Comparison of Methods for Analyzing Kinetic Data from Mechanism-Based Enzyme Inactivation: Application to Nitric Oxide Synthase

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ABSTRACT The goals of this study were (1) to investigate the performance of 2 classical methods of kinetic analysis when applied to data from enzyme systems in which mechanism-based inactivation and enzyme degradation are present, and (2) to develop and validate a nonlinear method of kinetic data analysis that may perform better under these situations. A composite equation was derived to link various parameters that govern the kinetics of mechanism-based inactivation, viz., enzyme activity, inhibitor-binding affinity (K_I), inactivation rate (k_{inact}), and enzyme degradation (k_{deg}). The relative accuracy and precision of parameter estimation by the Dixon and Kitz-Wilson methods and a new nonlinear method were evaluated by computer simulation. The behavior of these methods of analysis were validated experimentally, using the nitric oxide synthase enzyme, both in purified form and as expressed in murine macrophage cell cultures. We showed that the Dixon method, as expected, could not provide accurate estimates of K_I in the presence of either enzyme inactivation or instability. The Kitz-Wilson method could provide accurate estimates of these parameters; however, the precisions of these estimates were poorer than those obtained using the nonlinear method of analysis. We conclude that the nonlinear approach is superior to classical methods of data analysis for enzyme inhibitor kinetics, based on better efficiency, accuracy, and precision.

INTRODUCTION

Enzyme inhibition represents one of the most common strategies in the development of therapeutic drug candidates. The inhibitory potency and selectivity of these agents are important factors that govern the in vivo effect and risk of metabolic drug interactions. Because inhibitory potency is linearly related to the binding affinity (K_I), this parameter can be a valuable predictor of in vivo potency (1). In addition, K_I parameters are commonly used to screen inhibitors for isoform selectivity (2-4) and drug interaction potential (5). The reliability of K_I as an indicator of in vivo potency and selectivity is obviously dependent on the accuracy and precision of its in vitro estimation (5). Thus, it is essential that the method of data analysis provide accurate and precise estimates of K_I .

Classical methods frequently employed in screening inhibitors for K_I in vitro do not account for mechanism-based inactivation, which has been demonstrated for a wide variety of enzymes and inhibitors (6-11). Unlike competitive inhibitors, mechanism-based inactivators (MBEIs) are metabolized by the bound enzyme to a reactive intermediate that results in irreversible inactivation (12-14). Another process not accounted for by standard methods is enzyme degradation. Enzyme activity may diminish in vitro through enzymatic degradation (15). Use of the classical methods, in the presence of enzymatic instability or inactivation, may adversely influence the accuracy of K_I estimation. In addition, the linearization of data required by the classical methods may decrease the precision of K_I estimation.

The Dixon and Kitz-Wilson methods represent 2 of the most commonly used methods for the estimation of K_I (16,17). Both of these methods require the use

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of several assumptions and data linearization. The Dixon method requires an assumption of enzyme stability and of a strictly reversible binding mechanism of action. This method is based on the classical Michaelis-Menten equation for competitive inhibition.

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot \left(1 + \frac{[I]}{K_I}\right) + [S]}$$

Equation 1:

The Dixon method arises from a linearization of Equation 1 to afford an estimate of the K_I parameter.

$$\frac{1}{v} = \frac{1}{V_{max}} \cdot \left[1 + \frac{K_m}{[S]}\right] + \frac{K_m \cdot [I]}{V_{max} \cdot K_I}$$

Equation 2:

A plot of $1/v$ versus $[I]$ yields a straight line. When $1/v = 1/V_{max}$, Equation 2 reduces to Equation 3, allowing an estimation of the K_I parameter based on the absolute value of the inhibitor concentration at this point.

$$\text{Equation 3: } K_I = -[I]$$

An appropriate method for the kinetic analysis of mechanism-based inactivation has been described by Kitz and Wilson (16). This method is based on Equation 4, which describes the loss of functional enzyme with time. $E(0)$ represents the maximal enzyme activity following preincubation in the absence of inhibitor and $E(t)$ represents the maximal enzyme activity following preincubation with inhibitor for a certain time, t . The apparent inactivation rate (k_{app}) is described by the slope obtained when the left-hand side of Equation 4 is plotted against time. The value of k_{app} depends on $[I]$, K_I , and the first-order inactivation rate constant, k_{inact} .

$$\ln\left(\frac{E(t)}{E(0)}\right) = \frac{-k_{inact} \cdot t}{\left(1 + \frac{K_I}{[I]}\right)} = -k_{app} \cdot t$$

Equation 4:

Estimates of k_{inact} and K_I are obtained by linearizing the k_{app} portion of Equation 4 to obtain Equation 5.

