Crenolanib Inhibits the Drug-Resistant PDGFRA D842V Mutation Associated with Imatinib-resistant Gastrointestinal Stromal Tumors

Michael C. Heinrich1, Diana Griffith1, Arin McKinley7, Janice Patterson1, Ajia Presnell1, Abhijit Ramachandran2, Maria Debiec-Rychter3

1Portland VA Medical Center and OHSU Knight Cancer Institute, Portland, OR, USA
2AROG Pharmaceuticals LLC, Dallas, TX, USA
3Department of Human Genetics, K.U.Leuven and University Hospitals, Leuven, Belgium

Correspondence to:
Michael C. Heinrich, MD
Division of Hematology/Oncology
Departments of Medicine and Cell and Developmental Biology
Portland VA Medical Center and OHSU Knight Cancer Institute, Oregon Health & Science University
R&D-19 3710 U.S. Veterans Hospital Road
Portland, OR 97239
Tel.: (503) 220-3405
Fax: (503) 273-5158
E-mail: Heinrich@ohsu.edu
Statement of Translational Relevance:

Approximately 5-10% of GI stromal tumors (GIST) have pathogenetic activating mutations of the PDGFRA receptor tyrosine kinase. The most common of these mutant kinases, PDGFRA D842V, is resistant to inhibition by imatinib or sunitinib which are currently approved as first-line and second-line treatment of advanced GIST, respectively. In addition, there are other PDGFRA mutations that are also resistant to imatinib. Patients whose GIST have a PDGFRA D842V mutant kinase have a markedly inferior progression-free and overall-survival compared with patients with KIT-mutant GIST. In the current study, we identified crenolanib as a potent inhibitor of PDGFRA D842V mutant kinase with an IC50 of approximately 10 nM. Thus, crenolanib is 100-150 fold more potent than imatinib against PDGFRA D842V. In addition, crenolanib has similar potency against less common imatinib-resistant PDGFRA mutations. Based on these results, a phase 2 study of crenolanib for advanced PDGFRA D842V mutant GIST has been initiated (NCT01243346).
Abstract

Purpose: To determine the potential of crenolanib, a potent inhibitor of PDGFRA, to treat malignancies driven by mutant PDGFRA.

Experimental Design: The biochemical activity of crenolanib was compared to imatinib using a panel of PDGFRA mutant kinases expressed in several different cell line models, including primary GIST cells. The anti-proliferative activity of crenolanib was also studied in several cell lines with PDGFRA-dependent growth.

Results: Crenolanib was significantly more potent than imatinib in inhibiting the kinase activity of imatinib-resistant PDGFRA kinases (D842I, D842V, D842Y, DI842-843IM, and deletion l843). For example, crenolanib was 135-fold more potent than imatinib against D842V in our isogenic model system, with an IC50 of approximately 10 nM. The relative potency of crenolanib was further confirmed in BaF3 and primary GIST cells expressing PDGFRA D842V. In contrast, imatinib was at least 10-fold more potent than crenolanib in inhibiting the V561D mutation. For all other tested PDGFRA mutations, crenolanib and imatinib had comparable potency.

Conclusions: Crenolanib is a potent inhibitor of imatinib-resistant PDGFRA kinases associated with GIST, including the PDGFRA D842V mutation found in ~5% of GISTs. The spectrum of activity of crenolanib suggests that this drug is a type I inhibitor (inhibitor of activated conformation of kinase). Based in part on these results, a phase 2
clinical study of this agent to treat GIST with the PDGFRA D842V mutation has been initiated.
Introduction

Activating mutations of receptor tyrosine kinases KIT or PDGFRA are fundamental to the pathogenesis of most gastrointestinal stromal tumors (GISTs). More than 80% of GISTs express mutated, constitutively active KIT receptor, another 5% to 10% express mutated PDGFRA, while 10% to 15% of cases have no mutations in either of these kinases (wild-type GIST, WT).(1, 2)

