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Identification of 2-acylaminothiophene-3-carboxamides as potent inhibitors of FLT3

Raymond J. Patch, Christian A. Baumann, Jian Liu, Alan C. Gibbs, Heidi Ott, Jennifer Lattanze and Mark R. Player*

Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, L.L.C., 8 Clarke Drive, Cranbury, NJ 08512, USA

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Abstract—A series of 2-acylaminothiophene-3-carboxamides has been identified which exhibit potent inhibitory activity against the FLT3 tyrosine kinase. Compound **44** inhibits the isolated enzyme (IC_{50} = 0.027 μM) and blocks the proliferation of MV4-11 cells (IC_{50} = 0.41 μM). Structure–activity relationship studies within this series are described in the context of a proposed binding model within the ATP binding site of the enzyme.

Acute myeloid leukemia (AML) is the most common form of adult leukemia and comprises approximately 20% of childhood leukemias.[1] Current treatment for AML typically involves treatment with general cytotoxic agents such as Cytarabine (Ara-C) and Daunorubicin to induce remission of disease. Induction therapy is followed by a variety of post-induction treatments ranging from high dose Cytarabine to bone marrow transplant.[2] Such aggressive therapies are often poorly tolerated by elderly patients. Additionally, since less than 20% of diagnosed AML patients survive more than 4 years, improved treatment paradigms are needed.

Activating mutations of the class III receptor tyrosine kinase FLT3 have been detected in approximately 30% of patients with acute myelogenous leukemia and a small number of patients with acute lymphoblastic leukemia or myelodysplastic syndrome.[3] Patients with such FLT3 mutations have a poor prognosis, with decreased remission times and disease free survival.[4] Furthermore, >90% of blasts from AML patients express either wild-type or mutant FLT3.[5] This suggests that inhibition of FLT3 may be of significant therapeutic benefit for AML patients.

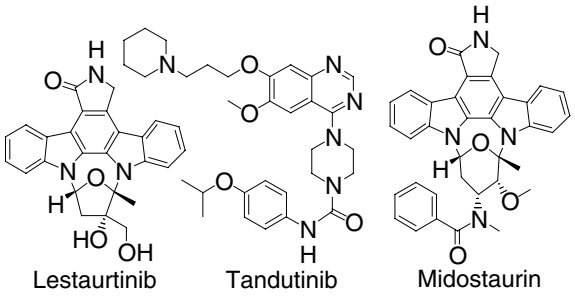

Figure 1. FLT3 inhibitors in clinical development.

Reported FLT3 inhibitors under advanced clinical development include the staurosporine structural analogues, lestaurinib and midostaurin, the quinazoline, tandutinib, and others.[6] These exhibit varying degrees of non-selectivity, particularly toward other receptor tyrosine kinases, and as such, may present undesirable side-effect profiles. Thus, the need still exists for further development of potent and selective FLT3 inhibitors (Fig. 1).

During the course of our screening efforts to discover FLT3 inhibitors derived from alternative chemotypes, we identified a class of 2-acylaminothiophene-3-carboxamides that exhibited potent inhibitory activities. Some compounds of this class had been reported to potentially inhibit a range of kinases in an earlier published patent application; however, no data were provided to support these claims, nor was FLT3 among the potentially

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* Corresponding author. Tel.: +1 610 458 6980; e-mail: mplayer@prdus.jnj.com

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affected kinases cited.[7] A more recent application cites inhibition of a broad panel of kinases (including FLT3) by various compounds within this class; however, data are presented for only a single inhibitory concentration (10 μM).[8] Herein, we report on initial structure–activity relationships for this series with the goal of mapping out a general binding motif for the inhibitory interaction with FLT3.[9] This information may serve in the development of FLT3 inhibitors exhibiting greater potencies and selectivity.