Plotting $1/k_{app}$ versus $1/[I]$ yields a straight line with an intercept of $1/k_{inact}$ and a slope of K_I/k_{inact} .

$$\frac{1}{k_{app}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{inact} \cdot [I]}$$

Equation 5:

If enzyme loss due to instability is similar in the presence or absence of inhibitor, then changes in the ratio of $E(t)$ and $E(0)$ should exclusively reflect mechanism-based inactivation. Therefore, enzyme instability should not influence the accuracy of this method. However, the use of the Kitz-Wilson method requires data linearization that may adversely affect its precision.

Because of the widespread application of the K_I parameter in pharmacodynamic and pharmacokinetic predictions, we have examined the accuracy and precision of its estimation by these 2 classical estimation methods. Competitive binding, enzyme instability, and mechanism-based inactivation frequently occur simultaneously under in vitro conditions. The K_I parameter estimated by the Dixon method, under these circumstances, may not reflect its true value (18). Despite the required assumptions of enzyme stability and competitive inhibition, the Dixon method has been used, inappropriately, in the kinetic analysis of mechanism-based inactivation (19-21). Here, we seek to characterize the consequences of inappropriate application of the Dixon method in the presence of mechanism-based inactivation. Because parameter estimation by both Dixon and Kitz-Wilson methods requires several linearization steps, we have also examined the relative precision of parameter estimation by these methods. The accuracy and precision of parameter estimates obtained by these methods were examined using computer simulations and experimentally. In this report, we describe a nonlinear method that permits efficient, accurate, and precise estimation of both K_I and k_{inact} parameters. The new kinetic method of analysis was validated by comparison with the Kitz-Wilson method, using nitric oxide synthase and its MBEI, L-N^G-methyl-arginine (L-NMMA) (21).

MATERIALS AND METHODS

Chemicals and Supplies

Chemicals used in the study were purchased from Sigma Chemical Co. (St Louis, MO). Murine macrophage nitric oxide synthase (NOS) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Murine J774 cells were purchased from the American Type Culture Collection (Rockville, MD).

Effect of Inactivation and Instability on the K_I Estimated by the Dixon Method

Based on a scheme for mechanism-based inactivation (see Appendix), an equation for enzyme velocity (Equation A9) can be derived. This equation was used to generate theoretical data, under various rates of k_{deg} and k_{inact} ranging from 0.0 to 0.2 minutes⁻¹, which were then treated according to the Dixon method. For simulations, K_m , V_{max} , K_I , and t parameters were fixed to 2.5 μ M, 37 pmol/minute, 2.5 μ M, and 15 minutes, respectively. To explore the influence of time, additional simulations were performed at time points of 2.5, 5, 10, 20, 30, 40, and 50 minutes and at a fixed k_{inact} of 0.05 minutes⁻¹. The mean parameter values chosen for simulations are consistent with literature reports (19-21) and our own experimental observations, illustrated here with regard to mechanism-based inactivation of NOS by L-N^G-methyl-arginine (L-NMMA).

Effect of Enzyme Inactivation on the K_I of L-NMMA Estimated by the Dixon Method for NOS

J774 cells were grown to confluence in 75-cm² flasks in 12 mL of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were then incubated for 18 to 24 hours in the presence of 2 μ g/mL of lipopolysaccharide from *Serratia Marcescens* in complete medium for the induction of NOS. Following induction, the medium was removed and cells were suspended in fresh complete medium. One-mL suspensions of 1×10^6 cells were then added to 5-mL gas-tight reaction vials containing 100 units of superoxide dismutase. Vials were capped immediately following the addition of L-NMMA for final concentrations of 0, 150, 300,

600, and 1200 μ M. Appearance of nitric oxide was assayed by chemiluminescence at 10, 20, 30, 40, 50, and 60 minutes, as previously reported (22). To observe the influence of inactivation on K_I estimation, Dixon plots were constructed at the selected time points.

