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VX-322: A Novel Dual Receptor Tyrosine Kinase Inhibitor for the Treatment of Acute Myelogenous Leukemia

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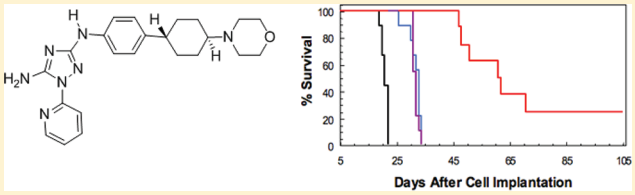
Supporting Information

ABSTRACT: In acute myelogenous leukemia (AML), the FLT3 receptor tyrosine kinase (RTK) is highly expressed with 30% of patients expressing a mutated, constitutively active form of this protein. To inhibit this receptor, VX-322 was developed and found to be very potent against both the FLT3 and c-KIT RTKs with enzyme K_i values of <1 nM and a cellular IC_{50} between 1 and 5 nM. It was efficacious in a FLT3-ITD dependent myeloproliferative mouse model, doubling survival compared to other FLT3 inhibitors, with 25% of the mice cured. Upon treatment of primary AML patient blast cells, the dual inhibition of FLT3 and c-KIT was superior to inhibitors targeting a single RTK. Thus, this compound may represent an improved pharmacologic and selectivity profile that could be effective in the treatment of AML.



INTRODUCTION

Acute myelogenous leukemia (AML) is the most common and aggressive form of leukemia, resulting from the uncontrolled proliferation of immature myeloid malignant cells that have lost the ability to differentiate.[1,2] Treatment options have changed little in the past 20 years, consisting of various combinations of cytotoxic agents with the overall goal of removing leukemic blast cells, returning the blood cell counts to normal levels.[3,4] This approach is effective in younger populations, as they are able to tolerate the high concentrations of these noncell type selective cytotoxics required to attain a normal blood count and induce complete remission. For AML patients under the age of 60, approximately 80% will attain remission, with 35% exhibiting disease-free survival for more than five years.[5,6] Patients over the age of 60 make up more than 50% of the AML population and can tolerate only low concentrations of the current cytotoxic agents used to treat this disease.[7] This results in only 50% attaining remission, with 10% achieving disease-free survival for greater than 5 years.[8,9] With an overall 5-year survival rate of 24%, a need for more effective and better tolerated therapies is paramount.

To better understand and treat this disease, proteins that are central mediators of leukemic blast cell survival have been identified that may represent susceptible targets for small-molecule inhibitors. Of the several proteins identified, the FMS-like tyrosine kinase III receptor (FLT3) appears to be among the most promising targets.[10,11] Approximately 25% of AML patients possess an internal tandem duplication (ITD) of the FLT3 juxtamembrane domain (JM), causing ligand independent, constitutive activation of the receptor.[12,13] A number of other FLT3 activating mutations in the kinase domain have been discovered as well.[14] When introduced into cell lines that are dependent upon the FLT3 ligand (FL) for growth, these FLT3 mutations cause ligand independent cell proliferation and transformation.[15] AML patients which harbor FLT3 activation mutations have a poor prognosis further supporting the importance of this target in AML.[16−18]

Several compounds that inhibit FLT3 activity have been evaluated in clinical trials which show a moderate and transient reduction in blast count, primarily in the periphery.[19−21]

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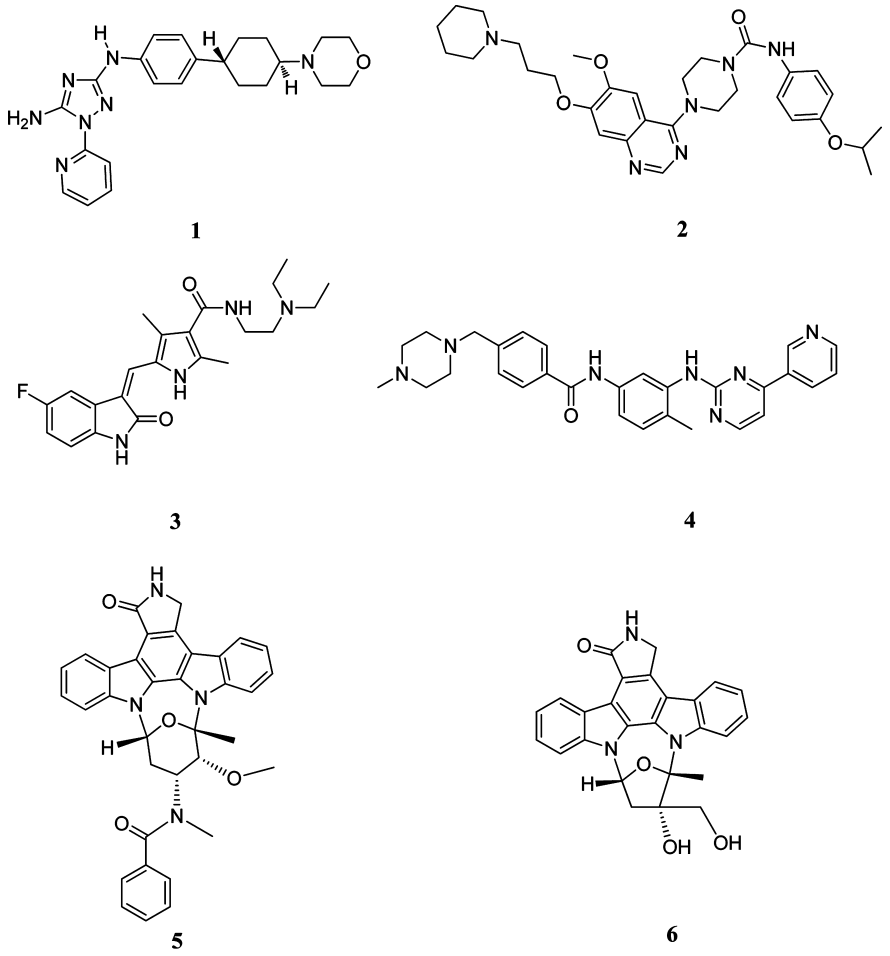

Figure 1. Structure of **1** and RTK inhibitors in the clinic.

