**Crenolanib is active against models of drug-resistant FLT3-ITD-positive acute myeloid leukemia**

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Running Title: Activity of crenolanib in FLT3-ITD-positive AML

Key Words: acute myeloid leukemia, FLT3-ITD, kinase domain mutations, crenolanib, sorafenib
Key Points

1. The tyrosine kinase inhibitor crenolanib has type 1 inhibitor properties and has potent activity against FLT3 activating mutations.

2. Crenolanib is active in vitro and in vivo against FLT3 inhibitor-resistant FLT3-ITD/D835 mutations.

Abstract

FLT3 kinase internal tandem duplication (ITD) mutations are common in acute myeloid leukemia (AML) and are associated with poor clinical outcome. While initial responses to FLT3 tyrosine kinase inhibitors (TKIs) are observed in FLT3-ITD-positive patients, subsequent relapse often occurs upon acquisition of secondary FLT3 kinase domain (KD) mutations, primarily at residues D835 and F691. Using biochemical assays, we determined that crenolanib, a novel TKI, demonstrates type I properties and is active against FLT3 containing ITD and/or D835 or F691 activating mutations. Potent activity was observed in FLT3-ITD-positive AML cell lines. Crenolanib delayed the outgrowth of MV4-11 cells in a xenograft mouse model, whereas in combination with the type II TKI sorafenib, a significant decrease in leukemic burden ($P < 0.001$) and prolonged survival ($P < 0.01$) was observed compared to either TKI alone. Crenolanib was active against Ba/F3 cells harboring FLT3-ITD and secondary KD mutations and sorafenib-resistant MOLM-13 cells containing FLT3-ITD/D835Y both in vitro and in vivo. In addition, crenolanib inhibited drug-resistant AML primary blasts with FLT3-ITD and D835H/Y mutations. These preclinical data demonstrate that crenolanib is effective against FLT3-ITD containing secondary KD mutations, suggesting that crenolanib may be a useful therapeutic agent for TKI-naive and drug-resistant FLT3-ITD-positive AML.
Introduction

Overall survival in children with acute myeloid leukemia (AML) has improved to 60-70%, exceeding survival rates of approximately 30-40% in adults.1-6 However, after disease recurrence, the likelihood of long-term survival is poor. Patients with activating FLT3 internal tandem duplication (ITD) mutations, which occur in ~15% of pediatric AML and ~30% of adult AML, are at high risk for disease relapse.7-9 Although therapy with FLT3 tyrosine kinase inhibitors (TKIs), such as sorafenib and quizartinib, initially produce clinical responses, most patients develop drug-resistant disease within a few months to a year of treatment.10-14 Therefore, new therapies are needed for newly-diagnosed and drug-resistant FLT3-ITD-positive AML.

Data from preclinical studies indicate that one mechanism of FLT3 TKI resistance is the acquisition of secondary point mutations in the FLT3 kinase domain (KD), which may alter drug binding and/or shift kinases to an auto-activated conformation.14 Recently, secondary FLT3 mutations have been observed in adults and children that developed resistance to sorafenib or quizartinib.10,12,13 Specifically, amino acid exchanges at residue D835 (D835F/H/V/Y) are the most commonly observed secondary FLT3 KD mutations, followed by the F691L mutation. A recent report by Smith and colleagues proposed that mutation of D835 destabilizes the inactive conformation of FLT3; therefore targeting these variants with type I TKIs that bind the FLT3 active conformation may be necessary.13 To our knowledge, this approach has not yet been investigated.

Crenolanib (CP-868,596) is a novel TKI that was developed as a selective and potent inhibitor of PDGFR α and β but also has high affinity for other type III receptor tyrosine kinases (RTKs), such as FLT3.15,16 Preclinical and clinical data have shown crenolanib to be active in imatinib-resistant gastrointestinal stromal tumor (GIST) with PDGFRα D842 mutations. As D842V mutations are thought to stabilize PDGFRα in the active conformation, this suggests that
crenolanib is a type I TKI. Most TKIs with activity against FLT3, such as quizartinib and sorafenib, are type II inhibitors that bind the inactive kinase conformation; these inhibitors show limited activity against the clinically-relevant FLT3 D835 secondary mutations because kinase activity overcomes inhibitor capacity. This suggests that crenolanib may be active against mutations at the analogous FLT3 D835 residue, with the potential to benefit therapy for drug-resistant AML.

In this report, various in vitro and in vivo studies demonstrate that crenolanib is a potent FLT3 inhibitor with type I properties and activity against FLT3-ITD-positive AML. Furthermore, we determined that combination of crenolanib with sorafenib, a type II inhibitor, potentiates anti-leukemic activity in a MV4-11 mouse xenograft model of AML. Finally, we show that crenolanib 1) decreases the viability of Ba/F3 cells expressing FLT3-ITD and TKI-resistant D835H/Y or F691L mutations and delays engraftment of FLT3-ITD/D835H cells in vivo; 2) decreases the viability of a TKI-resistant FLT3-ITD-positive MOLM-13 AML cell line harboring a D835Y mutation in vitro and prolongs survival in a mouse xenograft model; and 3) is active against TKI-resistant pediatric AML blast samples containing FLT3 ITD and D835H/Y mutations. Taken together, these results support clinical evaluation of crenolanib for treatment of AML patients harboring FLT3 activating mutations.

