Practical Evaluation of Time-Dependent Inhibition Kinetics Through Small-Volume Size-Exclusion Chromatography

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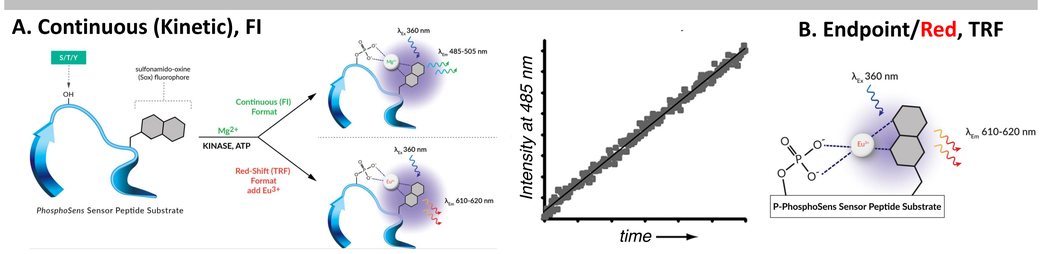
ABSTRACT

Accurate identification and characterization of the mechanism of time-dependent inhibitors (TDIs) is essential for reporting key kinetic parameters, which can significantly improve the efficiency of early-stage drug discovery campaigns. A practical, quantitative method that is amendable to integration into upstream lead characterization pipelines can provide this critical context, as classical potency determinations alone may be misleading if a time-dependent element of inhibitor binding is unaccounted for. Here we have developed a new method for the determination of compound binding reversibility and the quantitative assessment of reversible inhibitors for guiding optimization of the structure-kinetic relationship of candidate TDI compounds. Currently, a well-established strategy for determining the mechanism of inhibitor reversibility in a high-throughput, label-free manner involves rapid dilution of a saturated kinase-inhibitor complex to drive compound dissociation. The complex is created by incubating the kinase with a concentration of inhibitor well above an estimated IC_{50} value. From here, a continuous, kinetic assay format can be leveraged to measure activity recovery of the enzyme over time, through which the relative reversibility of the inhibitor can be visualized. In the case of reversible inhibitors, one can also quantify the residence time τ from this progress curve, further improving characterization. Despite the utility of the assay, however, this rapid dilution strategy – known as a "jump dilution" – faces significant challenges when deployed to characterize highly potent inhibitors. The high concentrations of enzyme in the preincubation step make it difficult to avoid the tight-binding limit of the assay. Further, the elevated inhibitor concentration in the assay – particularly if adjusted to account for the tight-binding limit – introduces the caveat of compound re-binding during the recovery of activity, necessitating further steps in the protocol. To eliminate for these complications, we demonstrate a novel application of small-volume size-exclusion chromatography (SEC) via desalting columns as a means to facilitate the removal of free compound from the kinase-inhibitor complex, driving dissociation in a manner similar to that of a jump dilution without requiring an elevation of kinase concentration. Following this SEC-facilitated compound clearance, we are able to detect and measure the recovery of active kinase using a continuous assay format. This allows us to directly observe reversibility and quantify off-rates through activity recovery in the progress curve. We demonstrate these quantitative applications through the side-by-side comparisons of well-known EGFR and ABL1 kinase inhibitors with various time-dependent characteristics. Through a combination of SEC-facilitated compound clearance and continuous kinetic monitoring, we are able to capture reversibility behavior and – for reversible inhibitors – determine residence times in agreement with the general literature.

