Supporting Information

A Comprehensive Guide for Assessing Covalent Inhibition in Enzymatic Assays Illustrated with Kinetic Simulations

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Supporting Figures

Linear regression methods are sometimes used to analyze preincubation-dependent enzyme inhibition (*Method III* and *IV*) because linear regression does not require dedicated graphical software, and benchmark protocols (Ito et al., 1998; Kitz & Wilson, 1962) promoting linear regression originate from a time that computation with nonlinear regression was not readily available. Here, observed reaction rate k_{obs} is calculated from the linear slope of the natural logarithm of the percentage remaining enzyme activity against preincubation time (Figure S1A). The straight line enables relatively simple visual inspection of the fit. For readers that prefer this visual output, we recommend to fit the data by nonlinear regression but plot the preincubation time-dependent enzyme activity on a semilog scale (Figure S1B).

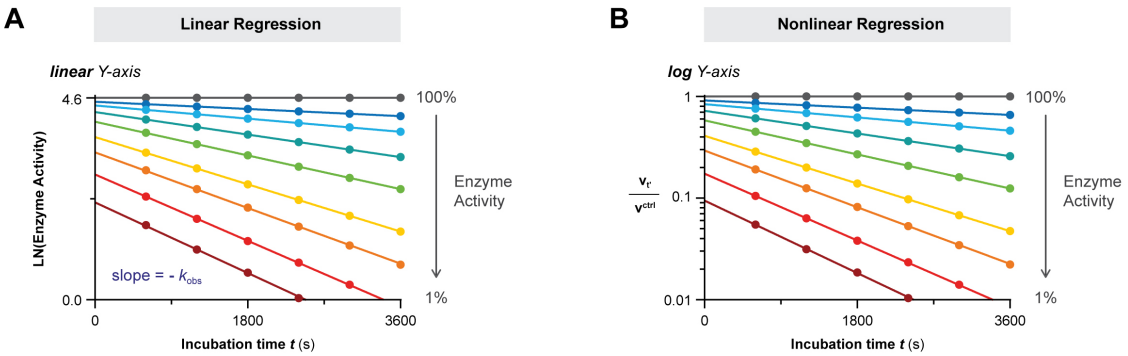

Figure S1. Comparison of linear and nonlinear regression. Illustrated with the data of inhibitor **C** in *Method III* (original data in Figure 15C). **A)** Linear regression. Reaction rate k_{obs} is obtained from the negative slope of the natural logarithm of percentage enzyme activity against preincubation time t'. LN(enzyme activity) = LN(100%·$v_{t'}/v^{ctrl}$). Negative values for LN(enzyme activity) corresponding with enzyme activity below 1% are excluded as assay sensitivity is normally insufficient to accurately distinguish 99% from 99.9% inhibition though this error will significantly affect the linear fit of k_{obs}. **B)** Nonlinear regression. Plot preincubation time-dependent enzyme activity against preincubation time on a semilog scale for visual inspection of the fit.

Supporting Tables

Table S1. Kinetic parameters for simulated inhibitors used in this work to illustrate methods

		Input				***Kinetic parameters*** [a]			
Substrate	**Mechanism**	k_1 ($M^{-1}s^{-1}$)	k_2 (s^{-1})	k_{cat} (s^{-1})		K_d (μM)		K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
S1	Substrate	10^8	100	1		1.00		1.01	$9.9x10^5$
L1	Ligand	10^8	100	0		1.00		-	-
Inhibitor	**Mechanism** [b]	k_3 ($M^{-1}s^{-1}$)	k_4 (s^{-1})	k_5 (s^{-1})	k_6 (s^{-1})	K_i (nM)	K_i^* (nM)	K_I (nM)	k_{inact}/K_I or k_{chem} ($M^{-1}s^{-1}$)
A	A	10,000	0.0001	0	0	10	-	-	-
B	B	10^8	10	0.001	0.0001	100	9.1	-	-
C	C	10^8	10	0.001	0	100	-	100	10^4
D	D	10,000	0	0	0	-	-	-	10^4

Reaction dynamics are illustrated in Table 1. [a] Calculated from microscopic rate constants in Table S2 and Table S3. [b] Mechanism in Figure 1.