Estimation of the K_I and k_{inact} Parameters of L-NMMA for NOS by the Kitz-Wilson and the Nonlinear Method

In a gas-tight reaction vial, murine macrophage NOS (0.1 units/200 μ L) was preincubated for 10 minutes at 37°C with L-NMMA (0, 1.25, 2.5, 5.0, 10, 20, 40, or 80 μ M) in 800 μ L of 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.4, containing 1 mM magnesium acetate, 100 units/mL superoxide dismutase (SOD), 0.1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 60 μ M H₄B, 833 μ M dithiothreitol (DTT), and 20% glycerol. Following preincubation, 0.1 units of the enzyme (200 μ L of the solution) was added to 800 μ L of 15 mM HEPES buffer, pH 7.4, containing a final concentration of 2 mM L-arginine, 1 mM magnesium acetate, 0.15 mM NADPH, 24 μ M H₄B, 333 μ M DTT, 100 units/mL SOD, and 20% glycerol. Following a 15-minute incubation period, the amount of NO in the headspace of the reaction vial was quantified by chemiluminescence. K_I and k_{inact} were estimated using the standard Kitz-Wilson analysis, and by nonlinear parameter estimation using Equation 6 and PCNONLIN 4.2 (Statistical Consultants Inc., Lexington, KY). Equation 6 was obtained from a re-arrangement of Equation 4.

$$\frac{\ln\left(\frac{E}{E(0)}\right)}{t} = -\left(\frac{k_{inact} \cdot [I]}{[I] + K_I}\right)$$

Equation 6:

The mean and standard deviation of the reported K_I and k_{inact} values were obtained by analyzing the data from triplicate experiments.

Evaluation of the Precision of Parameter Estimates Obtained From the Various Kinetic Methods of Analysis

The accuracy and precision of the various methods were assessed using simulated data with observation error. Simulated data points were obtained using Equation A9 and ADAPT II (Biomedical Simulations Resource, CA). A variance model, which caused random observation error based on a coefficient of variation of 15%, was used to simulate experimental variability. For all simulations, K_I , K_m , and $[V]^{(0)}_{\max}$ were fixed at 2.5 μM , 2.5 μM , and 100 pmol/minute, respectively. The chosen distribution of mean parameter values is consistent with literature reports (19-21) and our own experimental observations, illustrated here with regard to mechanism-based inactivation of NOS by L-NMMA.

Theoretical data were generated using Equation A9 for kinetic analysis by the Dixon method. Triplicate sets of data with variability were generated for five inhibitor concentrations (1.0-5.0 μM) at 3 different substrate concentrations (1.25, 2.5, and 5.0 μM), for a total of 45 simulated observations. Consistent with the required assumptions for use of the Dixon analysis, k_{inact} and k_{deg} parameters were fixed at 0.0 minute^{-1} . Under these conditions, Equation A9 reduces to the standard competitive inhibition equation. Simulations were performed in triplicate to attain 3 separate estimates of the K_I parameter. This procedure was performed to assess the ability of the Dixon method to predict a true value of K_I of 2.5 μM under ideal conditions, in which no underlying assumptions are violated.

Using Equation A9, data were also simulated for analysis by the Kitz-Wilson method. Simulated data were analyzed by the classical Kitz-Wilson method depicted in Equations 4 and 5. Triplicate sets of $E(t)$ data were generated for 3 time points (10, 20, and 40 minutes) and 5 inhibitor concentrations (0.625-80 μM) for a total of 35 simulated observations. Triplicate sets of $E(0)$ data were generated for 3 time points (10, 20, and 40 minutes) in the absence of inhibitor for a total of 9 simulated observations. Therefore, a total of 44 simulated observations were used for this analysis. Data were generated in

triplicate to obtain 3 separate estimates of K_I and k_{inact} . This procedure was performed to assess the ability of the Kitz-Wilson method to predict a true value of K_I and k_{inact} of 2.5 μM and 0.05 minutes^{-1} .

The same data, simulated for the Kitz-Wilson analysis, were used to estimate K_I and k_{inact} by the proposed nonlinear method. Three estimates of K_I and k_{inact} were made using PCNONLIN 4.2 to describe the relationship between inhibitor concentration and rate of loss of enzyme activity depicted in Equation 6.