The use of KIT/PDGFRA tyrosine kinase inhibitors (TKIs) has transformed the treatment of localized and advanced GIST. Front-line treatment of metastatic GIST with imatinib produces high rates of clinical benefit (PR + SD) and progression-free survival in the range of 10-24 months. In addition, median overall survival of patients with metastatic GIST is now approximately 50-60 months.(2-4) In the second-line setting, treatment with sunitinib increases progression-free- and overall-survival of patients with imatinib-resistant disease compared with placebo.(5) Patients with primary KIT exon 11 mutations have the longest progression-free- and overall-survival during imatinib treatment.(3, 6) Congruent with this observation, KIT exon 11 mutant-kinases are potently inhibited by imatinib and sunitinib in vitro.(7)

However, treatment of PDGFRA-mutant GIST with currently available PDGFRA TKIs (imatinib, sunitinib, etc) has yielded mixed results, with outcomes being closely correlated with the intrinsic sensitivity of different mutant PDGFRA oncoproteins to various inhibitors. In vitro, some PDGFRA-mutant kinases (e.g. V561D) are extremely
sensitive to imatinib and patients with these underlying imatinib-sensitive PDGFRA mutations seem to have similar clinical outcomes as patients with KIT exon 11 mutations. (7-10)

In contrast, the most common PDGFRA mutation associated with GIST, D842V, is strongly resistant to inhibition by imatinib or sunitinib. (7, 8, 11, 12) In addition to the D842V mutation, there are other less common PDGFRA mutant kinases that are also resistant to imatinib in vitro (e.g. PDGFRA D842Y or PDGFR D1842-843IM). (13) Patients with D842V-mutant GIST have a very low rate of clinical benefit from imatinib treatment. For example, Biron et al. conducted a survey of international GIST treatment centers and collected data on 20 patients with locally advanced or metastatic GIST treated with front-line imatinib. In this series, no objective responses were seen. In addition, the median progression free survival was a mere 2.8 months and the median overall survival was only 12.7 months. (14) Similar results were seen in smaller numbers of PDGFRA D842V-mutant GIST patients treated as part of phase 2-3 trials of imatinib for advanced GIST. (6, 8-10) The PDGFRA D842V mutant kinase is also strongly resistant to sunitinib in vitro. Limited clinical data suggest that sunitinib has no activity against GIST with primary or secondary PDGFRA D842V mutations. (7)

The above data indicate that patients with TKI-resistant PDGFRA mutant kinases do not benefit from standard TKI therapy. Based on the underlying molecular similarity of PDGFRA- and KIT-mutant GIST, it is predicted that GIST with drug resistant PDGFRA D842V mutations would respond to effective kinase inhibition. (15) However, currently
there is no proven effective medical treatment for patients with PDGFRA D842V mutant GIST.

Crenolanib (formerly CP-868,596), an orally bioavailable benzimidazole, is a selective and potent inhibitor of PDGFRA and PDGFRB. This agent has been tested in both phase 1 single agent and phase 1b combination therapy clinical studies and found to be well tolerated.(16, 17) We tested the activity of crenolanib against GIST-associated PDGFRA mutant kinases in a variety of models. We identified crenolanib as the most potent PDGFRA D842V kinase inhibitor described to date with an IC₅₀ in the range of 10 nM. Notably, crenolanib was at least 100-fold more potent than imatinib for biochemical inhibition of PDGFRA D842V kinase. We also report herein the biochemical activity of crenolanib against a larger panel of imatinib-sensitive and -resistant PDGFRA mutant kinases. Based on our results, we hypothesize that crenolanib could be an effective treatment for patients with PDGFRA mutant GIST, particularly those patients whose GIST express the PDGFRA D842V mutant oncogenic kinase.

Methods

DiscoveRx – KINOMEscan™: Kd ELECT

Crenolanib was profiled using the commercially available KINOMEscan™: Kd ELECT screening service (DiscoveRx–KINOMEscan™ San Diego, CA) as previously described.(18, 19) KINOMEscan™: Kd ELECT is based on a competition binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active-site directed ligand. The assay was performed by combining three components:
DNA-tagged kinase; immobilized ligand; and a test compound. The ability of the test compound to compete with the immobilized ligand was measured via quantitative PCR of the DNA tag. Inhibitor binding constants (Kd values) were calculated from duplicate 11-point dose-response curves. Measurements were made under optimized conditions that generate true thermodynamic Kd values which facilitate direct comparison of inhibitor affinity across kinases.

