Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants

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Running title: Crenolanib in FLT3-mutated AML

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Key Points

- Crenolanib has activity against several of the important kinase domain mutations (at position D835) found in FLT3
- Patients receiving crenolanib achieve FLT3-inhibitory plasma levels

Abstract

Mutations of the type III receptor tyrosine kinase (RTK) FLT3 occur in approximately 30% of acute myeloid leukemia (AML) patients and lead to constitutive activation. This has made FLT3 activating mutations an attractive drug target, as they are probable driver mutations of this disease. As more potent FLT3 inhibitors are developed, a predictable development of resistance-conferring point mutations, commonly at residue D835, has been observed. Crenolanib is a highly selective and potent FLT3 tyrosine kinase inhibitor (TKI) with activity against the internal tandem duplication (FLT3/ITD) mutants as well as against the FLT3/D835 point mutants. We tested crenolanib against a panel of D835 mutant cell lines and primary patient blasts and observed superior cytotoxic effects when compared to other available FLT3 TKIs such as quizartinib and sorafenib. Another potential advantage of crenolanib is its reduced inhibition of c-Kit compared to quizartinib. In progenitor cell assays, crenolanib was less disruptive of erythroid colony growth, which may result in relatively less myelosuppression than quizartinib. Finally, correlative data from an ongoing clinical trial demonstrate that AML patients can achieve sufficient levels of crenolanib to inhibit both FLT3/ITD and resistance-conferring FLT3/D835 mutants in vivo. Crenolanib is thus an important next-generation FLT3 TKI. This study is registered at clinicaltrials.gov, ID: NCT01657682.
Introduction

Approximately one third of AML patients harbor an internal tandem duplication (ITD) in the receptor tyrosine kinase FLT3. Point mutations of FLT3 at aspartate 835 (D835) are also observed in patients, although at a frequency of only about 7%. The FLT3/ITD mutations are known to confer a poor prognosis, while the prognostic impact of the D835 mutations is more controversial. Both types of mutations lead to constitutive activation of the tyrosine kinase function, which makes FLT3 an attractive drug target to improve outcomes for AML patients with FLT3 mutations. Over the past decade, several TKIs targeting FLT3 have been studied in the setting of clinical trials to treat AML with limited success. More recently, sorafenib and quizartinib have emerged as more potent FLT3 inhibitors and have significant clinical activity. Quizartinib in particular has been associated with high bone marrow response rates in relapsed and refractory FLT3/ITD AML patients, although there appeared to be a degree of myelosuppression in some patients, possibly the result of concomitant inhibition of c-KIT.

The BCR-ABL inhibitor imatinib has been in widespread use for CML and Ph+ ALL for a decade now. Soon after its introduction into this patient population, resistance-conferring point mutations in the ABL kinase domain emerged during therapy, leading to disease progression. This has been successfully countered with the introduction of second-generation BCR-ABL inhibitors such as nilotinib and dasatinib. In contrast, as clinical trials of FLT3 inhibitors were being conducted over the past decade, resistance-conferring point mutations in the FLT3 coding sequence were not routinely observed, except sporadically. A reasonable explanation for this is that high-level FLT3 inhibition in vivo was not generally achieved by the first generation FLT3 inhibitors. However, as the more potent inhibitors sorafenib and quizartinib have been more widely used to treat FLT3/ITD AML, both in clinical trials and in off-label use, point mutations...
have finally emerged during disease progression.\textsuperscript{11} These mutations are predominantly found at aspartate 835 (D835) in the activation loop, although mutations at phenylalanine 691 (F691) were also reported. Both mutations render the receptor resistant to the FLT3 inhibitor to a variable degree and are clearly associated with disease progression. Resistance-conferring FLT3 D835 mutations have also been reported to arise during sorafenib and sunitinib therapy.\textsuperscript{12,13} As the clinical development of FLT3 inhibitors proceeds into advanced phase trials, it has become obvious that these mutations will represent a new obstacle in the care of FLT3/ITD AML patients, at least when quizartinib or sorafenib are used as the initial TKI. Furthermore, FLT3/D835 mutations present at diagnosis in 7\% of AML patients and in some cases may represent a driver of the disease.\textsuperscript{14-16} While first generation FLT3 inhibitors such as midostaurin have activity against FLT3 tyrosine kinase domain mutations,\textsuperscript{17,18} their relative lack of potency has limited their utility as single agents.