RESULTS

Effects of the Presence of Enzyme Inactivation and Degradation on the K_I Estimated by the Dixon Method

Equation A9 represents enzyme activity in a system in which competitive binding, mechanism-based inactivation, and enzyme instability occur simultaneously. This equation represents the model from which all theoretical data were generated. Using computer simulations, we obtained the classical form of the Dixon plot, independent of inactivation rate or exposure time to inactivation (Figure 1 and Figure 2, insets). Similar to the classical plot, the simulated lines converged at the point in which $1/v = 1/V_{\max}$. However, this convergence point was no longer purely a function of the true K_I value but was influenced by exposure time and inactivation rate. Our results indicate that the apparent estimate of K_I will decrease exponentially with increasing inactivation rate or exposure time to inactivation (Figure 1 and Figure 2). Unlike inactivation, the presence of enzyme degradation did alter the general appearance of the Dixon plot, although apparent linearity was preserved. Although the simulated lines converged at the theoretical value of $\sim 2.5 \mu\text{M}$ on the x-axis, this intersection occurred above the horizontal $1/V_{\max}$ line (Figure 3). The degree of deviation from the $1/V_{\max}$ line became larger with increasing degradation rates (data not shown).

Figure 1. Effect of concurrent enzyme inactivation on the K_I estimate obtained with the Dixon method. Using Equation 6, enzyme activity was simulated over a range of k_{inact} values between 0.0 and 0.2 minutes⁻¹. Apparent K_I values were obtained from the simulated data, using the classical Dixon method. Inset: Simulated Dixon plot assuming an inactivation rate of 0.2 minutes⁻¹. Each line represents a different substrate concentration: (open circle) 1.25, (open square) 2.50, and (open triangle) 5.00 μM . The horizontal line represents the reciprocal value of the V_{max} parameter.

Figure 2. Effect of exposure time on the K_I estimate obtained with the Dixon method. Using Equation 6, enzyme activity was simulated over a range of exposure times between 0 and 60 minutes. For all simulations, k_{inact} was fixed at 0.05 minutes⁻¹. Apparent K_I values were obtained from the simulated data, using the classical Dixon method. Inset: Simulated Dixon plot assuming an inactivation rate of 0.05 minutes⁻¹ and an exposure time of 60 minutes. Each line represents a different substrate concentration: (open circle) 1.25, (open square) 2.50, and (open triangle) 5.00 μM . The horizontal line represents the reciprocal value of the V_{max} parameter.

Figure 3. Simulated Dixon plot assuming a degradation rate of 0.20 minutes⁻¹. Each line represents a different substrate concentration: (open circle) 1.25, (open square) 2.50, and (open triangle) 5.00 μM . The horizontal line represents the reciprocal value of the V_{max} parameter.

Effect of Enzyme Inactivation on the K_I of L-NMMA Estimated by the Dixon Method for Nitric Oxide Synthase

Figure 4. Effect of exposure time on the estimated K_I of L-N^G-methyl-arginine for nitric oxide synthase expressed in murine macrophages as determined by the Dixon method. Cells were exposed to L-NMMA (0, 150, 300, 600, and 1200 μM) for times of 10 (solid circle), 20 (open circle), 30 (solid square), 40 (open square), 50 (solid triangle), and 60 (open triangle) minutes before quantifying nitric oxide in the headspace by chemiluminescence. The horizontal line represents the reciprocal V_{max} of the system. Data are plotted in the standard Dixon format for each exposure time. Symbols represent the mean of triplicate experiments. The horizontal line represents the reciprocal value of the V_{max} parameter. Inset: Data points represent the K_I values obtained by Dixon analysis at each exposure time.

When the K_I was determined at different times of L-NMMA incubation with NOS in J774 cells, the resultant Dixon plots were also apparently linear, but the estimated K_I values were time dependent (Figure 4). As predicted by Equation A9, the apparent K_I values estimated by the Dixon method decreased exponentially with exposure time to L-NMMA (Figure 4, inset).

K_I and k_{inact} of L-NMMA for NOS, as Estimated by the Kitz-Wilson and the Nonlinear Method

The Kitz-Wilson method could be applied to the inhibition data of L-NMMA and NOS to allow estimation of both the K_I and k_{inact} parameters (Figure 5).

Figure 5. Kitz-Wilson plot for L-N^G-methyl-arginine and murine macrophage nitric oxide synthase. Symbols represent the raw data of triplicate mechanism-based inactivation experiments. The line represents the best-fit linear regression through the raw data.