A survey of simple 2-acylaminothiophene-3-carboxamides (Table 1) revealed a minimal tolerance for steric bulk at the carbon adjacent to the carbonyl of the secondary amide and a preference for sp^2 hybridization at this center. Thus, while acetamido and propionamido analogues **1** and **2**, respectively, inhibited FLT3 at low-micromolar levels, larger alkyl amides resulted in reduced activities. Of particular interest are the contrasting activities of the pseudo sp^2 isopropylcarboxamide **3** and cyclopropylcarboxamide **4**; the former exhibiting greatly diminished activity. Cyclohexylcarboxamide **5** was inactive at a concentration of 10 μM; however, its aromatic counterpart, **6** and 2-furoylamide **7**, exhibited significantly enhanced activities (IC_{50} = 0.36, 0.17 μM, respectively).[10] Pyridine carboxamides (**8**–**10**) exhibited a range of inhibitory potencies against the isolated enzyme with *p*- > *o*- > *m*-isomer (**10** > **8** > **9**), an order which may reflect important electronic ring effects as opposed to specific electrostatic interactions with the enzyme.

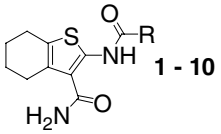
Substituent effects were evaluated on benzoylamide **6** (Table 2). Both steric and electronic factors affected activity in this series. Small substituents (methyl, **11**; methoxyl, **12**) at the 4-position afforded increased FLT3 enzyme inhibition. Somewhat larger substituents were tolerated (halo, **13**, **14**; acetyl, **15**; *t*-butyl, **16**), but with increasing size activity dropped precipitously (phenyl, **17**; phenoxyl, **18**). Similar effects on substitution of the 3-position were observed, with methyl and methoxyl substituents providing increased activity (**19**, **20**; IC_{50}s = 0.14 and 0.11 μM, respectively). Ortho substitution was uniformly detrimental to activity, likely due to induced conformational effects away from co-planarity.

Interestingly, while enhanced inhibitory activity was obtained with 3,4-dimethoxybenzoylamide **25** (IC_{50} = 0.042 μM), 3,4,5-trimethoxybenzoylamide **28** was devoid of activity.

The introduction of spacer atoms between the aryl ring and carbonyl carbon was also investigated (Table 3). The results are consistent with a requirement of coplanarity between these two groups. Thus, saturated homologues showed greatly reduced inhibitory activities compared with vinyl analogues. Again, methoxyl substitution was beneficial, particularly in the case of 3,4-dimethoxycinnamoyl analogue **39**.

Structure–activity relationships of the bicyclic thiophene-3-carboxamide core were also investigated (Table 4). Deletion of the fused cyclohexyl methylenes (**42**) resulted in a 50-fold reduction in FLT3 inhibitory activity, much of which could be recovered by

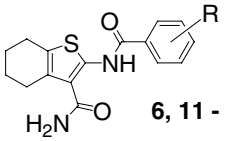
Table 1. FLT3 inhibitory activities for 2-acylaminothiophene-3-carboxamides **1**–**10**



Compound	R	FLT3 inhibition IC_{50}[a] (μM)	MV4-11 proliferation IC_{50}[b] (μM)
1	Methyl	2.0 (±0.21)	
2	Ethyl	2.1 (±0.92)	
3	*i*-Propyl	>10	
4	*c*-Propyl	1.3 (±0.37)	1.2
5	*c*-Hexyl	>10	
6	Phenyl	0.36 (±0.07)	>10
7	Furan-2-yl	0.17 (±0.05)	1.5
8	Pyridin-2-yl	0.64 (±0.10)	0.35
9	Pyridin-3-yl	1.1 (±0.41)	2.0
10	Pyridin-4-yl	0.11 (±0.05)	2.1

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] All dose–response data are calculated from an average of three replicates per dose.

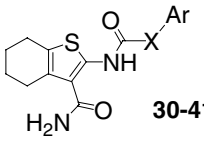
Table 2. FLT3 inhibitory activities for 2-phenacylaminothiophene-3-carboxamides **11**–**29**



