Supporting Information

for

Methods for Kinetic Evaluation of Reversible Covalent Inhibitors from Time-Dependent IC_{50} Data

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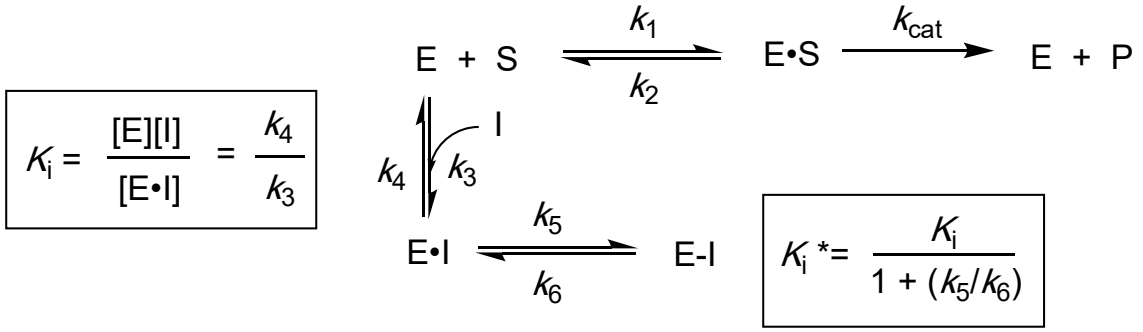
Derivation of explicit equation for product formation in the case of time-dependent covalent inhibition:

If a continuous assay is available, time-dependent (covalent) inhibition can be monitored conveniently, by incubating the enzyme in the presence of the inhibitor and the substrate of the continuous assay. The equilibria of the inhibition mechanism are illustrated in the following scheme:



The rate of the uninhibited reaction (v_0) is described by the Michaelis-Menten equation:

$$v_0 = \frac{d}{dt}[P] = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}} \qquad \textbf{(Eqn. S1)}$$

In the presence of a competitive inhibitor I, the rate of product formation will initially be inhibited to v_i given by the Michaelis-Menten equation, modified by multiplying K_M by the term $(1 + [I]/K_i)$:

$$v_i = \frac{d}{dt}[P]_i = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \frac{[I]}{K_i}\right)} \qquad \textbf{(Eqn. S2)}$$

$$\text{where } K_i = \frac{k_4}{k_3} = \frac{[E][I]}{[E \cdot I]} \qquad \textbf{(Eqn. S3)}$$

This inhibited rate v_i can be normalised against the uninhibited rate v_0 (equation (**Eqn. S1**)) as follows:

$$\frac{v_i}{v_0} = \frac{\dfrac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \dfrac{[I]}{K_i}\right)}}{\dfrac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}}} = \frac{[\mathrm{S}] + K_\mathrm{M}}{[\mathrm{S}] + K_\mathrm{M}\left(1 + \dfrac{[I]}{K_i}\right)} = \frac{[\mathrm{S}] + K_\mathrm{M}}{[\mathrm{S}] + K_\mathrm{M} + \dfrac{K_\mathrm{M}}{K_i}[I]}$$

$$= \frac{K_i([S] + K_M)}{K_i([S] + K_M) + K_\mathrm{M}[I]} = \frac{K_i\left(\dfrac{[S]}{K_M} + 1\right)}{K_i\left(\dfrac{[S]}{K_M} + 1\right) + [I]} = \frac{K_i^{app}}{K_i^{app} + [I]}$$

$$\text{where } K_i^{app} = K_i\left(\frac{[S]}{K_M} + 1\right) \qquad \textbf{(Eqn. S4)}$$

$$\frac{v_i}{v_0} = \frac{K_i^{app}}{K_i^{app} + [I]} = \frac{1}{1 + \dfrac{[I]}{K_i^{app}}}$$

$$v_i = \frac{v_0}{1 + \dfrac{[I]}{K_i^{app}}} \qquad \textbf{(Eqn. S5)}$$

After the final equilibrium is established, the more strongly inhibited rate v_s can be given by the Michaelis-Menten equation, modified by multiplying K_M by the term $(1 + [\mathrm{I}]/K_\mathrm{i}^*)$:

$$v_s = \frac{d}{dt}[P]_s = \frac{V_{max} \cdot [\mathrm{S}]}{[\mathrm{S}] + K_{\mathrm{M}}\left(1 + \frac{[I]}{K^*_i}\right)} \quad \textbf{(Eqn. S6)}$$

By analogy with **Eqn. S4**,

$$K^{*\,app}_{i} = K^*_i\left(\frac{[S]}{K_M} + 1\right) \quad \textbf{(Eqn. S7)}$$

By analogy with **Eqn. S5**,

$$v_s = \frac{v_0}{1 + \frac{[I]}{K^{*\,app}_{i}}} \quad \textbf{(Eqn. S8)}$$

In this context, K_{i}^* can be thought of as an apparent dissociation constant between all inhibitor-bound species (E·I and E-I) and the free enzyme and inhibitor:[1]

$$K^*_i = \frac{[E][I]}{[E \cdot I] + [E - I]} \quad \textbf{(Eqn. S9)}$$

From the kinetic scheme above, we see from the final equilibrium that

$$[E - I] = \frac{[E \cdot I]\, k_5}{k_6} \quad \textbf{(Eqn. S10)}$$

Substituting equation (**Eqn. S10**) into equation (**Eqn. S9**), we can write

$$K^*_i = \frac{[E][I]}{[E \cdot I] + \frac{[E \cdot I]\, k_5}{k_6}} = \frac{[E][I]}{[E \cdot I]\left(1 + \frac{k_5}{k_6}\right)}$$

