Effects of Crenolanib (CP-868596), a Highly Selective Platelet-derived Growth Factor Receptor (Pdgfr) Tyrosine Kinase Inhibitor, on the Proliferation of Interstitial Cell of Cajal (ICC) Precursors and Kit-mutant Gastrointestinal Stromal Tumor (GIST) Cells

Yujiro Hayashi,1,2 Michael R. Bardsley,1,2 Yoshitaka Toyomasu,1,2 Kyoung Moo Choi,2 Takahiro Taguchi,3 Abhijit Ramanchandran,4 Simon J Gibbons,2 Gianrico Farrugia,2 Tamas Ordog1,2

1Gastroenterology Research Unit, 2Enteric Neuroscience Program, Mayo Clinic, Rochester, MN, USA
3Division of Human Health Medical Science, Graduate School of Kuroshio Science, Kochi University, Kochi, Japan,
4AROG Pharmaceuticals, LLC, Dallas, TX, USA
Kit and Pdgfра are closely related type 3 receptor tyrosine kinases found in distinct interstitial cell types of the GI tunica muscularis:

- Kit is expressed in ICC and Pdgfра is expressed in ‘fibroblast-like cells’ (FLC)
  Sanders et al., Neurogastroenterol Motil 1999; 11: 311-38

- Most GISTs harbor oncogenic, mutually exclusive Kit or Pdgfра mutations, which are in most cases heterozygous
  Heinrich et al., Science 2003; 299: 708-10
  Negri et al., J Pathol 2009; 217: 103-112
However, both fetal and adult ICC stem cells (ICC-SC), as well as most GISTs co-express wild-type Kit and Pdgfra

Kurahashi et al., Neurogastroenterol Motil 2008; 20: 521-31
Bardsley et al., Gastroenterology 2010;139:942-52
Negri et al., J Pathol 2009; 217: 103-12

Signaling mediated by wild-type Kit/Pdgfra heterodimers contributes to GIST growth even after blocking oncogenic signaling by Kit/Pdgfra inhibitors such as imatinib

Negri et al., J Pathol 2009; 217: 103-112

Thus, Kit and Pdgfra signaling may not be as compartmentalized as previously suggested and Pdgfra inhibition may further inhibit Kit⁺ GIST growth
Aims

To investigate

- the co-expression of Kit, Pdgfra and Pdgfrb in cells of the ICC lineage and GIST;

and

- the effect of Pdgfr inhibition on cell proliferation in Kit$^+$ and Kit$^-$, Pdgfr-expressing cells
Detection of ICC, ICC-SC and FLC by Flow Cytometry

**ICC:** Kit⁺Cd44⁺Cd34⁻

**ICC-SC:** Kit⁻³⁄₄ Cd44⁺Cd34⁺ (e.g., Bardsley et al., Gastroenterology 2010)

**FLC:** Pdgfra⁺Kit⁻Cd44⁻Cd34⁻ (or Cd34⁻³⁄₄; Iino&Nojyo, Arch Histol Cytol 2009)
Validation of Flow Cytometry/FACS by qRT-PCR

Day 20 BALB/c corpus+antrum
Distribution of Pdgfra Immunoreactivity

Day 21 BALB/c corpus+antrum
Pdgfra is Expressed by Subsets of ICC & ICC-SC

Age: 8 days

Age: 6 weeks
Co-expression of Pdgfra and Kit

Colocalized: 4.6% of ICC
Summary 1

- **Kit and Pdgfra are co-expressed in a subset of murine ICC**

- The proportion of **Kit+Pdgfra+ ICC and ICC-SC** changes with age
Human GIST Cell Lines

- **GIST-T1**: Heterozygous *KIT* mutant, *KIT*+, imatinib-sensitive
- **GIST882**: Homozygous *KIT* mutant, *KIT*+, imatinib-sensitive
- **GIST48B**: Homozygous & heterozygous double *KIT* mutant, *KIT*−, imatinib-resistant
Both KIT$^+$ and KIT$^-$ GIST Cells Express PDGFR Isoforms

![Graph showing gene expression levels for different cell lines and protein bands for KIT, PDGFRA, and PDGFRB.]