Compound	R	FLT3 inhibition IC_{50}[a] (μM)	MV4-11 proliferation IC_{50}[b] (μM)
6	H	0.36 (±0.07)	>10
11	4-Me	0.052 (±0.01)	0.45
12	4-OMe	0.12 (±0.03)	1.3
13	4-Cl	0.31 (±0.05)	1.4
14	4-Br	0.31 (±0.13)	1.5
15	4-Ac	0.59 (±0.18)	1.8
16	4-*t*-Bu	0.63 (±0.09)	1.6
17	4-Ph	>10	
18	4-OPh	>10	
19	3-Me	0.14 (±0.03)	3.2
20	3-OMe	0.11 (±0.06)	1.5
21	3-Cl	0.63 (±0.10)	2.2
22	2-Me	3.0	
23	2-OMe	>10	
24	2-Cl	>10	
25	3,4-Di-OMe	0.042 (±0.03)	0.34
26	3,4-Methylenedioxy	0.14 (±0.01)	0.89
27	3,5-Di-OMe	1.7 (±0.62)	>10
28	3,4,5-Tri-OMe	>10	
29	3,4-Di-Me	0.54 (±0.40)	0.71

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] All dose–response data are calculated from an average of three replicates per dose.

Table 3. FLT3 inhibitory activities for homologated 2-acylaminothiophene-3-carboxamides **30–41**

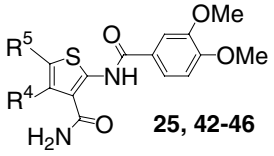


30-41

Compound	X	Ar	FLT3 inhibition IC_{50}[a] (μM)	MV4-11 proliferation IC_{50}[b] (μM)
30	$–CH_2–$	Ph	3.2 (±1.1)	>10
31	$–CH_2–O–$	Ph	>10	
32	$–CH_2–S–$	Ph	>10	3.8
33	$–CH_2–O–$	4-MeOPh	~10	
34	$–CH_2–CH_2–$	Ph	3.6 (±0.76)	
35	$–CH_2–CH_2–$	4-MeOPh	2.7	>10
36	–CH=CH–	Ph	0.25 (±0.02)	0.53
37	–CH=CH–	4-MeOPh	0.25 (±0.06)	1.8
38	–CH=CH–	4-*i*-PrPh	0.49 (±0.17)	2.7
39	–CH=CH–	3,4-Di-OMe	0.13 (±0.04)	2.0
40	–CH=CH–	Furan-2-yl	0.56 (±0.06)	0.48
41	–CH=CH–	5-Methylfuran-2-yl	1.0 (±0.23)	10

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] All dose–response data are calculated from an average of three replicates per dose.

Table 4. Effects of thiophene substitution upon FLT3 inhibitory activities for 2-phenacylaminothiophene-3-carboxamides **42–46**



25, 42-46

Compound	R^4, R^5	FLT3 inhibition IC_{50}[a] (μM)	MV4-11 proliferation IC_{50}[b] (μM)
42	H, H	2.1 (±1.3)	2.4
43	Me, Me	0.14 (±0.10)	0.33
44	$–(CH_2)_3–$	0.027 (±0.01)	0.41
25	$–(CH_2)_4–$	0.042 (±0.03)	0.34
45	$–(CH_2)_5–$	6.9 (±2.9)	
46	$–(CH_2)_6–$	>100	

[a] Values are means of three experiments, standard deviation is given in parentheses.
[b] All dose–response data are calculated from an average of three replicates per dose.

incorporation of methyl groups at R^4 and R^5 (**43**). Within the series of fused alkylene ring homologues, activity was inversely correlated with ring size, with optimal activity residing with cyclopentyl-fused thiophene **44**.

In order to evaluate the functional activities within this series, those inhibitors with approximate sub-micromolar IC_{50}s were assayed for antiproliferative effects on MV4-11 cells (a human acute myelogenous leukemia cell line expressing a constitutively activated mutant FLT3).[11] With only a few exceptions, activities between the two assays were well correlated. Thus, effective anti-proliferative concentrations (IC_{50}s) were roughly 10-fold less than corresponding inhibitory concentrations in the isolated enzyme assay. Notable exceptions to this general correlation were 2-pyridinylcarboxamide **8**, dimethylbenzoylamide **29**, cinnamoylamide **36**, furylacrylamide **40**, and 4,5-dimethylthiophene analogue **43**, each of which exhibited greater anti-proliferative effects than would have been anticipated from their FLT3 inhibitory activities. This could be the result of enhanced cell permeabilities of these analogues; alternatively, it is possible that additional anti-proliferative mechanisms were involved in these cases.