Recalling equation (**Eqn. S3**), this can be simplified further:

$$K_i^* = \frac{[E][I]}{[E \cdot I]} \cdot \frac{1}{\left(1 + \frac{k_5}{k_6}\right)} = K_i\left(\frac{1}{1 + \frac{k_5}{k_6}}\right) \quad \textbf{(Eqn. S11)}$$

The time-dependent change in the rate of product formation, due to the slow establishment of the final binding equilibrium, is described by a rate constant that represents the sum of the forward and reverse steps for that rate-limiting equilibrium. For the reverse step, the rate constant is given by k_6. In the case of the forward step, the fraction of the rate constant k_5 that is observed will show hyperbolic dependence on inhibitor concentration, reflecting the fraction of enzyme that is in the form of E·I, from the initial rapid binding equilibrium (see **Eqn. S3**). Taken together, this leads to the following observed rate constant for the establishment of the final binding equilibrium:

$$k_{obs} = k_6 + \left(\frac{k_5[I]}{[I] + K_i^{app}}\right) \quad \textbf{(Eqn. S12)}$$

Considering all these equations, the integrated rate law for product formation by a time-dependent inhibitor following a two-step binding mechanism (as shown on the kinetic scheme above) is given as:

$$[P]_{I(t)} = v_s t + \frac{(v_i - v_s)}{k_{obs}}\left(1 - e^{-k_{obs}t}\right) \quad \textbf{(Eqn. S13)}$$

Derivation of an implicit equation for the time dependence of incubation IC_{50} values, for time-dependent (reversible covalent) inhibition, allowing for fitting of k_5, k_6 and K_i^{app}:

When a continuous assay is not available, a discontinuous end-point assay is usually applied. According to this approach, typically the concentration of *product* formed after a defined incubation period is measured, as a function of inhibitor concentration. These data are used to measure time-dependent IC_{50} values. Formally, an IC_{50} value is determined as the concentration of inhibitor that gives 50% inhibition, relative to the reaction performed under the same conditions but in the absence of inhibitor. Functionally, 50% inhibition is defined as the end-point product concentration half-way between the upper and lower plateaus of the sigmoidal dose-response curve. If the lower plateau is zero (as is often the case, although not always), the product concentration corresponding to 50% inhibition is equal to half the concentration that would be observed in the absence of inhibitor. The rate of the uninhibited enzymatic reaction is given by the Michaelis-Menten equation (see **Eqn. S1**). Assuming substrate concentration does not vary significantly over the time course of the experiment, and that enzyme is stable over the same time period, the maximum concentration of product formed from the uninhibited reaction at any time *t* is given by:

$$[P]_{max}(t) = v_0 \cdot t \qquad \textbf{(Eqn. S14)}$$

From this we can define the product concentration at the IC_{50} inflection point as half-way between the maximum concentration and the lower plateau, which is assumed to be zero:

$$[P]_{IC50}(t) = \frac{1}{2}[P]_{max}(t) = \frac{1}{2} \cdot v_0 \cdot t \qquad \textbf{(Eqn. S15)}$$

When the inhibitor concentration is equal to IC_{50}, equation (**Eqn. S13**) can be set equal to equation (**Eqn. S15**):

$$[P]_{IC50(t)} = v_s t + \frac{(v_i - v_s)}{k_{obs}}\left(1 - e^{-k_{obs}t}\right) = \frac{1}{2} \cdot v_0 \cdot t \qquad \textbf{(Eqn. S16)}$$

Recalling **Eqn. S5** and **Eqn. S8**, **Eqn. S16** can be expanded, setting [I] = $IC_{50(t)}$, to give:

$$\frac{v_0}{1 + \frac{IC_{50}(t)}{K^{*app}_i}} \cdot t + \frac{\left(\frac{v_0}{1 + \frac{IC_{50}(t)}{K^{app}_i}} - \frac{v_0}{1 + \frac{IC_{50}(t)}{K^{*app}_i}}\right)}{k_{obs}} \cdot \left(1 - e^{-k_{obs}t}\right) = \frac{1}{2} \cdot v_0 \cdot t$$

$$\frac{2}{1 + \frac{IC_{50}(t)}{K^{*app}_i}} \cdot t + \frac{\left(\frac{2}{1 + \frac{IC_{50}(t)}{K^{app}_i}} - \frac{2}{1 + \frac{IC_{50}(t)}{K^{*app}_i}}\right)}{k_{obs}} \cdot \left(1 - e^{-k_{obs}t}\right) = t$$

$$\frac{2}{1 + \frac{IC_{50}(t)}{K^{*app}_i}} \cdot t + \left(\frac{2}{1 + \frac{IC_{50}(t)}{K^{app}_i}} - \frac{2}{1 + \frac{IC_{50}(t)}{K^{*app}_i}}\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs}} = t$$

$$2 \cdot t + \left(\frac{2\left(1 + \frac{IC_{50}(t)}{K^{*app}_i}\right)}{1 + \frac{IC_{50}(t)}{K^{app}_i}} - 2\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs}} = t\left(1 + \frac{IC_{50}(t)}{K^{*app}_i}\right)$$

$$2+\left(\frac{2\left(1+\frac{IC_{50}(t)}{K^{*app}_i}\right)}{1+\frac{IC_{50}(t)}{K^{app}_i}}-2\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=\left(1+\frac{IC_{50}(t)}{K^{*app}_i}\right)$$

$$1+\left(\frac{2\left(1+\frac{IC_{50}(t)}{K^{*app}_i}\right)}{1+\frac{IC_{50}(t)}{K^{app}_i}}-2\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=\frac{IC_{50}(t)}{K^{*app}_i}$$