Materials and methods

Cell culture and reagents

Human AML MV4-11, THP-1, U937, and OCI-AML3 (ATCC, Manassas, VA) cell lines and MOLM-13 and PL21 (DSMZ, Brunswick, Germany) cell lines were maintained in RPMI 1640 media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) at 37°C in a humidified atmosphere containing...
5% CO₂. Mouse Ba/F3 cells were derived and maintained as previously described (see supplemental materials).  

Antibodies against FLT3 (C-20), β-actin (C4), and phospho-tyrosine (pY99) were obtained from Santa Cruz Biotechnology (Dallas, TX); phospho-STAT5 (pY694), STAT5, phospho-ERK (pT202/pY204; 197G2), ERK (137F5), phospho-S6 (pS235/pS236; D57.2.2E), and S6 (5G10) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Crenolanib (AROG Pharmaceuticals LLC, Dallas, TX) and sorafenib (Chemietek, Indianapolis, IN) were formulated in DMSO for in vitro assays. Cytosine β-D-arabinofuranoside (AraC) was purchased from Sigma-Aldrich (St. Louis, MO) and reconstituted in water.

**Western blot analysis**

FLT3 immunoprecipitation and Western blot analysis was performed as previously described; details are provided in supplementary materials.

**Cell viability assessment**

The effects of drug treatment on the viability of AML cell lines, Ba/F3 cells and primary leukemic blast samples were assessed using the WST-1 or MTT cell proliferation reagents (Roche Applied Science; Indianapolis, IN) according to the manufacturer’s instructions; details are provided in supplementary materials.
Binding affinity for FLT3 and Abl variants

Binding of crenolanib and sorafenib to purified FLT3 and Abl kinase was performed using the commercially available KdELECT assay (DiscoveRx, Fremont, CA), as previously described. The apparent dissociation constant (Kd) was calculated for the following kinases: wildtype (WT) FLT3, FLT3 D835H, FLT3 D835Y, FLT3 D835V, FLT3-ITD, FLT3-ITD/D835V, FLT3-ITD/F691L, WT Abl, and Abl Q252H.

Crenolanib tolerability and pharmacokinetic studies

Crenolanib tolerability and pharmacokinetic studies were performed in non-leukemia-bearing and leukemia-bearing mice. Details of drug formulation and administration, blood sampling and processing, analytical methods, and pharmacokinetic data analysis are provided in the supplementary materials. All animal studies were approved by the Animal Care and Use Committee at St. Jude Children’s Research Hospital.

In vivo xenograft mouse models

Details on the cell engraftment and dosing of xenograft mouse models are provided in the supplementary materials.

Statistical Analysis

Prism (Graphpad, La Jolla, CA) software was used for non-linear regression analysis of cell viability dose-response data and Kaplan-meier analysis of animal survival. WinNonlin (Pharsight, Cary, NC) and NONMEM (Icon, Dublin, Ireland) software were used for
pharmacokinetic data analysis. CalcuSyn (Biosoft, Cambridge, UK) software was used for analysis of drug combination effects. Densitometry was measured using ImageJ software (NIH, Bethesda, MD). Animal images were analyzed using Living Image version 4.2 software (Perkin Elmer). Data statistical significance was determined by a Student's t-test with a $P$ value $< 0.05$.

**Results**

**Crenolanib is a FLT3 inhibitor with type I properties**

Crenolanib was developed as a potent inhibitor of PDGFR and is highly selective for other class III RTKs.\textsuperscript{16} In agreement with Heinrich et al., crenolanib bound WT FLT3 with high affinity ($K_d = 0.15$ nM; Table 1 and Supplemental Figure 4).\textsuperscript{15} Crenolanib had similar affinity to FLT3 variants containing activating mutations ($K_d$ range: 0.14-22 nM) compared to WT FLT3 (Table 1 and Supplemental Figure 4). For comparison, we tested the affinity of FLT3 variants to sorafenib, a TKI used to treat AML patients expressing FLT3-ITD.\textsuperscript{18,19} Of note, WT FLT3 as well as the FLT3 mutants had greater affinity for crenolanib compared to sorafenib, particularly for the dual ITD/835V and ITD/F691L mutants ($ITD/D835V$ $K_d$: 3.6 vs. 630 nM; $ITD/F691L$ $K_d$: 22 vs. 860 nM, crenolanib vs. sorafenib, respectively) (Table 1 and Supplemental Figure 4).