Sustained inhibition of FLT3 to a level of >80% was required for a clinical response.[22] It is unclear why these drug candidates had limited efficacy as a monotherapy. Several factors alone or in combination may contribute to the mild efficacy observed for these inhibitors including potency, narrow therapeutic index, development of resistance mutants, and/or pharmacokinetics.[19,21] Additionally, because AML is heterogeneous, blocking a single target such as mutant FLT3 will likely have partial efficacy against this disease. Because both the FLT3 and c-KIT receptors are found on >80% of AML blasts, a small molecule equally potent against these receptors may help to improve efficacy.[23,24]

Here we describe the in vitro and in vivo effects of the small molecule **1** (VX-322, Figure 1), an inhibitor of the FLT3 and c-KIT receptors. This inhibitor displays nanomolar potency in biochemical kinase and cellular assays against both RTKs. This translated to an in vivo efficacy, as assessed in a FLT3-ITD dependent myeloproliferative model,[25] where its potency and pharmacokinetic properties increased the survival time by 1.7-fold, compared to the RTK inhibitors Tandutinib (**2**) and Sutent (**3**). In addition, 25% of the mice treated did not succumb to the disease. To determine how important dual RTK inhibition would be in treating AML patients, a series of small-molecule inhibitors that were selective for inhibiting FLT3 or both FLT3 and c-KIT were evaluated. Efficacy was assessed against several primary AML patient blast samples. FLT3 selective inhibitors were cytotoxic, with an average IC_{50} value of 4.1 μM. Dual inhibition of FLT3 and c-KIT dramatically reduced the IC_{50} values to an average value of 0.11 μM. This synergistic effect was confirmed upon dosing FLT3 selective inhibitors with the c-KIT inhibitor Imatinib (**4**).

These results support the idea that the inhibition of multiple signaling pathways is necessary to strongly suppress the growth of the leukemic blast cells. In addition, having an improved pharmacological profile compared to other FLT3 inhibitors could further increase the efficacy in treating AML patients.

RESULTS

Enzymatic Characterization. Results from medicinal chemistry efforts led to **1**, a 3,5-diamino-1,2,4-triazole inhibitor of FLT3 and c-KIT (Figure 1).[26]

Compound **1** was found to be a reversible ATP competitive inhibitor of FLT3 with a K_i value of 0.17 nM. To evaluate its selectivity, the molecule was characterized against a panel of tyrosine kinases, including members of the class III RTK family: c-KIT, PDGFR-β, and FMS. It was found to have similar potency against c-KIT, with an estimated K_i value of less than 1 nM but demonstrated a >100-fold selectivity window against the other tyrosine kinases (Table 1). Additionally, compound **1** exhibited a >2000-fold window against a panel of serine/threoine kinases (Table S1, Supporting Information).

Compound 1 Is Cytotoxic to Cell Lines Expressing FLT3 or c-KIT. To assess the effectiveness and selectivity of compound **1** against type III RTKs in cells, a panel of TF1 cell lines were engineered that expressed each type III RTK family member (Table 2). In addition, TF1 cells expressing either the

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Table 1. Selectivity Profile of Compound 1 against a Panel of Tyrosine Kinases[a]

	FLT3	c-KIT	PDGFR-β	KDR	FGFR3	TIE2	AXL
1	0.00017	<0.001	0.2	0.085	0.4	0.2	0.04
	cMET	SYK	JAK3	SRC	LCK	LYN	ABL
1	0.13	0.22	0.28	0.77	0.4	0.4	0.4

[a] K_i values were determined by an in vitro kinase assay and are represented in μM.

Table 2. Nanomolar Cytotoxicity Exhibited by 1 against FLT3 and c-KIT Expressing Cell Lines[a]

	FLT3 WT	FLT3 ITD	FLT3 D835Y	c-KIT	PDGFR-β
1	0.0005	0.0013	0.0014	0.0016	0.33
	FMS	TF1 parental	MV4−11	Kasumi-1	THP-1
1	>4	>4	0.0002	0.03	>4

[a] IC_{50} values are represented in μM.

D835Y or ITD constitutively active mutants of FLT3 were also generated. Compound **1** was dosed at increasing concentrations, and cell viability was determined after 72 h. IC_{50} values in the range of 0.5 to 1.6 nM with >95% reduction in viability against wild-type FLT3, mutant FLT3, and wild-type c-KIT TF1 cells was observed (Figure 2a,b and Table 2). In contrast, **1** exhibited an IC_{50} value of 330 nM in the TF1-PDGFR-β cell line and was not cytotoxic against the TF1-FMS cell line or the parental TF1 cells, up to 4 μM (Table 2).

To assess the efficacy of **1** in FLT3 dependent AML cells, the compounds were tested in the MV4−11 leukemic cell line which is homozygous for the FLT3-ITD mutation (Table 2). Similar to the TF1 FLT3-ITD engineered line, treatment of MV4−11 cells with **1** reduced cell viability by >95% with an IC_{50} of 0.2 nM (Figure 2c and Table 2). c-KIT inhibition was also observed with **1** in the Kasumi-1 cell line, which is dependent upon a constitutively active mutation of residue N822K in c-KIT for survival.[27] Cell viability was reduced, with an IC_{50} of 30 nM (Table 2).