$$K^{*app}_i+K^{*app}_i\left(\frac{2\left(1+\frac{IC_{50}(t)}{K^{*app}_i}\right)}{1+\frac{IC_{50}(t)}{K^{app}_i}}-2\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t)$$

$$K^{*app}_i+\left(\frac{2K^{*app}_i\left(1+\frac{IC_{50}(t)}{K^{*app}_i}\right)}{1+\frac{IC_{50}(t)}{K^{app}_i}}-2K^{*app}_i\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t)$$

$$K^{*app}_i+\left(\frac{2\left(K^{*app}_i+IC_{50}(t)\right)}{1+\frac{IC_{50}(t)}{K^{app}_i}}-2K^{*app}_i\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t)$$

$$K^{*app}_i+\left(\frac{2K^{app}_i\left(K^{*app}_i+IC_{50}(t)\right)}{K^{app}_i+IC_{50}(t)}-2K^{*app}_i\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t)$$

$$K^{*app}_i+\left(\frac{\left(2K^{app}_iK^{*app}_i+2K^{app}_iIC_{50}(t)\right)}{K^{app}_i+IC_{50}(t)}-2K^{*app}_i\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t) \qquad \textbf{(Eqn. S17)}$$

Now recalling **Eqn. S11**, K_i^{*app} can be written in terms of K_i^{app}, reducing the number of parameters to be fitted:

$$K_i^{app}\left(\frac{k_6}{k_6+k_5}\right)+\left(\frac{\left(\left(\frac{2k_6}{k_6+k_5}\right)\left(K_i^{app}\right)^2+2K_i^{app}IC_{50}(t)\right)}{K_i^{app}+IC_{50}(t)}-2K_i^{app}\left(\frac{k_6}{k_6+k_5}\right)\right)\cdot\frac{\left(1-e^{-k_{obs}t}\right)}{k_{obs}\cdot t}=IC_{50}(t)$$

(**Eqn. S18**)

where k_{obs} is defined as per **Eqn. S12**, but in this case [I] = $IC_{50(t)}$, so

$$k_{obs}=k_6+\left(\frac{k_5IC_{50}(t)}{IC_{50}(t)+K_i^{app}}\right)$$

(**Eqn. S19**)

Plain text version:

Note that **Eqn. S18** is an implicit equation, since $IC_{50(t)}$ appears on both sides of the equation, but it can be solved by least squares regression and can be used to fit experimental values of $IC_{50(t)}$. The following plain text version of **Eqn. S18** can be entered as a user-defined implicit equation into any fitting software (e.g. GraphPad Prism), to allow regression fitting of IC_{50} values (Y) versus the time of their measurement (X) to provide the kinetic parameters K_i^{app}, k_5 and k_6:

Y = Kiapp*(k6/(k5 + k6)) + ((((2*k6/(k5 + k6))*(Kiapp^2)+(2*Kiapp*Y))/(Kiapp + Y)) – (2*Kiapp*(k6/(k5 + k6))))*(1 - EXP(-(k6 + (k5*Y/(Y + Kiapp)))*X))/((k6 + (k5*Y/(Y + Kiapp)))*X)

where Kiapp = Ki*(1 + [S]/Km)

and K_i^{*app} can be subsequently calculated as Kiapp*(k6/(k6 + k5)).

Limits of implicit equation

Recall the implicit equation **Eqn. S17**:

$$K^{*app}_{i} + \left(\frac{\left(2K^{app}_{i} K^{*app}_{i} + 2K^{app}_{i} IC_{50(t)} \right)}{K^{app}_{i} + IC_{50(t)}} - 2K^{*app}_{i} \right) \cdot \frac{\left(1 - e^{-k_{obs} t} \right)}{k_{obs} \cdot t} = IC_{50(t)} \qquad \textbf{(Eqn. S17)}$$

Zero time limit:

As t→0, only the term $\frac{\left(1 - e^{-k_{obs} t} \right)}{k_{obs} \cdot t}$ is affected, but $\lim_{t \to 0} \frac{\left(1 - e^{-k_{obs} t} \right)}{k_{obs} \cdot t}$ is indeterminate. So applying L'Hôpital's rule, we replace the numerator of this term with its derivative and the denominator with its derivative:

For the numerator, $\frac{d}{dt}\left(1 - e^{-k_{obs} t} \right) = k_{obs} \cdot e^{-k_{obs} t}$.

For the denominator, $\frac{d}{dt}(k_{obs} t) = k_{obs}$

This gives a new fraction whose limit can be calculated:

$$\lim_{t \to 0} \left(\frac{k_{obs} \cdot e^{-k_{obs} t}}{k_{obs}} \right) = \lim_{t \to 0} \left(e^{-k_{obs} t} \right) = 1$$

Substituting this value for the limiting term into **Eqn. S17** gives a new equation (**Eqn. S20**):

$$K^{*app}_{i} + \left(\frac{\left(2K^{app}_{i} K^{*app}_{i} + 2K^{app}_{i} IC_{50(0)} \right)}{K^{app}_{i} + IC_{50(0)}} - 2K^{*app}_{i} \right) = IC_{50(0)} \qquad \textbf{(Eqn. S20)}$$

$$\frac{\left(2K^{app}_{i} K^{*app}_{i} + 2K^{app}_{i} IC_{50(0)} \right)}{K^{app}_{i} + IC_{50(0)}} - K^{*app}_{i} = IC_{50(0)}$$

$$\frac{\left(2K^{app}_{i} K^{*app}_{i} + 2K^{app}_{i} IC_{50(0)} \right)}{K^{app}_{i} + IC_{50(0)}} = IC_{50(0)} + K^{*app}_{i}$$