Type II inhibitors preferentially bind kinases in the inactive conformation, whereas type I inhibitors do not discriminate between active and inactive kinase conformations.\textsuperscript{20} To gain insight into the conformational preference for crenolanib binding, we compared the crenolanib $K_d$ for non-phosphorylated (inactive) WT Abl kinase and phosphorylated (active) WT Abl; this method has been previously validated to predict kinase inhibitor binding properties.\textsuperscript{21} Crenolanib had greater affinity for active WT Abl versus inactive WT Abl with an active/inactive ratio of 0.33 (Table 1 and Supplemental Figure 5). This was confirmed by measuring the $K_d$ for active and inactive Abl Q252H (active/inactive $K_d$ ratio: 0.36; Table 1 and Supplemental Figure 5). Unlike
the type II inhibitors sorafenib and imatinib, which have active/inactive Kd ratios greater than 1.0, the active/inactive Kd ratio for crenolanib was similar to the type I inhibitors staurosporine and PD-173955 (Supplemental Figure 5). These data confirm preliminary work suggesting that crenolanib is a type I inhibitor. Overall, our data demonstrate that crenolanib is a TKI with type I properties and binds with high affinity to WT FLT3 and FLT3 mutants.

Crenolanib inhibits FLT3-ITD-positive AML cell viability in vitro

Gain-of-function FLT3-ITD mutations represent one of the most common gene alterations in AML and confer a poorer prognosis. We next tested whether crenolanib has activity against a panel of AML cell lines with FLT3-ITD mutations or WT FLT3. Crenolanib potently decreased the viability of FLT3-ITD-expressing MV4-11 and MOLM-13 cells, with an IC50 of 1.3 and 4.9 nM, respectively (Figure 1 and Supplemental Table 1). The sensitivity to crenolanib was similar to that of sorafenib (IC50: 4.9 and 17 nM for MV4-11 and MOLM-13, respectively; Supplemental Table 1). In contrast, cells expressing WT FLT3 were less sensitive to crenolanib and sorafenib treatment (IC50 range: 2.8-3.5 µM and 3.9-13 µM, respectively; Figure 1 and Supplemental Table 1). The loss of FLT3-ITD cell viability correlated with a decrease in FLT3-ITD pro-survival signaling (Figure 1).

We next determined the combination effects of crenolanib with AraC, the most widely used chemotherapeutic agent for AML, in FLT3-ITD-positive AML cells using a growth inhibition assay. After simultaneous treatment with both drugs for 72 h, a synergistic loss in cell viability was observed in MV4-11 and MOLM-13 cells, with combination index (CI) values ranging from 0.3 to 0.7 (Supplemental Figure 6). Synergy was also observed for additional sequences and schedules of drug exposure including: AraC before crenolanib, crenolanib before AraC, and
simultaneous drug treatment for 4 h followed by 68-h treatment with crenolanib (Supplemental Figure 6).

**Crenolanib pharmacokinetics in mice**

To test the *in vivo* activity of crenolanib, we first determined the pharmacokinetic properties of crenolanib in mice. Initial studies using non-leukemia-bearing FLT3-ITD transgenic C57Bl/6 mice demonstrated that intraperitoneal (i.p.) administration achieved greater crenolanib plasma concentrations compared to oral administration (Supplemental Figure 7). *NOD.Cg-Prkdc<sup>scid</sup> II2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice engrafted with MV4-11-luc cells were administered crenolanib at a maximally tolerated dose of 15 mg/kg i.p. (described in supplementary materials) and serial blood sampling was performed over a 24-h period. A summary of crenolanib pharmacokinetic parameters is presented in Supplemental Table 2, and population model-predicted plasma concentration-time plots are shown in Supplemental Figure 7. The median AUC<sub>(0-24)</sub> was approximately 2,140 ng/mL*h, and the Cmax was 1370 ng/ml (~3.1 µM). The AUC achieved at 15 mg/kg i.p. in mice is comparable with the exposure observed in adults receiving crenolanib 100 mg orally.²³

**Crenolanib has anti-leukemic activity in a FLT3-ITD-positive AML mouse model**

Evaluation of crenolanib in biochemical assays and AML cell lines demonstrated activity against FLT3-ITD. Therefore, we determined the *in vivo* anti-leukemic effects of crenolanib using a FLT3-ITD-positive MV4-11 AML xenograft mouse model. Crenolanib 15 mg/kg i.p. was administered to male NSG mice once daily (Monday - Friday) for 3 consecutive weeks beginning 10 days after cell injection. As depicted in Supplemental Figure 8, crenolanib
treatment suppressed MV4-11-luc bone marrow infiltration compared to vehicle-treated animals. A significant prolongation of survival was also observed after crenolanib treatment (median group survival: 55 and 71 days for vehicle and crenolanib, respectively; \( P = 0.0031 \)) (Supplemental Figure 8).