Table S2. Kinetic Parameters (pseudo-first order reaction conditions)

	1-step REV	**2-step REV**	**2-step IRREV**	**1-step IRREV**
K_i (M)	$\frac{k_4}{k_3}$	$\frac{k_4}{k_3}$	$\frac{k_4}{k_3}$	-
K_i^* (M)	-	$\frac{k_4}{k_3+\frac{k_3k_5}{k_6}}$	-	-
K_I (M)	-	-	$\frac{k_4+k_{\mathrm{inact}}}{k_3}$	-
k_{off} (s^{-1})	k_4	$\frac{k_4k_6}{k_3+k_5+k_6}$	0	0
k_{obs} (s^{-1})	$k_4+k_3[\mathrm{I}]$	$k_6+\frac{k_5[\mathrm{I}]}{\left(\frac{k_4}{k_3}\right)+[\mathrm{I}]}$	$\frac{k_{\mathrm{inact}}[\mathrm{I}]}{\left(\frac{k_4+k_{\mathrm{inact}}}{k_3}\right)+[\mathrm{I}]}$	$k_{\mathrm{chem}}[\mathrm{I}]$
Inactivation rate constant ($M^{-1}s^{-1}$)	-	-	$\frac{k_{\mathrm{inact}}}{\mathrm{K_I}}=\frac{k_3k_{\mathrm{inact}}}{k_4+k_{\mathrm{inact}}}$	k_{chem}
$t_{½}$ (s)	$\frac{\mathrm{LN}(2)}{k_4+k_3[\mathrm{I}]}$	$\frac{\mathrm{LN}(2)}{k_6+\frac{k_5[\mathrm{I}]}{\left(\frac{k_4}{k_3}\right)+[\mathrm{I}]}}$	$\frac{\left(\frac{k_4+k_{\mathrm{inact}}}{k_3}+[\mathrm{I}]\right)\mathrm{LN}(2)}{k_{\mathrm{inact}}[\mathrm{I}]}$	$\frac{\mathrm{LN}(2)}{k_{\mathrm{chem}}[\mathrm{I}]}$
$t_{½diss}$ (s)	$\frac{\mathrm{LN}(2)}{k_4}$	$\frac{(k_3+k_5+k_6)\mathrm{LN}(2)}{k_4k_6}$	0	0
τ (s)	$\frac{1}{k_4}$	$\frac{(k_3+k_5+k_6)}{k_4k_6}$	∞	∞

General formulas are provided in the Supporting Information.

Table S3. Similarities in parameters for kinetic description of substrate hydrolysis for enzymes complying with Michaelis-Menten kinetics, and inactivation potency of two-step covalent inhibitors.

Michaelis-Menten Enzyme		**Covalent 2-step Inactivation***		
Parameter (Unit)	*Formula*	*Parameter (Unit)*	*Formula*	*Meaning*
K_s or K_d (M)	$K_d = \frac{k_2}{k_1}$	K_i (M)	$K_i = \frac{k_4}{k_3}$	Dissociation constant for non-covalent ES or EI complex
K_M (M)	$K_M = \frac{k_2 + k_{cat}}{k_1} = K_d + \frac{k_{cat}}{k_1}$	K_I (M)	$K_I = \frac{k_4 + k_{inact}}{k_3} = K_i + \frac{k_{inact}}{k_3}$	Concentration at half-maximum; [S] at ½V_{max} or [I] at ½k_{inact}
k_{cat} (s^{-1})	$k_{cat} = k_{max}$	k_{inact} (s^{-1})	$k_{inact} = k_{max}$	Reaction rate of irreversible step at saturating [S] or [I]
k_{cat}/K_M ($M^{-1}s^{-1}$)	$\frac{k_{cat}}{K_M} = \frac{k_1 k_{cat}}{k_2 + k_{cat}}$	k_{inact}/K_I ($M^{-1}s^{-1}$)	$\frac{k_{inact}}{K_I} = \frac{k_3 k_{inact}}{k_4 + k_{inact}}$	Efficiency of substrate conversion/enzyme inactivation
V_{max} ($M \cdot s^{-1}$)	$V_{max} = k_{cat}[E]_0$	-	-	$[E]_0$-dependent maximum velocity of product formation

* Mechanism in Figure 1C.

K_d = dissociation constant for noncovalent E + S <-> ES equilibrium (M), k_1 = association rate constant for E + S <-> ES equilibrium ($M^{-1}s^{-1}$), k_2 = dissociation rate constant for E + S <-> ES equilibrium (s^{-1}), k_{cat} = rate constant for irreversible ES → E + P reaction (s^{-1}) and K_M = Michaelis constant for substrate hydrolysis (M). K_i = inhibition constant for noncovalent E + I <-> EI equilibrium (M), k_3 = association rate constant for E + I <-> EI equilibrium ($M^{-1}s^{-1}$), k_4 = dissociation rate constant for E + I <-> EI equilibrium (s^{-1}), k_{inact} = k_5 = association rate constant for EI → EI* reaction (s^{-1}) and K_I = inactivation constant for two-step inactivation (M).