$$\left(2K_i^{app}K^{*}{}_i^{app} + 2K_i^{app}IC_{50(0)}\right) = \left(IC_{50(0)} + K^{*}{}_i^{app}\right) \cdot \left(K_i^{app} + IC_{50(0)}\right)$$

$$2K_i^{app}K^{*}{}_i^{app} + 2K_i^{app}IC_{50(0)} = K_i^{app}IC_{50(0)} + IC_{50(0)}{}^2 + K_i^{app}K^{*}{}_i^{app} + K^{*}{}_i^{app}IC_{50(0)}$$

$$0 = -K_i^{app}IC_{50(0)} + IC_{50(0)}{}^2 - K_i^{app}K^{*}{}_i^{app} + K^{*}{}_i^{app}IC_{50(0)}$$

$$IC_{50(0)}{}^2 + \left(K^{*}{}_i^{app} - K_i^{app}\right) \cdot IC_{50(0)} - K_i^{app}K^{*}{}_i^{app} = 0$$

$$\left(IC_{50(0)} + K^{*}{}_i^{app}\right) \cdot \left(IC_{50(0)} - K_i^{app}\right) = 0 \qquad \textbf{(Eqn. S21)}$$

The two roots for **Eqn. S21** are $IC_{50(0)} = -K^{*}{}_i^{app}$ and $IC_{50(0)} = +K_i^{app}$, but only the latter makes physical sense. So, the limit at time zero is:

$$IC_{50(0)} = K_i^{app} \qquad \textbf{(Eqn. S22)}$$

<u>Infinite time limit:</u>

At infinitely long times, as t→∞, only the term $\frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t}$ is affected, and

$$\lim_{t \to \infty} \left(\frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t} \right) = 0$$

Substituting this value for the limiting term into **Eqn. S17** gives a new equation :

$$K^{*}{}_i^{app} + \left(\frac{\left(2K_i^{app}K^{*}{}_i^{app} + 2K_i^{app}IC_{50(\infty)}\right)}{K_i^{app} + IC_{50(\infty)}} - 2K^{*}{}_i^{app} \right) \cdot 0 = IC_{50(\infty)} \qquad \textbf{(Eqn. S23)}$$

$$K^{*}{}_i^{app} = IC_{50(\infty)}$$

So, the limit at infinite time is:

$$IC_{50(\infty)} = K^{*}{}_i^{app} \qquad \textbf{(Eqn. S24)}$$

Comparison with the "Krippendorff equation"

The equation relating the IC_{50} values of *irreversible* inhibitors to the incubation times at which they are measured was first published by Krippendorff *et al*.[2] We recently published a derivation of this equation (Eqn. S25), according to the approach shown above for equation Eqn. S18.[3]

$$IC_{50(t)} = K_I^{app}\left(\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} - 1\right) \qquad \textbf{(Eqn. S25)}$$

where

$$k_{obs} = \frac{k_{inact} \cdot IC_{50(t)}}{IC_{50(t)} + K_I^{app}} \qquad \textbf{(Eqn. S26)}$$

Irreversible covalent inhibition can be thought of as the ultimate physical limit for covalent inhibition, where residence time is infinite, since the rate constant for breaking the E-I covalent bond (k_6) is zero. This suggest**Eqn. S18** may simplify to **Eqn. S25**, on setting k_6 equal to zero. Let us first recall **Eqn. S18**:

$$K_i^{app}\left(\frac{k_6}{k_6 + k_5}\right) + \left(\frac{\left(\left(\frac{2k_6}{k_6 + k_5}\right)\left(K_i^{app}\right)^2 + 2K_i^{app}IC_{50(t)}\right)}{K_i^{app} + IC_{50(t)}} - 2K_i^{app}\left(\frac{k_6}{k_6 + k_5}\right)\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = IC_{50(t)}$$

And then replace k_6 with zero to give:

$$K_i^{app}(0) + \left(\frac{\left((0)\left(K_i^{app}\right)^2 + 2K_i^{app}IC_{50(t)}\right)}{K_i^{app} + IC_{50(t)}} - 2K_i^{app}(0)\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = IC_{50(t)} \qquad \textbf{(Eqn. S26)}$$

$$\left(\frac{\left(2K_i^{app}IC_{50(t)}\right)}{K_i^{app} + IC_{50(t)}}\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = IC_{50(t)}$$

$$\left(\frac{2K_i^{app}}{K_i^{app} + IC_{50(t)}}\right) \cdot \frac{\left(1 - e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = 1$$

$$K_i^{app} \cdot \frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} = K_i^{app} + IC_{50(t)}$$

$$K_i^{app} \cdot \frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} - K_i^{app} = IC_{50(t)}$$

$$K_i^{app}\left(\frac{\left(2 - 2e^{-k_{obs}t}\right)}{k_{obs} \cdot t} - 1\right) = IC_{50(t)} \qquad \textbf{(Eqn. S27)}$$

Eqn. S27 is identical to **Eqn. S25**.

Concerning k_{obs}, we can perform a similar simplification of **Eqn. S19** to compare it to **Eqn. S26**. For covalent reversible inhibition, we have:

$$k_{obs} = k_6 + \left(\frac{k_5 IC_{50(t)}}{IC_{50(t)} + K_i^{app}}\right) \qquad \textbf{(Eqn. S19)}$$

Setting k_6 to zero, in the case of irreversible inhibition, **Eqn. S19** becomes:

$$k_{obs} = 0 + \left(\frac{k_5 IC_{50(t)}}{IC_{50(t)} + K_i^{app}}\right) \qquad \textbf{(Eqn. S28)}$$

Eqn. S28 is identical to **Eqn, S26**, where k_5 is equivalent to k_{inact}.