Combination of type I and II FLT3 inhibitors potentiates anti-leukemic activity in vivo

It has been hypothesized that the combination of crenolanib with a type II inhibitor, such as sorafenib, may provide superior FLT3 inhibition by concomitant targeting of multiple kinase conformations.\(^{24}\) Combination treatment with crenolanib and sorafenib for 72 h had additive effects on inhibiting the \textit{in vitro} viability of MV4-11 and MOLM-13 cells (CI range: 0.89-1.0 (Figure 2). Profiling FLT3-ITD pro-survival signaling in MV4-11 cells found that crenolanib had more potent inhibitory effects compared to sorafenib, which is consistent with the cell viability data (Supplemental Table 1), and the drug combination caused greater inhibition of signaling compared to each drug alone (Figure 2).

We next tested this hypothesis by determining the \textit{in vivo} effects of crenolanib in combination with sorafenib in the MV4-11-luc xenograft mouse model. Leukemia-bearing female NSG mice were treated with crenolanib 15 mg/kg i.p. once daily, sorafenib 15 mg/kg orally once daily, or the drug combination (crenolanib 15 mg/kg i.p. and sorafenib 15 mg/kg orally) once daily (Monday - Friday) for 3 consecutive weeks. For this study, treatment began 17 days after cell injection to allow for considerable tumor engraftment (Supplemental Figure 1). Both TKIs given alone significantly delayed the outgrowth of MV4-11-luc cells compared to vehicle-treated mice (\( P < 0.001 \)), whereas the drug combination resulted in tumor regression and a significant decrease in leukemia burden compared to groups treated with vehicle or single-agent TKI (\( P < 0.001 \)) (Figure 3). Sorafenib or crenolanib monotherapy prolonged survival for more than 2
weeks beyond vehicle-treated animals (median survival: 34, 53, and 51 days for vehicle, sorafenib, and crenolanib, respectively; \( P = 0.0017 \) for crenolanib or sorafenib versus vehicle), whereas the drug combination conferred the greatest survival advantage (median survival: 62 days; \( P = 0.0017 \) for the drug combination versus vehicle; \( P = 0.0021 \) and \( P = 0.0026 \) for the drug combination versus crenolanib or sorafenib alone, respectively). Overall, these data suggest that inhibition of multiple FLT3 kinase conformations by simultaneous treatment with type I and type II kinase inhibitors results in greater anti-leukemic efficacy compared to either inhibitor alone and represents a novel treatment strategy for FLT3-ITD-positive AML.

**Crenolanib is active against FLT3 secondary drug-resistant mutations in Ba/F3 cells in vitro and in vivo**

A commonly described mechanism of resistance to FLT3 inhibitors in preclinical models, and more recently in patients with FLT3-ITD-positive AML, is the acquisition of secondary KD point mutations primarily at residues D835 and F691.\(^{10,12-14}\) In addition, Ba/F3 cells expressing these mutations alone or in combination with an ITD mutation were resistant to sorafenib (Supplemental Table 3).\(^{10,13}\) Using this same model, we determined that crenolanib potently inhibited the viability of cells expressing single FLT3 D835H or D835Y mutations (IC\(_{50}\): 0.3 and 0.06 nM, respectively) and moderately inhibited cells expressing double FLT3 mutants containing the ITD and D835H/Y or F691L (IC\(_{50}\) range: 35-62 nM; Figure 4 and Supplemental Table 3); reduction in Ba/F3 cell viability correlated with a decrease in FLT3 phosphorylation (Figure 4).

Cross-resistance to multiple FLT3 inhibitors, including sorafenib, quizartinib and ponatinib, develops upon acquisition of a secondary FLT3 KD mutation at residue D835.\(^{10,13,25}\) Therefore, an inhibitor with activity against FLT3 D835 mutations might provide clinical benefit
for patients with TKI-resistant FLT3-ITD-positive AML. We tested the in vivo activity of crenolanib in female NSG mice engrafted with Ba/F3 FLT3-ITD/D835H-luc cells. Due to the shorter half-life of crenolanib compared to sorafenib in mice (1.2 vs. 3.4 h, respectively)\textsuperscript{26}, we administered crenolanib twice daily in this study involving highly aggressive and FLT3 inhibitor-resistant Ba/F3 cells. Mice received crenolanib 15 mg/kg i.p. twice daily or sorafenib 15 mg/kg orally once daily for 10 consecutive days beginning on day 1. In contrast to sorafenib, crenolanib treatment resulted in a significant decrease in Ba/F3 FLT3-ITD/D835H-luc bone marrow infiltration ($P = 0.0013$ and $P = 0.0157$ on days 7 and 10 after cell injection, respectively), as determined by noninvasive luciferase imaging (Figure 4).