Derivatization of Equilibrium Constants

Noncovalent equilibrium constant K_i

E + I <-> EI

Rates of EI formation and dissociation are at equilibrium

$$k_3[\mathrm{E}][\mathrm{I}] = k_4[\mathrm{EI}]$$

Express [E][I] as function of [EI]

$$\mathrm{K_i} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} = \frac{\frac{k_4}{k_3}[\mathrm{EI}]}{[\mathrm{EI}]} = \frac{k_4}{k_3}$$

Steady-state equilibrium constant K_i^*

E + I <-> EI <-> EI*

Rate of EI formation/dissociation is at equilibrium step 1 (E+I <-> EI) and step 2 (EI <-> EI*)

$$k_3[\mathrm{E}][\mathrm{I}] = k_4[\mathrm{EI}]$$
$$k_5[\mathrm{EI}] = k_6[\mathrm{EI}^*]$$

Express [E][I] and [EI*] as function of [EI]

$$\mathrm{K_i}^* = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}^*] + [\mathrm{EI}]} = \frac{\frac{k_4[\mathrm{EI}]}{k_3}}{\frac{k_5[\mathrm{EI}]}{k_6} + [\mathrm{EI}]}$$

Divide all by [EI]

$$\mathrm{K_i}^* = \frac{\frac{k_4[\mathrm{EI}]}{k_3[\mathrm{EI}]}}{\frac{k_5[\mathrm{EI}]}{k_6[\mathrm{EI}]} + \frac{[\mathrm{EI}]}{[\mathrm{EI}]}} = \frac{\frac{k_4}{k_3}}{\frac{k_5}{k_6} + 1} = \frac{\mathrm{K_i}}{\frac{k_5}{k_6} + 1}$$

Inactivation constant K_I

E + I <-> EI → EI*

Rate of EI formation is equal to the rate of dissociation

$$k_3[\mathrm{E}][\mathrm{I}] = (k_4 + k_5)[\mathrm{EI}]$$

Express [E][I] as a function of [EI]

$$\mathrm{K_I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]} = \frac{\frac{(k_4 + k_5)}{k_3}[\mathrm{EI}]}{[\mathrm{EI}]} = \frac{k_4 + k_5}{k_3}$$

General Algebraic Equations

Association half-life under pseudo-first order reaction conditions ($[I]_0 > 10[E]_0$)

$$t½_{ass} = \frac{LN(2)}{k_{obs}}$$

Dissociation half-life

$$t½_{diss} = \frac{LN(2)}{k_{off}}$$

Target residence time

$$\tau = \frac{1}{k_{off}}$$

Preincubation Time-dependent Enzyme Occupancy

E + I <-> EI → EI*

Expected covalent occupancy $[EI^*]_{t'}/[E]_0$ after preincubation t'

$$\frac{[EI^*]_{t'}}{[E]_0} = 1 - e^{-k_{obs}t'}$$

Minimum k_{obs} to detect minimum covalent occupancy $[EI^*]_{t'}/[E]_0 \geq 0.6$ after preincubation t'

$$k_{obs} = \frac{LN\left(1 - \frac{[EI^*]_{t'}}{[E]_0}\right)}{t'} = \frac{LN(1 - 0.6)}{t'}$$

Minimum preincubation time t' to reach covalent occupancy $[EI^*]_{t'}/[E]_0 \geq 0.6$

$$t' = \frac{LN\left(1 - \frac{[EI^*]_{t'}}{[E]_0}\right)}{k_{obs}} = \frac{LN(1 - 0.6)}{k_{obs}}$$

Uninhibited Enzyme Activity

E + S <-> ES → E + P

Uninhibited product formation velocity under steady-state conditions ($[S]_0 \geq 10[E]_0$ and $[P]_t < 0.1[S]_0$).

$$v^{ctrl} = \frac{k_{cat}[E]_0[S]}{K_M + [S]}$$

Maximum enzyme concentration $[E]_0$ for 10% substrate conversion during incubation in the uninhibited control ($[S]_0 \geq 10[E]_0$)

$$[E]_0^{max} = \frac{0.1([S] + K_M)}{k_{cat}t} = \frac{[S] + K_M}{10k_{cat}t}$$

Maximum incubation time t for 10% substrate conversion in the uninhibited control ($[S]_0 \geq 10[E]_0$)

$$t = \frac{[S] + K_M}{10k_{cat}[E]_0}$$

Inhibited Enzyme Activity at Noncovalent Equilibrium

E + S <-> ES → E + P
E + I <-> EI

Inhibited product formation velocity after noncovalent equilibrium has been reached ($[I]_0 \geq 10[E]_0$ and $[S]_0 \geq 10[E]_0$ and $[P]_t < 0.1[S]_0$).

