Supporting Information

for

A Practical Guide for the Assay-Dependent Characterisation of Irreversible Inhibitors

Lavleen K. Mader, Jessica E. Borean, Jeffrey W. Keillor*

Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, Ontario K1N 6N5, Canada.

* Corresponding author: jkeillor@uottawa.ca

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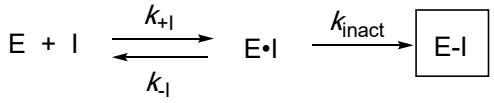
Equations from Direct Observation Methods

The reaction of an enzyme with an irreversible inhibitor, in the absence of any substrate, typically takes place by the following two-step kinetic scheme (shown as **Scheme 1** in the manuscript):



From this scheme it is evident that the total concentration of catalytically competent enzyme (i.e. $[E]_{cat} = [E] + [E•I]$) is converted irreversibly into the catalytically inactive species E-I, as a function of time. The conservation of mass equation for all enzyme species is:

$$[E]_0 = [E]_{cat} + [E - I] \quad \text{(i)}$$

The kinetic scheme above provides the rate law

$$\frac{d}{dt}[E - I] = k_{inact}[E•I]$$

where the total concentration of enzyme does not change ($\frac{d}{dt}[E]_0 = 0$) so we can write:

$$\frac{d}{dt}[E]_{cat} = -k_{inact}[E•I] \quad \text{(ii)}$$

The steady state approximation can then be applied:

$$k_{+I}[E][I] = k_{-I}[E•I] + k_{inact}[E•I]$$

$$K_I = \frac{[E][I]}{[E•I]} = \frac{k_{-I} + k_{inact}}{k_{+I}}$$

$$[E] = \frac{K_I[E•I]}{[I]}$$

From which $[E]_{cat} = \frac{K_I[E•I]}{[I]} + [E•I] = [E•I]\left(1 + \frac{K_I}{[I]}\right) = [E•I]\left(\frac{[I] + K_I}{[I]}\right)$

$$[\mathrm{E{\bullet}I}] = [\mathrm{E}]_{\mathrm{cat}} \cdot \frac{[\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{I}}} \qquad \text{(iii)}$$

To obtain a rate law for disappearance of active enzyme in terms of the kinetic parameters for irreversible inhibition, one can substitute (iii) into (ii) to give:

$$\frac{d}{dt}[\mathrm{E}]_{cat} = -k_{inact} \cdot [\mathrm{E}]_{\mathrm{cat}} \cdot \frac{[\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{I}}} \qquad \text{(iv)}$$

Integration of equation (iv), using the equality $[\mathrm{E}]_{\mathrm{cat}} = [\mathrm{E}]_0$ at time zero, gives:

$$[\mathrm{E}]_{cat(t)} = [E]_0 e^{-k_{inact} \cdot \frac{[\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{I}}} \cdot t} \qquad \text{(v)}$$

The time-dependent formation of modified enzyme (E-I) can be written explicitly as:

$$[\mathrm{E\text{ - }I}] = [E]_0 \left(1 - e^{-k_{inact} \cdot \frac{[\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{I}}} \cdot t}\right) \qquad \textbf{(Eqn. S1)}$$

The observed rate constant for formation of E-I can be defined as k_{obs}, as follows:

$$k_{obs} = \frac{k_{inact} \cdot [\mathrm{I}]}{[\mathrm{I}] + K_{\mathrm{I}}} \qquad \textbf{(Eqn. S2)}$$

Eqn. S2 also shows the hyperbolic dependence of the observed rate constant on inhibitor concentration.

Eqn. S1 describes a mono-exponential association curve, which is commonly included in most fitting software packages. Otherwise, it can be entered as the following plain text equation:

Y = Y0 + (Plateau-Y0)*(1-EXP(-kobs*X))

where Y0 is typically zero, after correction for background signal.

Eqn. S2 may also be manipulated to generate a double reciprocal version, for linear regression:

$$\frac{1}{k_{obs}} = \frac{K_I}{k_{inact}} \cdot \frac{1}{[I]} + \frac{1}{k_{inact}} \qquad \textbf{(Eqn. S3)}$$

However, *fitting to this equation can be highly error-prone* and is not recommended. To illustrate this, the three data sets shown in **Figure S1** were simulated for a low solubility inhibitor and include ≤10% random relative error in each rate constant. These data sets were then fitted to **Eqn. S3**.