Crenolanib is active against drug-resistant MOLM-13 AML cells with a FLT3-ITD/D835Y mutation in vitro and in vivo

To test crenolanib efficacy in a model of drug-resistant FLT3-ITD-positive AML, we generated MOLM-13-RES-luc cells, which express FLT3-ITD/D835Y, by continuous treatment with the FLT3 inhibitor tandutinib. Similar to Moore et al., no alteration in FLT3 allele number was apparent in the MOLM-13-RES-luc cells (Supplemental Figure 9).\textsuperscript{27} In addition, PCR-based phase analysis determined that the D835Y mutation was expressed on FLT3-ITD alleles (Supplemental Table 4). In comparison to the parental MOLM-13-luc cell line, MOLM-13-RES-luc cells displayed increased FLT3-ITD phosphorylation and activation of downstream signaling (Supplemental Figure 9). In vitro cell viability assays determined that the MOLM-13-RES-luc cells were resistant to sorafenib (IC50: 3.1 and 785 nM in MOLM-13-luc and MOLM-13-RES-luc cells, respectively) (Figure 5). In contrast, MOLM-13-RES-luc cells were sensitive to crenolanib, with an IC50 value similar to the parental MOLM-13-luc cell line (IC50: 1.2 and 3.9 nM in MOLM-13-luc and MOLM-13-RES-luc cells, respectively). The relative sensitivity of the MOLM-
13-luc and MOLM-RES-luc cells to crenolanib correlated with loss of FLT3-ITD phosphorylation (Figure 5). Additionally, we observed synergistic activity of crenolanib combined with AraC against MOLM-13-RES-luc cells following simultaneous drug exposure for 72 h (Supplemental Figure 6).

We next determined whether MOLM-13-RES-luc cells were sensitive to crenolanib treatment in vivo. NSG mice engrafted with MOLM-13-RES-luc cells received sorafenib 15 mg/kg orally once daily or crenolanib 15 mg/kg i.p. once or twice daily (Monday - Friday) for 3 consecutive weeks beginning on day 3. MOLM-13-RES-luc leukemic infiltration was significantly reduced in mice treated with crenolanib twice daily compared to vehicle-treated animals ($P < 0.0001$ on day 7; $P = 0.0028$ and 0.0002 on days 12 and 19, respectively), whereas mice treated with sorafenib or crenolanib once daily showed infiltration comparable to the vehicle group (Figure 5 and Supplemental Figure 10). Notably, once and twice daily crenolanib treatment produced a significant increase in survival compared to vehicle-treated mice (median survival: 27, 23, and 20 days for crenolanib twice daily, crenolanib once daily, and vehicle treatment groups, respectively; $P < 0.0001$; Figure 5). In contrast, sorafenib treatment produced no survival advantage over vehicle-treated controls (median survival = 20 days; Figure 5). Overall, these data suggest that crenolanib may be beneficial in the context of drug-resistant FLT3-ITD-positive AML, particularly after acquisition of a FLT3 D835 secondary mutation. This may have clinical implications for the use of crenolanib in drug-resistant FLT3-ITD-positive AML.

**Activity of crenolanib against TKI-resistant FLT3-ITD-positive AML primary blast samples**

We recently described the development of clinical TKI resistance after sequential therapy with sorafenib and sunitinib in FLT3-ITD-positive pediatric AML patients; the loss in TKI sensitivity was associated with acquisition of FLT3 D835H/Y mutations.$^{10}$ We next determined
the sensitivity of primary blast samples taken from two TKI-resistant patients to crenolanib and sorafenib ex vivo (Supplemental Table 5) in a MTT assay. Crenolanib treatment produced moderate inhibition at low nanomolar concentrations (average inhibition range: 34-46% at 20 nM crenolanib and 48-52% at 80 nM crenolanib; Figure 6); in contrast, sorafenib produced minimal effects (average inhibition range: 1-3% at 20 nM sorafenib and 4-18% at 80 nM sorafenib; Figure 6). Two recent preliminary reports show similar effects of crenolanib and sorafenib on primary blast samples from two adult AML patients harboring FLT3 D835V/F mutations.28,29 In agreement with our in vitro and in vivo studies, these data suggest that crenolanib has activity against leukemic cells with FLT3-ITD containing D835 mutations.

Discussion

We describe the biochemical and pharmacological characterization of crenolanib, a selective inhibitor of type III RTKs, in preclinical models of FLT3 mutant AML. In binding assays, crenolanib demonstrated type I kinase inhibitor properties and potent activity against FLT3-ITD. In cellular assays, crenolanib inhibited the viability of FLT3-ITD-positive AML cell lines. In vivo, crenolanib delayed tumor infiltration and prolonged survival in a mouse xenograft model of FLT3-ITD-positive AML. Furthermore, the combination of crenolanib and the type II inhibitor sorafenib produced tumor regression and prolonged survival compared to either TKI administered alone. Crenolanib demonstrated potent inhibition of TKI-resistant single FLT3 D835 mutants and retained activity against double FLT3-ITD/D835 mutants in binding assays and Ba/F3 cells. Finally, crenolanib inhibited the viability of primary AML blast samples harboring FLT3-ITD and D835 mutations and demonstrated anti-leukemic activity against a sorafenib-resistant FLT3-ITD-positive AML cell line with a D835Y mutation in vitro and in vivo. Consistent with our findings, a preliminary report by Smith et al. showed that crenolanib binds
FLT3-ITD with high affinity, displays potent activity against FLT3-ITD-positive AML cells, and exhibits type I properties in vitro.\textsuperscript{22}

