enzymlogic

Exploring activation state-dependent kinetics to discover novel drugs

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A substantial challenge encountered in the development of ATP-competitive kinase inhibitors is obtaining sufficient specificity for the target kinase, a consequence of the high degree of conservation in the active site among the >500 members of the kinase superfamily.

Crystallographic studies of active and inactive kinases have revealed a remarkable plasticity in the kinase domain that allows the adoption of distinct conformations in response to interactions with specific regulatory domains, proteins and inhibitors. This implies that tuning the drug interaction across multiple activation states opens new inhibition possibilities with improved efficacy, selectivity or higher barrier to resistance development[1].

Avoid missing promising compounds using KINETICfinder®

Features and benefits

- Accurate.
- Robust.
- Reproducible.
- Sensitive.
- Broad dynamic range.
- Activated and non-activated targets.
- Rapid turnaround.

Applications

- Modify the on- and off-target kinetics (k_{on}, k_{off}, residence time and K_d).
- Design drugs with improved efficacy, selectivity or sensitivity to mutations.
- Identify novel chemotypes and binding modes.
- Use binding kinetics to interpret structural data.

How KINETICfinder® assays work

KINETICfinder® is a highly tuned TR-FRET Kinetic Assay. Real-time binding of an active-site directed fluorescent probe is detected using a labeled anti-tag antibody, which binds to the target of interest. The binding of the probe and antibody to the target increases the TR-FET signal, whereas the displacement of the probe with a compound decreases the TR-FRET signal.

Assay process

1. **Performance:**
    - 384 microplates containing the target of interest, a fluorescent probe and labelled antibody.
    - 4-point 10-fold serial dilutions of test compounds.
    - A reference compound.
    - Up to 32 total binding and non-specific binding controls.
2. **Detection:** Reaction is monitored over time at room temperature.
3. **Analysis:** Specific TR-FRET signals are fitted to the Motulsky-Mahan equation. Association (k_{on}) and dissociation (k_{off}) rate constants of test and reference compounds are determined and the K_d and residence time (τ) values are calculated:

$$K_d = \frac{k_{off}}{k_{on}} \qquad \tau = \frac{1}{k_{off}}$$

4. **Quality control:** S/B, Z-value and MSR (within and between-run variability) for each assay.

Approved FLT3 drugs are kinetic and conformation selective

The FLT3 receptor tyrosine kinase consists of an extracellular domain, a transmembrane region and a cytoplasmic juxtamembrane (JM) region and kinase domain.

- JM domain acts as an autoinhibitory loop that interacts with the catalytic domain (JM-in), stabilizing FLT3 in an inactive, autoinhibited state in which the activation loop is closed (DFG-out).
- JM domain phosphorylation leads to the non-autoinhibited state (JM-out) where the DFG motif is unlocked and able to switch from the inactive (DFG-out) to the open activated state (DFG-in)[2].

Internal tandem duplication (ITD) mutations within the JM domain contribute to the majority of FLT3 activating mutations in acute myelogenous leukemia (AML).

- These mutations disrupt the JM autoinhibitory activity, resulting in constitutive FLT3 activation.
- Type I (sunitinib, midostaurin, gilteritinib) and type II inhibitors (foretinib) can target ITD mutations.
- Midostaurin and gilteritinib are the only FDA approved inhibitors for the treatment of AML[3].

Our data establish that the activation states of FLT3 can have a large impact on inhibitor affinity and binding kinetics, conferring to FDA-approved inhibitors optimal clinical efficacy and safety profile.