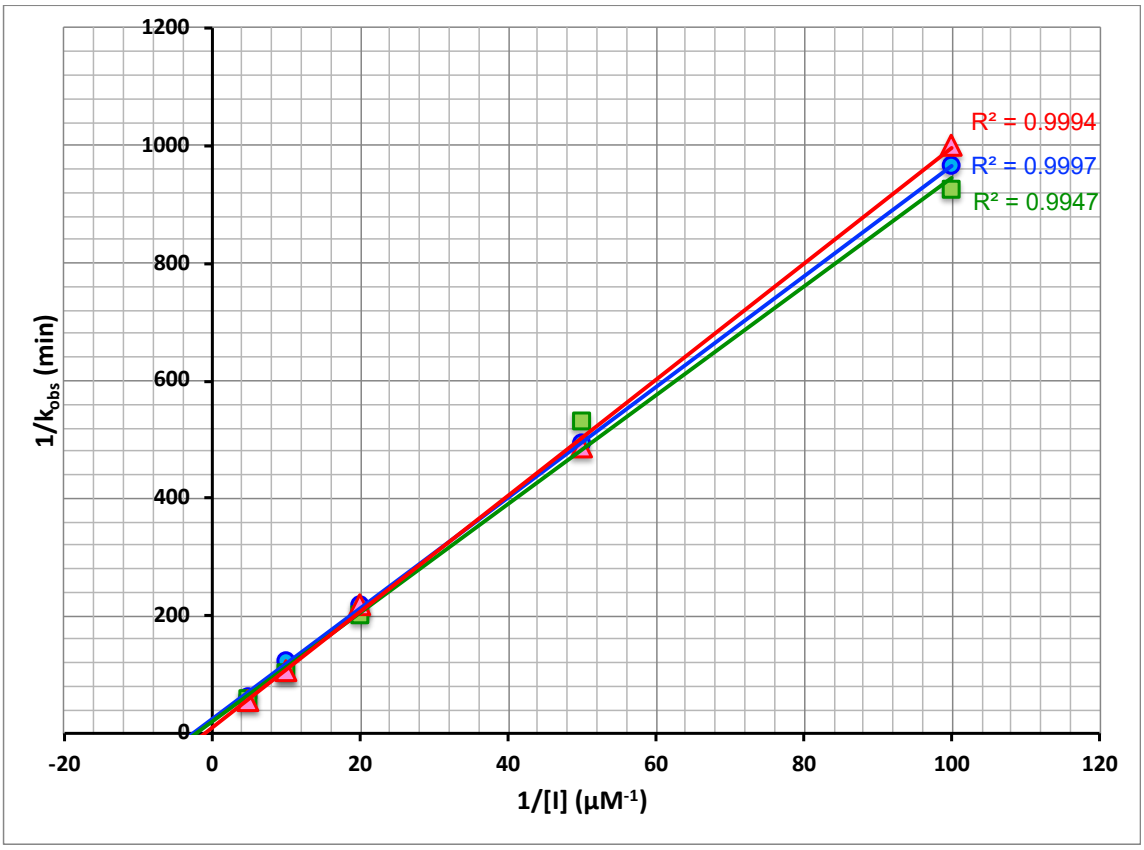


Figure S1. Simulated data points that incorporate less than 10% random error in each rate constant, which were then fitted to double reciprocal **Eqn. S3**. The fitted values of k_{inact}, K_I and k_{inact}/K_I are shown in **Table S1**.

While the fitting shown in **Fig. S1** appears to be excellent for each data set ($R^2 > 0.99$), the values derived from the fitting are highly erroneous. The same data points from **Fig. S1** are shown in **Fig. S2**, where the solid lines represent the theoretical curves corresponding to the authentic kinetic parameters used to generate the simulated data.