To verify that the cytotoxicity exhibited by **1** was through the inhibition of FLT3 and c-KIT, the inhibitor was tested against the THP-1 AML cell line. These cells do not express any constitutively active forms of FLT3 or c-KIT, and their growth and survival is factor independent. No cytotoxicity was observed in this cell line at concentrations greater than 4 μM, confirming that the cellular effects of **1** were selective for FLT3 and c-KIT expressing cells (Table 2).

Cytotoxicity Occurs through Apoptosis, And Potency Is Retained in the Presence of Human Serum. The mechanism of cell death induced by **1** was shown to be through apoptosis, as measured by an increase in annexin V surface binding. Thirty to forty percent of the MV4−11 cells bound annexin V after a 24 h incubation with the compound, increasing to 70% after 48 h (Figure 2d). Consistent with our results, inhibition with other known FLT3 inhibitors has been shown to induce apoptosis.[28]

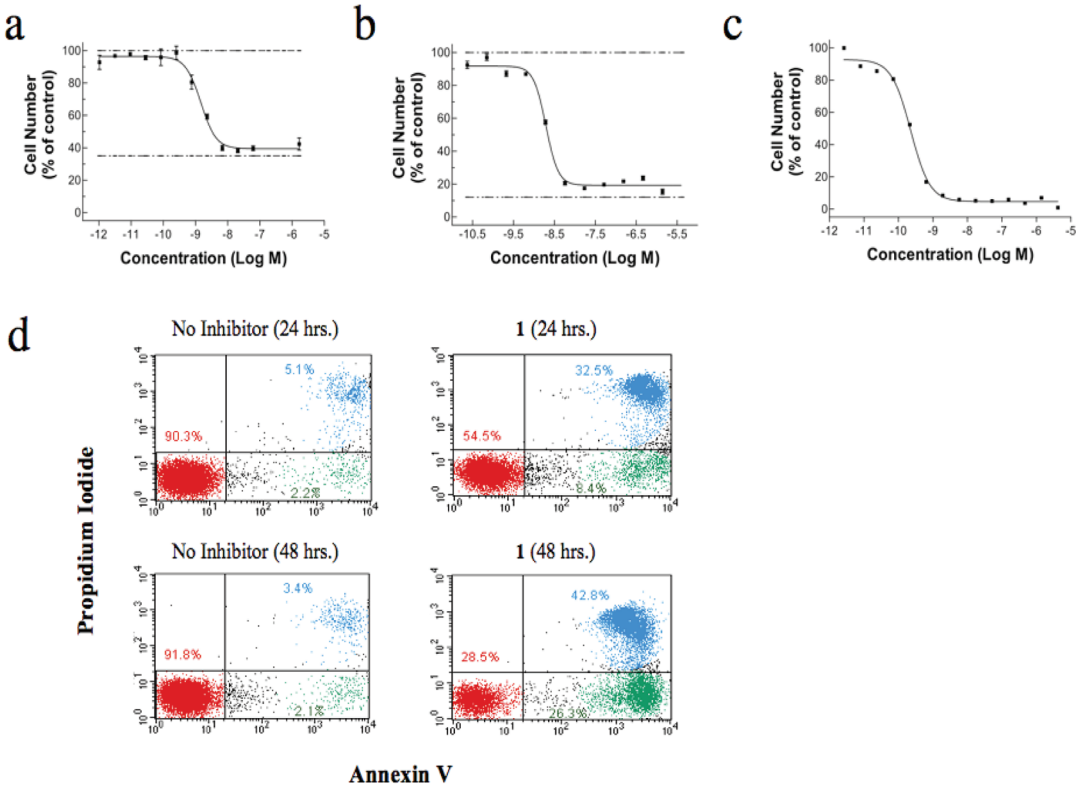

Figure 2. Cell viability is decreased in FLT3 and c-KIT dependent cell lines. (a) TF1 cells expressing the wild-type FLT3 receptor were incubated in the presence of the 25 ng/mL of the FLT3 ligand (FL) with varying concentrations of **1** and viability measured after 72 h. Dashed lines represent the boundaries for FL dependent growth in the absence of compounds. (b) TF1 cells expressing c-KIT were incubated in the presence of 25 ng/mL of SCF ligand with **1** and viability measured after 72 h. (c) MV4−11 cells were incubated in the presence of inhibitor for 72 h. and viability determined. (d) MV4−11 cells were incubated with 100 nM of inhibitor and apoptosis measured by annexin V and propidium iodide staining. For each panel, nonapoptotic cells are indicated in red while the annexin V positive and propidium iodide/annexinV positive apoptotic cells are shown in green and blue, respectively.

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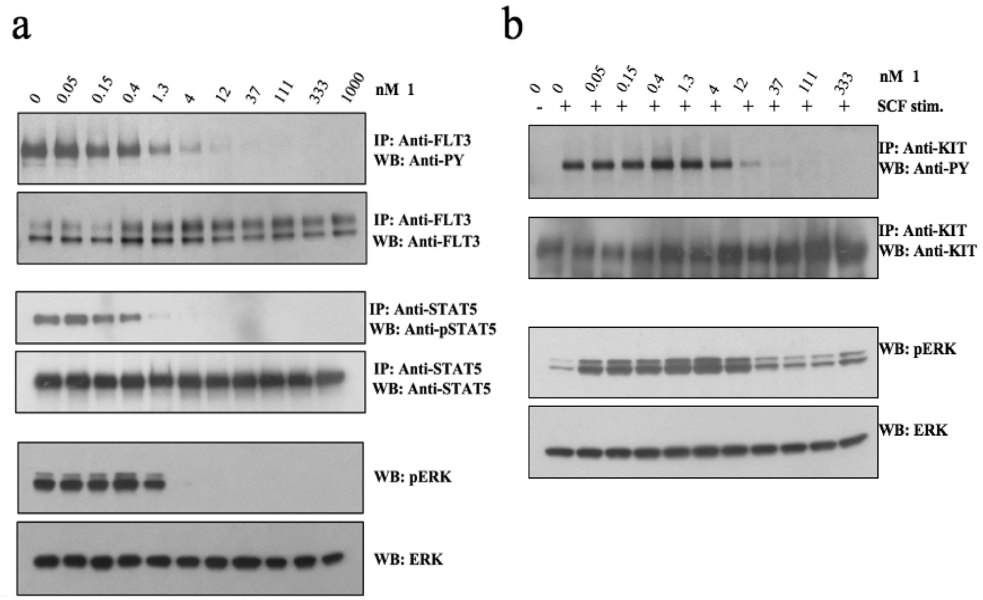