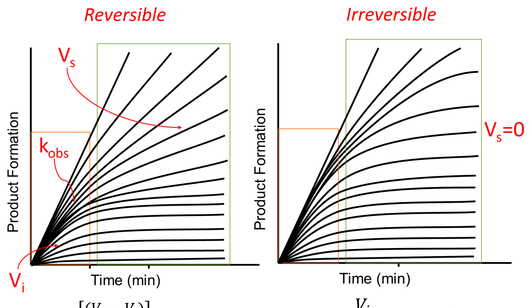
FIGURE 1. THE CORE OF THE PHOSPHOSENS® CONTINUOUS ASSAY FORMAT



Peptide sequences are synthesized using solid-phase methods with the Sox fluorophore coupled through the sulfhydryl group of a cysteine residue proximal to a protein kinase phosphorylation site, such as a tyrosine, serine, or threonine. Upon addition of a kinase, the peptide is phosphorylated. In the presence of magnesium ion, a chelation complex is formed with the phosphate group, resulting in fluorescence enhancement of the Sox fluorophore that can be **monitored continuously** as fluorescence intensity **(A)**. Kinase inhibitors prevent phosphorylation and thus fluorescence. At any point, Europium ion can be added, to displace the magnesium ion, resulting in a long wavelength, time-resolved fluorescence (TRF) **endpoint/Red format (B)** that is useful for high-throughput or structure activity relationship (SAR) applications.

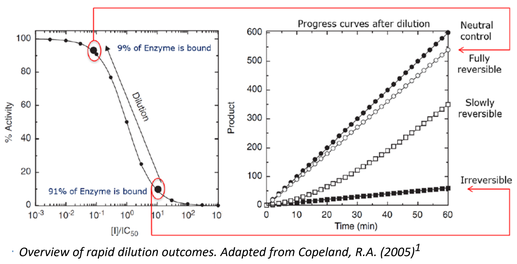
FIGURE 2. IMPLICATIONS OF EARLY-STAGE TDI CHARACTERIZATION

- To capture key time-dependent inhibition (TDI) kinetics of early-stage lead candidates, **binding reversibility must be explicitly determined** to enable determination of appropriate parameters (k_{off} for reversible inhibitors; k_{inact}/K_I for irreversible) and aid in the design of the follow-on experiments.
- Continuous activity assays enable such characterization. However, determining reversibility with forward progress curves alone can be difficult for even moderate k_{obs} values. One can instead directly observe reversibility through monitoring dissociation kinetics.
- To do so, one must **assume that k_{obs} approximates k_{off}** (by **saturating the kinase** with excess inhibitor, and yet **minimizing inhibitor rebinding** during dissociation)[1]



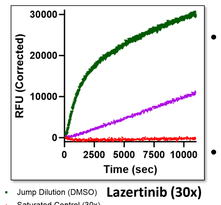
$$P = V_s t + \left[\frac{(V_i - V_s)}{k_{obs}}\right](1 - e^{-k_{obs}t}) \qquad P = \frac{V_i}{k_{obs}}(1 - e^{-k_{obs}t})$$

FIGURE 3. QUANTITATIVE CONSIDERATIONS OF RAPID DILUTION STRATEGIES



Overview of rapid dilution outcomes. Adapted from Copeland, R.A. (2005)[1]

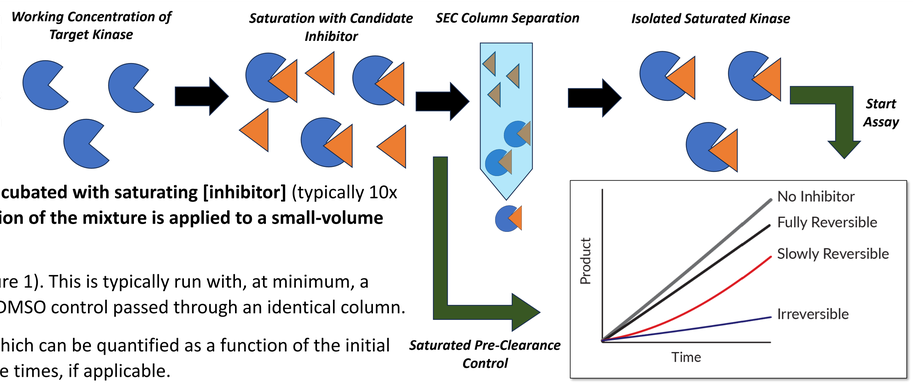
A common method by which to visualize reversibility in an enzymological fashion involves rapid dilution of a saturated enzyme/inhibitor complex. This technique, known as a "jump dilution," involves **incubating the kinase target with excess inhibitor** (typically 10-fold above an IC_{50} value determined under identical conditions), **then rapidly diluting via low-volume transfer** into a final assay volume[1]. For compounds with compatible potencies, this method has worked well to drive enzymological assessments of compound reversibility. However, several critical caveats exist that can complicate assessments of potent inhibitor candidates:



- The pre-incubation step of the **jump dilution necessitates a kinase concentration 100-fold above working concentration**, **increasing the nominal tight-binding limit**. Incomplete saturation can complicate quantitation of residence times; at worst, this can make irreversible inhibition appear to be fast-off reversible without the appropriate controls to provide context (bottom figure).
- For potent compounds, **increasing [inhibitor]** to accommodate incomplete saturation can lead to [inhibitor] that is too high following the jump dilution; **this can lead to non-trivial compound rebinding**, which in turn compromises the approximation of k_{off} through k_{obs}, further obscuring residence times.

