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A Proven Activity-based Workflow for the Identification and Characterization of Time-Dependent Kinase Inhibitors using a Continuous Assay Format

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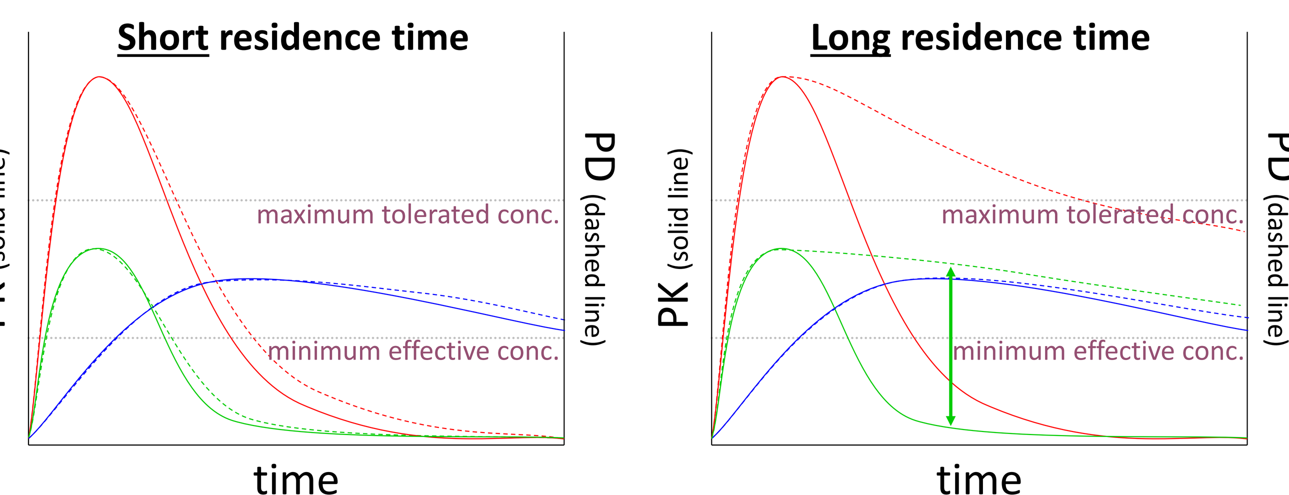
ABSTRACT

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: after 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 IC_{50}s for TDIs will not suffice, and can, indeed, also 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 IC_{50} from the preincubated experiment are indicative of TDI. In the absence of TDI, simple IC_{50}s are reported with, if possible, K_i values. If TDI is present, a second experiment is conducted to assess compound reversibility using either a jump-dilution protocol or a novel free-compound clearance 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 k_{inact}/K_I, and, if possible, k_{inact} and K_I 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).

FIGURE 1. WHY TDI?

Three different types of inhibitors dosed 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.



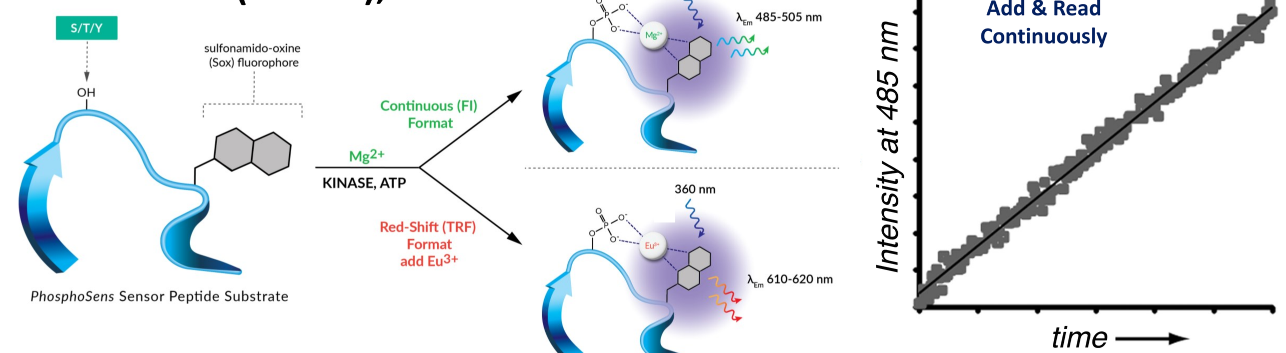
For inhibitors with a short residence time, PD (pharmacodynamics = target coverage) quickly follows PK (pharmacokinetics = blood levels). The best target coverage follows the longest blood exposure: **AUC (area under the curve)-driven efficacy**

