Time-dependent inhibitors (TDIs) of enzyme targets offer distinct advantages for the development of potent and selective compounds with favorable pharmacokinetic and pharmacodynamic properties. Such inhibitors are characterized by non-linear progress curves: an initial inhibited velocity, a rate constant governs the transition to a final steady-state reaction rate of the inhibited enzyme. A final rate of zero indicates irreversible inhibition, whereas a non-zero final rate indicates slow-binding inhibition. Characterizing these inhibitory modes of action is enabled with a continuous assay format that avoids the common pitfalls and misleading results seen with end-point assays. A continuous assay format enables efficient and robust determination of the kinetic parameters required to drive structure-activity relationship optimization to streamline the development of more effective drugs. It is important to note that simple IC₅₀ for TDIs will not suffice, and can, indeed, be misleading. We have developed a robust three-step workflow based on kinetic catalytic activity measurements to quickly identify and characterize TDIs. First, dose-response experiments are conducted with and without an enzyme-inhibitor preincubation step. The curvature of the reaction progress curve in the non-preincubated experiment and a shift in IC₅₀ from the preincubated experiment are indicative of TDIs. In the absence of TDIs, simple IC₅₀ are reported with, if possible, Kᵢ values. If TDIs is present, a second experiment is conducted to assess compound reversibility using either a jump-dilution protocol or a novel free-compound clearing method that uses gel filtration spin columns or spin plates. In either protocol, forward progress curve analysis is used to monitor the recovery of enzymatic activity after dilution of inhibitor in solution. Lastly, the potency of the inhibitor is evaluated using kinetic experiments tailored to the nature of the inhibition – either reversible or irreversible. If reversible, then the rate constant from the reversibility experiment is used to determine the residence time of the molecule. If irreversible, then a 24-point dose-response experiment with serial 1.5-fold dilutions is performed, and all the progress curves are globally fit to determine kᵢat/Kᵢ, and, if possible, kᵢat and Kᵢ separately. The method will be fully described through the characterization of known EGFR inhibitors of three inhibition types: fast-off (Gefitinib), slow binding (Lapatinib), and irreversible (Osimertinib).

Three different types of inhibitors doped in an animal model: two with short blood half lives dosed either high (red) or low (green), and one with long blood half-life dosed at a relatively low level (blue). PK and PD are indicated for short and long residence time inhibitors.

For inhibitors with a short residence time, PD (pharmacodynamics = target coverage) quickly follows PK (pharmacokinetics = blood levels). The best target coverage follows the longest blood exposure: AUC (area under the curve)-driven efficacy

For inhibitors with a long residence time or for irreversible inhibitors, PD extends beyond PK. Best target coverage can come from shorter exposure, so less chance for off-target toxicity: kᵢat (maximum blood level)-driven efficacy

Thus, measure PD vs. tox, rather than PK vs. tox to assess the therapeutic window of time-dependent inhibitors.

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 (J). 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.

Network models 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 (J). 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.

The progress curve for product formation is measured in the presence of increasing inhibitor concentration, and data are fit globally to determine: kᵢat/Kᵢ = the inactivation efficiency, the most important parameter to describe irreversible inhibitor potency Kᵢ, Concentration of inhibitor required for half maximal rate of covalent bond formation.

kᵢat = Observed maximal rate of inactivation of the enzyme.

The rate constant that describes the transition from the initial rate to the steady-state rate, kᵢat approximates the off rate Kᵢ of the inhibitor.

P = f(1 − exp(−kᵢat t))

Caveats:
• Re-binding can be an issue: want [inhibitor] as low as possible; conduct at several concentrations
• Pre-incubation is done at 100x enzyme concentration; may run into tight-binding limit for potent compounds

Coming soon: direct clearing of unbound compound in solution via size-exclusion chromatography with spin columns/plates. Both caveats of jump dilutions are avoided!

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