From the Kitz-Wilson analysis, we estimated K_I and k_{inact} to be $1.55 \pm 1.15 \mu\text{M}$ and $0.07 \pm 0.01 \text{ minutes}^{-1}$, respectively. Using Equation 6 and PCNONLIN, we were able to describe the same data set obtained for L-NMMA and NOS (Figure 6). Using this nonlinear method, we obtained K_I and k_{inact} estimates of $1.35 \pm 0.22 \mu\text{M}$ and $0.07 \pm 0.00 \text{ minutes}^{-1}$, respectively. Estimates obtained by the 2 methods were not significantly different, as determined by Student's *t* test ($P < 0.05$).

Figure 6. Plot of the rate of loss of murine macrophage nitric oxide synthase activity versus L-N^G-methyl-arginine concentration. Symbols represent the raw data of triplicate mechanism-based inactivation experiments. The line represents the best-fit nonlinear regression through the raw data using the proposed nonlinear model.

Precision of Parameter Estimates Obtained From the Various Methods

Under ideal conditions, all 3 methods yielded accurate estimates of K_I ($2.5 \mu\text{M}$) and k_{inact} ($0.05 \text{ minutes}^{-1}$) from the simulated data with variability. However, the highest precision of estimation was obtained by the nonlinear method, as evidenced in the smaller coefficient of variation of the parameter estimates (Table 1).

Table 1. Accuracy and precision of parameter estimation by the Dixon, the Kitz-Wilson, and the nonlinear method, using simulated data with variability. Data were simulated using ADAPT. Random observation error was applied to the data based on 15% coefficient of variation. $k_{deg} = 0.0 \text{ minutes}^{-1}$ for all simulations. N/A = not applicable. Data are expressed as mean $\pm$ SD of triplicate simulations. Values in parentheses represent the CV% of the estimate.

Method	True K_I (μM)	K_I estimate (μM)	True k_{inact} (min^{-1})	k_{inact} estimate (min^{-1})
Dixon	2.5	2.13 ± 0.74 (35)	0.00	N/A
Kitz-Wilson	2.5	2.82 ± 0.65 (23)	0.05	0.043 ± 0.010 (25)
Nonlinear	2.5	2.36 ± 0.48 (20)	0.05	0.052 ± 0.006 (9)

DISCUSSION

Accurate and precise estimation of an enzyme inhibitor binding affinity for selected enzymes is an important step in predicting in vivo potency, selectivity, and potential for metabolic interaction. The binding affinity is sometimes estimated in vitro under conditions in which many enzymes are unstable. In addition, metabolism of inhibitors can result in mechanism-based enzyme inactivation. Because many kinetic methods of analysis do not account for the simultaneous effects of reversible binding, mechanism-based inactivation, and enzyme degradation, the K_i parameter estimated in such systems may be inaccurate. In addition, the linear transformation of data required by classical methods may result in imprecise estimates of K_i by distorting the observed experimental error (23). To gain an appreciation of the kinetic processes governing these systems, we have derived an equation for enzyme activity that accounts for k_{inact} and k_{deg} (Equation A9). This mathematical relationship is consistent with the known kinetic behavior of mechanism-based inactivation systems. Equation A9 was used to generate theoretical data, which were analyzed by several methods of analysis to determine accuracy and precision of parameter estimation. The Dixon and Kitz-Wilson methods were chosen because of their widespread use in the analysis of the kinetics of enzyme inhibition. Results obtained from these methods were compared to that obtained using a nonlinear method.

When the Dixon method was applied to the theoretical data, we observed that the inactivation rate (k_{inact}) and exposure time significantly influenced the resultant K_i estimate. Increasing inactivation rates or exposure times to inactivation led to an exponentially increasing degree of inaccuracy (Figure 1 and Figure 2). Despite the greater than 10-fold estimation error observed at the highest inactivation rates or exposure times, the general appearance of the Dixon plot remained unaltered (Figure 1 and Figure 2, insets). Consistent with these predictions, we have observed a time-dependent decrease in the apparent K_i parameter of L-NMMA for NOS in cell culture (Figure 4, inset). This exponential decline in the K_i estimate occurs despite the observation of linear