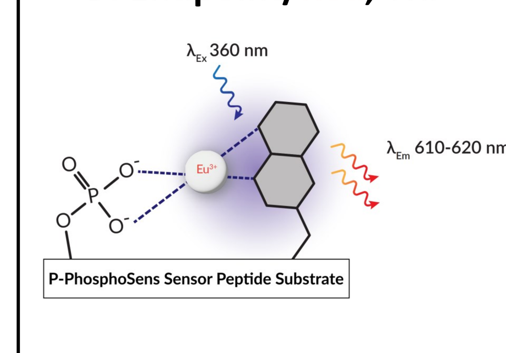
For inhibitors with a long residence time or for irreversible inhibitors, PD extends beyond PK. Best target coverage can come from shorter exposure, so less chance for off-target toxicity: **C_{max} (maximum blood level)-driven efficacy**

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

FIGURE 2. THE PHOSPHOSENS® ASSAY – CONTINUOUS & ENDPOINT/RED FORMATS

A. Continuous (Kinetic), FI





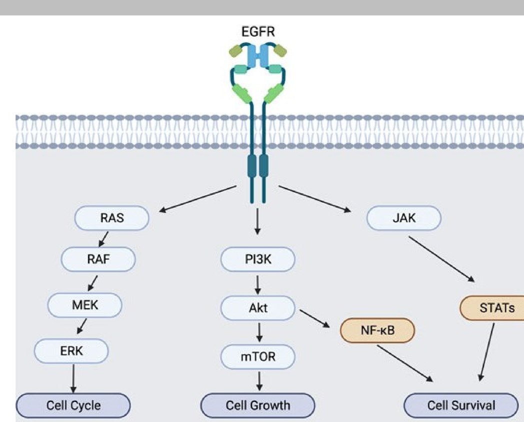
B. Endpoint/Red, TRF

P-PhosphoSens Sensor Peptide Substrate

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 3. TARGET AND INHIBITORS

EGFR is a receptor tyrosine kinase overexpressed in many human cancers (e.g., lung and breast), leading to inappropriate activation of several signaling pathways eventually leading to uncontrolled cell proliferation