Crenolanib is a benzimidaine quinolone derivative originally developed as an inhibitor of platelet-derived growth factor receptor (PDGFR).\textsuperscript{19} In a phase 1 trial of crenolanib enrolling solid tumor patients, eight patients achieved stable disease but no objective responses were reported, despite micromolar plasma concentrations being achieved in tolerable fashion.\textsuperscript{19} Subsequent analysis using an in vitro kinase assay indicated that the compound had activity against FLT3. We report here the characterization of crenolanib as a novel TKI that has potent activity against both the FLT3/ITD-mutated receptor as well as to FLT3/D835-mutated receptors, including those arising in the context of FLT3/ITD mutations following treatment with sorafenib and quizartinib.
Methods

Cell culture and Reagents

All cell lines and primary blasts were cultured in RPMI culture medium containing 10% fetal bovine serum (Millipore), at 37°C in 5% CO₂. Molm14 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Crenolanib was supplied by Arog Pharmaceuticals. Quizartinib was supplied by Ambit Biosciences. Sorafenib was obtained from LC Laboratories (Woburn, MA). Drugs were dissolved in DMSO at a stock concentration of 10µM. Ba/F3 mouse lymphoid cells were transfected by nucleofection with the Amaxa Kit V (Lonza, Walkersville, MD) using pCI-neo mammalian expression vector (Promega, Madison, WI) containing either WT FLT3 DNA or FLT3 ITD DNA isolated from a patient according to manufacturer’s protocol. DNA was manipulated using QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) to create D835F, D835H, D835V, and D835Y point mutants according to manufacturer’s protocol.

Cytotoxicity

Cytotoxicity was assessed using a dimethyl-thiazol diphenyl tetrazolium bromide (MTT) assay (Roche Diagnostics, Indianapolis, IN) and with the Annexin V assay for apoptosis (Pharmingen, San Diego, CA) as described previously.²⁰

Colony-forming Assays of Normal Human Bone Marrow

Mononuclear cells were isolated from bone marrow samples from normal donors (n=3) by Ficoll-Paque PLUS (GE Healthcare, Piscataway, N.J.) centrifugation. Mononuclear cells were extracted from the monolayer, washed, and plated in methylcellulose medium MethoCult H4435 Enriched (Stem Cell Technologies, Vancouver, B.C, Canada) containing various concentrations
of crenolanib or quizartinib at 100,000 cells/mL in 35mm dishes in quadruplicate. Dishes were incubated for 10-12 days and visually assessed using an Olympus CKX31 inverted microscope for abundance of erythrocyte burst forming units (BFU-E) and granulocyte-macrophage colony forming units (CFU-GM).

**Immunoblotting**

Electrophoresis and immunoblotting were performed as previously described. Anti-phosphotyrosine antibody (4G10) was obtained from Millipore (Bedford, MA). Antibodies for pAKT (Thr308), AKT, pMAPK (p-p44/42), phospho-STAT5, and MAPK (p44/42) were obtained from Cell Signaling (Danvers, MA). The antibodies for FLT3, cKit, and STAT5 and were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Plasma inhibitory assay was performed as previously described.

**DNA sequencing**

Genomic DNA from patients was isolated with QIamp Mini Kit (Qiagen, Valencia, CA). FLT3 mutations were identified, and allelic burden was estimated as previously described. This DNA was then analyzed by Sanger sequencing using the primer GAATTCATTTCACTCTGAAGAT as previously described.

**Patient samples**

Plasma inhibitory activity (PIA) assays and pharmacokinetic data were obtained from samples from patients enrolled on an institutional review board (IRB) approved phase II study of crenolanib (NCT01657682) conducted at MD Anderson Cancer Center (Principal Investigator J.C.). Primary AML patient leukemia cells were collected separately under the auspices of the institutional (IRB approved) Tumor and Cell Procurement Bank at Johns Hopkins, supported by the Regional Oncology Research Center Grant #2 P30 CA 006973-44. Whole blood and bone
marrow aspirates from healthy donors were also collected under this procurement protocol, including bone marrow cells for colony-forming assays isolated from unused remainders of normal donor harvests at the Johns Hopkins Hospital. All samples used in this study were from patients who gave informed consent according to the Declaration of Helsinki.