Figure 3. Compound **1** inhibits receptor autophosphorylation and the phosphorylation of its downstream substrates. (a) The level of FLT3 receptor autophosporylation, ERK, and STAT5 phosphorylaton were determined in the MV4−11 cell line by immunoblot analysis. To control for loading, the blots were probed with FLT3, STAT5, and ERK antibodies. (b) The effect of **1** on c-KIT phosphorylation was determined in the TF1 c-KIT expressing cell line.

Two FLT3 inhibitors currently in the clinic, Midostaurin (**5**, Figure 1) and Lestaurtinib (**6**, Figure 1), have been reported to bind tightly to the human serum protein α-1-acidic glycoprotein, which resulted in 1000-fold decrease in potency against FLT3.[29] To assess if the potency of **1** would shift in the presence of human serum, the MV4−11 survival assay was run in 20% human serum instead of FBS (Figure S1, Supporting Information). In the presence of human serum, **5** demonstrated a 1000-fold decrease in potency compared to 10% FBS.[30] In contrast, **1** demonstrated a modest 5-fold decrease in potency with human serum compared to FBS. Therefore, this limited protein binding effect on compound activity should result in a higher fraction of free drug to inhibit FLT3 and c-KIT in AML patients.

Cytotoxicity is a Direct Result of FLT3 and c-KIT Inhibition. The biochemical assay indicated that **1** was potent against FLT3 and c-KIT but also inhibited AXL with a K_i of 40 nM. Because inhibitors of AXL have shown cytotoxicity in cancer cell lines and animal models, it was important to verify that the mechanism of cell death was directly linked to the inhibition of the FLT3 and c-KIT RTKs.[31] A series of studies were conducted to measure the effects of **1** on the receptor kinase activity by assessing the level of receptor autophosphorylation and the effects on the phosphorylation of downstream substrates. Autophosphorylation of FLT3-ITD was blocked in a dose-dependent manner with an estimated IC_{50} of 0.4−1.3 nM, comparable to its cytotoxicity value (Figure 3a). Because both ERK1/2 and STAT5 are downstream effectors of FLT3-ITD signaling, their phosphorylation state was followed in the presence of **1** to verify that the downstream signaling pathways were inhibited.[32] Phosphorylation of both ERK1/2 and STAT5 was inhibited with an estimated IC_{50} of 1.3 nM, similar to the value observed for FLT3-ITD autophosphorylation (Figure 3a).

To verify that **1** also induced cytotoxicity through the inhibition of c-KIT, the effects of **1** on the level of receptor phosphorylation was determined. The SCF induced phosphorylation of c-KIT was blocked in a dose-dependent manner with an estimated IC_{50} of 4−12 nM, comparable to its cytotoxicity value of 1.6 nM (Figure 3b). This agreement was further verified by following the phosphorylation of the c-KIT substrate, ERK1/2. Inhibition of phosphorylation also occurred between 4 and 12 nM (Figure 3b). These results demonstrate a strong mechanistic link between receptor inhibition, signal transduction, and cell viability.

Compound 1 Dramatically Increased Survival in a Leukemic Mouse Model. The PK profile for compound **1** was shown to have a high oral bioavailability and a half-life of 15.5 h in mice.[26] To evaluate the in vivo efficacy, a mouse model using murine Ba/F3 cells engineered to express FLT3-ITD was generated. In this model, proliferation of the introduced Ba/F3 FLT3-ITD cells leads to a lethal myeloproliferative disease characterized by marked leukocytosis and splenomegaly. The effectiveness of **1** for treating the disease was compared to FLT3 inhibitors, compounds **2** and **3**, which have advanced to clinical trials. Compound **2** is equipotent against FLT3, c-KIT, and PDGFR with IC_{50}s of 200, 220, and 170 nM, respectively.[28] Compound **3** is also equipotent across multiple RTKs, including FLT3, c-KIT, PDGFR, and VEGFR2, with the potency ranging from 8 to 10 nM.[33,34] Thus **1** and **3** display a similar potency profile for FLT3 and c-KIT. The Ba/F3-FLT3-ITD cells were implanted in Balb/c mice and allowed to proliferate for five days prior to treatment with either vehicle, **1**, **2**, or **3**.

The average survival for mice treated with vehicle was 20 days. At the maximum tolerated dose for **2** and **3**, the median survival was extended to 33 and 36 days, respectively (Figure 4a). Dose escalation studies with **1** resulted in a median survival of 62 days when a dose of 5 mg/kg was administered QD, nearly double that of **2** and **3** (Figure 4a). Furthermore, 25% of the animals in this dosing group survived 105 days of treatment and did not succumb to disease after being without drug for another 100 days.