FIGURE 4. LEVERAGING SEC SEPARATION FOR HIGH-THROUGHPUT, ACTIVITY-DRIVEN REVERSIBILITY ASSESSMENTS

Small-volume size-exclusion chromatography (SEC) is a proven means to separate free unbound small molecule from saturated protein targets, taking advantage of significant mass differential.[2] While primarily paired with various biophysical assays to assess dissociation, this separation technique can also be exploited in an enzymological context while retaining throughput:

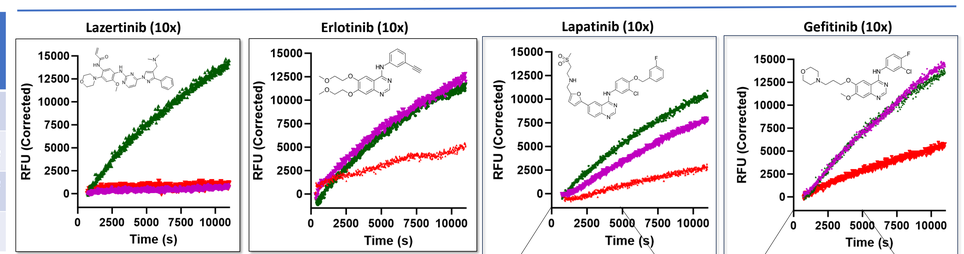


- A concentration of **kinase near working assay concentration is incubated with saturating [inhibitor]** (typically 10x IC_{50} at conditions mirroring the final assay). Following this, a **portion of the mixture is applied to a small-volume SEC column** (such as a desalting spin column).
- The resulting **eluate is then assayed in a continuous format** (Figure 1). This is typically run with, at minimum, a saturated control taken from the incubated mixture, as well as a DMSO control passed through an identical column.
- The resulting **progress curve is evaluated for activity recovery,** which can be quantified as a function of the initial rate V_o and final steady-state rate V_s (Figure 2) to extract residence times, if applicable.

FIGURE 5. SPIN COLUMN SEPARATION ENABLES DETERMINATION OF EGFR INHIBITOR BINDING MODALITIES

Inhibitor (xIC50 @ 60 min. preincubation, 1mM ATP)	Trade Name	Company	Generation	Reversibility (residence time)
Lazertinib (10x)	Leclaza	Yuhan/ Janssen	Third	Irreversible
Erlotinib (10x)	Tarceva	OSI/ Genentech	First	Rapidly reversible
Gefitinib (10x)	Iressa	AZ	First	Rapidly reversible (< 5 min.)
Lapatinib (10x)	Tykerb	GSK	First	Reversible (45-60 min.)

EGFR INHIBITORS @ 1mM ATP, 30°C (0.3 nM EGFR WT)



Scalable separation techniques can **enable at-a-glance assessments** of compound reversibility:

- Maintaining [EGFR] close to working assay concentration **ensures better saturating conditions** where free [inhibitor] and total [inhibitor] remain approximately equal in cases of high potency.
- **Avoidance of high-concentration** EGFR in small-volume incubation conditions **may also limit [enzyme]-dependent behaviors such as oligomerization** that may emerge and complicate compound binding and activity readouts.
- Even at an **early qualitative screening stage**, progress curves may still be analyzed for residence times (contrast Gefitinib and Lapatinib recovery; 3rd and 4th from left) that translate into measurable cellular outcomes.[3]

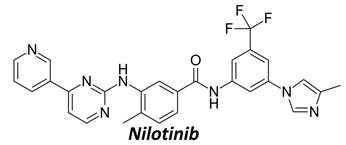
DMSO (column)
10x (no column)
10x (column)

FIGURE 6. QUANTITATIVE ASSESSMENT OF ABL INHIBITOR DRUG-TARGET RESIDENCE TIMES

High-potency reversible inhibitors can be more rigorously evaluated through more flexible modulation of incubation and assay conditions enabled by an SEC-driven separation step.

