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Discovery of a potent and selective c-Kit inhibitor for the treatment of inflammatory diseases

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ABSTRACT

A potent and selective c-Kit inhibitor **20** was identified through a structure–activity relationship study. In an in vivo mouse model of mast cell activation, **20** blocked the SCF-induced histamine release with an EC_{50} of 26 nM.

The KIT gene encodes for a tyrosine kinase transmembrane protein (c-Kit), which functions as the receptor for stem cell factor (SCF).[1,2] C-Kit was first found to be expressed on the surface of mast cells in 1989.[3] Later it was shown to play a critical role in modulating histamine release from mast cells.[4,5] Binding of SCF to the c-Kit receptor mediates various functions of the mast cell. It has been demonstrated that SCF induces mast cells survival in vitro, adhesion to extracellular matrix and degranulation, leading to expression and release of histamine.[6] Inhibition of c-Kit may have therapeutic utility in mast cell associated inflammatory and autoimmune diseases.[7] In a recent study using a murine asthma model, either down regulation of SCF expression or inhibition of c-Kit resulted in reduced inflammatory response.[8,9] Therefore, blocking c-Kit signaling may present a potential therapeutic approach for treating human inflammatory diseases related to mast cells.

Gleevec is a commercially available multi-kinase inhibitor (c-Kit, bcr-abl, and PDGFR) that has been approved for the treatment of chronic myeloid leukemia[10] and GISTs.[11,12] Preclinical studies have shown that Gleevec also inhibits mast cells and is efficacious in rodent arthritis models.[13,14] Our goal was to identify a more potent and selective c-Kit inhibitor compared to Gleevec for the treatment of chronic inflammatory illnesses. Herein, we report our efforts towards identifying a molecule that fulfills these criteria.

From the screening of our kinase-preferred collection, we identified compound **1** as a potent small-molecule c-Kit inhibitor. Further profiling of this molecule through the MAP kinase and tyrosine kinase panels revealed a superb selectivity against KDR (Table 1, 460-fold), yet moderate to poor selectivity against other kinases, such as p38α, Lck, and Abl. Compared to Gleevec, **1** showed better Lck and Abl selectivity, but the p38α activity needed to be attenuated.

P38α mitogen-activated protein kinase (MAPK) has been shown to play a crucial role in regulating the biosynthesis of proinflammatory cytokines including tumor necrosis factor (TNFα) and interleukin-1 (IL-1β).[15] Inhibition of p38α is a proven therapeutic strategy in suppressing inflammation, which we intend to avoid in our c-Kit inhibitor program for treating similar diseases.

Examination of the structure of **1** reveals some potential liabilities, one of those being the orientation of the two amide groups, which might lead to amide hydrolysis. Indeed, both amide groups of **1** were hydrolyzed extensively in vivo (rat), resulting in the release of aniline-type metabolites.[16] To avoid these metabolites, the amide orientations were reversed to yield **2** and **3**. The inhibitory

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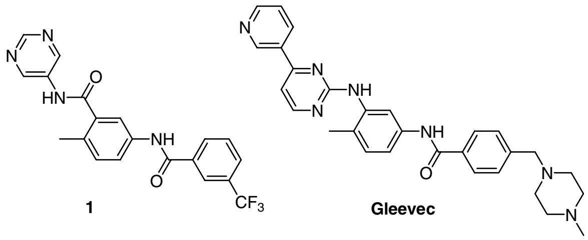
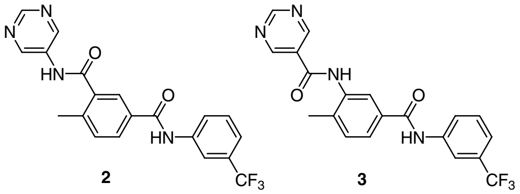
Table 1
Compound **1** vs. Gleevec as potent c-Kit inhibitor


1 **Gleevec**

	c-Kit[a] IC_{50} (μM)	p38α[a] IC_{50} (μM) (fold[b])	KDR[a] IC_{50} (μM) (fold[b])	Lck[a] IC_{50} (μM) (fold[b])	Abl[a] IC_{50} (μM) (fold[b])
1	0.014	0.20 (14×)	>8.33 (>460×)	0.24 (17×)	0.24 (17×)
Gleevec	0.052	13.7 (244×)	>25 (>481×)	0.32 (5×)	0.11 (2×)

[a] IC_{50} values are means of two or more separate determinations.
[b] Fold difference was calculated between enzyme assays.