In this sense, the Krippendorff equation (**Eqn. S25**) can be considered a simplified version of **Eqn. S18**, for the special limiting case where k_6 equals zero.

Table S1: Differential equations implicated in reversible covalent inhibition and used to develop EPIC-CoRe.

Species	Differential equation	Note
Pre-incubation phase		
E·I	$[E \cdot I] = \frac{([E]_{tot} + [I] + K_i) - \sqrt{([E]_{tot} + [I] + K_i)^2 - 4[E]_{tot}[I]}}{2}$	Fraction of unmodified enzyme bound by inhibitor; assumes rapid equilibrium but accounts for tight binding
E-I	$\frac{d[E-I]}{dt} = k_5 \cdot [E \cdot I] - k_6[E-I]$	No competition prior to substrate addition
E_{tot}	$\frac{d[E]_{tot}}{dt} = -\frac{d[E-I]}{dt}$	Total [E] is depleted as E-I is formed
I	$\frac{d[I]}{dt} = -\frac{d[E-I]}{dt}$	Total [I] is depleted as E-I is formed

Incubation phase		
E·I	$[E \cdot I] = \frac{\left([E]_{tot} + [I] + K_i\left(1 + \frac{[S]}{K_M}\right)\right) - \sqrt{\left([E]_{tot} + [I] + K_i\left(1 + \frac{[S]}{K_M}\right)\right)^2 - 4[E]_{tot}[I]}}{2}$	Fraction of unmodified enzyme bound by inhibitor; assumes rapid equilibrium in competition with substrate but accounts for tight binding
E-I	$\frac{d[E-I]}{dt} = k_5[E \cdot I] - k_6[E-I]$	Competition accounted for in [E·I]
E_{tot}	$\frac{d[E]_{tot}}{dt} = -\frac{d[E-I]}{dt}$	Total [E] depleted as E-I is formed
I	$\frac{d[I]}{dt} = -\frac{d[E-I]}{dt}$	Total [I] depleted as E-I is formed
P	$\frac{d[P]}{dt} = \frac{k_{cat} \cdot [S]}{\left([S] + K_M\left(1 + \frac{[I]}{K_i}\right)\right)} \cdot [E]_{tot}$	Accounts for competitive inhibition by inhibitor, as well as depletion of $[E]_{tot}$ due to covalent modification
S	$\frac{d[S]}{dt} = -\frac{d[P]}{dt}$	[S] depleted as P is formed

Code for numerical simulation of product formation

The iterative calculations of the concentration of free enzyme, covalently modified enzyme, inhibitor, substrate and product, over the course of the biphasic *pre-incubation* experiment, are shown below. The following code was written in Visual Basic, so that it could be implemented as the function 'PreIncRevEndPoint' in Microsoft Excel, as a broadly available software platform. This allows the rapid calculation of a predicted end-point concentration to be incorporated into a least-squares regression approach, for the fitting of k_5, k_6 and K_i, from which K_i^* can then be calculated. This code, and/or the differential equations of Table S1, could alternatively be implemented using more sophisticated fitting software (e.g. KinTek Explorer),[4] but these require commercial licenses and/or competency in computer language.

```
Function PreIncRevEndPoint(PreIncTime, DilFact, IncTime, AddSub, EnzConc, kcat, Km, InhConc, Ki, k5, k6)
Dim i As Integer
Dim j As Integer

dPreTime = PreIncTime / 100    'This sets granularity of each simulation phase to 100 time intervals
dIncTime = IncTime / 100

'Set some values at the beginning of the experiment:
SubConc = 0
ProdConc = 0
EIstarConc = 0
dSPConc = 0
dEIConc = 0
```

```
' ** Note that in this code, the non-covalent inhibitor-bound complex (E.I) is called EI and the covalent complex (E-I) is called EIstar **

'Pre-incubation phase
For i = 1 To 100    'Where each iteration i represents a time interval dPreTime
   'First calculate the rapid equilibrium concentration of EI (using quadratic equation to allow for cases where EnzConc may be close to value of Ki)
   EIConc = ((EnzConc + InhConc + Ki) - ((EnzConc + InhConc + Ki) ^ 2 - (4 * EnzConc * InhConc)) ^ 0.5) / 2
   'Then calculate instantaneous rate of formation of EIstar
   EIstarRate = (k5 * EIConc) - (k6 * EIstarConc)

    'Now calculate incremental changes in concentrations, multiplying rates by time interval (dPreTime)
    dEIstarConc = EIstarRate * dPreTime     'EIstar increases by this amount
    If dEIstarConc > EIConc Then
       dEIstarConc = EIConc      'This protects from EIConc going below zero
    End If

    'Then calculate new concentrations, at the end of this time interval, to account for decrease of free enzyme and inhibitor due to covalent modification:
    EnzConc = EnzConc - dEIstarConc  'The change is subtracted as a conc decrease due to formation of the covalent E-I complex.
    InhConc = InhConc - dEIstarConc  'The change is subtracted as a conc decrease due to formation of the covalent E-I complex.
    EIstarConc = EIstarConc + dEIstarConc  'The change is added as a conc increase due to formation of the covalent E-I complex.
```

```
Next i

'Now account for addition of substrate and dilution of all species
SubConc = SubConc + AddSub
EnzConc = EnzConc * DilFact
InhConc = InhConc * DilFact
EIstarConc = EIstarConc * DilFact

'Now Incubation phase
For j = 1 To 100    'Where each iteration j represents a time interval dIncTime
    'First calculate instantaneous rate of product formation, at instantaneous enzyme concentration,
accounting for competitive inhibition:
    ProdRate = kcat * EnzConc * (SubConc / (SubConc + Km * (1 + InhConc / Ki)))
    'Then calculate the rapid equilibrium concentration of EI, accounting for competition with substrate
(using quadratic eqn to allow for cases where EnzConc may be close to value of Ki)
    EIConc = ((EnzConc + InhConc + (Ki * (1 + SubConc / Km))) - ((EnzConc + InhConc + (Ki * (1 + SubConc /
Km))) ^ 2 - (4 * EnzConc * InhConc)) ^ 0.5) / 2
   'Then calculate instantaneous rate of formation of EIstar
   EIstarRate = (k5 * EIConc) - (k6 * EIstarConc)

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

    dEIstarConc = EIstarRate * dIncTime    'EIstar increases by this amount
```

```
    If dEIstarConc > EIConc Then
       dEIstarConc = EIConc      'This protects from EIConc going below zero
    End If

    'Then calculate new concentrations, at the end of this time interval:
    SubConc = SubConc - dSPConc      'The change is subtracted as a conc decrease for substrate.
    ProdConc = ProdConc + dSPConc   'The change is added as a conc increase for product.
    EnzConc = EnzConc - dEIstarConc  'The change is subtracted as a conc decrease due to formation of the
covalent E-I complex.
    InhConc = InhConc - dEIstarConc  'The change is subtracted as a conc decrease due to formation of the
covalent E-I complex.
    EIstarConc = EIstarConc + dEIstarConc  'The change is added as a conc increase due to formation of the
covalent E-I complex.

Next j

PreIncRevEndPoint = ProdConc              'Return final product concentration
End Function
```

