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A novel cell-based assay to identify the FLT3 kinase construct used in enzyme assays that best represents the activity of cellular FLT3

Lunda Shen, Ada Chen, Hema Singh, Inna Kurilyak, Patricia Fabila, Joseph Kulusich, Julia Ong, Cesar A. Meleza, Anne Van Abbema, Juan José Fung, Xiaoning Zhao, David W. Green

Arcus Biosciences, Inc., 3928 Point Eden Way, Hayward, CA 94545, USA

Abstract

FMS-like tyrosine kinase 3 (FLT3) is a type III receptor tyrosine kinase (RTK) that plays a key role in hematopoiesis. Mutations of this protein are associated with hematologic malignancies[1]. Our focus was to develop a FLT3 enzymatic selectivity assay for another RTK program. FLT3 consists of six domains: the extracellular domain (ED), transmembrane domain (TD), juxtamembrane domain (JMD), tyrosine kinase domain, kinase-insert domain, and C-terminal intracellular domain[2]. Here, we describe the characterization of FLT3 enzyme assays using two FLT3 proteins, one with a partial JMD and one with full-length JMD. RTK inhibitors yielded significantly different IC_{50} values between the two FLT3 constructs, suggesting a role of the JMD in FLT3 inhibitor activity. To further explore the role of the JMD, we tested known type I and II kinase inhibitors. Type II inhibitors (which bind to the inactive kinase) demonstrated large potency differences between the two FLT3 constructs, being more potent towards the FLT3 protein with full-length JMD, while type I inhibitors (which bind to the inactive and active kinase) did not show significant potency differences. To identify the FLT3 kinase protein that better represents the cellular activity of the entire FLT3 protein, we developed a novel cell-based assay that measures RTK phosphorylation using a pLISA luminescence detection method. This assay demonstrated that results from the FLT3 protein containing the full-length JMD correlated better with those from full length cellularly expressed FLT3, and is therefore a better enzyme assay predictor of *in vitro* cellular rank order potency.

Domains Of FLT3

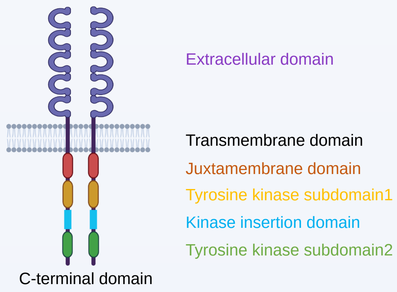

Fig. 1. Topological domains of FLT3

FLT3 consists of six domains: an extracellular domain containing 5 immunoglobulin-like folds, a transmembrane domain, a juxtamembrane domain where internal tandem duplications occur, a tyrosine kinase domain interrupted by a kinase insert domain, and a C-terminal domain.

Biochemical Potency Differences Between Different FLT3 Proteins

Table 1. Conditions of the FLT3 biochemical assays

	Enzyme	ATP Km	Amino Acid Range	Reaction Time	Substrate	Assay
FLT3-Partial-JMD	2.5 nM	17 µM	571-993	2hr	TK	HTRF
FLT3-FL-JMD	0.2nM	17 µM	564-958	2hr	TK	HTRF

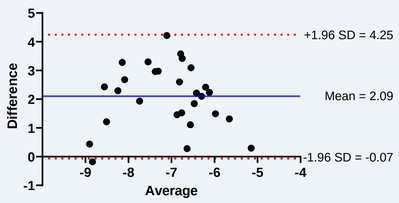

Fig. 2. Bland-Altman plot of the two FLT3 biochemical assays

In the Bland-Altman analysis, Difference = [FLT3-Partial-JMD] – [FLT3-FL-JMD] for each compound, with IC_{50} log10 transformed. Most compounds showed significantly larger IC_{50} values with the FLT3-Partial-JMD.

Table 2. Type I and II inhibitor potency is FLT3 construct dependent

		FLT3 Biochemical IC_{50} (nM)	
Known Inhibitor Type	Molecule Name	FLT3-FL-JMD	FLT3-Partial-JMD
Type I	Midostaurin/PKC 412	0.88	0.42
Type I	Avapritinib/BLU-285	167	325
Type I	Crenolanib	1.93	2.24
Type I or II	Sunitinib	0.86	2.15
Type II	Imatinib	> 10,000	> 10,000
Type II	Sorafenib	2.03	178
Type II	Ripretinib	2.87	> 10,000
Type II	Regrorafenib	3.23	1570
Type II	Quizartinib (AC220)	1.61	12.5

Type II inhibitors demonstrated large potency differences between the two FLT3 constructs, with higher potency values obtained in the FLT3 protein with full-length JMD, while type I inhibitors did not show significant potency differences.

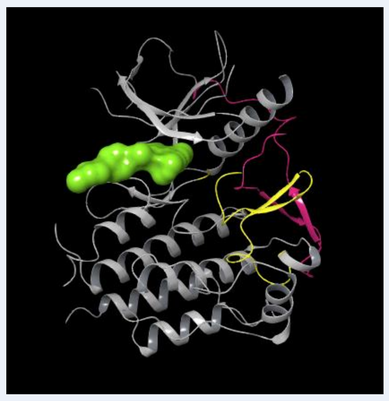

Fig. 3. Crystal structure of FLT3 (PDB 6JQR)

FLT3 is shown in gray, with the juxtamembrane domain in pink and the activation loop in yellow. Green is an example type I / II inhibitor. Type I inhibitors bind to the ATP pocket. Type II inhibitors bind to the similar region but interact with an additional hydrophobic pocket next to the ATP site associated with the DFG-out conformation, in which the Asp and Phe of the Asp-Phe-Gly (DFG) motif on the activation loop swap from the DFG-in conformation.