Reagents and antibodies
Imatinib was obtained commercially from LC Laboratories (Woburn, MA) and reconstituted in phosphate buffered saline (PBS) to yield a 10 mM stock. Crenolanib was obtained from AROG Pharmaceuticals, LLC and reconstituted in DMSO to yield a 10 mM stock. Working solutions of imatinib or crenolanib were diluted in growth media or PBS for use in experiments. For PDGFRA immunoprecipitation experiments, we used rabbit polyclonal anti-PDGFRα antibody (Santa Cruz Biotechnology, SC-20, Santa Cruz, CA) and Protein A/G beads (Santa Cruz). For detection of phospho-PDGFRα and total PDGFRA we used anti-phosphotyrosine monoclonal antibody (PY-20, 1:500, BD Transduction Labs, Sparks, Maryland) and anti-PDGFRα rabbit polyclonal antibody (SC-20, 1:500, Santa Cruz), respectively. KIT immunoprecipitation experiments were performed as previously described.(7) For detection of phospho- and total-KIT, we used rabbit polyclonal antibody to P-KIT Tyr 719 (Cell Signaling Technology, cat#3391, 1:500, Danvers, MA) or total KIT (Santa Cruz, C-19, 1:500), respectively.

Cell Lines
The BaF3 V561D and BaF3 D842V cell lines have been previously described.(13) These cell lines were generated by transfection of mutant PDGFRA isoforms into BaF3
cells. Clones were selected for G418 resistance and IL-3 independence. DNA from stable lines was checked to ensure that the expected mutant PDGFRA sequence was present, and expression was verified by immunoblotting for total and tyrosyl-phosphorylated forms of PDGFRA. The GIST T1 cell line was a generous gift of Dr. Taguchi (Kochi University, Kochi, Japan). This cell line was established from an untreated GIST, and expresses an exon 11 deletion constitutively active KIT kinase.(20) The EOL-1 cell line was derived from a patient with chronic eosinophilic leukemia and expresses the constitutively activated FIP1L1-PDGFRA fusion kinase (DSMZ Tissue Bank, Germany).(21) The H1703 non-small cell lung cancer cell line was obtained from the American Tissue Type Collection (Manassas, VA). The H1703 cell line has been previously reported to have 24-fold amplification of the 4q12 region that contains the PDGFRA locus.(22)

**Biochemical assessment of PDGFRA/KIT kinase activity**

Chinese hamster ovary (CHO) cells were transiently transfected with mutated KIT or PDGFRA cDNA constructs and treated with various concentrations of imatinib or crenolanib as previously described.(7, 13) Experiments involving recombinant DNA were performed using biosafety level 2 conditions in accordance with published guidelines. Protein lysates from cell lines were prepared and subjected to immunoprecipitation using anti-KIT or anti-PDGFRA antibodies followed by sequential immunoblotting for phospho-KIT and total KIT, or phosphotyrosine or total PDGFRA, respectively, as previously reported.(7, 13) Densitometry was performed to quantify drug effect using Photoshop 5.1 software, with the level of phospho-KIT or phospho-PDGFRA normalized to total protein. Densitometry and proliferation experimental
results were analyzed using CalcuSyn 2.1 software (Biosoft, Cambridge, UK) to mathematically determine the IC_{50} values. The Wilcoxon Rank Sum Test was used to compare the IC_{50} values of imatinib and crenolanib for a given mutation.

**Ex vivo assay using primary GIST cells**

Surgical specimens of primary GIST from imatinib-naive patients were used for the primary cell cultures, as previously described.\(^{(12)}\) The DNA was isolated and PDGFRA D842V mutation was identified by direct sequencing, according to standard procedures. For Western immunoblotting, primary GIST cells obtained from collagenase-disaggregated tumor specimens were seeded in duplicate at 80% confluence in 25 mm diameter cell culture dishes (Corning Inc, Corning, NY) and grown in Dulbecco's modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 1.0 mM non-essential amino acids, and 1.0 mM sodium pyruvate for 24 hours at +37°C. Next, the cells were exposed to a crenolanib in different concentrations, or to vehicle alone (DMSO) and incubated for 2 hours at +37°C. After a wash in ice-cold PBS cells were lysed and immunoblotted using anti-phospho-PDGFRA(Tyr754) and anti-PDGFRA antibodies.

