6H); LC/MS (ESI) calcd for $C_{20}H_{28}N_7O_3$ $(MH)^+$ 414.2, found 414.2.

Compound 25. A mixture of 4-(6-amino-5-formyl-pyrimidin-4-yl)-piperazine-1-carboxylic acid (4-isopropoxy-phenyl)-amide (Ref. 18) (20.9 mg, 0.054 mmol) and *O*-(2-morpholin-4-yl-ethyl)-hydroxylamine dihydrochloride salt (Ref. 18) (12 mg, 0.054 mmol) in MeOH (1 mL) was heated at 100 °C for 0.5 h and the solvent was removed. The residue was partitioned between EtOAc and water. The organic extracts were dried with Na_2SO_4, evaporated, and the residue was purified by preparative TLC (5% MeOH/EtOAc) to yield compound **25**. ^{1}H NMR (CD_3OD) δ 8.24 (s, 1H), 8.08 (s, 1H), 7.21 (d, J = 8.79 Hz, 2H), 6.83 (d, J = 9.03 Hz, 2H), 4.52 (m, 1H), 4.34 (t, J = 5.63 Hz, 2H), 3.71 (t, J = 4.84 Hz, 4H), 3.63 (m, 4H), 3.43 (m, 4H), 2.75 (t, J = 5.60 Hz, 2H), 2.57 (t, J = 4.96 Hz, 4H), 1.28 (d, J = 6.05 Hz, 6H); LC/MS (ESI) calcd for $C_{25}H_{37}N_8O_4$ $(MH)^+$ 513.2, found 513.3.

21. Compounds **27**–**29** were prepared as outlined in Scheme 1 using *O*-ethylhydroxylamine hydrochloride in place of *O*-methylhydroxylamine hydrochloride in step B.