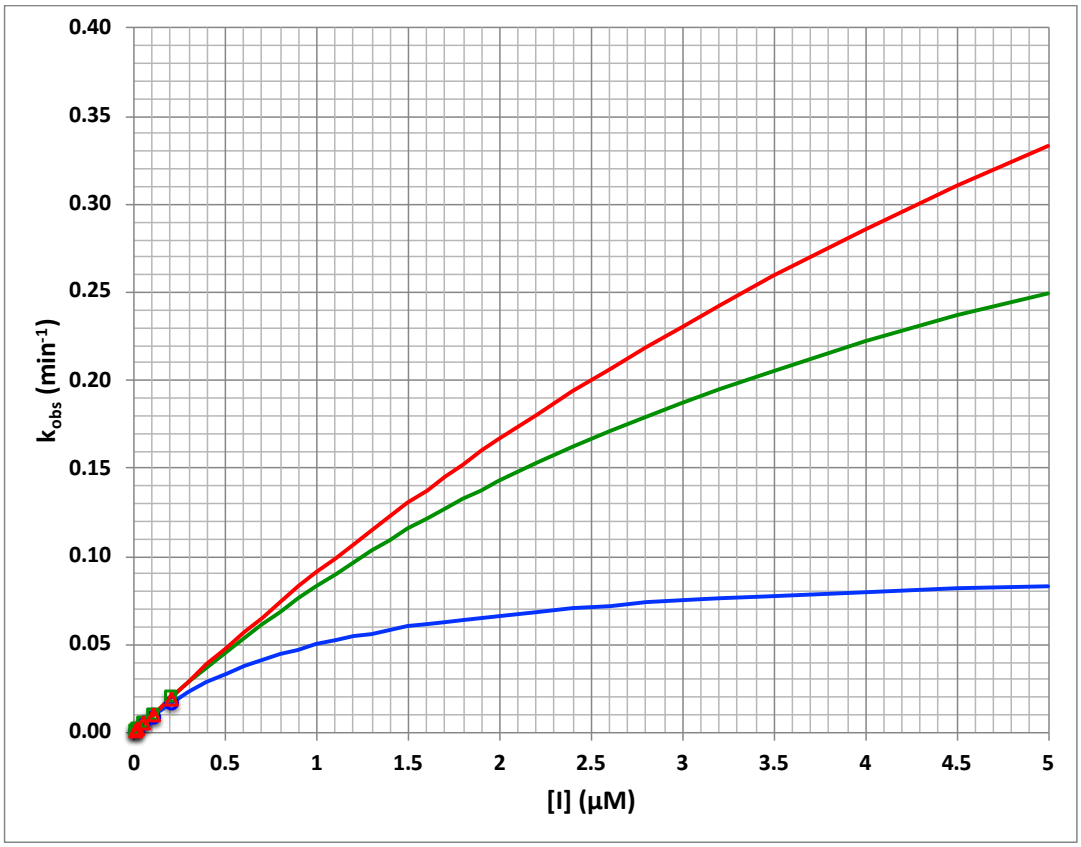


Figure S2. Hyperbolic curves corresponding to the authentic kinetic parameters listed in **Table S1**, which were used to generate the simulated data sets, also shown in **Figure S1**.

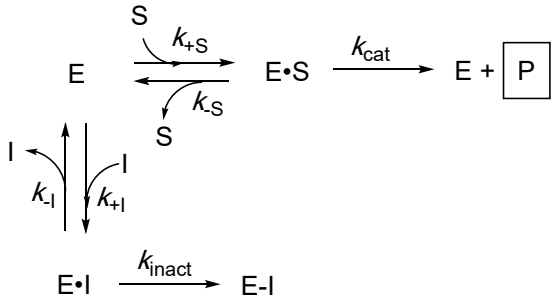
Table S1. Comparison of authentic kinetic parameters used to generate simulated datasets (**Figs. S1** and **S2**) and those derived from double reciprocal fitting (**Fig. S1**).