The efficacy observed for **1** was dose-dependent and correlated with the plasma steady-state (Css) concentration

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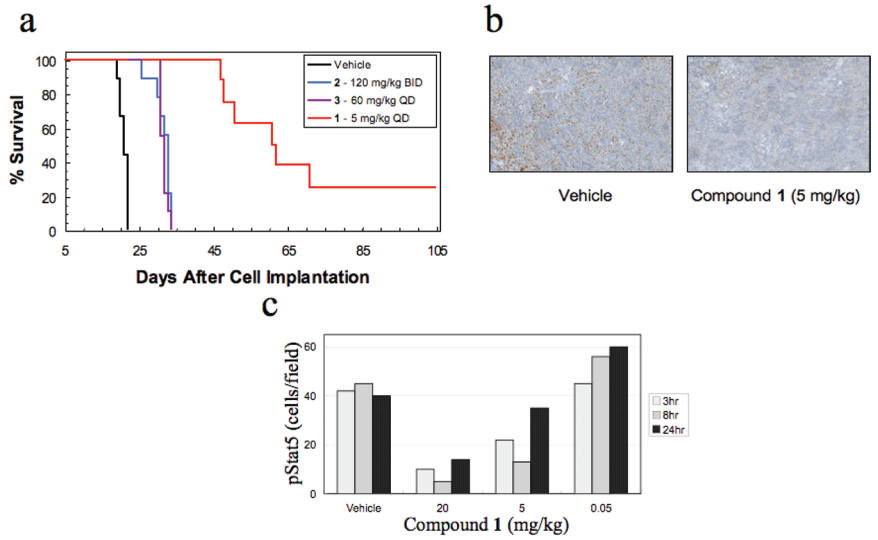

Figure 4. Improved efficacy observed for **1** in a myeloproliferative mouse model compared to compounds in the clinic. (a) A significant increase in median survival is observed with **1** over the vehicle control, as well as **2** and **3**. (b) Representative micrographs are shown for the fixed spleen sections stained for phospho-Stat5 in the vehicle and for the 5 mg/kg **1** treated mice. (c) Analysis of the fixed spleen sections stained with the p-STAT antibody exhibit a dose dependent decrease in p-STAT levels for **1** over a 24 h period. Spleens were harvested at 3, 8, and 24 h after treatment with either 0.05, 5, or 20 mg/kg.

(Table 3). Upon comparing the steady-state plasma concentrations for compounds **1** and **3**, at the dose that gave similar survival times (34 days for compound **1**, and 32 days for **3**), **1** demonstrated a steady state plasma exposure level that was 15-fold lower in concentration than **3** (Table 3). Thus, even though **3** was at a much higher concentration in the blood, it did not translate to increased survival over **1**.

Table 3. Summary of Dose Escalation Results for Compounds 1 and 3 in the Ba/F3 FLT3-ITD Myeloproliferative Mouse Model

treatment group	total dose (mg/kg/day)	daily dose schedule	median survival (days)	Css (ng/mL)	*P* value
1	5	QD	62	641 ± 61	<0.0001
1	1	QD	34	91 ± 29	<0.0001
1	0.25	QD	25	22 ± 3	<0.0001
1	0.05	QD	24	4 ± 0.3	0.0003
3	60	QD	36	N/A	<0.0001
3	20	QD	32	1378 ± 301	<0.0001

To verify that the in vivo efficacy seen with **1** correlated with the inhibition of FLT3 signaling, the phosphorylation level of the downstream substrate STAT5 was determined in cells from the spleen. Ba/F3-FLT3-ITD cells were implanted in Balb/c mice, and the disease was allowed to progress for 15 days before dosing different amounts of compound in the animals. The mice were dosed once a day for two days, and upon receiving the last dose, samples were taken from 3 to 24 h. At each time point, spleens were collected and sections prepared for immunohistochemical staining. The sections were stained with a phospho-specific STAT5 antibody and the number of positive cells counted. A significant dose-dependent reduction in phosphorylated STAT5 was observed for **1**, with maximal inhibition occurring at 8 h (Figure 4b,c). This clear correlation between the level of efficacy and inhibition of STAT5 indicates that the improvement in survival is due to the inhibition of FLT3-ITD.

Dual Inhibition Significantly Increases Cytoxicity in Primary AML Patient Blasts. To address the impact of a dual inhibitor of FLT3 and c-KIT, two distinct types of FLT3 inhibitors were evaluated. The first displayed characteristics represented by **10**, where both FLT3 and c-KIT were inhibited with equivalent potency (Table 4).

This compound was able to inhibit the wild-type and mutant FLT3 receptor with an average IC_{50} value of 0.0093 μM. The inhibition of c-KIT occurred with a similar IC_{50} of 0.018 μM, resulting in a c-KIT/FLT3 selectivity of only 1.9-fold (Table 4). The second type of inhibitor was selective for FLT3 over c-KIT and is represented by **9** (Figure 5 and Table 4). This compound was able to inhibit mutant and wild-type FLT3 with an average IC_{50} value of 0.011 μM but had an IC_{50} value of 1.16 μM for c-KIT. This corresponded to a selectivity of 128 fold for FLT3 over c-KIT.

To determine which inhibitor profile would be most effective, primary leukemic blasts isolated from the peripheral blood of AML patients were screened against multiple members of the two classes of compounds. The patient samples were first characterized for FLT3 mutations by RT-PCR and for cell-surface expression of FLT3 and c-KIT by flow cytometry. Cells from three of the patients expressed wild-type FLT3 (patients 1, 4, 5), one displayed the D835Y activating mutation in the kinase domain (patient 2), and one had the ITD mutation (patient 3).

The dual inhibitor, **1**, decreased cell survival by >80% with an IC_{50} value of 0.012 μM when dosed against patient 3 (Figure 5a). Similar values were also observed in the wild-type FLT3 expressing blast cells isolated from patient 4, where an IC_{50} value of 0.02 μM was obtained (Figure 5b). This potent cytotoxicity occurred with all of the dual inhibitors, with IC_{50} values ranging from 0.02 to 0.31 μM and were >80% cytotoxic (Table 5).