**Pharmacokinetics**

Venous blood samples from patients enrolled in the crenolanib phase II trial were taken prior to the initial dose, and at hours 0.5, 1, 2, 4, 8, and 24 post-dose of cycle 1 day 1. Samples were also taken on cycle 1 day 15 at pre-dose, 0.5, and 6 hours post-dose. Samples were processed within 30 minutes of collection by centrifugation for 10 minutes at 1,500x g under refrigeration (~4°C). The resultant serum was stored at –70°C until subsequent analysis for crenolanib concentrations using a validated liquid chromatography/tandem mass spectrometry (LC/MS/MS) method performed by MicroConstants (San Diego, CA). Briefly, at the day of the analysis, serum samples were thawed and 50 µL of each sample were transferred to an appropriately labeled 5mL polypropylene centrifuge tube with snap-in cap. 5µLs of the internal standard was added to each sample. The sample was vortexed thoroughly and 1.5 mL of tertiary-butyl methyl ether (TBME) was added to each sample and vortexed for 5 min. The samples were then centrifuged at 4000 rpm for 5 minutes. The TBME layer was transferred to another clean, labeled 5mL polypropylene centrifuge tube and dried with nitrogen gas for 20 minutes. The sample were reconstituted in mobile phase (30% Acetonitrile, 70% water, A-0.16% Ammonium Formate, 1.25% Citric Acid, 0.2% Formic acid) and analyzed by HPLC using a Waters XSelect CSH C18 column. The mobile phase was nebulized using heated nitrogen in a Z-spray source/interface set to electrospray positive ionization mode. The ionized compounds were detected using MS/MS.
Pharmacokinetic analysis of these samples was performed using Phoenix® WinNonlin version 6.3 software (Pharsight, Mountain View, CA).
Results

Crenolanib has activity against FLT3/ITD AML cells

Crenolanib (Figure 1A) was originally developed as an inhibitor of PDGFRB. In a commercially-available assay of kinase selectivity (KinomeScan, DiscoveRx, San Diego, CA), crenolanib was demonstrated to have a high degree of selectivity for FLT3 relative to other kinases (Figure 1B). We compared the FLT3 inhibitory activity of crenolanib with sorafenib and quizartinib using cell lines expressing mutant and wild type FLT3 (Figure 2). In immunoblot assays examining FLT3 autophosphorylation and phosphorylation of downstream signaling proteins (Figure 2A-B), crenolanib inhibited autophosphorylation of both wild type and ITD-mutated FLT3 to a similar degree, with an IC50 of approximately 2 nM. In MTT assays, crenolanib exhibited a cytotoxic effect against Molm14 and MV4-11 cells in culture medium to a similar degree as sorafenib and somewhat less so compared with quizartinib (Figure 2C). This effect was also observed in assays of apoptosis using Annexin V (Figure 2D). Likewise, crenolanib inhibited FLT3 autophosphorylation in primary FLT3/ITD AML blasts (from a patient with a FLT3/ITD mutation at an allelic ratio of 0.95) in culture with an IC50 of 2.4 nM (Figure 2E).

Crenolanib has activity against FLT3/D835 point mutants

Point mutations at residue D835 can arise in the wild type FLT3 receptor or within the context of an existing ITD mutation (i.e., within the same allele). In order to test the inhibitory activity of crenolanib against both categories of D835 mutations, we generated a series of transfectants of the murine lymphoid cell line Ba/F3. As shown in Table 1, crenolanib and quizartinib have
similar potency against a FLT3/ITD receptor lacking any kinase domain mutation. However, crenolanib was significantly more potent than quizartinib against the D835Y, D835F, and D835V mutant receptors. Interestingly, crenolanib and quizartinib were of equal potency against the D835H and D835N variants. Crenolanib has minimal activity against the F691L variant.