Dataset	*Authentic Values*			*Fitted Values*		
	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}min^{-1}$)	k_{inact} (min^{-1})	K_I (μM)	k_{inact}/K_I ($\mu M^{-1}min^{-1}$)
Blue	0.1	1.0	0.10	0.043	0.410	0.106
Green	0.5	5.0	0.10	0.051	0.525	0.097
Red	1.0	10	0.10	0.132	1.35	0.098

Even a cursory inspection of the values in **Table S1** reveals that the individual values of k_{inact} and K_I derived from the double reciprocal fitting shown in **Fig. S1** can differ from the authentic values by nearly an order of magnitude. However, the ratios of the fitted values (k_{inact}/K_I) all compare favourably to the authentic values of 0.1. Intuitively, it is obvious from **Fig. S2** that the datasets can only provide a good estimate of the k_{inact}/K_I ratio, as the linear portion of the hyperbolic curves, defined at the lowest inhibitor concentrations. However, it may not be as apparent from the double reciprocal plot shown in **Fig. S2** that the individual fitted values of k_{inact} and K_I may be highly unreliable. This illustrates an important caveat: For datasets that do not show saturation, and cannot be fitted to a hyperbolic curve, fitting to a double reciprocal equation does *not* necessarily provide independent values for k_{inact} and K_I that are any more reliable.

Equations from Evaluation by Continuous Activity Assays

When a continuous activity assay is available, irreversible inhibition can be monitored by incubating the enzyme in the presence of both the irreversible inhibitor and the substrate of the reporter assay. The kinetic scheme representing this experiment is shown below, presented as **Scheme 2** in the manuscript:



The concentration of remaining free enzyme E is indicated by its reaction with substrate S to form product P. In the presence of a competitive inhibitor such as I, product formation will proceed at an inhibited rate v_I given by the following equation, where the effective K_M is increased by the term $(1 + [I]/K_I)$:

$$v_I = \frac{d}{dt}[P]_I = \frac{k_{cat} \cdot [S] \cdot [E]_0}{[S] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad \text{(vi)}$$

Over the course of the experiment, the total concentration of active enzyme decreases in a time-dependent fashion. This results in a the time-dependent change in the rate of product formation, such that $[E]_0$ in equation (vi) must be replaced with $[E]_{cat(t)}$:

$$v_{I(t)} = \frac{d}{dt}[P]_{I(t)} = \frac{k_{cat} \cdot [S] \cdot [E]_{cat(t)}}{[S] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad \text{(vii)}$$

Equations (iv) and (v) describe the concentration of catalytically active enzyme in the presence of an irreversible inhibitor, but in the context of the simultaneous reaction with substrate, these equations must be modified to account for competition between inhibitor and substrate (see Scheme 2). This is easily managed by multiplying K_I by the term $\alpha = 1 + ([S]/K_M)$:

$$[\mathrm{E}]_{cat(t)} = [E]_0 e^{-k_{obs} \cdot t} \qquad \textbf{(Eqn. S4)}$$

where

$$k_{obs} = \frac{k_{inact} \cdot [\mathrm{I}]}{[\mathrm{I}] + K_\mathrm{I}\left(1 + \frac{[S]}{K_M}\right)} \qquad \textbf{(Eqn. S5)}$$

The substitution of **Eqn. S4** into equation (vii) provides an equation that shows how the *rate* of product formation changes as a function of the time-dependent disappearance of active enzyme:

$$v_{I(t)} = \frac{d}{dt}[P]_{I(t)} = \frac{k_{cat} \cdot [\mathrm{S}] \cdot [E]_0 e^{-k_{obs} \cdot t}}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \frac{[I]}{K_I}\right)} = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \frac{[I]}{K_I}\right)} \cdot e^{-k_{obs} \cdot t} \qquad \text{(viii)}$$

The integration of equation (viii) from the beginning of the reaction (t=0, P=0), provides an explicit equation for the concentration of product formed at any time t, due to the reaction of enzyme with substrate, with simultaneous irreversible inhibition:

$$\int_0^{\mathrm{P}} d[\mathrm{P}]_{\mathrm{I(t)}} = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \frac{[I]}{K_I}\right)} \cdot \int_0^t e^{-k_{obs} \cdot t} \cdot dt \qquad \text{(ix)}$$

$$[P]_{I(t)} = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \frac{[I]}{K_I}\right)} \cdot \left(\frac{1 - e^{-k_{obs}t}}{k_{obs}}\right) \qquad \text{(x)}$$

Equation (x) can be simplified by substitution from equation (vii):

$$[P]_{I(t)} = \frac{V_I}{k_{obs}} \cdot \left(1 - e^{-k_{obs}t}\right) \quad \textbf{(Eqn. S6)}$$