Table 2
Reversed amides of **1** and their kinase activities


2 **3**

	c-Kit[a,b] IC_{50} (μM)	UT-7 prolif[a] IC_{50} (μM)	p38α[a] IC_{50} (μM) (fold[c])	KDR[a] IC_{50} (μM) (fold[c])	Lck[a] IC_{50} (μM) (fold[c])	Src[a] IC_{50} (μM) (fold[c])
1	0.014	0.012	0.20 (14×)	>8.33 (>595×)	0.43 (31×)	0.24 (17×)
2	0.037	0.030	1.75 (47×)	3.53 (95×)	0.546 (15×)	1.21 (33×)
3	0.108	0.031	0.455 (4×)	>5.0 (>46×)	2.76 (26×)	3.76 (35×)

[a] IC_{50} values are means of two or more separate determinations.
[b] The ATP concentration in this experiment is 50 μM.
[c] Fold difference was calculated between enzyme assays.

activities in a variety of enzymatic assays as well as the c-Kit-driven, cell-based assay[17] are shown in Table 2. For the lower-reversed amide **2**, the selectivity against p38α improved (47-fold, vs. 14-fold), even though the enzyme and cell-based potency for c-Kit decreased by about 3-fold. The doubly-reversed amide **3** was a weaker and less selective c-Kit inhibitor. In rat hepatocytes, the lower amide of **2** did not appear to hydrolyze as readily compared to **1**. Amide **2** was selected for additional studies due to its greater selectivity against p38α and improved metabolic profile.

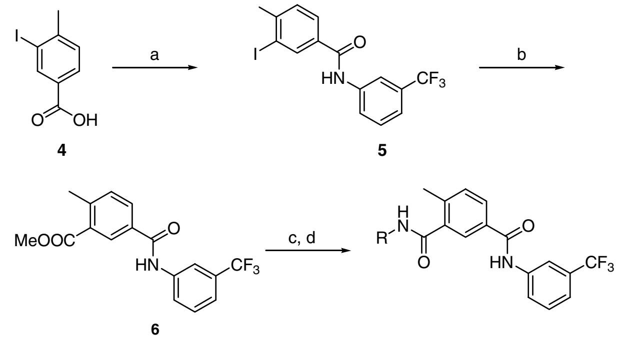
The metabolic stability and pharmacokinetic profile of amide **2** were examined next. The results of this study are shown in Table 3. The microsomal stability and pharmacokinetic profile following intravenous (iv) administration of compound **2** were similar to those of **1**; however, when dosed orally, the compound did not show significant oral bioavailability (~1% vs. 73% of **1**) in rats. Driven by these unfavorable properties, we next focused on improving the PK attributes of amide **2** in addition to optimizing the potency and selectivity.

We hypothesized that the 5-pyrimidyl unit of compound **2** might contribute to the metabolic liability of the molecule. Therefore, we set out to identify more stable replacements for this element. This region of the inhibitor interacts with the hinge region of the c-Kit protein; thus, it is expected that new groups containing an H-bond acceptor would have the highest probability of retaining potency.[18] Therefore, a series of heterocyclic amines were installed following the synthetic sequence shown in Scheme 1. Coupling of 3-iodo-4-methyl benzoic acid (**4**) with 3-(trifluoromethyl)aniline under standard EDC coupling conditions gave

Table 3
Metabolic stability and pharmacokinetic parameters in male Sprague–Dawley rats[a] for compounds **1**, **2** and analogs

	Metabolic stability[b]		iv[c]			po[d]		
	RLM (μl/min/mg)	HLM (μl/min/mg)	Cl (mL/h/kg)	V_{ss} (mL/kg)	$t_{1/2}$ (h)	AUC (ng*h/mL)	C_{max} (ng/mL)	F (%)
1	<50	<50	1624	1385	1.4	2286	861	73
2	48	38	1680	1543	1.1	75	8	1
9	<50	<100	854	1350	3.9	6153	956	53
11	<100	<100	922	1382	3.3	n/d	n/d	n/d
20	30	<14	4083	5364	4.4	1922	518	78

[a] $n = 3$ animals per study.
[b] In vitro intrinsic clearance after incubation with rat/human liver microsomes. Compound concentration = 1 μM, microsomal protein = 0.25 mg/mL.
[c] Dosed at 2 mg/kg as a solution in DMSO.
[d] Dosed at 10 mg/kg as a solution in 2% HPMC/1% Tween 80.