Gly-Pro-pNA Kinetics with DDPIV

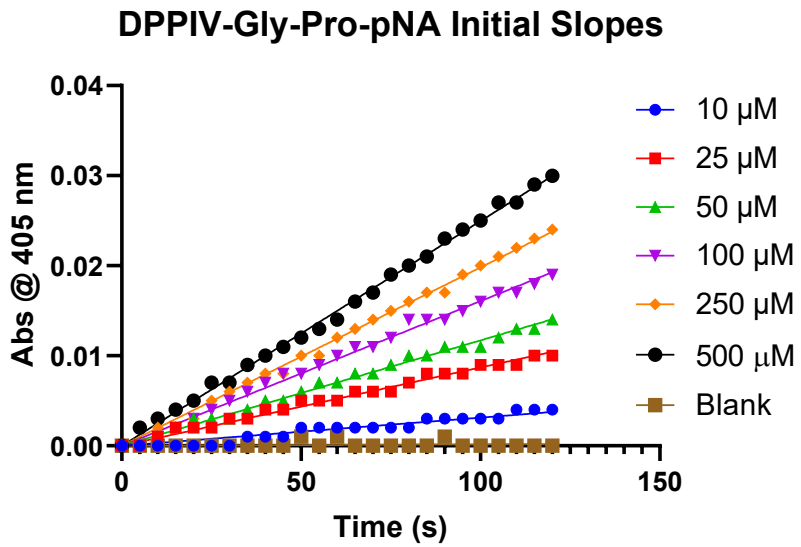


Figure S1. Initial rate data for reaction between DPPIV and Gly-Pro-pNA.

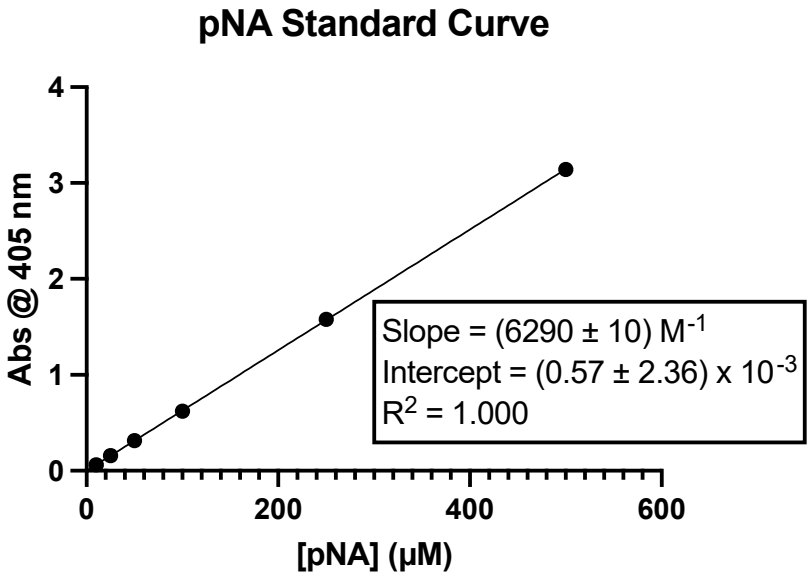


Figure S2. Standard absorbance *vs.* concentration curve for product pNA.

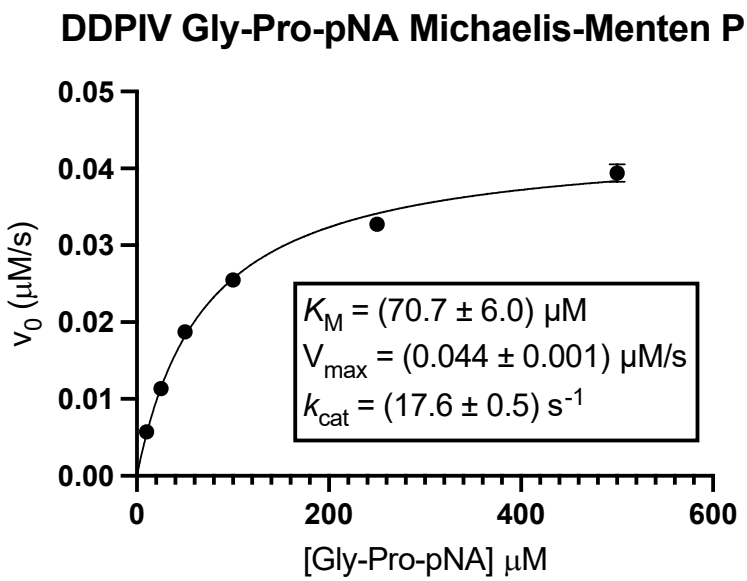


Figure S3. Michaelis-Menten plot for determination of K_M and V_{max}. Rates were measured using 2.5 nM of DPPIV.