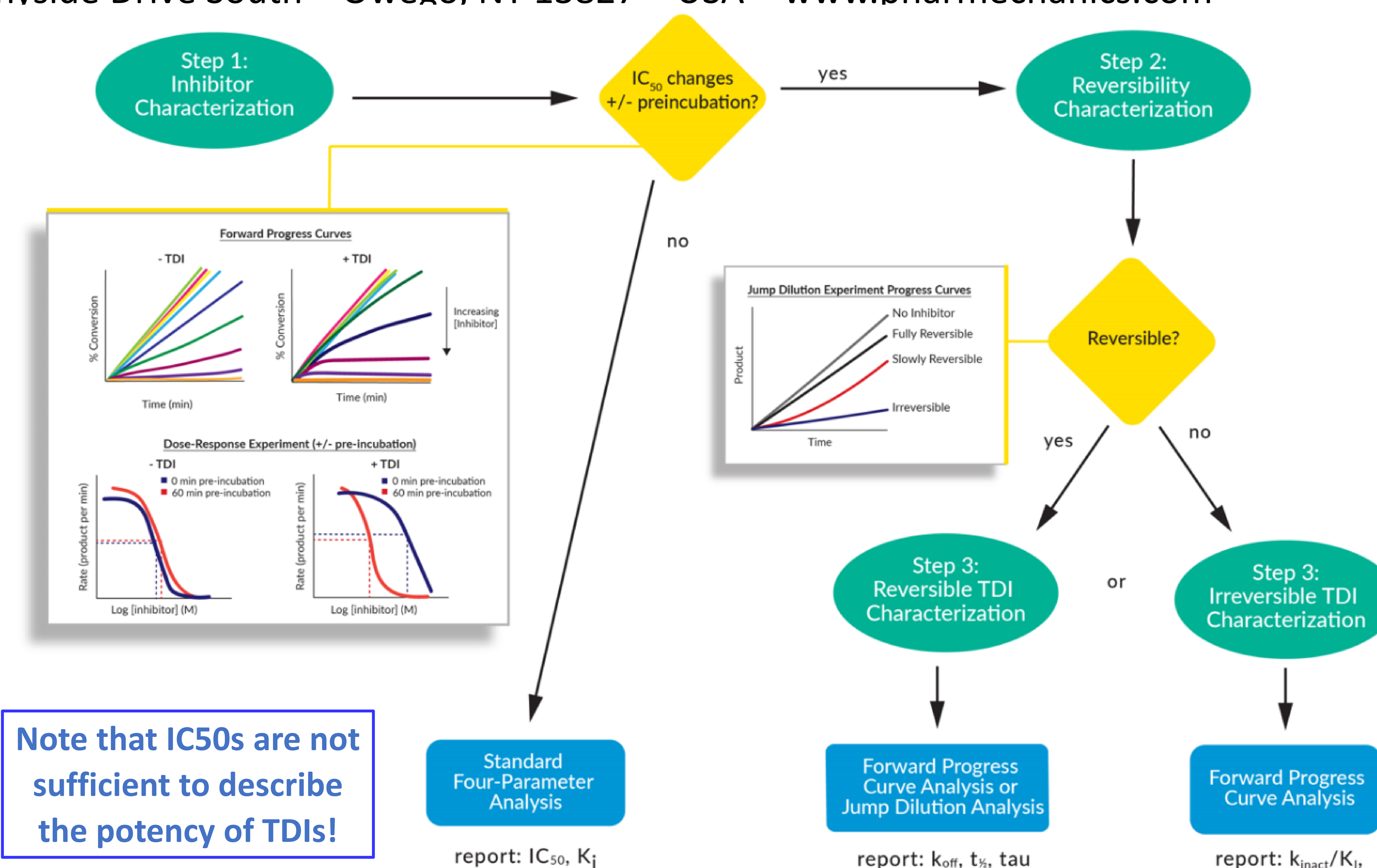
Front Pharmacol (2022) 13:1050758

	Trade Name	Company	Generation	Target	Cancer
Gefitinib	Iressa	AZ/Teva	First	EGFR	BrCa, NSCLC, other
Lapatinib	Tykerb	GSK	First	EGFR/ErbB2	BrCa
Osimertinib	Tagrisso	AZ	Third	EGFR T790M	NSCLC

FIGURE 4. THE TIME-DEPENDENT INHIBITION ASSAY WORKFLOW

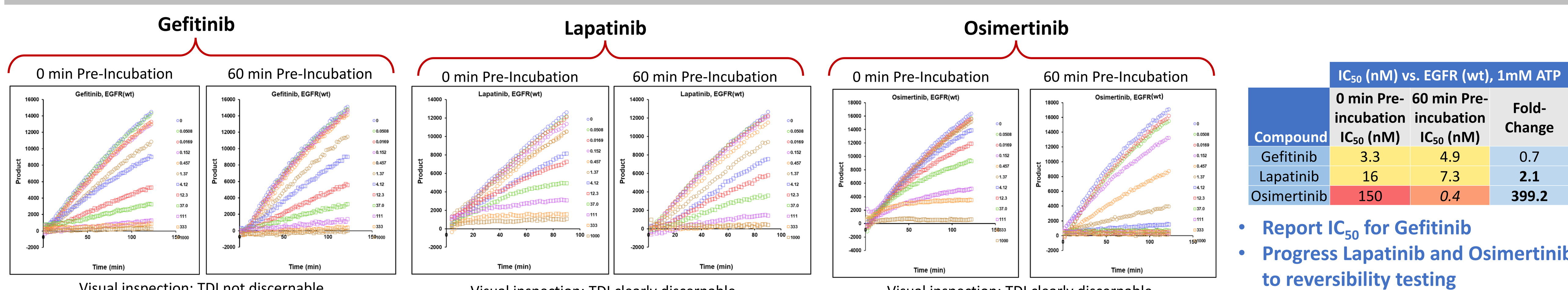
A workflow for the identification, characterization, and potency determination of time-dependent inhibitors

- Step 1: TDI identification. Titrate the inhibitor with and without pre-incubation of compound with enzyme. A shift in IC50 together with deviation from progress curve linearity indicates TDI. If no TDI is apparent, report IC_{50} and K_i.
- Step 2: Reversibility determination. Assess recovery of activity with rapid inhibitor concentration shift from 10x to 0.1x the IC_{50}.
- Step 3: Potency determination of the TDI
  - Step 3A: for reversible inhibitors, a careful rapid dilution experiment will quantify the off-rate or residence time
  - Step 3B: for irreversible inhibitors, a global fit to progress curves from an in-depth dose-response experiment will yield the inactivation efficiency, k_{inact}/K_I



Note that IC50s are not sufficient to describe the potency of TDIs!