Phospho-LISA Assay

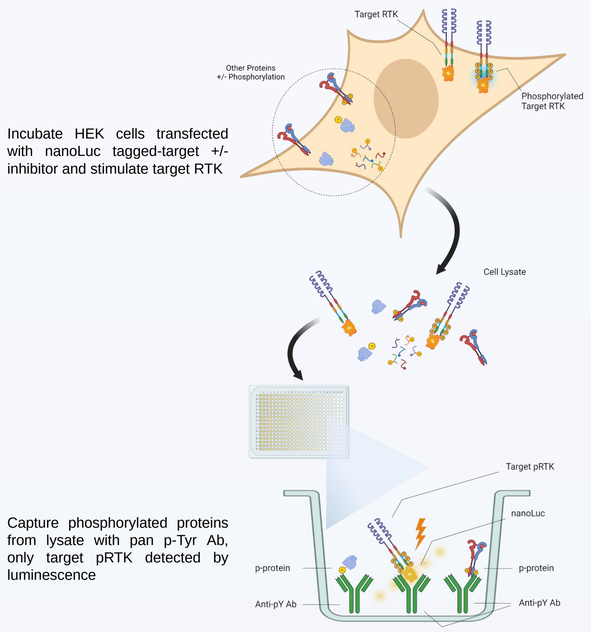

Fig. 4. pLISA RTK phosphorylation assay

The RTK of interest is expressed in HEK cells as a nanoLuc (Promega) hybrid protein and cells are treated +/- inhibitor. Overexpression can activate (i.e., receptor dimerization and autophosphorylation) the RTK or activation can be done by adding the RTK ligand. The cells are lysed and tyrosine-phosphorylated proteins are captured with an anti-phosphotyrosine antibody. Although many phosphorylated proteins will be captured, only the target RTK with the nanoLuc tag will be detected.

FLT3-FL-JMD Biochemical Potency Correlates With Phospho-LISA

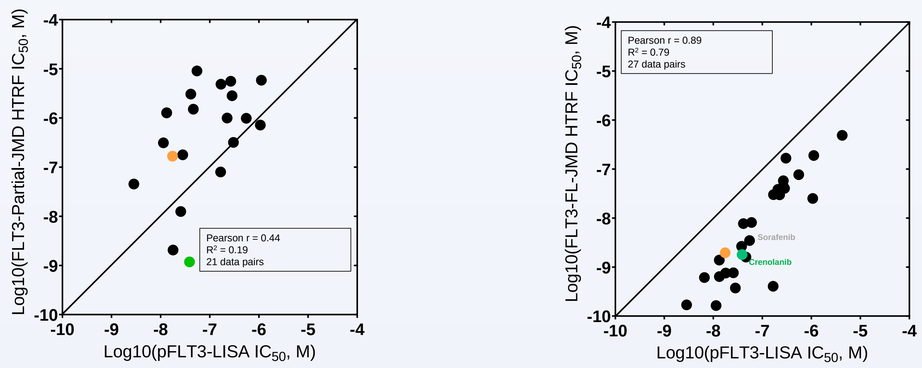

Figure 5. Correlation between FLT3 construct biochemical potency and pLISA

FLT3-Partial-JMD (left) did not have as good a correlation with full-length FLT3 in pLISA as construct FLT3-FL-JMD (right), suggesting that FLT3-FL-JMD is a better predictor of cellular rank order potency. Crenolanib, a known FLT3 type I inhibitor, is shown in green. The IC_{50} of Crenolanib was 2.24 ± 0.95 nM (N=4) with FLT3-Partial-JMD, 1.93 ± 0.97 nM (N=2) with FLT3-FL-JMD, and 41.3 ± 20.3 nM (N=2) in the pLISA assay. Sorafenib, a known FLT3 type II inhibitor, is shown in orange. The IC_{50} of Sorafenib was 178 ± 52 nM (N=4) with FLT3-Partial-JMD, 2.03 ± 0.75 (N=2) with FLT3-FL-JMD, and 18.5 ± 8.77 (N=2) nM in the pLISA assay.

Summary

- Arcus compounds have significant biochemical IC_{50} potency differences between FLT3-FL-JMD and FLT3-Partial-JMD.
- Type I inhibitors tested did not show potency differences between the two FLT3 enzymes; type II inhibitors tested showed a significant potency difference, being more potent towards the FLT3 protein with full-length JMD.
- A novel cell-based assay that measures RTK phosphorylation using a pLISA luminescence detection method was developed for detection of phospho-FLT3 in cells.
- FLT3 pLISA derived IC_{50}s correlate better with those from FLT3-FL-JMD biochemical assay.

References

1) Gilliland D.G., et al. Blood. 2002;100(5):1532-1542.
2) Haage, T.R., et al. Cancers 2023, 15, 2991.
3) Figures 1 and 4 created with BioRender.com (2024).