**Proliferation Assays**

Cells were added to 96-well plates at densities of 20,000 cells/well and incubated with imatinib or crenolanib for 72 hours before measuring cellular proliferation using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT)-based assay (Roche Molecular Biochemicals, Indianapolis, IN).\(^{(13)}\)

**RESULTS**
DiscoveRx – KINOMEscan™, Kd ELECT of Crenolanib

Crenolanib was developed as an orally available inhibitor of PDGFR kinases with >100 fold selectivity versus a limited number of profiled kinases (VEGFR2, TIE-2, FGFR-2). To better characterize the activity of crenolanib, we performed competitive binding assays to quantify the affinity of crenolanib for a panel of recombinant kinases (DiscoveRx – KINOMEscan™, Kd ELECT).

The results demonstrate that crenolanib is a specific and potent inhibitor of class III receptor tyrosine kinases (RTKs). The inhibitor binding constant ($K_d$) of crenolanib for the wild-type receptors PDGFRA, PDGFRB, and FLT3 was 3.2 nM, 2.1 nM, and 0.74 nM, respectively (Table 1). In contrast to other known inhibitors of PDGFR (e.g. imatinib, sunitinib, dasatinib), crenolanib has 25 fold more affinity for PDGFRA/B compared to KIT. Besides class III RTKs, crenolanib does not inhibit any other known tyrosine or serine/threonine kinases at clinically achievable concentrations. Notably, crenolanib was identified in this study as an extremely potent inhibitor of FLT3; the activity of crenolanib against FLT3 has not been previously reported.

**In-vitro Measure of IC50 Activity of Crenolanib or Imatinib against Specific Mutants in an Isogenic Model System**

The above results indicate that crenolanib is a fairly specific kinase inhibitor with only minimal predicted “off-target” effects. However, these binding assays are not always predictive of biochemical activity. (23) Therefore, we sought to confirm these results in biochemical assays using full length kinase. To determine the potential clinical efficacy
of crenolanib in the treatment of GIST, we measured the activity of crenolanib against gain-of-function PDGFRA mutations associated with GIST. In particular, we analyzed the activity of crenolanib against imatinib-resistant PDGFRA oncoproteins.

Mutant isoforms corresponding to selected human PDGFRA mutations were created by site-specific mutagenesis and expressed in Chinese hamster ovary cells by transient transfection as previously described. (8, 13) All of the expressed mutants demonstrated phosphorylation of tyrosine residues in the absence of PDGF-AA ligand, implying constitutive activation of their kinase domains. We directly compared the biochemical potency of imatinib and crenolanib to inhibit PDGFRA activity (as assessed by inhibition of autophosphorylation) in the same experiment.

Consistent with previous data, both imatinib and crenolanib are potent inhibitors of PDGF-AA ligand stimulated PDGFRA. (7, 8, 16) Both compounds have IC_{50}s in the very low nanomolar range with no difference in potency between these drugs (Table 2, Figure 1). Imatinib is 6-20 times more potent than crenolanib for inhibiting PDGFRA mutations involving the juxtamembrane domain (V561D, RV560-561RERV). There is no difference in potency between imatinib or crenolanib for inhibiting an ATP binding pocket activating PDGFRA mutation (N659K, exon 14).

We and others have previously noted that mutations involving PDGFRA exon 18, which encode the PDGFRA activation loop, can be divided into groups of mutations that are sensitive to imatinib (e.g. deletion DIMH842-845) and those that are resistant (e.g.
D842V). (8, 11, 12) For the imatinib-sensitive mutations that we tested (deletion DIMH842-845, deletion I843, deletion HDSN845-848R, D846Y, and N848K), there was no significant difference between imatinib and crenolanib for inhibition of kinase activity (Table 2, Figure 1). In contrast, crenolanib is significantly more potent than imatinib for inhibiting mutant kinases that were previously reported to be imatinib-resistant (PDGFRA D842I, D842V, D842Y, and D1842-843IM). Notably, crenolanib is approximately 135-fold more potent than imatinib for inhibiting the PDGFRA D842V mutation in this model system.