When compared to other FLT3 inhibitors in development, crenolanib displays greater binding affinity to FLT3.\textsuperscript{30} Crenolanib is selective to type III RTKs and shows similar potency as the selective inhibitor quizartinib and the multi-kinase inhibitor sorafenib against FLT3-ITD-expressing MV4-11 cells.\textsuperscript{13,29,30} However, unlike crenolanib, quizartinib and sorafenib display type II inhibitor characteristics and are not active against FLT3-ITD/D835 mutations observed clinically.\textsuperscript{10,12,13} On the other hand, midostaurin shows type I properties but lacks selectivity and potency against FLT3-ITD in binding and cellular assays.\textsuperscript{30,31} Like most TKIs showing high binding in human plasma (range: 95 - >99.5%), crenolanib exhibits high protein binding (95.9%).\textsuperscript{16} However, crenolanib inhibited FLT3 phosphorylation at clinically achievable concentrations in a plasma inhibitory assay.\textsuperscript{28} Therefore, crenolanib as a type I inhibitor may be advantageous compared to other selective and potent FLT3-targeted TKIs in development, which are primarily type II inhibitors.

Enhanced anti-leukemic activity observed with the combination of crenolanib and sorafenib suggests that total inhibition of multiple FLT3-ITD conformations (e.g., active and inactive) by simultaneous type I and type II inhibitor therapy may be an effective treatment strategy for FLT3-ITD-positive AML. Previous studies have evaluated the combination of type I and type II kinase inhibitors in other tyrosine kinase-mutant cancers. For example, the combination of the BCR-Abl/cKit inhibitors imatinib and dasatinib (type II and type I, respectively) resulted in greater anti-tumor effects compared to imatinib monotherapy in a mouse model of GIST containing a cKit V558 deletion mutation.\textsuperscript{32} Additionally, the combination of ponatinib (type II) with midostaurin (type I) had synergistic effects on neoplastic mast cells containing the cKit D816V mutation.\textsuperscript{33} Furthermore, it has been shown that imatinib and dasatinib combination therapy suppresses the outgrowth of imatinib-resistant BCR-Abl
mutations in vitro.\textsuperscript{34} Whether treatment with the combination of crenolanib and sorafenib similarly suppresses the development of sorafenib-resistant clones with FLT3 KD mutations is currently being tested. It also remains to be determined if type I and II inhibitors can be combined safely clinically due to the potential for drug-drug interactions and increased toxicity.

While some success has been observed with FLT3 inhibitor therapy in FLT3-ITD-positive AML, the acquisition of secondary FLT3 KD mutations often diminishes the response to TKIs.\textsuperscript{10,12,13} In particular, emergence of mutations at residues D835 and F691L is associated with clinical resistance to sorafenib and the second generation FLT3 inhibitor quizartinib.\textsuperscript{10,12,13} Recent in vitro studies showed that substitutions at residue D835 also confer a high degree of resistance to the novel TKI ponatinib, while ponatinib retained modest activity against F691L mutants.\textsuperscript{25} D835 mutation is hypothesized to indirectly change the conformation of the kinase activation loop and stabilize the active, DFG-in conformation, similar to the corresponding D186V mutant of cKit\textsuperscript{35}; whereas F691 is analogous to the “gatekeeper” residue T315 in BCR-Abl, mutation of which is predicted to impair drug binding.\textsuperscript{10,13} Modeling these mutations in a Ba/F3 cell line, we demonstrated potent activity of crenolanib against single FLT3 D835H/Y mutants, and modest activity against cells expressing double FLT3-ITD/D835H/Y or FLT3-ITD/F691L mutations. Similarly, recent preliminary reports describe crenolanib activity against quizartinib-resistant single FLT3 D835V/Y and double FLT3-ITD/D835V/Y/F mutants modeled in Ba/F3 or 32D cells.\textsuperscript{22,28} Additionally, adult AML primary blast samples expressing a FLT3 D835V/F mutation were sensitive to crenolanib, whereas cross-resistance was observed to quizartinib and sorafenib.\textsuperscript{28,29} Our data are consistent with these reports and extend their findings by demonstrating that crenolanib has activity against 1) Ba/F3 cells harboring clinically relevant sorafenib-resistant FLT3-ITD/D835H mutations in vitro and in vivo; 2) a TKI-resistant MOLM-13 AML cell line containing double FLT3-ITD/D835Y mutations in vitro and in vivo; and 3) TKI-resistant pediatric AML primary blasts harboring double FLT3-ITD/D835H/Y mutations.
In conclusion, crenolanib is a novel FLT3 inhibitor with type I properties and potent activity against FLT3-ITD as well as FLT3 D835 mutations, which are resistant to currently available FLT3 inhibitors. Our results support the evaluation of crenolanib in patients with TKI-naive and TKI-resistant FLT3-ITD-positive AML. To this effect, several phase II studies of crenolanib for the treatment of adults with relapsed/refractory AML and FLT3 activating mutations are ongoing (NCT01522469 and NCT01657682).