In order to assess kinase selectivity, compound **25** was tested against a standard panel of kinases (Table 5). At a concentration of 3 μM, only the human Abl-related gene kinase Arg(h) was inhibited at a level approaching 50%.

Table 5. Kinase selectivity screen for compound **25**

Kinase	% Inh[a]
Abl(h)	21
AMPK(r)	−4
Arg(h)	49
Axl(h)	−10
Bmx(h)	9
BTK(h)	14
CDK2/cyclinA(h)	−22
CDK7/cyclinH/MAT1(h)	−1
CHK1(h)	−7
c-RAF(h)	−1
EphB2(h)	−11
Fes(h)	−12
Fyn(h)	33
GSK3ß(h)	−2
JNK3(h)	−6
Lyn(h)	21
Met(h)	13
PKCμ(h)	3
PKD2(h)	−7
Rsk1(h)	3
TrkB(h)	39
Fes(h)	−12

[a] Compound **25** was tested at single concentration of 3 μM; ATP concentration was 10 μM. Values are averages of duplicate experiments.

The low level of inhibition of TrkB in this assay is also noteworthy, given the reported potent inhibitory activity of lestaurtinib against Trk isoenzymes A–C ($IC_{50} \sim 4$ nM).[12]

To help rationalize the aforementioned structure–activity relationships and drive future syntheses, a homology model of the FLT3 kinase domain was constructed and compound docking studies were carried out. The kinase domain of FGF receptor 1 (pdb Accession No. 2FGI) was chosen as the template for FLT3 homology modeling because of the high homology and identity (66% and 50%, respectively) between the two enzymes.

Multiple conformations of representative compounds from this series were generated, each with an internal hydrogen bond between the primary amide CO and the secondary amide NH fixed in place (Fig. 2).[13] The conformers were then subjected to rigid body docking in order to determine a low-energy binding mode. (In these studies, we assume that the intramolecular hydrogen bond is retained upon enzyme binding.) Figure 2 illustrates a common low-energy binding mode, which correlates well with the SARs observed for this series (compound **25**). The figure shows a cut-away of the active site between the N- and C-terminal lobes of the kinase. The compounds are oriented so that the primary amide engages in a hydrogen bonding interaction with the backbone carbonyl of glutamate 692—the so-called 'hinge region' of receptor tyrosine kinases. The secondary amide projects its carboxylate-derived substituent into a narrow cleft underneath phenylalanine 691 (in the region of ATP binding). This is consistent with a preference for aromatic or extended aromatic (vinylaryl) systems that could participate in π-interactions with Phe 691 while at the same time avoiding steric clashes in this narrow region. This binding mode also illustrates the preference for (1) co-planarity of secondary amide substituents and (2) a free primary amide capable of hydrogen bonding to the hinge region of the backbone.

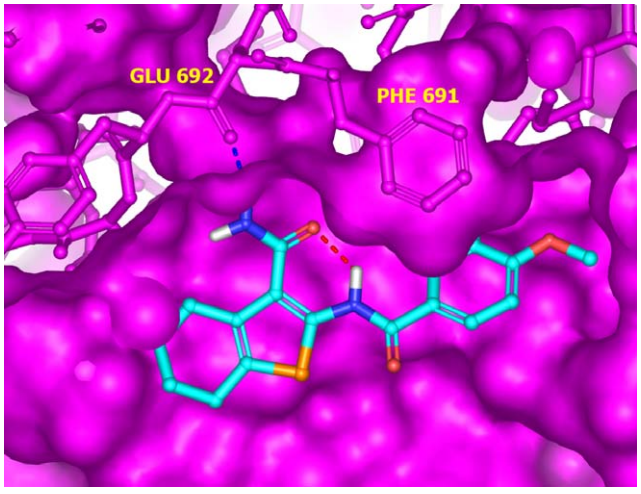


Figure 2. Cut-away illustration of the active site between the N- and C-terminal lobes (magenta). Predicted low-energy binding mode of compound **25** (cyan). Intra-molecular (red dash) and inter-molecular (blue dash) hydrogen bonds are shown. Only ligand polar hydrogens are shown.