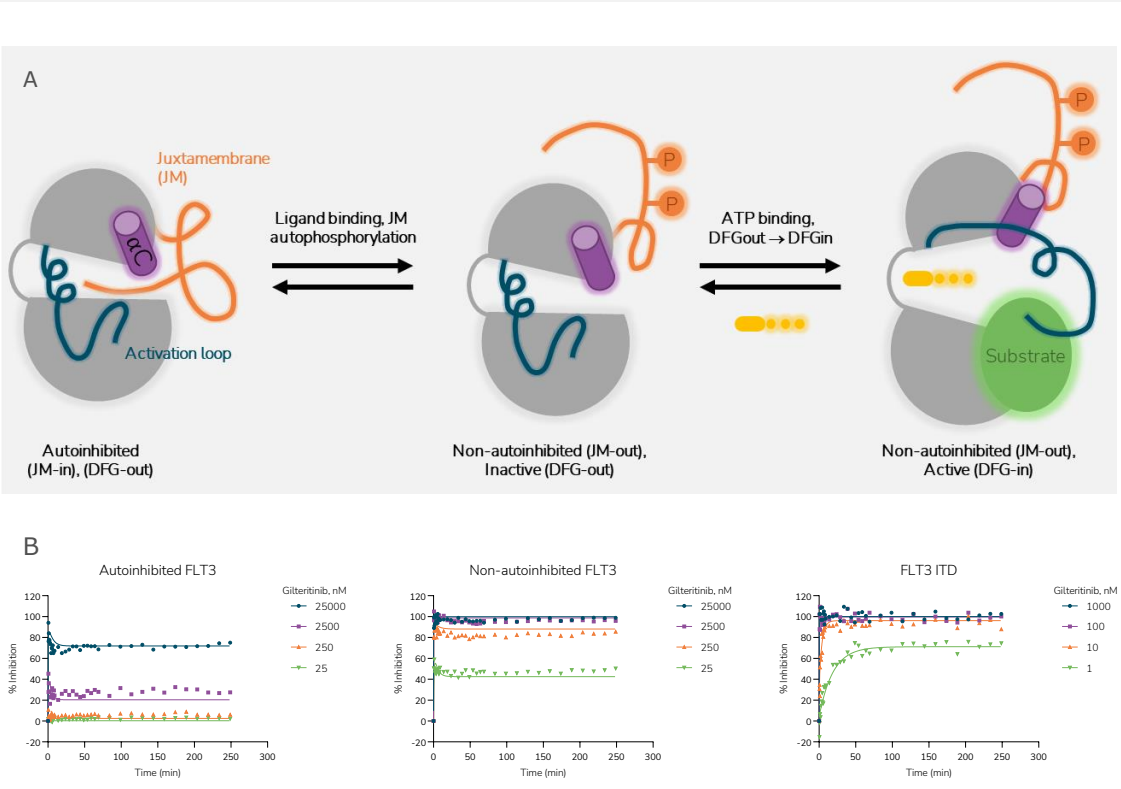

Figure 1. A) Model of FLT3 kinase activation and B) kinetic curves of gilteritinib for the autoinhibited, non-autoinhibited and constitutive active states of FLT3 obtained with KINETICfinder® showing different binding kinetics depending on the conformation of FLT3.

Clinically effective drugs display long-lasting inhibition of FLT3-ITD

All inhibitors bind to both FLT3-ITD and non-autoinhibited FLT3 forms (Table 1).

- Midostaurin and sunitinib have similar kinetic profiles and affinities for the activating mutation and non-autoinhibited state.
- Gilteritinib is 60 times more potent with FLT3-ITD due to a substantial increase in the residence time (30 times).
- Type II foretinib has 4-fold lower affinity for the activating mutation caused by a shorter residence time. Our results confirm the preferential binding of foretinib to the inactive (DFG-out) non-autoinhibited state.
- Midostaurin and gilteritinib are long-lasting inhibitors (>60 min) of FLT3-ITD.