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Authorship


Conflict-of-interest disclosure: A.R. is an employee of AROG Pharmaceuticals, LLC. All other authors declare no conflicts of interest.

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References


### Table 1. Crenolanib and sorafenib in vitro kinase binding

<table>
<thead>
<tr>
<th>FLT3 Variant</th>
<th>Crenolanib $K_d$ (nM)</th>
<th>Sorafenib $K_d$ (nM)</th>
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<th>Abl Variant</th>
<th>Crenolanib $K_d$ (nM)</th>
<th>Crenolanib Active/Inactive $K_d$ Ratio</th>
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<tr>
<td>Inactive Abl</td>
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<td>-</td>
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<tr>
<td>Active Abl (Q252H)</td>
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<td>Inactive Abl (Q252H)</td>
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* - Data obtained from KdELECT screening.
** - As reported in Davis et al.17

Abbreviations: ITD, internal tandem duplication.
Figure Legends

Figure 1. Crenolanib potently inhibits FLT3-ITD AML cell viability and FLT3 signaling. AML cells were treated with DMSO or increasing concentrations of crenolanib (A) for 72 h and cell viability was measured or (B) for 1 h and lysed. Western blot analysis was performed on FLT3 immunoprecipitation eluent or whole cell lysate using the indicated antibodies (IP: immunoprecipitation). Cell viability measurements represent the mean ± SEM of 3 experiments with six replicates each (n = 18).

Figure 2. Drug combination effects on MV4-11 cell viability and FLT3 signaling. (A) MV4-11 and MOLM-13 cells were treated with DMSO or increasing concentrations of crenolanib, sorafenib, or both at a fixed concentration ratio for 72 h and cell viability was measured. CalcuSyn combination index (CI) values were generated for drug combination effects on cell viability. Cell viability experiments were performed in triplicate with six replicates each (n = 18) (CI value >1.0: antagonism; CI value = 1.0: additivity; CI value <1.0: synergism). (B) MV4-11 cells were treated for 1 h with DMSO or the indicated concentration of crenolanib, sorafenib, or both and lysed. Western blot analysis was performed on whole cell lysate using the indicated antibodies. Densitometry measurements were normalized to total protein amount. Abbreviations: CRE, crenolanib; SOR, sorafenib.

Figure 3. Anti-leukemic effects of the combination of crenolanib and sorafenib in a MV4-11 xenograft AML mouse model. Female NSG mice engrafted with MV4-11-luc cells were treated with vehicle, crenolanib 15 mg/kg i.p., sorafenib 15 mg/kg orally, or crenolanib (15 mg/kg, i.p.) plus sorafenib (15 mg/kg, orally) once daily (Monday - Friday) for 3 consecutive weeks beginning on day 17. (A-B) Leukemic cell bone marrow infiltration was monitored by noninvasive luciferase imaging (*, P < 0.05; **, P < 0.01; ***, P < 0.001). (C) Kaplan-meier analysis of animal survival (**, P = 0.0017 for crenolanib versus vehicle; P = 0.0017 for
sorafenib versus vehicle; \( P = 0.0017 \) for the drug combination versus vehicle; \( P = 0.0021 \) and \( P = 0.0026 \) for the drug combination versus crenolanib or sorafenib alone, respectively). Black bar denotes treatment period.

**Figure 4. Crenolanib activity against Ba/F3 cells expressing various FLT3 mutants in vitro and in vivo.** (A-B) Ba/F3 cells expressing FLT3 mutants were treated with DMSO or increasing concentrations of crenolanib (A) for 72 h and viability was measured or (B) for 1 h and lysed. Western blot analysis was performed on FLT3 immunoprecipitation eluent using the indicated antibodies. Cell viability measurements represent the mean ± SEM of 2-4 experiments with six replicates each (\( n = 12-24 \)). (C-D) Male NSG mice engrafted with Ba/F3 FLT3-ITD/D835H-luc cells were treated with vehicle, crenolanib 15 mg/kg i.p. twice daily, or sorafenib 15 mg/kg orally once daily for 10 consecutive days beginning on day 1 (5 mice per treatment group). (C) Ba/F3 FLT3-ITD/D835H-luc cell bone marrow infiltration was monitored by noninvasive luciferase imaging. Black bar denotes treatment period (*, \( P = 0.0157 \); **, \( P = 0.0013 \)). (D) Representative whole body luciferase images from each treatment group are shown.