This equation shows that the formation of product from a continuous assay reaction, with concomitant irreversible inhibition, follows a mono-exponential association curve, to an upper plateau of (V_I / k_{obs}). In practice, V_I can be very difficult to measure accurately, over the initial ~10% of the reaction, corresponding to a time period as short as $0.1/k_{obs}$. It is also inconvenient to measure V_I and include its *different, independent* value as a constraint for the fitting of *each* reaction curve. For these practical reasons, this fitting constraint is rarely performed, and the upper plateau is fitted as an independent parameter, according to the following equation:

$$[P]_{I(t)} = [P]_{I(\infty)} \cdot \left(1 - e^{-k_{obs}t}\right) \quad \textbf{(Eqn. S7)}$$

As shown for **Eqn. S1**, **Eqn. S7** also describes a mono-exponential association curve, commonly included in most fitting software. Alternatively, it can be entered as the following plain text equation:

Y = Y0 + (Plateau-Y0)*(1-EXP(-kobs*X))

where Y0 is typically zero, after correction for background signal.

If reaction progress is followed by monitoring substrate depletion, rather than product formation, the following alternative equation can be used to fit for k_{obs}:

$$[S]_{I(t)} = \left([S]_{I(0)} - [S]_{I(\infty)}\right) \cdot \left(e^{-k_{obs}t}\right) + [S]_{I(\infty)} \quad \textbf{(Eqn. S7b)}$$

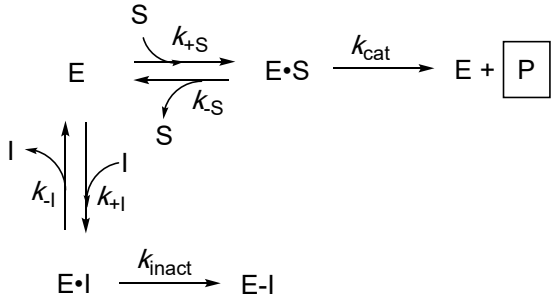
Likewise, the corresponding plain text version that can be used for fitting software is:

Y = Plateau + (Y0-Plateau)*(EXP(-kobs*X))

where Y0 is the signal for the initial substrate concentration, and Plateau corresponds to the final substrate concentration after complete enzyme inhibition.

Implicit Equation for the Time-Dependence of Incubation IC_{50} Values

For many enzymes, no continuous assay exists for monitoring their activity in real time. When a continuous assay is not available, it becomes necessary to apply a discontinuous end-point assay instead. The kinetic scheme describing this experiment is identical to that shown in the preceding section (namely **Scheme 2** from the manuscript):



However, importantly, product concentration is measured once, as an end-point, after a defined period of incubation. Inhibitor concentration is then varied, and the experiment is repeated, leading to a different end-point product concentration. These [product] *vs* [inhibitor] data are then used to construct IC_{50} plots, at varied incubation times. The IC_{50} value derived from each plot is defined as the concentration of inhibitor that gives 50% inhibition relative to the uninhibited reaction performed under the same conditions. Practically speaking, 50% inhibition is defined as the end-point product concentration that is half of the upper plateau of the sigmoidal dose-response curve (assuming the lower plateau has been normalised to zero). The upper plateau represents the concentration of product generated by the uninhibited enzymatic reaction, whose rate is given by the following equation:

$$v = \frac{d}{dt}[P] = \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}} \qquad \text{(xi)}$$

Typically, these assays are performed under conditions where substrate concentration does not vary significantly over the time course of the experiment, such that the maximum concentration of product formed from the uninhibited reaction at any time *t* is given by:

$$[P]_{max(t)} = \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}} \cdot t \qquad \text{(xii)}$$

From this the product concentration at the IC_{50} inflection point can be defined as:

$$[P]_{IC_{50}(t)} = \frac{1}{2}[P]_{max(t)} = \frac{1}{2} \cdot \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}} \cdot t \qquad \text{(xiii)}$$

Eqn. S5, from the preceding section, also tells us the concentration of product formed, in the presence of inhibitor. So, when the inhibitor concentration is equal to IC_{50}, **Eqn. S5** can be set equal to equation (xiii):

$$[P]_{IC_{50}(t)} = \frac{V_I}{k_{obs}} \cdot \left(1 - e^{-k_{obs}t}\right) = \frac{1}{2} \cdot \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}} \cdot t \qquad \text{(xiv)}$$