Dixon plots (Figure 4). Unlike inactivation, the presence of enzyme degradation did affect the general form of the Dixon plot. Higher rates of degradation (k_{deg}) led to a convergence at points farther above the horizontal line at $1/V_{\text{max}}$ (Figure 3). These observations have several implications for the use and interpretation of the Dixon analysis. Because the inherent assumption of competitive antagonism is violated, it is well known to experienced kineticists that the Dixon method should not be applied to MBEIs. However, results of our study indicate that the observation of a linear Dixon plot does not exclude the presence of mechanism-based inactivation. In fact, this work demonstrates that the classical Dixon plot can be obtained despite extensive inactivation. Consistent with our observation, classical Dixon plots and Kitz-Wilson plots have both been applied in the literature for reporting the kinetics of MBEIs of NOS (19,24). In addition, the observed form of the Dixon plot resulting from enzyme degradation may erroneously be interpreted as resulting from a noncompetitive inhibitory mechanism.

The Kitz-Wilson method accounts for enzyme inactivation and is insensitive to enzyme instability. Therefore, it is a relatively more robust method for the estimation of the K_i parameter. However, this method requires data linearization and parameter estimation by a 2-step linear regression (Equations 4 and 5), which may adversely influence the accuracy and precision of estimation, particularly when the data contain substantial variabilities. Our nonlinear method offers several advantages in determining the kinetics of these systems. From our experimental data of in vitro NOS inhibition by its MBEI, L-NMMA, the Kitz-Wilson method provided estimates of K_i and k_{inact} of $1.55 \pm 1.15 \mu\text{M}$ and $0.07 \pm 0.01 \text{ minutes}^{-1}$, respectively (Figure 5). In comparison, the nonlinear method provided estimates of K_i and k_{inact} of $1.35 \pm 0.22 \mu\text{M}$ and $0.07 \pm 0.00 \text{ minutes}^{-1}$, respectively (Figure 6). These estimates were not significantly different from one another ($P > 0.05$, Student's *t* test), but the precision appeared to be improved using the nonlinear method.

To further evaluate the precision of the 3 methods, we used ADAPT II to place random measurement

errors on the simulated observations. Under conditions in which no underlying assumption was violated, all 3 methods provided accurate estimates of the inhibitory parameters (data not shown). However, the highest precision was observed using the nonlinear method (Table 1). The higher precision observed with the nonlinear method is, at least in part, a result of using nonlinear parameter estimation instead of the linear transformation and regression used for the other 2 methods.

In conclusion, these results indicate that the Dixon analysis would yield plots of classical appearance in spite of the presence of mechanism-based inactivation. Because the K_i estimate becomes time-dependent under these circumstances, estimation of this parameter at 2 time points may be an efficient way to detect the presence of enzyme inactivation. Our work also suggests that a nonlinear method can provide more precise parameter estimates than the classical Kitz-Wilson method in the presence of mechanism-based enzyme inactivation. Precision may be further enhanced using nonlinear parameter estimation programs such as ADAPT II that allow estimation of appropriate data weighing functions. Enhanced precision, as observed with this nonlinear estimation method, may afford an improved ability to tease out the relative potency and selectivity of inhibitor candidates, and to allow more efficient screening by minimizing the number of data points required for parameter estimation. In addition, the form of the nonlinear equation lends itself to more efficient parameter estimation. Equation 6 requires data acquired at only 1 time point, unlike the several time points required for the Kitz-Wilson method. Both the Dixon and Kitz-Wilson methods were developed before the widespread availability of computers and software designed for nonlinear parameter estimation. Now that these resources are widely available, nonlinear methods, such as the one described here, should be used to obtain more accurate and precise estimates of kinetic parameters in these systems.