Scheme 1. Reagents and conditions: (a) 3-(trifluoromethyl)aniline, EDC, DCM, DMF, 78%; (b) $Pd(OAc)_2$, 1,3-bis(diphenylphospino)propane, Et_3N, MeOH, CO, 90 °C, 93%; (c) LiOH, THF; (d) RNH_2, HATU, Et_3N, DMF.

amide **5** in 78% yield. Carbonylation was carried out with $Pd(OAc)_2$ and DPPP in methanol under an atmosphere of CO to give 93% yield of the methyl ester **6**. After ester hydrolysis, the liberated acid was coupled to selected amines using standard conditions to give the final products.

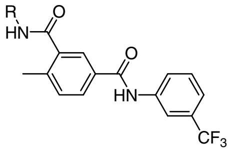
The biological activity of a select set of analogs bearing heteroaromatic head groups are shown in Table 4. By changing the pyrimidine (**2**) to pyridine (**7**), we observed ~2-fold increase in c-Kit potency; however, selectivity for p38α and KDR decreased. With the 5-amino group added to the pyrimidine (**8**) and pyridine (**9**), the c-Kit enzymatic activity improved by 7- and 3-fold, respectively, while p38α selectivity remained poor for both compounds. Notably, we observed improved KDR selectivity in the pyridine pair (for **9**, 67-fold vs. for **7**, 16-fold), and a slight decrease of selectivity in the pyrimidine pair (for **8**, 41-fold vs. for **2**, 95-fold). We speculated that the additional NH may allow for a bidentate H-bond interaction to the linker binding region, which improved affinity to all kinases, with the exception of KDR. Encouraged by this result, we then examined the acetylated amino pyrimidine **10** and pyridine **11**. For **10**, although we observed a ~10-fold decrease in c-Kit potency compared to **8**, selectivity against p38α improved, however, KDR selectivity diminished. For pyridine **11**, the c-Kit potency was maintained or slightly improved compared to **9**, selectivity against p38α improved, whereas selectivity against KDR dropped slightly. We concluded that the overall profiles of compounds **9** and **11** were improved with respect to c-Kit potency and off-target activity and therefore selected them for detailed PK studies.

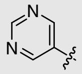
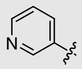
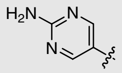
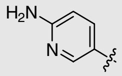
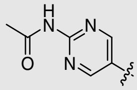
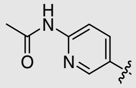
Table 3 shows a comparison of in vitro and in vivo rat PK profiles for compounds **2**, **9**, and **11**. When dosed intravenously, aminopyridine **9** exhibited lower clearance (0.85 L/h/kg) and an elongated half life (3.9 h) compared to **2**. Following oral administration, **9** showed an oral bioavailability of 53%. The acetylated aminopyridine **11** gave an iv PK profile similar to that of **9**.

Encouraged by the PK properties of **9**, we decided to use the aminopyridine head group as a starting point for further SAR studies aimed at improving the selectivity profile by modifying the right-hand side of **9**.

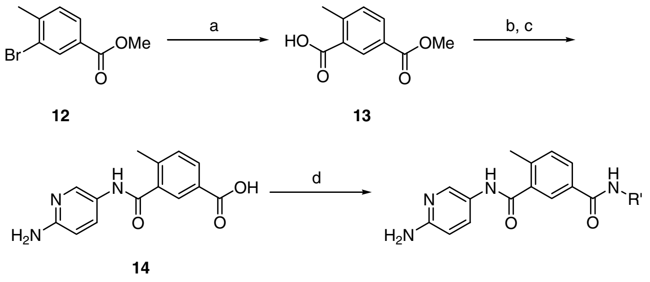
To prepare these analogs, we utilized the modified synthetic sequence shown in Scheme 2. Hydroxycarbonylation was carried out on methyl 3-bromo-4-methylbenzoate (**12**) with $Pd(OAc)_2$ and triphenylphospine in DMF and water under an atmosphere of CO to give the corresponding benzoic acid **13** in 22% yield. Coupling of acid **13** with 2,5-diaminopyridine under standard EDC coupling conditions, followed by hydrolysis of the methyl ester using