Equation (xiv) can be expanded, using equation (vi) and setting $[\mathrm{I}] = IC_{50(t)}$, to give:

$$\frac{\dfrac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}\left(1 + \dfrac{IC_{50}(t)}{K_I}\right)}}{k_{obs}} \cdot \left(1 - e^{-k_{obs}t}\right) = \frac{1}{2} \cdot \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}} \cdot t$$

$$\frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}\left(1 + \dfrac{IC_{50(t)}}{K_I}\right)} \cdot \frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{[\mathrm{S}] + K_{\mathrm{M}}}$$

$$\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = \frac{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0}{k_{cat} \cdot [\mathrm{S}] \cdot [\mathrm{E}]_0} \cdot \frac{[\mathrm{S}] + K_{\mathrm{M}}\left(1 + \dfrac{IC_{50(t)}}{K_I}\right)}{[\mathrm{S}] + K_{\mathrm{M}}}$$

$$\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=\frac{[\mathrm{S}]+K_{\mathrm{M}}\left(1+\frac{IC_{50(t)}}{K_I}\right)}{[\mathrm{S}]+K_{\mathrm{M}}}$$

$$\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=\frac{[\mathrm{S}]+K_{\mathrm{M}}+K_{\mathrm{M}}\frac{IC_{50(t)}}{K_I}}{[\mathrm{S}]+K_{\mathrm{M}}}$$

$$([\mathrm{S}]+K_{\mathrm{M}})\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=[\mathrm{S}]+K_{\mathrm{M}}+K_{\mathrm{M}}\frac{IC_{50(t)}}{K_I}$$

$$([\mathrm{S}]+K_{\mathrm{M}})\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}-([\mathrm{S}]+K_{\mathrm{M}})=K_{\mathrm{M}}\frac{IC_{50(t)}}{K_I}$$

$$([\mathrm{S}]+K_{\mathrm{M}})\left(\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}-1\right)=K_{\mathrm{M}}\frac{IC_{50(t)}}{K_I}$$

$$IC_{50(t)}=\frac{K_I}{K_{\mathrm{M}}}([\mathrm{S}]+K_{\mathrm{M}})\left(\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}-1\right)$$

$$IC_{50(t)}=K_I\left(1+\frac{[\mathrm{S}]}{K_{\mathrm{M}}}\right)\left(\frac{\left(2-2e^{-k_{obs}t}\right)}{k_{obs}\cdot t}-1\right) \qquad \textbf{(Eqn. S8)}$$

It should be noted that this equation was first published by Krippendorff *et al*.[1] In **Eqn. S8**, k_{obs} is defined as per equation (**Eqn. S4**), but [I] is equal to $\mathrm{IC}_{50(t)}$, such that

$$k_{obs}=\frac{k_{inact}\cdot IC_{50(t)}}{IC_{50(t)}+K_{\mathrm{I}}\left(1+\frac{[S]}{K_M}\right)} \qquad \text{(xv)}$$

This means that **Eqn. S8** is an *implicit* equation, since $\mathrm{IC}_{50(t)}$ appears on both sides of the equation. However, it can be solved easily by least squares regression, and the implicit equation can also be used to fit experimental values of $\mathrm{IC}_{50(t)}$, measured at different incubation times. The plain text

version of **Eqn. S8** can be entered into most fitting software packages, as a user-defined implicit equation. More specifically, data sets of time-dependent IC_{50} values (Y) versus the 'incubation' time of their measurement (X) can be fitted by non-linear least squares regression to provide the kinetic parameters k_{inact} and K_I^{app} :

Y=KIapp*(((2-2*EXP(-kinact*X*Y/(Y+KIapp)))/(kinact*X*Y/(Y+KIapp)))-1)

Note that in this version of the equation, $K_I^{app} = K_I\left(1 + \frac{[S]}{K_M}\right)$.