Fitting of incubation time-dependent IC50 datasets with EPIC-CoRe

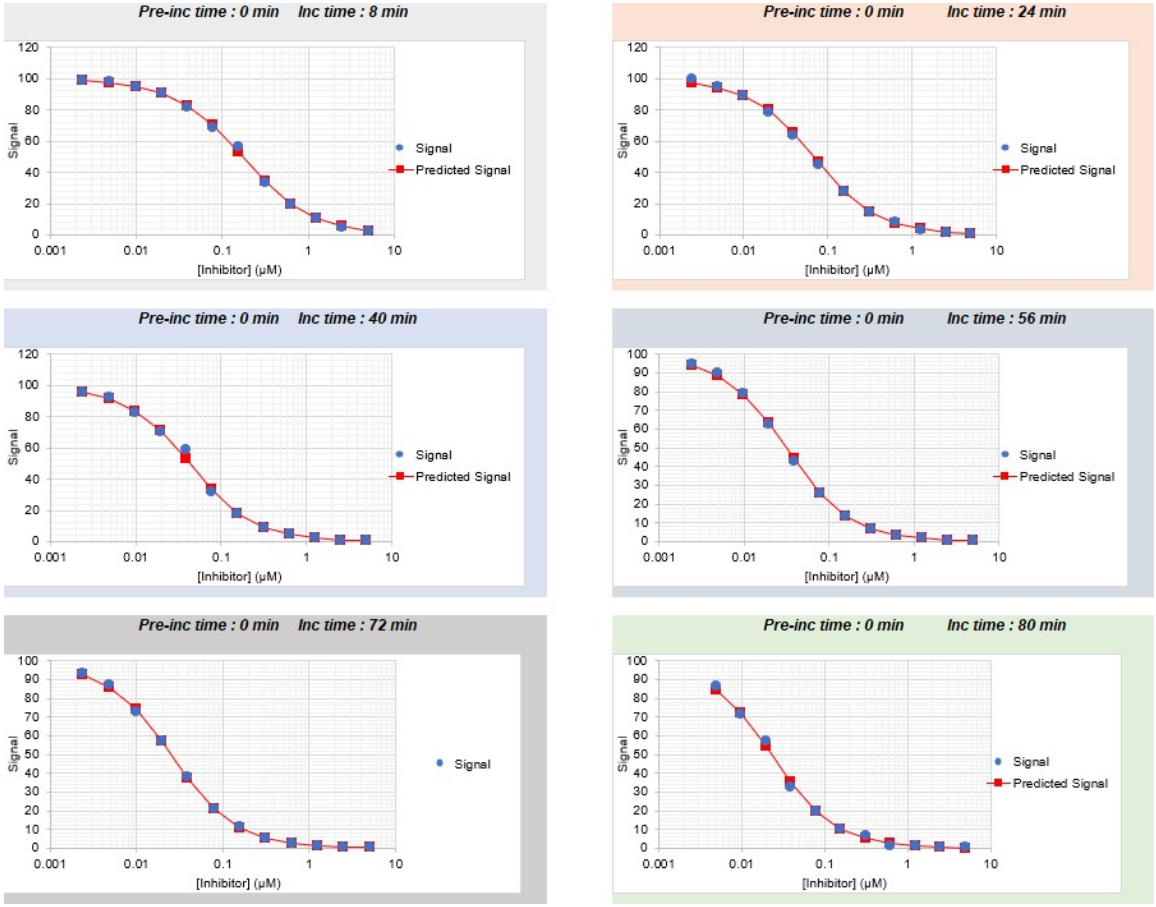


Figure S4. Global fitting of incubation time-dependent IC_{50} datasets obtained for saxagliptin, using EPIC-CoRe.