We next tested crenolanib against a series of primary AML samples with D835 mutations arising both spontaneously and in the context of FLT3 inhibitor therapy. Figure 3A shows a dose response experiment using 3 primary AML samples in vitro, each of which harbored a D835 mutation (in a wild type FLT3 background, no ITD mutation). Patients 2 and 3 harbored D835Y mutations and were newly-diagnosed, while patient 4, harboring a D835V mutation, was collected at relapse. Crenolanib inhibited FLT3 autophosphorylation in all three samples, with IC50’s of 1.2, 8.1, and 2.0 nM, respectively. No significant cytotoxicity was induced by the drug in the two diagnostic specimens, while the relapsed specimen did appear to respond (Figure 3B). This is similar to what has been observed with FLT3/ITD-mutated samples, in that newly-diagnosed cases are not generally responsive to highly selective FLT3 inhibition, while in the relapsed setting the response is more predictable.22 Examining this relapsed patient sample further, Figure 3C shows that crenolanib is more effective than either sorafenib or quizartinib at inhibiting FLT3 autophosphorylation and at inducing cytotoxicity. Finally, the utility of crenolanib against a tyrosine kinase domain mutation in the background of a FLT3/ITD mutation is demonstrated in Figure 3D. A FLT3/ITD AML patient (with an allelic ratio of 2.65) who was being maintained on sorafenib developed clinical progression, and was found to harbor a D835F mutation which was not present at the start of sorafenib therapy. Again, of the three inhibitors, only crenolanib induced a significant effect against these blasts in vitro (there were insufficient blasts from this sample to perform immunoblotting).
c-Kit inhibition and effects on hematopoietic colony formation

FLT3 inhibitors often have inhibitory activity against the closely-related RTK, c-KIT. This may account to some degree for the myelosuppression, particularly the anemia and thrombocytopenia that is often observed in patients receiving FLT3 inhibitors in clinical trials. An extensive literature supports the notion that suppression of c-KIT activity would be expected to result in impaired red cell and platelet production. A relative therapeutic index, therefore, would be expected to exist for inhibitors of class III RTKs such as crenolanib and quizartinib. We compared the activity of both drugs against c-KIT using immunoblot assays with the erythroleukemia cell line, TF-1, which expresses wild type c-KIT (Figure 4A). Quizartinib was the more potent inhibitor of c-KIT in this assay. In hematopoietic progenitor cell assays of normal donor marrow, quizartinib, but not crenolanib, was generally more suppressive of erythroid activity at concentrations consistent with those associated with c-KIT inhibition (Figure 4B). Crenolanib, therefore, has an advantage over quizartinib in this respect, in that the relative degree of myelosuppression it induces in patients may be less.

Crenolanib achieves inhibitory activity against FLT3/ITD and FLT3/D835 AML in vivo

Crenolanib is currently being studied in a phase II clinical trial for patients with relapsed/refractory AML with FLT3 activating mutations (NCT01657682). Patients are treated with 100 mg crenolanib orally every eight hours. However, on study day 1, patients were given a single dose of drug so that single-dose pK studies could be conducted. We used plasma samples collected from patients on day 1 of treatment with crenolanib for analysis of in vivo FLT3 inhibition using the PIA assay, and pK analysis was carried out using serum from these time points to determine serum drug concentrations at the corresponding PIA time points.
(Figures 5A-B). No significant difference was seen between experiments performed in plasma compared to serum which showed IC$_{50}$s against phospho-FLT3 of 48 nM and 40 nM, respectively (Supplemental Figure 1). Comparing the PIA results with the pK results, we observed that FLT3 inhibition was readily achieved throughout the dosing period. A trough serum concentration of 100-200 nM appears to be required to maintain inhibition of FLT3 autophosphorylation to 10-15% of baseline. This PIA assay was performed using Molm14 cells, which harbor a FLT3/ITD mutation. To provide evidence for the in vivo activity of crenolanib against kinase domain mutations, we took blasts from patient 4 (described above), which contained a D835V mutation, and used them in a PIA assay of trough plasma samples from several different trial patients. As shown in Figure 5C, steady state (Day 15) levels of crenolanib were quite sufficient to profoundly inhibit this mutant FLT3 receptor. These clinical correlative data indicate that AML patients treated with 100 mg crenolanib every eight hours are able to achieve sufficient concentrations of the drugs to effectively inhibit the target, whether it is a FLT3/ITD mutation or a FLT3/D835 mutation. Data regarding the safety, tolerability, and efficacy of crenolanib in these patients is not yet available, as accrual is ongoing.
Discussion