Limit of Implicit Equation as Incubation Times Approach Zero

As shown in the manuscript (see **Figure 6**), end-point IC_{50} values increase markedly at shorter incubation times, but **Eqn. S8** predicts a finite limiting value (i.e. a y-axis intercept) at infinitely short times. This value can be calculated by deriving the limit of **Eqn. S8**, as $t \rightarrow 0$.

$$IC_{50(t)} = K_I\left(1 + \frac{[\mathrm{S}]}{K_\mathrm{M}}\right)\left(\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} - 1\right) \qquad \textbf{(Eqn. S8)}$$

Examination of **Eqn. S8** (above) shows that as $t \rightarrow 0$, the term $\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t}$ becomes indeterminate. By applying L'Hôpital's rule, the numerator of this term can be replaced with its derivative and the denominator of the term with its derivative.

$$\frac{d}{dt}\left(2 - 2e^{-k_{obs}t}\right) = 2k_{obs}e^{-k_{obs}t} \qquad \text{(xvi)}$$

$$\frac{d}{dt}(k_{obs}t) = k_{obs} \qquad \text{(xvii)}$$

These derivatives of the numerator (xvi) and denominator (xvii) can then be substituted back into the indeterminate term to give:

$$\lim_{t\to 0}\left(\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t}\right) = \lim_{t\to 0}\left(\frac{2k_{obs}e^{-k_{obs}t}}{k_{obs}}\right) = \lim_{t\to 0}\left(2e^{-k_{obs}t}\right) = 2 \qquad \text{(xviii)}$$

Equation (xviii) indicates that the value of 2 should be substituted for the indeterminate term in calculating the limit of **Eqn. S7**, as follows:

$$\lim_{t\to 0} \mathrm{IC}_{50(t)} = \lim_{t\to 0}\left(K_I\left(1 + \frac{[\mathrm{S}]}{K_\mathrm{M}}\right)\left(\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} - 1\right)\right)$$

$$= \left(K_I\left(1 + \frac{[\mathrm{S}]}{K_\mathrm{M}}\right)(2 - 1)\right)$$

$$\lim_{t \to 0} \mathrm{IC}_{50(t)} = K_I\left(1 + \frac{[\mathrm{S}]}{K_\mathrm{M}}\right) \qquad \textbf{(Eqn. S9)}$$

Eqn. S9 indicates that the limiting value for $\mathrm{IC}_{50(t)}$ as the incubation time approaches zero is equal to $K_\mathrm{I}^{\mathrm{app}}$, which is the apparent K_I value, under the specific experimental conditions (namely, at the concentration of competitive substrate used in the experiment).

Intuitively, as the incubation time approaches zero, the only form of inhibition that would be manifested would be due to rapid equilibrium binding of inhibitor to form E•I, in the first step of the two-step inhibition mechanism. Prior to any time-dependent inactivation (to form E-I), any observed inhibition would be due to non-covalent binding alone. This explains why the lower limit of $\mathrm{IC}_{50(t)}$ is defined by $K_\mathrm{I}^{\mathrm{app}}$ alone and is independent of k_{inact}.

Table S2: Differential equations used in the numerical simulation of the EPIC-Fit method.

Species	Differential equation	Note
Pre-incubation phase		
E-I	$\frac{d[E-I]}{dt} = \frac{k_{inact} \cdot [I]}{([I] + K_I)} \cdot [E]$	no competition prior to substrate addition; *cf* equation (iv)
E	$\frac{d[E]}{dt} = -\frac{d[E-I]}{dt}$	[E] depleted as E-I is formed
I	$\frac{d[I]}{dt} = -\frac{d[E-I]}{dt}$	[I] depleted as E-I is formed
Incubation phase		
E-I	$\frac{d[E-I]}{dt} = \frac{k_{inact} \cdot [I]}{\left([I] + K_I\left(1 + \frac{[S]}{K_M}\right)\right)} \cdot [E]$	accounts for competition with substrate; *cf* **Eqn. S5**
E	$\frac{d[E]}{dt} = -\frac{d[E-I]}{dt}$	[E] depleted as E-I is formed
I	$\frac{d[I]}{dt} = -\frac{d[E-I]}{dt}$	[I] depleted as E-I is formed
P	$\frac{d[P]}{dt} = \frac{k_{cat} \cdot [S]}{\left([S] + K_M\left(1 + \frac{[I]}{K_I}\right)\right)} \cdot [E]$	accounts for competitive inhibition by inhibitor; see also equation (vii)
S	$\frac{d[S]}{dt} = -\frac{d[P]}{dt}$	[S] depleted as P is formed