	FLT3 ITD			Non-autoinhibited FLT3		
	On-rate ($M^{-1}s^{-1}$)	Residence time (min)	K_d (nM)	On-rate ($M^{-1}s^{-1}$)	Residence time (min)	K_d (nM)
Sunitinib	$1.2x10^7$	4	0.3	$7.9x10^6$	7	0.3
Midostaurin	$1.0x10^4$	61	27	$1.9x10^4$	42	21
Gilteritinib	$3.5x10^6$	62	0.1	$1.7x10^6$	2	4.7
Foretinib	$4.3x10^4$	33	12	$5.4x10^4$	111	2.8

Table 1. Association rate, residence time and affinity values of sunitinib, midostaurin, gilteritinib and foretinib for the activating mutation and non-autoinhibited state.

JM domain substantially impacts binding kinetics and affinity of gilteritinib and midostaurin

Binding affinity is consistently reduced by the insertion of the JM domain into the active site of FLT3 because of a significant reduction in the association rate (Fig. 2).

- Sunitinib binds to all the activation sates with high affinity. However, the JM domain slightly interferes with sunitinib binding, suggesting steric hindrances.
- Our results reveal that rearrangement of the JM domain in the autoinhibited conformation is required for midostaurin and gilteritinib binding. In fact, these inhibitors have 100- and 20,000-fold greater affinity for the activating mutation.
- Midostaurin and gilteritinib present exquisite selectivity for the fully active state (JM-out, DFG-in) relative to the autoinhibited state, minimizing undesired effects on normal cells.

These data exhibit that the prolonged interaction of midostaurin and gilteritinib with the FLT3-ITD conformation along with the high selectivity relative to the autoinhibited FLT3 conformation, contribute to their clinical success in the treatment of AML.

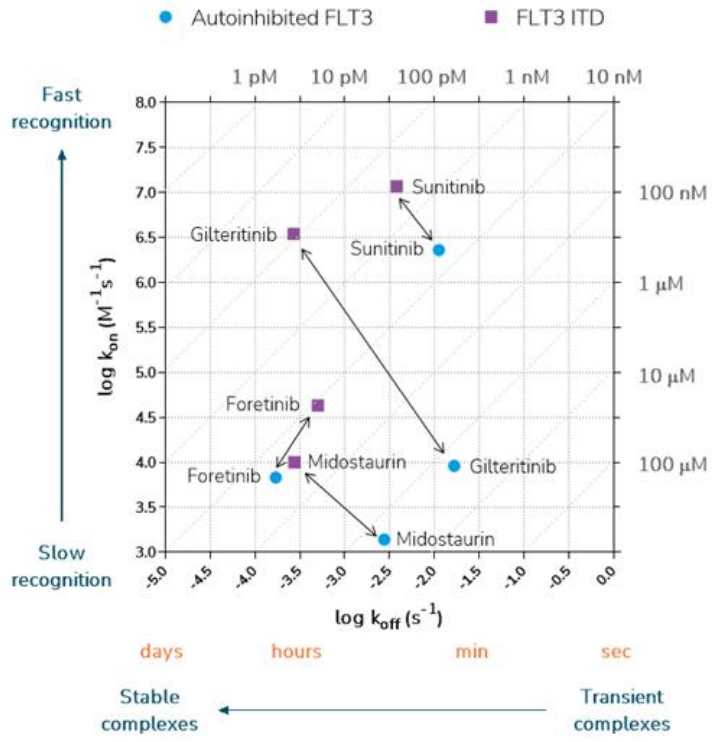

Figure 2. Comparison of the association (k_{on}) and dissociation (k_{off}) rates of sunitinib, foretinib, midostaurin and gilteritinib on activated and autoinhibited FLT3 obtained with KINETICfinder®. The dashed lines refer to the K_d values.

References

1. Copeland R.A. (2016) The drug-target residence time model: a 10-year retrospective. Nat Rev Drug Discov.15(2):87-95.
2. Griffith J. et al. (2004) The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell 30;13(2):169-78.
3. Stanchina M. et al (2020) Advances in Acute Myeloid Leukemia: Recently Approved Therapies and Drugs in Development. Cancers (Basel) 12(11):3225.