**Figure 5. Crenolanib activity against MOLM-13-RES cells in vitro and in vivo.** (A-B) MOLM-13-luc and MOLM-13-RES-luc cells were treated with DMSO or increasing concentrations of crenolanib or sorafenib (A) for 72 h and viability was measured or (B) for 1 h and lysed. Western blot analysis was performed on FLT3 immunoprecipitation eluent using the indicated antibodies. Cell viability measurements represent the mean ± SEM of 2-3 experiments with six replicates each (\( n = 12-18 \)). (C-D) Female NSG mice engrafted with MOLM-13-RES-luc cells were treated with vehicle, sorafenib 15 mg/kg orally once daily, or crenolanib 15 mg/kg i.p. once or twice daily (Monday - Friday) for 3 consecutive weeks beginning on day 3. (C) Leukemic cell bone marrow infiltration was monitored by noninvasive luciferase imaging (**, \( P < 0.01 \); ***, \( P < 0.001 \)). (D) Kaplan-meier analysis of animal survival (***, \( P < 0.0001 \) for crenolanib once daily versus
vehicle;***, $P < 0.0001$ for crenolanib twice daily versus vehicle). Black bar denotes treatment period.

**Figure 6. Crenolanib activity against TKI-resistant pediatric AML primary blasts ex vivo.** Primary blast samples were treated with DMSO or increasing concentrations of crenolanib or sorafenib for 72 h. Cell viability measurements represent the mean ± SEM of four replicates.
Fig 1

A

Cell Viability (% control)

Crenolanib (nM)

MV4-11
MOLM-13
PL21
OCI-AML3
THP-1
U937

B

IP: FLT3

<table>
<thead>
<tr>
<th>Crenolanib (nM):</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>50</th>
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</thead>
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<tr>
<td>MV4-11</td>
<td></td>
<td></td>
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<td></td>
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<td>MOLM-13</td>
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</table>

| pFLT3            |    |    |    |    |    |            |
| FLT3             |    |    |    |    |    |            |
| pSTAT5           |    |    |    |    |    |            |
| STAT5            |    |    |    |    |    |            |
| pERK             |    |    |    |    |    |            |
| ERK              |    |    |    |    |    |            |
| pS6              |    |    |    |    |    |            |
| S6               |    |    |    |    |    |            |
| β-actin          |    |    |    |    |    |            |
**Fig 2**

A. **Combination index**

**ED50** | **ED75** | **ED90**
---|---|---
0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 1.1 | 1.2

**MV4-11: CRE + SOR**
**MOLM-13: CRE + SOR**

B. **Western Blot Analysis**

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<tr>
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<td>STAT5</td>
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<td>CRE</td>
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<td>1.00</td>
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</tbody>
</table>
A

Vehicle
Sorafenib
Crenolanib
Combination

Day Post-tail vein injection
Avg. Whole Body Luminescence log(p/s/cm²/sr)

Day 33
Day 47

***
**
*
***

Vehicle
Sorafenib
Crenolanib
Combination

C

Overall survival (%)

Day Post-tail vein injection

Number at risk Day 40

Day 0
5 5 5 5

Day 40
5 5 4 3

Day 50
5 5 3 0

Day 60
5 5 5 3

Fig 3
Fig 4

A

Cell Viability (% control)

0.001 0.01 0.1 1 10 100 1000 10000

0
25
50
75
100
125

D835H
D835Y
ITD D835H
GFP + IL3
ITD
ITD D835Y

Crenolanib (nM): GFP + IL3, D835H, D835Y, ITD, ITD D835H, ITD D835Y, ITD F691L

B

Crenolanib (nM):

ITD

D835H

D835Y

pFLT3
FLT3

Crenolanib (nM):

ITD F691L

ITD D835H

ITD D835Y

pFLT3
FLT3

C

Avg. Whole Body Radiance (p/s/cm²/sr)

0 2 4 6 8 10 12

0
2.0 × 10⁷
4.0 × 10⁷
6.0 × 10⁷
8.0 × 10⁷

Vehicle
Sorafenib
Crenolanib

D

Vehicle
Sorafenib
Crenolanib

Fig 4
A. Cell Viability (% control) vs. Drug (nM)

B. Western Blot images for pFLT3 and FLT3

C. Avg. Whole Body Radiance (p/s/cm²/sr) vs. Day Post-tail vein injection

D. Overall survival (%) vs. Day Post-tail vein injection

Vehicle
Sorafenib
Crenolanib once daily
Crenolanib twice daily

Fig 5
Fig 6

Patient 1

- Crenolanib
- Sorafenib

Patient 2

- Crenolanib
- Sorafenib

Drug (nM)

% control

0 100 200 300

0

25

50

75

100
Crenolanib is active against models of drug-resistant FLT3-ITD-positive acute myeloid leukemia