Code for Numerical Simulation of Product Formation

The concentration of enzyme, inhibitor, substrate and product can be calculated iteratively, over the simulation of a biphasic pre-incubation inhibition reaction. These iterative calculations have been written Visual Basic, allowing them to be implemented as the function 'PreIncEndPoint' in Microsoft Excel.[2] This function allows for the rapid calculation of a predicted end-point product concentration, based on input of experimental parameters and predicted k_{inact} and K_I values. The predicted end-point values can then be compared against observed end-point values, allowing least-squares regression in the fitting of k_{inact} and K_I.

```
Function PreIncEndPoint(PreIncTime, DilFact, IncTime, AddSub, EnzConc, kcat,
Km, InhConc, kinact, KI)

'Set the granularity of each phase of the simulation to 100 finely-divided
time intervals
dPreTime = PreIncTime / 100
dIncTime = IncTime / 100

'Pre-incubation phase
For i = 1 To 100    'For each time interval dPreTime

   'First calculate instantaneous rate of E-I formation
   EIRate = kinact * InhConc / (InhConc + KI) * EnzConc

    'Now calculate incremental changes in concentrations, multiplying rates
by time interval (dPreTime)
    dEIConc = EIRate * dPreTime     'E and I both decrease by the same amount
```

```
    If dEIConc > EnzConc Then
       dEIConc = EnzConc         'This protects from EnzConc going below zero
    End If
    If dEIConc > InhConc Then
       dEIConc = InhConc         'This protects from InhConc going below zero
    End If

    'Then calculate new concentrations, at the end of this time interval:
    EnzConc = EnzConc - dEIConc  'Conc decrease for enzyme.
    InhConc = InhConc - dEIConc  'Conc decrease for inhibitor.

Next i

'Now account for addition of substrate and dilution of Enz and Inh
SubConc = SubConc + AddSub
EnzConc = EnzConc * DilFact
InhConc = InhConc * DilFact

'Now simulate Incubation phase
For j = 1 To 100    'For each time interval dIncTime

   'First calculate instantaneous rate of prod formation, accounting for
competitive inhibition:
    InstRate = kcat * EnzConc * SubConc / (SubConc + Km * (1 + InhConc / KI))
   'Then calculate instantaneous rate of E-I formation, accounting for
competition with substrate:
    EIRate = kinact * (InhConc / (InhConc + KI * (1 + SubConc / Km))) *
EnzConc
```

```
      'Now calculate incremental changes in concentrations, multiplying rates
by time interval (dIncTime)
    dSPConc = InstRate * dIncTime  'Sub and Prod change by the same amount
    If dSPConc > SubConc Then
       dSPConc = SubConc         'This protects from SubConc going below zero
    End If

    dEIConc = EIRate * dIncTime     'E and I both decrease by the same amount
    If dEIConc > EnzConc Then
       dEIConc = EnzConc         'This protects from EnzConc going below zero
    End If
    If dEIConc > InhConc Then
       dEIConc = InhConc         'This protects from InhConc going below zero
    End If

    'Then calculate new concentrations, at the end of this time interval:
    SubConc = SubConc - dSPConc     'Conc decrease for substrate
    ProdConc = ProdConc + dSPConc   'Conc increase for product
    EnzConc = EnzConc - dEIConc     'Conc decrease for enzyme
    InhConc = InhConc - dEIConc     'Conc decrease for inhibitor

Next j

PreIncEndPoint = ProdConc     'Return final product concentration

End Function
```

References

1. Krippendorff, B.-F.; Neuhaus, R.; Lienau, P.; Reichel, A.; Huisinga, W. Mechanism-Based Inhibition: Deriving K_I and k_{inact} Directly from Time-Dependent IC_{50} Values. *Journal of Biomolecular Screening* **2009**, *14* (8), 913–923.
2. Mader, L. K.; Keillor, J. W. Fitting of k_{inact} and K_I Values from Endpoint Pre-Incubation IC_{50} Data. *ACS Medicinal Chemistry Letters* **2024**, *15* (5), 731–738.