FLT3 inhibitors have been under investigation for over a decade. First generation inhibitors, such as lestaurtinib, midostaurin, and sunitinib, were re-purposed multi-targeted compounds with minimal clinical activity as single agents because they lacked potency and/or tolerability. Second generation inhibitors such as quizartinib and PLX3397 are more selective, and therefore presumably better tolerated at concentrations necessary to fully inhibit FLT3 in vivo. This has led to more impressive clinical results, particularly with quizartinib, but has also not unexpectedly led to the emergence of resistance-conferring point mutations, most commonly at residue D835. The emergence of these mutations in the context of FLT3 inhibition represents evidence that FLT3 mutations- either the ITD mutation at diagnosis or the ITD/D835 combination- represent driver mutations for this disease. Furthermore, while isolated FLT3/D835 mutations (i.e., those occurring in the absence of a FLT3/ITD mutation) overall have an uncertain prognostic impact, in some cases they probably also function as driver mutations (e.g., see Figure 3), and therefore warrant targeting.

Crenolanib has been studied in patients with solid tumors, and is currently being studied in patients with PDGFR-driven gastrointestinal stromal tumors (NCT01243346) and in pediatric patients with gliomas (NCT01393912), as well as in AML patients with FLT3 activating mutations (NCT01522469; NCT01657682). Preliminary clinical and pharmacokinetic data from these studies indicate that the drug is cytochrome P450-metabolized, has an approximate terminal half-life ($t_{1/2}$) of 8 hours, and is well-tolerated at 100 mg three times daily (Arog Pharmaceuticals, personal communication). This $t_{1/2}$ is considerably shorter than that of sorafenib (~24 hours) or quizartinib (1.5 days), necessitating thrice daily dosing.
Crenolanib has no activity against the F691 point mutations, which also emerged during quizartinib, albeit at a lower frequency than the D835 mutations, and we did not test the drug exhaustively against all of the other relatively uncommon FLT3 point mutations that have been previously reported. It is likely that as this field progresses, a number of compounds will emerge that have overlapping activity against FLT3 variants, similar to the case with inhibitors of BCR-ABL. However, FLT3/ITD and FLT3/D835 mutations, either arising spontaneously or in response to treatment with a FLT3TKI, constitute the vast bulk of clinically important FLT3 activating mutations, and we have demonstrated here that crenolanib not only has in vitro activity against them, oral dosing of the drug can achieve inhibitory concentrations against these mutations in AML patients.

Myelosuppression can be a challenging clinical parameter to evaluate in any trial involving AML patients. Most AML patients enrolled on early phase studies are already myelosuppressed from their disease burden. Nonetheless, suppression of bone marrow function to some degree is almost always a feature of TKIs that have activity against c-KIT, and the relatively limited activity of crenolanib against c-KIT may offer a unique advantage of this drug over others in this class.

In summary, our results indicate that crenolanib represents the next generation of FLT3 TKIs, one with a broader range of activity than the other agents in development. Phase 2 trials of this drug in FLT3-mutated AML patients are ongoing (NCT01657682 and NCT01522469), and phase 3 trials are currently in the planning stage.
Authorship

Contribution: A.G. and M.L. designed the study, performed experiments, analyzed the data, and wrote the manuscript. H.M. performed experiments and helped edit the manuscript. T.R. performed experiments. A.R. coordinated the pK analysis and helped write the manuscript. D.S. contributed to study design and helped edit the manuscript. J.C. contributed to study design, enrolled patients and coordinated sample collection, and helped edit the manuscript.

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

Acknowledgements

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References


17. Knapper S, Mills KI, Gilkes AF, Austin SJ, Walsh V, Burnett AK. The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. *Blood*. 2006;108(10):3494-3503.


Table 1

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Table 1. Inhibitory activity of crenolanib against different FLT3 TKD mutations. Ba/F3 cell lines were treated with quizartinib or crenolanib for one hour, then cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3. Densitometry analysis was performed using Quantity One software (version 4.5.0). IC$_{50}$s were calculated by regression analysis after linear conversion (CalcuSyn software).
Figure Legends

Figure 1. Crenolanib. (A) The structure of crenolanib, a benzimidane quinolone derivative. (B) KinomeScan results for crenolanib. Shown are the results of all kinases in the panel inhibited 50% or more (compared with baseline activity) by 100 nM crenolanib.