Conversely, FLT3 selective inhibitors were found to not be very effective at reducing primary blast cell viability, with IC_{50} values in the μM range. In some instances, viability was reduced by only 50% at the highest concentration tested (15 μM)

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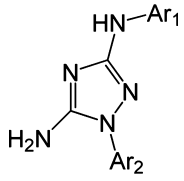
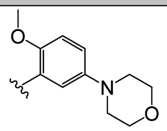
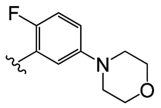
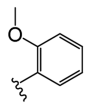
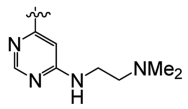
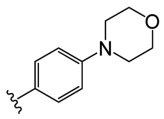
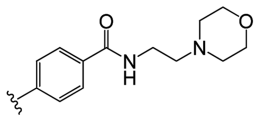
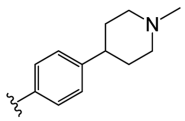
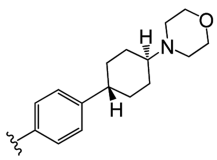
Table 4. Characterization of FLT3 Selective and FLT3/c-KIT Selective Compounds



Compound	Ar1	Ar2	FLT3	FLT3 ITD	FLT3 D835Y	c-KIT	Ratio c-KIT/FLT3
7			0.036	0.089	0.37	2.3	64
8			0.027	0.028	N.D.	0.76	28
9			0.009	0.014	0.0007	1.16	128.9
10			0.01	0.007	0.11	0.018	1.8
11			0.019	0.006	0.015	0.023	1.2
12			0.0035	0.005	0.003	0.004	1.1
1			0.0005	0.0013	0.0014	0.0016	3.2

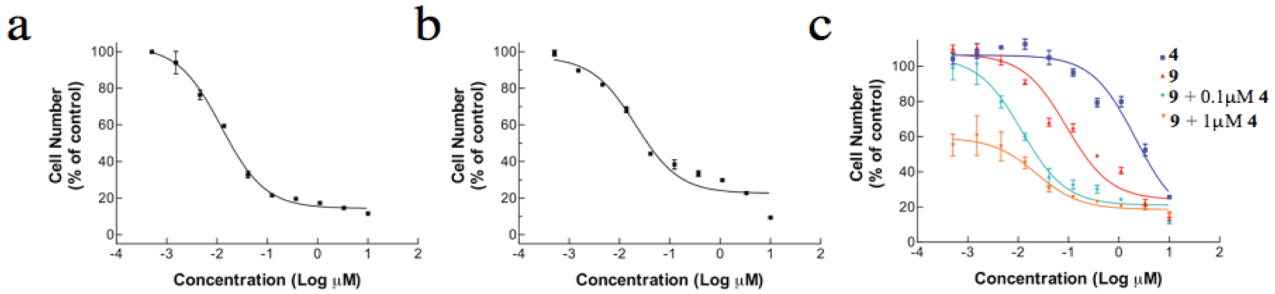


Figure 5. Dual FLT3/KIT inhibitors are significantly more potent against AML patient blast cells than FLT3 or c-KIT selective inhibitors. (a) Viability of AML blast cells isolated from the FLT3-ITD positive patient 3 (b), and FLT3 wild-type patient 4, was measured in the presence of **1** (0.0005 to 10 μM) with human serum and growth factors. (c) The FLT3 selective molecule, **9**, in combination with **4**, improved efficacy on AML patient samples.

(Table 5). Efficacy of a c-KIT selective inhibitor was assessed using **4**, which showed limited cytotoxcity in most cases, reducing cell viability by 30% at 15 μM.

Compared to the RTK inhibitors evaluated in the leukemic mouse model, the dual inhibitors were more potent than **2** and displayed IC_{50} values comparable to the multi-RTK inhibitor **3** (Table 5).

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Table 5. Characterization of Inhibitor Profiles Across a Panel of Primary AML Patient Blast Cells

inhibitor class	compound	patient 1 (WT)	patient 2 (D835Y)	patient 3 (ITD)	patient 4 (WT)	patient 5 (WT)
FLT3/c-KIT	**1**	0.1	0.22	0.012	0.02	0.025
FLT3/c-KIT	**12**	ND	0.3	0.004	0.011	0.017
FLT3/c-KIT	**10**	0.26	0.31	0.03	0.1	0.11
FLT3/c-KIT	**11**	0.21	0.24	0.03	0.016	ND
clinical	**2**	0.64	3.4	0.29	0.64	ND
clinical	**3**	ND	1.1	0.03	0.03	0.05
FLT3	**7**	0.6 (50%)	5.9	1.1	1.3	ND
FLT3	**8**	6.1	10.5	0.41	0.47	ND
FLT3	**9**	9.1	8.9	0.09	3.5	0.12
c-KIT	**4**	0.1 (30%)	0.5 (30%)	ND	0.2 (30%)	0.5 (50%)

The degree to which FLT3 and c-KIT receptors control the survival and proliferation of AML isolates was investigated through measuring the potency of **9** in the presence of fixed concentrations of **4**. The isolates from patient 3 were dosed with **9**, which gave an IC_{50} value of 0.092 μM (Figure 5c and Table 6).

Table 6. Inhibition Constants for Compound 9 Dosed with Increasing Concentrations of Compound 4[a]

compd	TF1-FLT3	TF1-cKIT	patient 3	patient 4
4	>5	0.013	2.1	0.7
9	0.008	1.1	0.092	1.6
9 + 0.1 μM **4**	N/A	N/A	0.011	0.5
9 + 1 μM **4**	N/A	N/A	0.022	0.11

[a] IC_{50} values are in μM.

Upon dosing **9** with 0.1 μM **4**, the cellular IC_{50} decreased by 9-fold. Compound **4** on its own had no cytotoxic effect at 0.1 μM. Upon increasing the concentration of **4** to 1 μM, a 40% reduction in cell viability occurred at the lowest dose of **9** tested (0.3 nM). In the absence of **9**, 1 μM of **4** was only able to reduce viability by 20%. This relationship was also observed with leukemic blasts from patient 4. Incubation of these cells with **9** and increasing concentrations of **4** resulted in increased potency coupled with a higher percentage of cell death (Table 6).