Table 4
Structure–activity relationship: left-hand modification of **2**



	R	c-Kit[a,b] IC_{50} (μM)	UT-7 prolif[a] IC_{50} (μM)	p38α[a] IC_{50} (μM) (fold[c])	KDR[a] IC_{50} (μM) (fold[c])
2		0.037	0.030	1.75 (47×)	3.53 (95×)
7		0.018	0.011	0.219 (12×)	0.294 (16×)
8		0.005	0.018	0.087 (12×)	0.205 (41×)
9		0.007	0.011	0.024 (3×)	0.470 (67×)
10		0.058	0.057	1.73 (30×)	0.748 (13×)
11		0.002	0.012	0.045 (22×)	0.114 (57×)

[a] IC_{50} values are means of two or more separate determinations.
[b] The ATP concentration in this experiment is 50 μM.
[c] Fold difference was calculated between enzyme assays.

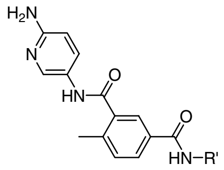


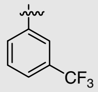
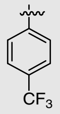
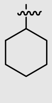
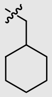
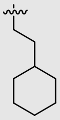
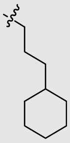
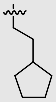
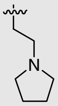
Scheme 2. Reagents and conditions: (a) Pd(OAc)$_2$, Ph$_3$P, Bu$_3$N, CsOAc, H$_2$O, DMF, CO, 90 °C, 22%; (b) 2,5-diaminopyridine, EDC, HOBt, *N,N*-diisopropylethylamine; (c) LiOH, THF, 55% (two steps); (d) R′NH$_2$, PyBop, *N,N*-diisopropylethylamine, DMF.

lithium hydroxide, gave acid **14** in 55% yield over two steps. The subsequent coupling step was carried out using PyBop and Hünig's base in DMF to provide the final products in good yields.

Most anilines bearing various substitutions on the phenyl group exhibited a potency and selectivity profile similar to **9**. When *m*-CF$_3$ was replaced with *p*-CF$_3$ (Table 5, compound **15**), a ∼17-fold decrease in c-Kit potency and > 5 μM activity for KDR was observed. We therefore envisioned that increasing the steric demand of substituents in the linear direction may eliminate KDR activity. Hence, we turned to saturated ring systems (e.g., cyclohexyl) to replace the phenyl group and systematically reached out by incorporating a carbon chain of variable length. When cyclohexylamine was directly attached (compound **16**), the c-Kit activity was dramatically reduced, whereas introduction of a methylene linker (**17**) led to a 4-fold improvement. Strikingly, the ethylene-linked analog **18** demonstrated an IC$_{50}$ value of 35 nM for c-Kit, and was inactive against KDR (up to 25 μM). Following this trend, the propylene derivative **19** showed another 4-fold increase in c-Kit potency. However, the selectivity against p38α and KDR did not improve.

Table 5
Structure–activity relationship: right-hand modification of **9**



	R′	c-Kit[a,b] IC$_{50}$ (μM)	UT-7 prolif[a] IC$_{50}$ (μM)	p38α[a] IC$_{50}$ (μM) (fold[c])	KDR[a] IC$_{50}$ (μM) (fold[c])
9		0.007	0.011	0.024 (3×)	0.470 (67×)
15		0.120	n/a	n/a	>5 (>42×)
16		4.47	0.761	n/a	n/a
17		1.18	0.143	n/a	n/a
18		0.035	0.014	0.181 (5×)	>25 (>714×)
19		0.009	0.0003	0.0578 (6×)	>5 (>556×)
20		0.077	0.023	0.998 (13×)	>25 (>325×)
21		3.64	n/a	n/a	n/a

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[a] IC$_{50}$ values are means of two or more separate determinations.
[b] The ATP concentration in this experiment is 50 μM.
[c] Fold difference was calculated between enzyme assays.