Previous studies of crenolanib have indicated that it is significantly less potent against KIT than against PDGFRA. (16) To confirm these results, we compared the activity of imatinib and crenolanib against KIT mutant isoforms associated with GIST or mastocytosis (Table 2). The IC50 for crenolanib for a KIT exon 11 deletion mutant kinase is >1000 nM vs. 8 nM for imatinib. Neither crenolanib nor imatinib had any significant biochemical activity against the D816V mutation, which is homologous to the PDGFRA D842V mutation associated with mastocytosis (IC50 > 1000 nM for both). (15)

The molecular mechanisms underlying the secondary clinical progression of imatinib-sensitive PDGFRA-mutant GIST are poorly understood. However, one reported secondary mutation has been described—acquisition of a secondary PDGFRA D842V mutation. (7) This same mutation has also been described as secondary drug resistance mutation in patients with hypereosinophilic syndrome with a FIP1L1-PDGFRA fusion who are treated with imatinib. (24) In addition, the secondary
gatekeeper mutation (PDGFRA T674I) has been reported in a patient with FIP1L1
PDGFRA-positive hematologic neoplasm treated with PDGFRA inhibitors.(25)

We expressed the compound PDGFRA mutants V561D + T674I (exon 12 +
gatekeeper), V561D + D842V (exon 12 + 18), and T674I + D842V (gatekeeper + exon 
18) in CHO cells and compared the activity of imatinib or crenolanib against these
compound mutant kinases (Table 3, Figure 2). Neither imatinib nor crenolanib has any
activity against the V561D + T674I mutant kinase (IC$_{50}$ >1000 nM for each). However,
crenolanib has low nanomolar potency against the V561D + D842V mutant kinase that
is similar to its potency against the isolated D842V mutation. This was unexpected as
the isolated V561D mutation is relatively resistant to crenolanib. We also found that
addition of the gatekeeper T674I mutation to the D842V mutation had no significant
effect on the potency of crenolanib, indicating that crenolanib binding is not dependent
upon the T674 residue.

**Comparative Activity of Crenolanib and Imatinib in Cellular Models**

The above studies were conducted in a CHO transient transfection system that allows
us to directly compare the activity of different TKIs on the same mutant kinase in an
isogenic system. To extend these results, we also compared the potency of crenolanib
and imatinib in a panel of PDGFRA-dependent cell lines. These cell lines allowed us to
not only assess biochemical potency of the two inhibitors, but also to measure the effect
of PDGFRA kinase inhibition on cellular proliferation.
The EOL-1 cell line is derived from a patient with chronic eosinophilic leukemia and expresses the constitutively activated FIP1L1-PDGFRα fusion kinase.\cite{21} Both imatinib and crenolanib potently inhibit the kinase activity of the fusion oncogene with IC\textsubscript{50}s of 1 and 21 nM, respectively (p=0.13) (Figure 3A). In addition, both compounds potently inhibit the proliferation of EOL-1 cells with a 50% growth inhibitory concentration of 0.2 pM (Figure 3B).

We also tested the comparative potency of imatinib and crenolanib to inhibit the activation of V561D or D842V-mutant kinases expressed in BaF3 cells. Consistent with our results in the transient transfection model (CHO cells), imatinib is more potent than crenolanib in inhibiting the activity of V561D (IC\textsubscript{50} 11 vs. 85 nM, p=0.05). In addition, we confirm that crenolanib is 100-fold more potent than imatinib for inhibiting activity of the D842V mutation (IC\textsubscript{50} 2 vs. 272 nM, p=0.002) (Figure 3A). Similar to the biochemical data, crenolanib is significantly more potent than imatinib for inhibiting proliferation of the BaF3 D842V cell line, with IC\textsubscript{50}s of 22 and 1510 nM, respectively (Figure 3B).