Together with the results from the selective FLT3 and FLT3/KIT inhibitors, the combination of FLT3 and c-KIT inhibition is significantly more efficacious than inhibiting either RTK alone.

DISCUSSION AND CONCLUSION

Improving efficacy and reducing side-effects is an essential goal for targeted therapies aimed at treating cancer. Existing therapies have centered on dosing strategies with various chemotherapeutic drug combinations to obtain an increase in the rate of remission. This has been successful in the younger AML population, but maintaining remission still remains a major issue, with death occurring within five to ten months upon relapse.[8,35]

FLT3 is one of the main signaling molecules associated with blast proliferation and survival. It is not only highly expressed in AML cells, but activating FLT3 mutations occur in approximately one-third of AML patients. In addition, c-KIT is expressed in a majority of AML patient blasts. In mouse models, leukemic stem cells with high expression of c-KIT have been shown to result in a high frequency of leukemia developing.[36] This led to the development of **1**, a subnanomolar inhibitor of FLT3 and c-KIT, with a greater than 100-fold selectivity against any other tyrosine or serine/threonine kinase tested, as assessed by enzymatic activity. This activity was found to translate to cytotoxicity in both wild-type and mutant FLT3 and c-KIT dependent cell lines.

A small-molecule inhibitor targeting FLT3 should strongly suppress leukemic blast proliferation and survival.[33,37,38] Recent phase I and II clinical trials with FLT3 inhibitors in patients that have relapsed after standard therapy show that a reduction in the peripheral and bone marrow blast count can be obtained.[4,29,39,40] Unfortunately, the response occurs only for a subset of patients and is short-lived, lasting from one to three months. The lack of clinical efficacy could be due the selectivity profile and suboptimal pharmacokinetic/pharmacodynamic (PK/PD) properties. Both of these properties are issues with the indolocarbazole class of FLT3 inhibitors (**5** and **6**), which are potent against FLT3 but are >100-fold less potent against c-KIT and both strongly bind α-1-acidic glycoprotein.[30,41] This binding can shift the potency of a molecule by more that 1000-fold. This was observed in the MV4−11 cell line, where the IC_{50} values for **5** went from the nanomolar range to >10 μM in the presence of 20% human serum. This shift in potency translated to the clinic, where patients receiving **6** needed a plasma concentration of 10 μM in order to inhibit FLT3, even though the in vitro IC_{50} was 2−3 nM.[30,41] This tight binding to serum proteins not only reduces the amount of drug that can interact with the blasts in the peripheral blood but may also reduce the concentration of drug in other tissues such as the bone marrow. The nonserum bound drug in the plasma would reduce the initial blast count, but because treatment of the bone marrow is not optimal, the blasts are able to proliferate.

The poor clinical efficacy seen for Tandutinib (**2**) could be due to the combination of serum binding but also a lack of potency against FLT3 and c-KIT. In cell lines, this inhibitor had IC_{50} values in the 100−200 nM range. Furthermore, **2** does not potently inhibit the FLT3 D835 point mutation, which is found in 7−14% of AML patients.[14,42] In contrast, **3** was shown to have single-digit nanomolar potency against FLT3, c-KIT, PDGFR, and VEGFR2 expressing cell lines.[34] Clinical trial data demonstrated a reduction in blast count for all patients exhibiting FLT3 mutations, while only a fraction of patients expressing wild-type FLT3 had a response. Increasing the dose from 50 to 75 mg resulted in toxicity, which may be due to the inhibition of multiple signaling pathways, in addition to the RTK pathways.

If serum binding and dose limiting toxicity are causing the limited efficacy of these inhibitors in treating AML, then inhibitors with the pharamacological profile of **1** should be able to address this. This compound is selective for only FLT3 and c-KIT and does not exhibit a dramatic shift in potency when in

the presence of 20% human serum, as only a 5-fold shift in potency was observed.

In vivo **1** demonstrated an oral bioavailability of 100% with a half-life of 15 h in mice. Animals dosed for up to 100 days showed no drop in body weight or signs of anemia or neutropenia. Administration, in a myeloproliferative mouse model prolonged survival by greater than 30 days when compared to animals given **2** or **3**. It is unclear why **3** was not more efficacious in vivo because it had similar potency as **1** in vitro and in vivo had higher plasma exposure levels (Table 4). Some possibilities may include nonoptimal tissue distribution, poor physical properties of the molecule, or the differences reported between inhibition of FLT3 and cytotoxicity. This latter explanation may be more valid because the amount of **3** required to inhibit FLT3-ITD autophosphorylation occurs at a concentration ~10 times greater than that required for cytotoxicity.[33,43] Therefore, at the maximally tolerated dose, the concentration of drug may not have been high enough to completely block FLT3 signaling in the Ba/F3 FLT3-ITD mouse model. This would allow for the continued proliferation of the Ba/F3 cells, resulting in a reduction in survival.

Finally, unlike chronic mylogenous leukemia (CML), which is due to the BCR/ABL oncogene, AML is a heterogeneous disease.[44] The recent understanding in the role that FLT3 and its activating mutations play in the uncontrolled growth of leukemic blast cells represents only the beginning in the search for the underlying mechanisms for this disease. Inhibitors have recently been developed that are highly selective for FLT3.[43] On the basis of the results presented here, targeting only FLT3 would be predicted to have limited efficacy, while targeting both receptors may improve clinical response in AML patients.