FIGURE 5. STEP 1: IDENTIFICATION OF TIME-DEPENDENT INHIBITION

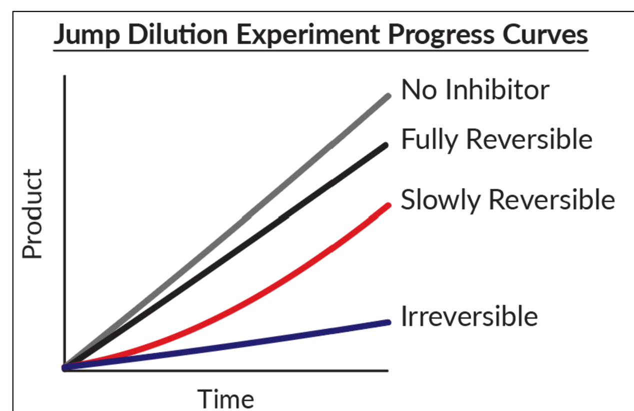


Compound	IC_{50} (nM) vs. EGFR (wt), 1mM ATP: 0 min Pre-incubation IC_{50} (nM)	60 min Pre-incubation IC_{50} (nM)	Fold-Change
Gefitinib	3.3	4.9	0.7
Lapatinib	16	7.3	**2.1**
Osimertinib	150	*0.4*	**399.2**

- **Report IC_{50} for Gefitinib**
- **Progress Lapatinib and Osimertinib to reversibility testing**

FIGURE 6. STEP 2: REVERSIBILITY ASSESSMENT

Jump dilution protocol:
- Pre-incubate inhibitor (10x IC_{50}) with enzyme (100x)
- Rapidly dilute reaction 100-fold to 0.1x IC_{50}
- Start reaction with ATP; monitor recovery of activity



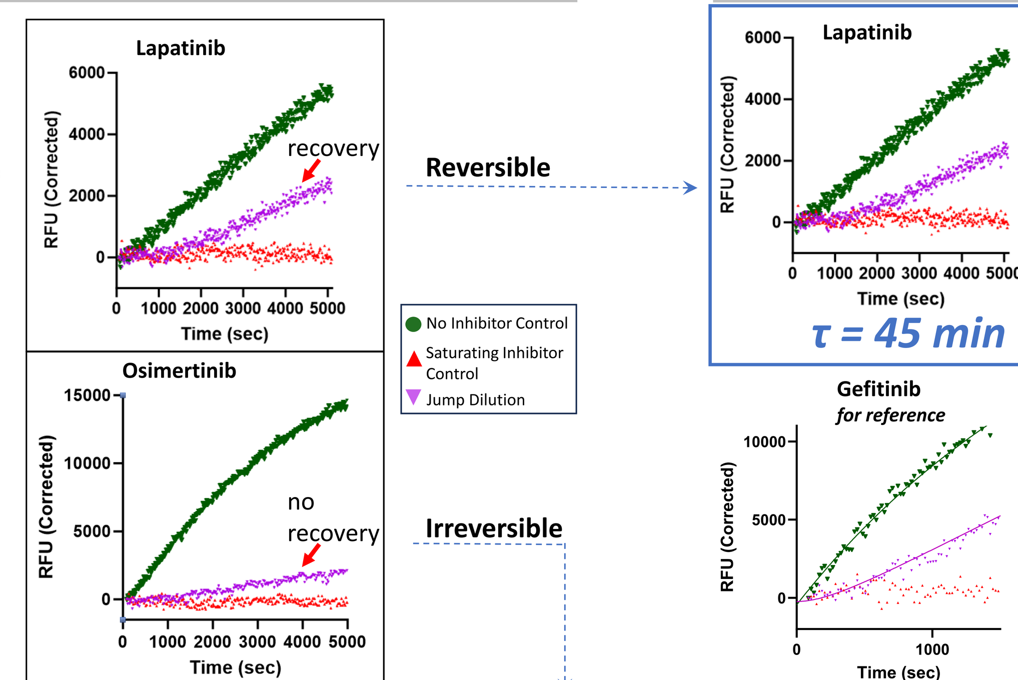


FIGURE 7. STEP 3A: REVERSIBLE INHIBITION POTENCY

The rate constant that describes the transition from the initial rate to the steady-state rate, k_{obs}, approximates the off-rate (k_{off}) of the inhibitor:

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

Robert A Copeland, et al. Anal Biochem. 2011 416(2):206

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!***

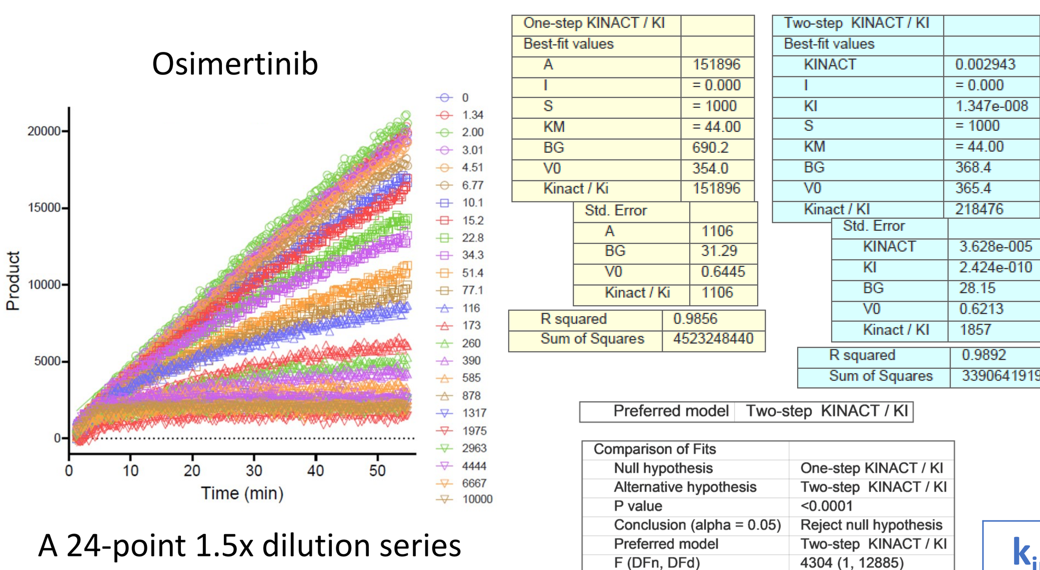
FIGURE 8. STEP 3B: IRREVERSIBLE INHIBITION POTENCY

The progress curve for product formation is measured in the presence of increasing inhibitor concentration, and data are fit globally to determine:

k_{inact}/K_I – the inactivation efficiency, the most important parameter to describe irreversible inhibitor potency.

K_I - Concentration of inhibitor required for half maximal rate of covalent bond formation.

k_{inact} - Observed maximal rate of inactivation of the enzyme.



One-step KINACT / KI	
Best-fit values	
A	151896
I	= 0.000
S	= 1000
KM	= 44.00
BG	690.2
V0	354.0
Kinact / Ki	151896
Std. Error	
A	1106
BG	31.29
V0	0.6445
Kinact / Ki	1106
R squared	0.9856
Sum of Squares	4523248440

Two-step KINACT / KI	
Best-fit values	
KINACT	0.002943
I	= 0.000
KI	1.347e-008
S	= 1000
KM	= 44.00
BG	368.4
V0	365.4
Kinact / KI	218476
Std. Error	
KINACT	3.628e-005
KI	2.424e-010
BG	28.15
V0	0.6213
Kinact / KI	1857
R squared	0.9892
Sum of Squares	3390641919

Preferred model	Two-step KINACT / KI

Comparison of Fits	
Null hypothesis	One-step KINACT / KI
Alternative hypothesis	Two-step KINACT / KI
P value	<0.0001
Conclusion (alpha = 0.05)	Reject null hypothesis
Preferred model	Two-step KINACT / KI
F (DFn, DFd)	4304 (1, 12885)

A 24-point 1.5x dilution series

$$[P] = \frac{v_i}{k_{obs}}[1 - \exp(-k_{obs} t)]$$

From the global fit, k_{inact}/K_I, is extracted in a one-step model relationship with k_{obs}, while k_{inact} and K_I are separately determined in a two-step model.

These two fits (one-step vs. two-step) are statistically compared, and the results from the model that best represents the data are reported. Note: this method offers many advantages over the hyperbolic fit of k_{obs} = f([I])

The **Osimertinib** data fit best to the two-step model:

k_{inact}/K_I = 2.18 $M^{-1}sec^{-1}$ | **k_{inact} = 2.94 $msec^{-1}$** | **K_I = 13.5 nM**

CONCLUSIONS

	TDI	Reversible	Potency Determination: IC50	Residence Time	k_{inact}/K_I	k_{inact}	K_I
Gefitinib	no	-	3.3 nM	-	-	-	-
Lapatinib	yes	yes	-	45 min	-	-	-
Osimertinib	yes	no	-	-	2.18 $M^{-1}sec^{-1}$	2.94 $msec^{-1}$	K_I = 13.5 nM

- Only for fast-off inhibitors can SAR be driven by IC_{50}s
- Only continuous assays can provide potency for kinetic modalities of kinase inhibitors
- For slow-off reversible inhibitors SKR is driven by off-rates, half-lives, and residence times
- For irreversible inhibitors SKR is driven by k_{inact}/K_I, k_{inact}, and K_I
- Capturing kinetic mode of action in kinome selectivity profiling is also available!