Figure 2. Activity of crenolanib against wild type and ITD-mutated FLT3 in vitro. (A) FLT3/ITD cell lines (Molm14 and MV411) and FLT3 WT cell lines (SEMK2) were treated with crenolanib for one hour, then cells were lysed, immunoprecipitated for FLT3 and analyzed by immunoblotting for phospho- and total FLT3. (B) An aliquot of the lysate of Molm14s treated with crenolanib from panel A was reserved for analysis of downstream signaling molecules including pAKT, pMAPK, pSTAT5, as well as the corresponding total protein content. (C) Cytotoxicity of these doses of crenolanib in FLT3/ITD cell lines was analyzed by MTT assay. HL-60 cells which express extremely limited WT FLT3 were used as a control. (D) Apoptosis induced by crenolanib and sorafenib 48 hrs after treatment of Molm14 cells with drug was analyzed by Annexin V flow cytometry. (E) Patient blasts containing a FLT3/ITD mutation were treated with crenolanib, lysed, and immunoblotted for phospho- and total FLT3.

Figure 3. Inhibitory activity of crenolanib against FLT3 TKD mutations in primary AML samples. (A) Blasts from patient 2 (de novo D835Y), patient 3 (de novo D835Y), and patient 4 (relapsed D835V) were incubated for one hour with crenolanib, lysed, immunoprecipitated for FLT3, and analyzed for phospho- and total FLT3 by immunoblotting. (B) Blasts from the same patients in panel two were treated with crenolanib in 96 well plates for 72 hours and cytotoxicity was analyzed by MTT. (C) Blasts from patient 4 were also treated with quizartinib and sorafenib in this analysis. In a separate immunoblot experiment (inset), blasts from patient 4 were treated
with 20 nM of quizartinib, crenolanib, and sorafenib for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho- and total FLT3. (D) Patient 5 had a FLT3/ITD mutation and was treated with sorafenib, responded, and then relapsed. Blasts collected after relapse harbored a D835F mutation, along with the original FLT3/ITD mutation. These blasts were treated with crenolanib, quizartinib, or sorafenib in 96 well plates for 72 hours and cytotoxicity was analyzed by MTT.

**Figure 4. Inhibition of c-KIT and erythropoiesis.** (A) TF-1 cells, which express WT c-Kit, were treated with crenolanib or quizartinib for one hour. In the last five minutes of drug treatment, 20 ng of SCF was added. The cells were lysed, immunoprecipitated for c-Kit and analyzed by immunoblot for phospho- and total c-Kit. (B) Normal human donor bone marrow (n=3) was collected and diluted to a concentration of 100,000 cells per mL in MethoCult. Various concentrations of crenolanib or quizartinib were added and cells were plated in quadruplicate in 35 mm dishes. Each dish was view under a light microscope and total numbers of CFU-GM and BFU-E colonies were recorded.

**Figure 5. Crenolanib inhibits FLT3 in vivo.** (A) Plasma samples from patient 6 and patient 7 were collected as part of the phase II clinical trial of crenolanib. Patients received a single dose of drug on Day 1, and samples were collected for PIA and pK analysis. Molm14 cells were incubated with each plasma sample for one hour, lysed, immunoprecipitated for FLT3, and analyzed by immunoblot for phospho and total FLT3. (B) The same time course samples from these patients were analyzed by mass spectroscopy for serum drug concentrations of crenolanib. (C) Blasts from patient 4 (relapsed D835V) were utilized for a PIA assay with steady state plasma samples from five different patients enrolled on the crenolanib trial. Blasts were incubated in plasma for one hour, lysed, immunoprecipitated for FLT3, and analyzed by
immunoblot for phospho- and total FLT3. Densitometry was performed on this blot and results are described under %FLT3 inhibition. Corresponding serum concentrations for the chosen sample are listed below.
FIGURE 4

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Quizzartinib

IC₅₀: 35 nM

pKIT

KIT

B

Average Number of Colonies per Plate

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FIGURE 5

C

<table>
<thead>
<tr>
<th>% pFLT3 inhibition</th>
<th>Control</th>
<th>9</th>
<th>10</th>
<th>8</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum crenolanib concentration (nM)</td>
<td>753</td>
<td>66</td>
<td>331</td>
<td>664</td>
<td>504</td>
<td></td>
</tr>
</tbody>
</table>

The figure shows a Western blot analysis of pFLT3 and FLT3 expression levels under different conditions. The table below lists the % pFLT3 inhibition and the corresponding Serum crenolanib concentration (nM) for each sample.
Crenolanib is a potent inhibitor of FLT3 with activity against resistance-conferring point mutants

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