PDGFRA genomic amplification leading to constitutive PDGFRA activation has been reported in a number of malignancies including non-small cell lung cancer and gliomas.\cite{22, 26-28} We tested the activity of imatinib and crenolanib against the H1703 non-small cell lung cancer cell line that has been previously reported to have 24-fold amplification of the 4q12 region that contains the PDGFRA locus. This cell line is dependent upon PDGFRA kinase activity for proliferation as evidenced by inhibition of
growth by shRNA against PDGFRA or PDGFRA TKIs (imatinib, sunitinib). Both imatinib and crenolanib inhibited PDGFRA activation in this cell line with IC50s of 93 and 26 nM, respectively (Figure 3B, p=0.4). Both drugs also inhibited the proliferation of these cells in a similar dose range (data not shown).

Finally, we tested the biochemical activity of crenolanib in two primary GIST cells with PDGFRA D842V mutation by ex vivo assay. Crenolanib, at a concentration of 10 nM, completely inhibits PDGFRA D842V activation as assessed by autophosphorylation (Figure 4). Due to the limited number of primary cells available, we did not perform comparative studies against imatinib nor perform experiments to assess the effect of crenolanib on cellular proliferation. Notably, in previously published studies using these cells, concentrations of imatinib up to 5000 nM had only a minimal effect on PDGFRA D842V autophosphorylation.

DISCUSSION

TKI inhibitor therapy of GIST has revolutionized the treatment of advanced metastatic or unresectable GIST. Prior to the introduction of TKI therapy, there was no effective medical therapy for GIST as these tumors are uniformly resistant to chemotherapy. However, the benefits of TKI therapy strongly correlate with the underlying biology of the tumor. Notably, the best results are seen for treatment of KIT exon 11 mutant GISTs which express mutant kinases that are extremely sensitive to in vitro inhibition by imatinib or sunitinib.
Although KIT is the most commonly mutated kinase in GIST, approximately 5-10% of GISTs express exclusively a mutated PDGFRA kinase.\(^2\) The response of patients with PDGFRA-mutant GIST to TKIs correlates with intrinsic sensitivity of the mutant kinase expressed by tumor cells. Patients whose GIST have an imatinib-sensitive PDGFRA mutation (e.g. PDGFRA exon 12 V561D mutation) seem to have similar clinical outcomes as patients whose tumor has a KIT exon 11 mutation. In contrast, the most common PDGFRA mutation associated with GIST (PDGFRA exon 18 D842V) has been shown to be resistant to imatinib and sunitinib \textit{in vitro}. Consequently, patients with PDGFRA D842V mutant GIST treated with conventional GIST TKIs have a very low rate of clinical benefit and the median overall survival for these patients is approximately 1 year, compared with the median of 4-5 years seen for patients with KIT exon 11 mutant GIST. In addition to the PDGFRA D842V mutation, there are also a number of less common PDGFRA mutations that also are resistant to imatinib \textit{in vitro}.\(^6,8-10,13,14\)

We tested the activity of crenolanib, a potent PDGFRA/B TKI to inhibit a panel of PDGFRA mutant kinases. Crenolanib was significantly more potent than imatinib in inhibiting the kinase activity of imatinib-resistant PDGFRA kinases (D842I, D842V, D842Y, DI842-843IM, and deletion I843). For example, crenolanib was 135-fold more potent than imatinib at D842V kinase activity in our isogenic model system. The relative potency of crenolanib was further confirmed in BaF3 and primary GIST cells expressing PDGFRA D842V. In contrast, imatinib was 6-10 fold more potent than crenolanib in
inhibiting the V561D mutation. For all other tested PDGFRA mutations, crenolanib and imatinib had comparable kinase activity.

The D842V mutation is the most common primary PDGFRA mutation found in GIST, but can also develop as a secondary resistance mutation following imatinib treatment of PDGFRA-mutant tumors. The crenolanib potently inhibited the activity of the compound V561D + D842V mutant kinase and retained activity when the gatekeeper T674I mutation was added to the D842V mutation.