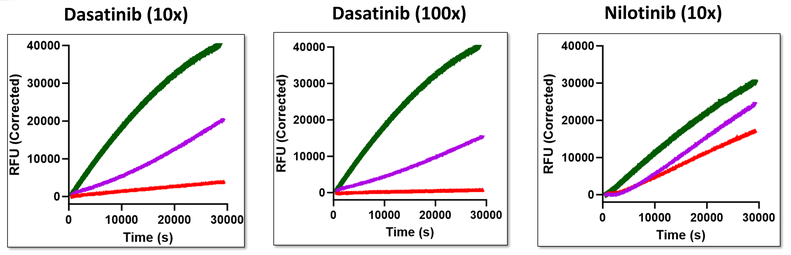
- Dasatinib and Nilotinib constitute second-line ABL inhibitors with **distinct off-rates that translate into differential duration** and dosing requirements.[4]
- Separation in a small-volume spin column permits **adjustment of [inhibitor] to ensure saturation, with little impact on residence time**
  - Such increases in [inhibitor] can be used to mitigate ligand displacement in the saturated control – most notably displacement of ATP-competitive inhibitors – while leaving post-column [inhibitor] unaffected (contrast the 10x plots of Dasatinib/Nilotinib with that of 100x Dasatinib)
- **Longer residence times** can be extracted **through modulation of concentration of stable kinase**, following confirmation of minimal kinase retention.

Dasatinib



Nilotinib

BCR-ABL INHIBITORS @ 1mM ATP, 30°C (0.25 nM BCR-ABL)

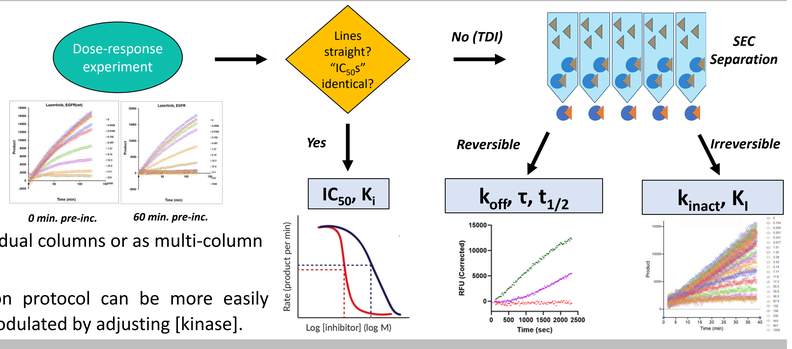


DMSO (column)
10/100x (no column)
10/100x (column)

Inhibitor (xIC50 @ 60 min. preincubation, 1mM ATP)	Trade Name	Company	Generation	Residence Time (min.)
Dasatinib (10x)	Sprycel	BMS	Second	130 min.
Dasatinib (100x)	Sprycel	BMS	Second	150 min.
Nilotinib (10x)	Tasigna	Novartis	Second	50 min.

CONCLUSIONS: SEPARATION TECHNIQUES IN A COMPREHENSIVE, HIGH-THROUGHPUT TDI WORKFLOW

Incorporation of separation techniques to power activity-driven assessments of time-dependent inhibition can enable early detection of desirable binding kinetics among early lead candidates. This can be leveraged into **an informed, efficient downstream discovery and optimization program** while ensuring that **correct kinetic metrics are captured** to best assess relevant parameters:



- Separation experiments can be introduced following dose-response experiments that combine progress curve analysis (Figure 2) with detection of IC_{50} shifts as a function of preincubation prior to instantaneous V_i calculation.
- SEC-driven separation can be scaled to achieve desired throughput, either as individual columns or as multi-column plates, to facilitate large-scale TDI assessment through activity-based readouts.
- The tight-binding limitation imposed by elevated [kinase] in the jump dilution protocol can be more easily circumvented, as final [inhibitor] is tied to binding site concentration and can be modulated by adjusting [kinase].

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