$$v_i = \frac{k_{cat}[E]_0[S]}{\left(1 + \frac{[I]}{K_i}\right)K_M + [S]}$$

Morrison's quadratic equation. Inhibited product formation velocity after noncovalent equilibrium has been reached ($[S]_0 \geq 10[E]_0$ with $[P]_t < 0.1[S]_0$), with correction for inhibitor depletion.

$$v_i = v^{ctrl}\left(1 - \frac{\left([E]_0 + [I]_0 + K_i\left(1 + \frac{[S]}{K_M}\right)\right) - \sqrt{\left([E]_0 + [I]_0 + K_i\left(1 + \frac{[S]}{K_M}\right)\right)^2 - 4[E]_0[I]_0}}{2[E]_0}\right)$$

Equilibrium Concentrations at (Initial) Noncovalent Equilibrium

E + I <-> EI

Equilibrium concentration of noncovalent enzyme-inhibitor complex in absence of competitor. ($[I]_0 \geq 10[E]_0$)

$$[EI]_{eq} = \frac{[E]_0[I]}{K_i + [I]}$$

Morrison's quadratic equation. Equilibrium concentration of noncovalent enzyme-inhibitor complex in absence of competitor with correction for inhibitor depletion.

$$[EI]_{eq} = \frac{([E]_0 + [I]_0 + K_i) - \sqrt{([E]_0 + [I]_0 + K_i)^2 - 4[E]_0[I]_0}}{2}$$

E + S <-> ES → E + P
E + I <-> EI

Equilibrium concentration of noncovalent enzyme-inhibitor complex in presence of competitor. ($[S]_0 \geq 10[E]_0$ and $[I]_0 \geq 10[E]_0$)

$$[EI]_{eq} = \frac{[E]_0[I]}{K_i\left(1 + \frac{[S]}{K_M}\right) + [I]}$$

Morrison's quadratic equation. Equilibrium concentration of noncovalent enzyme-inhibitor complex in presence of competitor ($[S]_0 \geq 10[E]_0$), with correction for inhibitor depletion.

$$[EI]_{eq} = \frac{\left([E]_0 + [I]_0 + K_i\left(1 + \frac{[S]}{K_M}\right)\right) - \sqrt{\left([E]_0 + [I]_0 + K_i\left(1 + \frac{[S]}{K_M}\right)\right)^2 - 4[E]_0[I]_0}}{2}$$

Equilibrium Concentrations at Steady-state Equilibrium

E + I <-> EI <-> EI*

Equilibrium concentrations of bound enzyme in absence of competitor. ($[I]_0 \geq 10[E]_0$)

$$[EI + EI^*]_{eq} = \frac{[E]_0[I]}{K_i^* + [I]}$$

$$[EI^*]_{eq} = [EI + EI^*]_{eq}\left(1 - \frac{K_i^*}{K_i}\right) = \frac{[E]_0[I]}{K_i^* + [I]}\left(1 - \frac{K_i^*}{K_i}\right)$$

$$[EI]_{eq} = [EI + EI^*]_{eq}\left(\frac{K_i^*}{K_i}\right) = \frac{K_i^*}{K_i}\left(\frac{[E]_0[I]}{K_i^* + [I]}\right) = \frac{K_i^*[E]_0[I]}{K_i(K_i^* + [I])} = \frac{[E]_0[I]}{K_i + \frac{K_i[I]}{K_i^*}}$$

E + I <-> EI <-> EI*
E + S <-> ES → E + P

Equilibrium concentrations of bound enzyme in presence of competitor. ($[I]_0 \geq 10[E]_0$ and $[S]_0 \geq 10[E]_0$)

$$[EI + EI^*]_{eq} = \frac{[E]_0[I]}{K_i^*\left(1 + \frac{[S]}{K_M}\right) + [I]}$$

$$[EI^*]_{eq} = [EI + EI^*]_{eq}\left(1 - \frac{K_i^*}{K_i}\right) = \frac{[E]_0[I]}{K_i^*\left(1 + \frac{[S]}{K_M}\right) + [I]}\left(1 - \frac{K_i^*}{K_i}\right)$$

$$[EI]_{eq} = \frac{K_i^*}{K_i}\left(\frac{[E]_0[I]}{K_i^* + [I]}\right) = \frac{K_i^*}{K_i}\left(\frac{[E]_0[I]}{K_i^*\left(1 + \frac{[S]}{K_M}\right) + [I]}\right)$$