Zhang et al. have classified TKIs into Type I and Type II inhibitors. Type I inhibitors can only bind to the active kinase conformation. In contrast, Type II inhibitors, such as imatinib and sunitinib, can only bind to the inactive conformation of the kinase and function by preventing the enzyme from assuming the active conformation. Of note, the D842V mutation is felt to stabilize the PDGFRA activation loop in the active conformation; this conformation cannot bind imatinib or sunitinib. However, in the current study we found that this mutation is very potently inhibited by crenolanib.

The spectrum of activity of crenolanib suggests that it is a Type I inhibitor. The ability of both imatinib and crenolanib to inhibit certain mutations with equivalent potency (e.g. del DIMH842-845), suggests that these particular mutant kinases are in an equilibrium with roughly equal populations of inactive and active kinase isoforms, thus allowing either Type I and II inhibitors to bind and thereby inhibit kinase activity. Based on this model, the relative potency of imatinib and crenolanib against PDGFRA mutant isoforms may
provide insight into how different mutations affect the distribution of protein isoforms between the inactive and active state. In addition, further structure based studies of crenolanib and related compounds may aid in the development of even more potent type I PDGFRA kinase inhibitors.

We hypothesize that crenolanib may be useful for the treatment of GIST with an associated PDGFRA mutation. In phase 1-2 studies, orally administered crenolanib was well tolerated and exhibited favorable pharmacokinetic properties. The dose limiting toxicities included hematuria, increased liver function tests, and nausea/vomiting. (16, 17) Based in part on these results and the prior clinical experience with crenolanib, a multi-center phase 2 study of crenolanib for treatment of PDGFRA D842V-mutant GIST has been initiated (NCT01243346).

In addition to a subset of GIST, genomic alterations resulting in PDGFRA or PDGFRB activation have been reported in other human malignancies including myeloproliferative disorders (e.g. F1P1L1-PDGFRA, BCR-PDGFRA, ETV6-PDGFRB), gliomas (amplification of PDGFRA), dermatofibrosarcoma protuberans (activation of PDGFRB due to paracrine secretion of COL1A1-PDGFB fusion protein), and non-small cell lung cancer (genomic amplification of PDGFRA). Also, PDGFRB activation may contribute to tumor angiogenesis either directly (endothelial cells) or indirectly (support of pericytes). Therefore, crenolanib may be useful in the treatment of cancers that have abnormal activation of PDGFRA or PDGFRB. Based on prior studies of the role of PDGFRA in the pathogenesis/biology of gliomas, several phase 2 studies of crenolanib
for treatment of pediatric or adult glioma have been initiated (NCT01229644, NCT01393912).(22, 26-28, 33, 34)

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Reference List


Ref Type: Abstract


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<table>
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**Table 1.** Crenolanib was profiled using the commercially available KINOMEscan™. Kd ELECT screening service as previously described.\(^{(18, 19)}\) Kd measurements were based on a competition binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active-site directed ligand. Inhibitor binding constants (Kd values) were calculated from duplicate 11-point dose-response curves. Measurements were made under optimized conditions that generate true thermodynamic Kd values which facilitate direct comparison of inhibitor affinity across kinases.
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<tr>
<td>PDGFRA deletion HDSN845-848P</td>
<td>18</td>
<td>CHO</td>
<td>266 ± 105</td>
<td>87 ± 52</td>
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<tr>
<td>PDGFRA D846Y</td>
<td>18</td>
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<td>29 ± 13</td>
<td>18 ± 6</td>
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<tr>
<td>PDGFRA N848K</td>
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<td>22 ± 7</td>
<td>39 ± 16</td>
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<tr>
<td>KIT exon 11 deletion</td>
<td>11</td>
<td>GIST-T1</td>
<td>8 ± 4</td>
<td>&gt;1000</td>
<td>0.05**</td>
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<tr>
<td>KIT D816V</td>
<td>17</td>
<td>CHO</td>
<td>&gt;1000</td>
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<tr>
<td>PDGFRA V561D</td>
<td>12</td>
<td>BaF3</td>
<td>13 ± 12</td>
<td>134 ± 71</td>
<td>0.03**</td>
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<tr>
<td>PDGFRA D842V</td>
<td>18</td>
<td>BaF3</td>
<td>272 ± 163</td>
<td>2 ± 2</td>
<td>0.002**</td>
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**Table 2. Biochemical IC₅₀ values for inhibition of kinase activity in cells expressing single mutation kinases.** The values for crenolanib and imatinib represent the biochemical IC₅₀ expressed in nM units ± the standard error of the mean. Values represent the data from at least three replicate experiments per mutation. ** p<0.05 by Wilcoxon Rank Sum Test.
Table 3. *Biochemical IC*$_{50}$ values for inhibition of PDGFRA kinase activity in CHO cells expressing compound mutant kinases.* The values for crenolanib and imatinib represent the biochemical IC$_{50}$ expressed in nM units ± the standard error of the mean. Values represent the data from at least three replicate experiments per mutation.