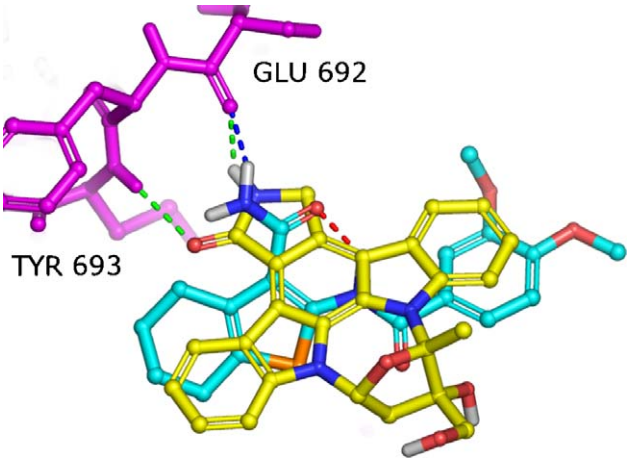


Figure 3. Cut-away illustration of the active site with superimpositions of low-energy binding modes for lestaurtinib (yellow) and compound **25** (cyan). Hydrogen bonds between the hinge region of the backbone and lestaurtinib are shown in green; that with compound **25** is shown in blue. Only polar hydrogens are shown.

We were interested in comparing this hypothetical binding mode with one that might be predicted for the FLT3 inhibitor, lestaurtinib. Docking lestaurtinib into FLT3 (Fig. 3) revealed a preferred orientation similar to many binding modes of staurosporine-like compounds found in kinase co-crystal structures (e.g., pdb Accession Nos. 1AQ1, 1BYG, and 1NVQ). Lestaurtinib binds in a typical bi-dentate fashion to the protein backbone (Glu 692, Tyr 693), whereas the acylaminothiophenecarboxamides bind with a mono-dentate interaction (Glu 692 only). However, the relative positions of the molecules within the active site are very similar. Both chemotypes are structurally quite flat and occupy the same plane within the active site. As shown in Figure 3, the ring systems of both molecules overlap significantly; however, the methoxyl substituents of the benzamide allow for further extension into the binding region than is accessed by lestaurtinib's unsubstituted indolocarbazole core. Such substitution may confer a different kinase selectivity profile on this chemotype than is observed with staurosporine-based molecules. Finally, this modeled overlap highlights potential regions where future synthetic plans might be directed to afford FLT3 inhibitors with greater potencies and enhanced clinical effectiveness.

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9. Compounds for this study could be readily synthesized from the corresponding 2-aminothiophene-3-carboxamide by reaction with the appropriate acid chloride under basic conditions as follows: To a solution of the acid chloride (0.5 mmol) in THF (2 mL) was added the 2-aminothiophene-3-carboxamide (0.5 mmol) followed by DIEA (1 mmol), and the mixture was stirred at room temperature for 2 h. The mixture was then diluted with ether and filtered. The resulting precipitate was suspended in DCM, extracted with water (to remove DIEA·HCl), and filtered again to afford the pure product (LCMS; ^{1}H NMR).
10. (FLT3 kinase assay protocol). To determine the activity of the compounds of the present invention in an in vitro kinase assay, inhibition of the isolated kinase domain of the human FLT3 receptor (a.a. 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 PhosphoTyrosine 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 $MgCl_2$, and 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.
11. MV4-11 cell-based assay. (a) Quentmeier, H.; Reinhardt, J.; Zaborski, M.; Drexler, H. G. *Leukemia* **2003**, *17*, 120(b) MV4-11 (ATCC Number: CRL-9591) cells were plated at 10,000 cells per well in 100 μL of in 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.
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13. Correlations between proton NMR chemical shifts and calculated proton magnetic shielding tensors (via DFT calculations at the B3LYP, 6-311+G[2d,p] level of theory) support the existence of this internal hydrogen bond for compounds within this class. This is manifested primarily by extensive deshielding of the secondary amide NH ($\delta \sim 13$ ppm in DMSO-d_6).