EPIC-CoRe Fitting Results with Fewer and Shorter Time Points

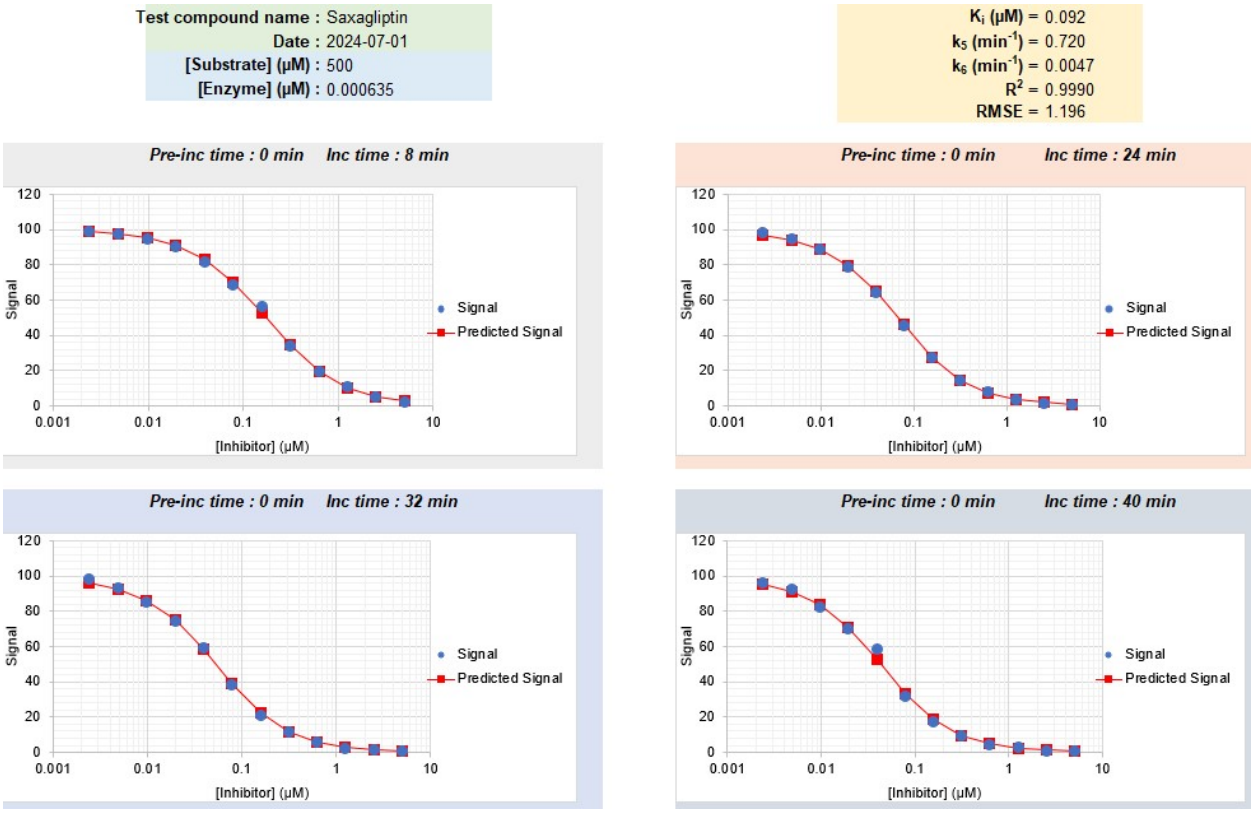


Figure S5. Average fitting results of incubation time-dependent IC_{50} datasets using only 4 time-points, up to only 40 minutes.

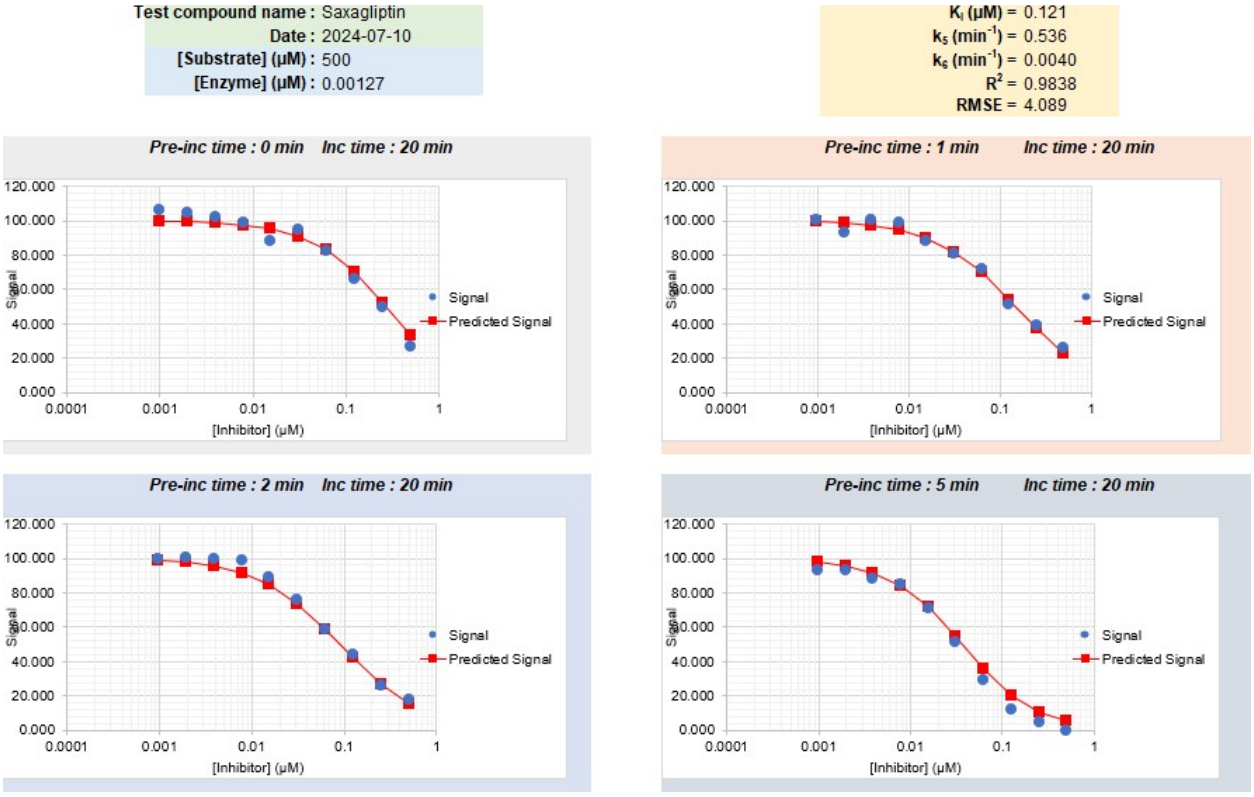


Figure S6. Average fitting results of pre-incubation time-dependent IC_{50} datasets using 4 time-points up to 5 minutes.

References

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