<table>
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<tr>
<th>Kinase</th>
<th>Exons</th>
<th>Imatinib</th>
<th>Crenolanib</th>
<th>p-value</th>
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<tr>
<td>V561D + T674I</td>
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<td>&gt;1000</td>
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<tr>
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<td>24 ± 12</td>
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Figure Legends

Figure 1. Sensitivity of single mutant PDGFRA isoforms to imatinib or crenolanib. Chinese hamster ovary cells were transfected with PDGFRA constructs and 24 hrs later were incubated for 90 minutes in the absence or presence of imatinib or crenolanib at the indicated concentrations. PDGFRA was immunoprecipitated from cell extracts and analyzed by immunoblotting using anti-PDGFR (for total PDGFRA) and anti-phosphotyrosine (for phospho-PDGFR) antibodies. Representative results from a minimum of three replicate experiments per mutant kinase are shown.

Figure 2. Sensitivity of compound mutant PDGFRA isoforms to imatinib or crenolanib. Chinese hamster ovary cells were transfected with PDGFRA constructs and analyzed exactly as described in Figure 1. Representative results from a minimum of three replicate experiments per mutant kinase are shown.

Figure 3A. Biochemical activity of imatinib or crenolanib to inhibit mutant kinases expressed in PDGFRA-dependent cell lines. Cell lines were incubated for 90 minutes in the absence or presence of imatinib or crenolanib at the indicated concentrations. PDGFRA was immunoprecipitated from cell extracts and analyzed by immunoblotting using anti-PDGFR (for total PDGFRA) and anti-phosphotyrosine (for phospho-PDGFR) antibodies as in figure 1. FIP1L1-PDGFR panel indicates experiments using EOL-1 cells. Representative results from a minimum of three replicate experiments per mutant kinase are shown.
Figure 3B. **Anti-proliferative activity of imatinib or crenolanib against mutant kinases expressed in PDGFRA-dependent cell lines.** Cell lines were incubated for 72 hours in the absence or presence of imatinib or crenolanib at the indicated concentrations. Proliferation/cell number was assessed using an XTT-based assay system as previously described. Error bars indicate the standard deviation for the depicted experimental conditions. Each TKI drug concentration was assayed in triplicate. Representative results from a minimum of three independent experiments per cell line are depicted.

Figure 4. **Crenolanib inhibits PDGFRA D842V in primary GIST cells by ex vivo assay.** Two independently established primary GIST cell lines were exposed to a crenolanib in different concentrations, or to vehicle alone (DMSO), and incubated for 2 hours at 37°C. Cell extracts were analyzed by western blotting using anti-PDGFRα (for total PDGFRA) and anti-phospho-PDGFRα(Tyr754) antibodies.
Figure 2

V561D + T6741

H313L + D842V

Inhibitor (nM) 0 1000 100 10 1

Crenolanib Imatinib

P-PDGFRA PDGFRA

Inhibitor (nM) 0 1000 100 10 1

Crenolanib Imatinib

P-PDGFRA PDGFRA
Figure 4

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<tr>
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<td>P-PDGFRα</td>
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<tr>
<td>PDGFRA</td>
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Crenolanib Inhibits the Drug-Resistant PDGFRA D842V Mutation Associated with Imatinib-resistant Gastrointestinal Stromal Tumors

Michael C Heinrich, Diana J Griffith, Arin McKinley, et al.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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