- KIT: 145 kDa and 120 kDa
- PDGFRA: 190 kDa
- GAPDH: 36 kDa
Crenolanib besylate (CP-868,596)

- **Crenolanib** (AROG pharmaceuticals) is an orally bioavailable *Pdgfra/b* tyrosine kinase inhibitor
  
  *Lewis et al., J Clin Oncol 2009; 27: 5262-9*

- **Highly selective** for *Pdgfra/b* over other tyrosine kinases including Kit: >100x

- **Highly potent**: IC$_{50}$: *Pdgfra*: 1.7 nM; *Pdgfrb*: 0.67 nM
Crenolanib Effectively Inhibits the Proliferation of KIT+ But Not KIT− GIST Cells (MTS Assay)

GIST-T1: Min=0.06; IC$_{50}$=5.74 nM
GIST882: Min=0; IC$_{50}$=5.35 nM
GIST48B: Min=0.47; IC$_{50}$=77.4 nM
Mechanisms of Crenolanib’s Effect on GIST-T1 Cells

Crenolanib inhibits PDGFRA phosphorylation but not KIT phosphorylation.

Crenolanib inhibits PDGFRA & KIT expression.

- **PDGFRA**: 190 kDa
- **KIT**: 145 kDa, 120 kDa
- **GAPDH**: 36 kDa

**Graphs**

- **Phosphoprotein / Total protein (fold change vs. DMSO)**
  - PDGFRA: IC_{50}=5.1 nM
  - KIT: IC_{50}=infinity

- **Target gene / GAPDH (fold change vs. DMSO)**
  - PDGFRA:
    - 34: P=0.028
    - 145 kDa: P=0.008
  - KIT:
    - 145 kDa: P<0.001
    - 120 kDa: P=0.025
Crenolanib inhibited the proliferation of KIT$^+$ human GIST cells with an IC$_{50}$ comparable to that of imatinib.

Crenolanib’s anti-proliferative effect in these cells paralleled the inhibition of Pdgfr phosphorylation but appeared to require expression of KIT protein.
Cell Lines Derived From Murine ICC Lineage

- **ICL2A**: Conditionally immortalized ICC
- **D2211B, 2xSCS70**: Spontaneously transformed, Kit\(^{\text{low/−}}\) ICC-SC lines
- **2xSCS2F10**: Wild-type Kit\(^{\text{low/−}}\) ICC-SC line
ICC-Related Cells Lacking Full-Length Kit Express PDGFR Isoforms

Legend:
- Kit 145 kDa
- Pdgfra 190 kDa
- Pdgfrb 195 kDa
- Gapdh 36 kDa

Samples:
1: BALB/c corpus+antrum
2: ICL2A
3: D2211B
4: 2xSCS70
5: 2xSCS2F10
Crenolanib Poorly Inhibits the Proliferation of KIT-ICC-Related Cells

- **ICL2A**: Min = 0.05; IC$_{50}$ = 667 nM
- **2xSCS2F10**: Min = 0; IC$_{50}$ = 1050 nM
- **D2211B**: Min = 0.04; IC$_{50}$ = 992 nM
- **2xSCS70**: Min = 0; IC$_{50}$ = 2751 nM

Formazan absorbance (fold change vs. Veh)

Crenolanib besylate (nM); 3 d
Expression of Wild-type but not Constitutively Active Kit in Pdgfr\(^{+}\) ICC-SC Increases Their Sensitivity to Crenolanib

2xSCS2F10 cells (1) were retrovirally transduced with full-length wild-type murine Kit (2xSCS2F10-mKit; 2) or murine Kit bearing the activating mutation K641E (2xSCS2F10-mKit\(^{K641E}\); 3)

![Western Blot Image]

- **Kit**: 145/120 kDa
- **truncated**: 36 kDa
- **Gapdh**: 36 kDa
- **GFP**: 27 kDa

![Graph Image]

- 2xSCS2F10; Min=0.07; IC\(_{50}\)=1157 nM
- 2xSCS2F10-mKit; Min=0; IC\(_{50}\)=121 nM
- 2xSCS2F10-mKit\(^{K641E}\); Min=0.04; IC\(_{50}\)=710 nM

Crenolanib besylate (nM); 3 d
Crenolanib did not effectively inhibit the proliferation of Kit\(^{-}\) cells expressing wild-type Pdgfра and/or Pdgfrb.

Retroviral transduction with wild-type murine Kit increased the cells’ sensitivity to crenolanib.
Conclusion

- Inhibition of wild-type PDGFRA expressed in some KIT mutant, KIT-addicted GIST can potently inhibit cell proliferation.

- The inhibitory effect of crenolanib in cells expressing wild-type PDGFR isoforms appears to require the presence of KIT protein.

- Co-expression of Pdgfr with wild-type Kit may enhance the effects of crenolanib.
Acknowledgements

The authors thank

Dr. Gregory J. Gores (Mayo Clinic) for the use of the LI-COR Odyssey scanner and
Dr. Brian Rubin (Cleveland Clinic) for the retroviral Kit vectors

Supported in part by

NIH/NIDDK R01 DK58185