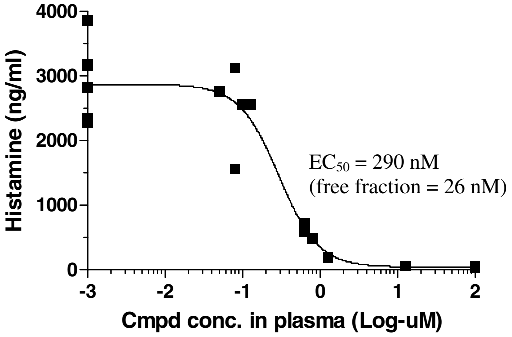

Figure 1. The correlation of serum histamine levels with compound **20** concentration in plasma.

From this study, we concluded that the ethylene linker provides an optimal balance of c-Kit potency and off-target activity. We then studied the effect of the ring size on biological activity and found that cyclopentyl (**20**) resulted in good c-Kit potency (IC_{50}: 77 nM for the enzyme and 23 nM for the UT7 cell) and excellent selectivity against the other kinases (IC_{50}: p38α, 1.0 μM; KDR, >25 μM; Lck, 1.9 μM; Src, 5.0 μM). Substituting the cyclopentyl group with a basic pyrrolidine (**21**) resulted in loss of c-Kit potency.

Based on the encouraging in vitro kinase activity profile, we studied the PK properties of compound **20**. When dosed iv, the compound had a clearance of 4.1 L/h/kg and a half life of 4.4 h. Following oral administration (10 mg/kg), it exhibited a C_{max} of 518 ng/mL and AUC of 1922 ng*h/mL, with an estimated oral bioavailability of 78%. The rat and mouse plasma protein binding were determined by ultracentrifugation to be 82% and 91%, respectively.

The PK profile of **20** was deemed sufficient to conduct a proof-of-concept study in vivo. For this, compound **20** was tested in an in vivo murine model of mast cell activation and systemic anaphylaxis. Stem cell factor, when administered systemically, activates mast cells, and provides a physiologically relevant and clear mechanistic assessment of c-Kit inhibitors.[19] Mice[20] were dosed with **20** orally 1 h before an intravenous injection of SCF.[21] Fifteen minutes after SCF treatment, the mice were euthanized by CO_2 inhalation and blood was collected by cardiac puncture for histamine measurement and PK analysis. SCF-induced serum histamine was quantified, and ED_{50} values were calculated from a dose-response curve (Fig. 1). From this study,[22] we concluded that compound **20** dose-dependently inhibited SCF-induced histamine release with an EC_{50} value of 26 nM (free fraction), similar to the UT7 cell-based IC_{50}.

In summary, we have identified a potent and selective small-molecule c-Kit inhibitor through a SAR study based on the screening hit **1** of our kinase-preferred compound collection. Optimization for metabolic stability, PK, and potency yielded compound **9**. To ensure good safety margin from angiogenesis and inflammation pathways, we further optimized KDR and p38α kinase selectivity to yield **20**. Compound **20** exhibited an IC_{50} of 23 nM in the c-Kit-driven UT7 cell-based assay and is significantly less active against several other kinases ($IC_{50} > 1\ \mu$M for the enzyme assay). Compound **20** also showed a good oral PK profile in rats, and is efficacious in a murine model of mast cell activation blocking histamine release in vivo with an EC_{50} of 26 nM.

Acknowledgment

The authors thank Dr. Alan Allgeier for scaling up intermediate **13** for our SAR studies.

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16. **1** was dosed intravenously in rat at 5 mg/kg. The plasma, bile and urine samples were collected and analyzed for metabolites at time points of 1, 4, and 8 h.
17. UT-7 is a factor dependent human megakaryoblastic leukemia cell line that carries wild type c-Kit receptor. The purpose of this assay is to test the general anti-proliferative effect of small molecule compounds on SCF-stimulated UT-7 cells.
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20. C57 Bl/6 female mice were obtained from Charles River Laboratories and were housed under pathogen-free conditions at Amgen animal facility in Thousand Oaks. 10–15 weeks old female mice were used for experiments.
21. SCF was obtained from Protein Science at Amgen Inc. Lot # was 08032, 0.95 mg/ml in PBS. ELISA kit for histamine measurement was obtained from Oxford Biomedical Research (EA31).
22. Gleevec was also tested in the SCF challenge model. Mice were dosed with 100 mg/kg of compound po 1 h prior to the SCF injection. Histamine release was inhibited by 88%.