Binding kinetics: high throughput assay for kinase inhibitors

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- **Microplate-based TR-FRET assay determines target binding kinetic rates k_{on} and k_{off} for kinase inhibitors**
- **Speed of the PHERAstar® offers high temporal resolution and high throughput kinetic characterization**
- **Screening of interaction between multiple inhibitors and kinases relates k_{off} and clinical success**

Introduction

Drug development is a lengthy and costly process with high attrition rates. More than half of the drug candidates going into phase 2 clinical trials fail mainly due to lack of effectiveness[1].
A feature associated to the efficacy and safety of drug candidates is how fast they bind (k_{on}), and dissociate (k_{off}) from their targets, and accordingly for how long they can occupy them (residence time). Ideally these kinetic parameters are determined in the preclinical stages, creating a need for high-throughput methods to profile hundreds and thousands of compounds. Moreover, comprehensive retrospective profiling of existing molecules from different clinical development stages can provide insights into the impact of target binding kinetics on the pharmacological profiles of drugs.
Here, we present the determination of k_{on} and k_{off} values in a PHERAstar *FS* microtiter plate reader using time-resolved FRET (TR-FRET) for a panel of 40 kinase drug targets in cancer therapies against 270 inhibitors.

Assay Principle

The kinetic probe competition assay (kPCA) detects the time resolved energy transfer (TR-FRET) from a lanthanide-based donor fluorophore linked to the target molecule via affinity reagents, to an acceptor dye conjugated tracer known to bind to the target of interest (and consequently found within Foerster distance).
In order to determine the kinetics of binding, target and fluorescent tracer pairs are first characterized by monitoring the TR-FRET signals during binding and dissociation of both interaction partners.
Once association and dissociation rates of the tracer are known, the same parameters can be determined for unlabelled compounds in a competitive setup where tracer and competitor molecules are simultaneously exposed to the target. In this context, binding of the competitor molecule alters the signal curve of interaction between tracer and target in a dose-dependent fashion.
Kinetics and affinity parameters of tracer and competitors can be derived by fitting the resulting signal to appropriate mathematical models[3] (Fig. 1).

Materials & Methods

- Kinase Inhibitors (SelleckChem)
- Alexa 647 labeled kinase tracers (ThermoScientific)
- Biotinylated kinases (Carna Biosciences)
- Streptavidin-Terbium (Cisbio)
- black 384 well small volume microplate (Greiner)
- PHERAstar® *FS* (BMG LABTECH)

Experimental procedure
For a detailed description please refer to Georgi *et al.* (2018)[4]. Briefly, assay plates were prepared to contain 5 µl of the fluorescent tracer with different concentrations of the competitive molecule. The reaction was started and immediately monitored by adding 5 µl of terbium-labelled kinase to the wells with a PHERAstar *FS* (with a sample injection unit).

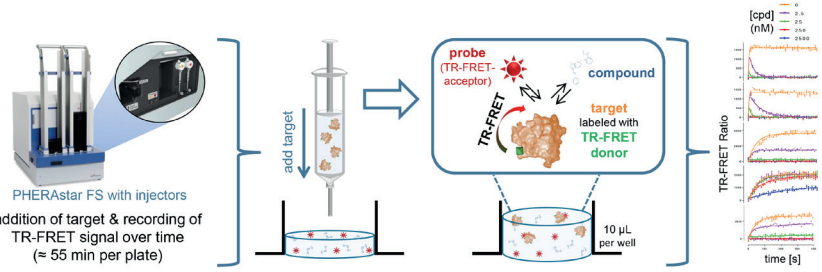

Fig. 1: **Principle of the kinetic probe competition assay.** Competitive compound and fluorescent tracer are placed in the microplate and the reaction is started by adding the Tb-coupled kinase (left). TR-FRET signal is recorded immediately after target addition and depends on the presence of competitive kinase inhibitors (middle). The deviation of the tracer-only curve to the tracer + competitor curve provides information about the binding kinetics of the unlabeled competitor molecule (right).

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Instrument settings

Optic settings	TR-FRET, plate mode kinetic read in octants, Top optic	
	Filters	HTRF optic module
General settings	Excitation source	Laser
	Number of flashes	5
	Integration start	100 µs
	Integration time	400 µs
	Settling time	0 s
Kinetic settings	Number of cycles	41
	Cycle time	10 s

Results & Discussion

In a first step the association and dissociation kinetics of a fluorescent tracer binding to a kinase (e.g. ABL was analysed. TR-FRET signals increase with times, indicating increased tracer-kinase binding, whereas the signal decreases when being displaced by an excess of unlabelled competitor (Fig. 2).

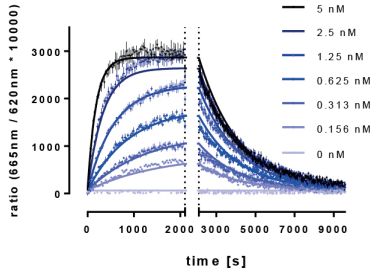

Fig. 2: Tracer Characterization. Association was monitored by TR-FRET after combining Tb-labelled kinase and a fluorescent kinase tracer. Dissociation was recorded after addition of excess staurosporine to the tracer sample.

Next, an unlabeled compound was analyzed using the kinetic probe competition assay. The reaction contained fluorescent tracer 178 and the indicated concentrations of staurosporine, an unlabelled ABL-binder. The reaction was started by adding Tb-labelled ABL resulting in a signal increase for the trace without competitor (Fig. 3, grey). In contrast, the signal in presence of the competitor is lower the higher its concentration, and the initial signal increase is followed by a decrease in signal over time. The latter indicates that due to slower binding kinetics the competitor equilibrates more slowly than the tracer.

The method was then used to determine on- an off-rates of 270 kinase inhibitors at different stages of pharmaceutical development. Their binding kinetic parameters for the interaction with different kinase on- and off-targets (in total 3230 interactions were quantified) is shown in Fig. 4. The further the compound is in clinical development, the greater the frequency of slow dissociating interactions

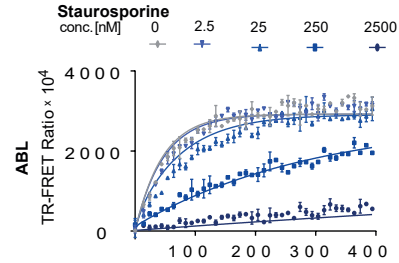

Fig. 3: TR-FRET ratio of competitive binding of Tracer 178 and unlabeled staurosporine to ABL. Motulsky-Mahan[3] analysis allows calculation of k_{on} and k_{off} of the unlabeled compound.

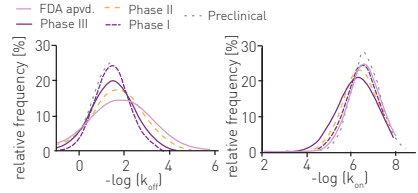

Fig. 4: Comprehensive analysis of 3230 kinase (40) vs. inhibitor (270) interactions (on and off targets) depending on their pharmaceutical development. Kinetic parameters were determined using the TR-FRET based kinetic probe competition assay. Adapted from Georgi *et al.* (2018)[4].

(indicated by high negative decadic off-rate logarithm). On the other hand, the graph illustrates that the association rate of preclinical compounds is barely different from that of approved drugs. All in all, these data suggest that the longer a kinase is occupied by a compound, the greater the likelihood for it to become a clinically effective drug.

Conclusion

The TR-FRET-based kinetic probe competition assay determines binding kinetics of unlabeled compounds in high throughput. The method allows linking binding parameters to clinical success, and the selection of new drug candidates for development according to their binding kinetic properties.

References

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