EXPERIMENTAL SECTION

Biochemical Kinase Assays. All compounds were assessed for purity with the analytical data presented in the preceding paper.[26] Once stucture and purity were determined, the kinase activity of the compounds against FLT3 or c-KIT was determined by radiometric assays using a recombinant human FLT3 or c-KIT kinase domain. The assay was carried out as previously described.[26]

Cell Lines. All cell lines were purchased from ATCC and cultured according to their recommendations. Recombinant human GMCSF (R&D Systems, Minneapolis MN) was added to TF-1 cells at 2 ng/mL, and 0.5 ng/mL IL3 (R&D Systems, Minneapolis MN) was added to Ba/F3 cells. TF-1 cells were transduced with retroviral expression vectors coding for FLT3-ITD or FLT3-D835, and cell populations were selected for growth in the absence of GM-CSF. TF-1 cells were also transduced with wild-type FLT3, c-KIT, or PDGFR-β receptor and selected for ability to grow without GM-CSF in the presence of FL, SCF, or PDGF, respectively. Ba/F3 cells were transduced with a retroviral expression vector coding for FTL3-ITD and a blasticidin-resistance gene as a selection marker.

AML Patient Blast Assay. Isolated AML patient blast cells were cultured in RPMI 1640 supplemented with 10% characterized FBS, Pen/Srep, Glutamax, and sodium pyruvate in the presence of 2 ng/mL GM-CSF, 25 ng/mL Fl, and 25 ng/mL SCF. Then 100 μL of cells were dispensed at 100 cells/μL in 96-well plates and allowed to incubate with compounds for 72 h prior to cytotoxicity analysis with ATP luciferase (Promega). Data was obtained by measuring luminescence with an LJL Acquest.

Resazurin Cell Viability Assay. IC_{50} values for the inhibitors were determined by measuring cell viability using the dye resazurin (Sigma-Aldrich, St. Louis, MO). The dye was dissolved in PBS to create a solution of 440 μM and stored at 4 °C prior to use. Cells were incubated with compound for 72 h prior to the addition of resazurin, with the final concentration of dye at 80 μM. The plates were then incubated at 37 °C for 1−2 h before detection by measuring the fluorescence emission at 590 nm.

Immunopreciptiation and Immunoblotting. To measure receptor phosphorylation levels, cells were placed into Optimem supplemented with 1% fetal bovine serum (FBS), incubated with compound for 1 h, harvested, and washed in cold PBS. The cells were lysed, and immunoprecipications carried out as previously described.[33] For immunoprecipitation and Western blotting the antibodies against FLT-3 (S-18), c-KIT (C-19-G), and phospho-tyrosine (PY99) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

For Western blotting, cells were lysed directly with lamelli sample buffer and boiled for 5 min. Samples were separated by gel electrophoresis as described above and immunoblotting carried out using antiphospho-STAT5A/B antibody, clone 8-5-2 (Upstate, Lake Placid, NY), antiphospho-Erk (E-4), or anti-Erk and anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vivo Ba/F3-FLT3-ITD Model. Male BALB/c mice (19−23 g) from Charles River Laboratories (Wilmington, MA) were handled in accordance with the National Institutes of Health (NIH) guidelines. For in vivo efficacy and biomarker studies, Ba/F3-FLT3-ITD cells were IV injected via tail vein with a 400 μL suspension of 1×10^6 cells. After 5 days post implantation, mice were dosed orally by gavage with vehicle or compound. Compounds **1** and **2** were suspended in 0.5% methylcellulose (MC) (Sigma, St Louis, MO) at the desired concentration and sonicated for 5 min. Compound **3** was dissolved in 50 mM citrate buffer (pH 3.5). For the biomarker study, mice were dosed beginning on day 16 post implantation with either vehicle or compound. Compound **1** was dosed twice in mice, and spleens were harvested after the second dose at different intervals, 3, 8, 24, and 48 h for immunohistochemistry (IHC) analysis.

Immunohistochemistry Stain for Phospho-Stat5. Paraffin-embedded spleen sections (5 μm) were cut using a RM2135 microtome (Leica Microsystems, Wetzlar, Germany) and stained for pSTAT5 (Y694; BD Transduction Laboratories, San Diego, CA) in the Ventana Discovery System (Ventana Medical Systems, Inc., Tucson, AZ, USA). A single paraffin section was analyzed for each spleen.

The pSTAT5 antibody was diluted 1:50 in 10% normal goat serum in phosphate buffered saline (PBS). The diluted pSTAT5 polyclonal antibody (100 μL) was titrated onto the slides for 2 h at room temperature. A secondary goat anti-rabbit polyclonal antibody diluted 1:500 in 10% normal goat serum/PBS was added, and the slides were incubated for 16 min at 37 °C followed by hematoxylin counterstain for 2 min with bluing for 2 min. Slides were placed in soapy distilled water to remove the liquid coverslip, dehydrated in a TissueTek DRS2000 automated slide stainer (Sakura, Torrance, CA), and coverslipped using a Sakura TissueTek Glass automated coverslipper (Sakura, Torrance, CA). Counts of pSTAT5 were performed on the IHC-stained sections using an Axioplan 2 microscope (Zeiss, Thormwood, NY), and an automated driven-stage system (Stereo Investigator software, MicroBrightField, Williston, VT) was used to select the random fields. The following criteria were used for cell counts: the computer was programmed to select multiple fields in order to have 20 possible fields (40× magnification) that covered the section and the first 10 fields with <20% white pulp were counted.

ASSOCIATED CONTENT

Supporting Information

Table of inhibition constants against Ser/Thr kinases and cytotoxicity data for **1** and **5** in the presence of human serum are shown. This information is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

FLT3, FMS-like tyrosine kinase; AML, acute myelogenous leukemia; RTK, receptor tyrosine kinase; ITD, internal tandem duplication; JM, juxtamembrane domain; ATP, adenosine triphosphate; FBS, fetal bovine serum; PDGFR-β, platelet derived growth factor-β; FL, FLT3 ligand; ERK, extracellular-signal-regulated kinases; VEGFR, Vascular endothelial growth factor; CML, chronic mylogenous leukemia

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