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REFERENCES

1. Cheng Y, Prusoff WH. Relationship between the inhibition constant K_i and the concentration of inhibitor which causes 50 per cent inhibition I_{50} of an enzymatic reaction. *Biochem Pharmacol*. 1973;22:3099-3108.
2. Furfine ES, Harmon MF, Paith JE, Knowles, RG, Salter M, Kiff, RJ, et al. Potent and selective inhibition of human nitric oxide synthases. Selective inhibition of neuronal nitric oxide synthase by S-methyl-L-thiocitrulline and S-ethyl-L-thiocitrulline. *J Biol Chem*. 1994;269:26677-26683.
3. Garvey EP, Oplinger JA, Tanoury GJ, Sherman PA, Fowler M, Marshall S, et al. Potent and selective inhibition of human nitric oxide synthases. Inhibition by non-amino acid isothioureas. *J Biol Chem*. 1994;269:26669-26676.
4. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ, et al. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J Biol Chem*. 1997;272:4959-4963.
5. Bertz RJ, Granneman GR. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet*. 1997;32:210-258.
6. Ghosh SS, Said-Nejad O, Roestamadj J, Mobashery S. The first mechanism-based inactivators for angiotensin-converting enzyme. *J Med Chem*. 1992;35:4175-4179.
7. Kalgutkar AS, Castagnoli Jr N. Synthesis of novel MPTP analogs as potential monoamine oxidase B MAO-B inhibitors. *J Med Chem*. 1992;35:4165-4174.
8. Wade ML, Voelkel NF, Fitzpatrick FA. "Suicide" inactivation of prostaglandin I_2 synthase: characterization of mechanism-based inactivation with isolated enzyme and endothelial cells. *Arch Biochem Biophys*. 1995;321:453-458.
9. Wolff DJ, Gauld DS, Neulander MJ, Southan G. Inactivation of nitric oxide synthase by substituted aminoguanidines and aminoisothioureas. *J Pharmacol Exp Ther*. 1997;283:265-273.
10. Marks GS, McCluskey SA, Mackie JE, Riddick DS, James CA. Disruption of hepatic heme biosynthesis after interaction of xenobiotics with cytochrome P-450. *FASEB J*. 1988;2:2774-2783.
11. Bondon A, Macdonald TL, Harris TM, Guengerich FP. Oxidation of cycloalkylamines by cytochrome P-450. Mechanism-based inactivation, adduct formation, ring expansion, and nitrene formation. *J Biol Chem*. 1989;264:1988-1997.
12. Rando RR. Mechanism-based enzyme inactivators. *Pharmacol Rev*. 1984;36:111-142.
13. Rando RR. Chemistry and enzymology of kcat inhibitors. *Science*. 1974;85:320-324.
14. Palfreyman MG, Bey P, Sjoerdsma A. Enzyme-activated/mechanism-based inhibitors. *Essays Biochem*. 1987;23:28-81.
15. Chen PF, Tsai AL, Wu KK. Cysteine 99 of endothelial nitric oxide synthase NOS-III is critical for tetrahydrobiopterin-

dependent NOS-III stability and activity. *Biochem Biophys Res Comm.* 1995;215:1119-1129.

16. Kitz R, Wilson IB. Esters of methanesulfonic acid as irreversible inhibitors of Acetylcholinesterase. *J Biol Chem.* 1962;237:3245-3249.
17. Dixon M. The determination of enzyme inhibitor constants. *Biochem J.* 1952;55:170-171.
18. Silverman R. Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology. Boca Raton, FL: CRC Press; 1988.
19. Reif DW, McCreedy SA. N-nitro-L-arginine and N-monomethyl-L-arginine exhibit a different pattern of inactivation toward the three nitric oxide synthases. *Arch Biochem Biophys.* 1995;320:170-176.
20. Olken NM, Marletta MA. NG-allyl- and NG-cyclopropyl-L-arginine: two novel inhibitors of macrophage nitric oxide synthase. *J Med Chem.* 1992;35:1137-1144.
21. Olken NM, Rusche KM, Richards MK, Marletta MA. Inactivation of macrophage nitric oxide synthase activity by NG-methyl-L-arginine. *Biochem Biophys Res Comm.* 1991;177:828-833.
22. Boje KM, Fung HL. Endothelial nitric oxide generating enzymes in the bovine aorta: subcellular location and metabolic characterization. *J Pharmacol Exp Ther.* 1990;253:20-26.
23. Motulsky HJ, Ransnas LA. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* 1987;1:365-374.
24. Olken NM, Marletta MA. NG-methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry.* 1993;32:9677-9685.

APPENDIX

Derivation of Equation A9

For the purposes of the derivation, it was assumed that enzyme degradation occurs at the same rate for free and bound concentrations of the enzyme (illustrated below). Although the validity of this assumption may be questionable, because of the stabilizing properties of many ligand-protein systems, this assumption is applied here for all methods of kinetic analysis. The implications of violating this assumption are beyond the scope of this work. Another assumption made in this derivation is that a rapid equilibrium occurs for both substrate and inhibitor binding. Therefore, additional considerations should be made for the rate constants of association and disassociation when this rapid equilibrium assumption is violated.

From the reaction scheme, [E], [EI], [ES], [E]* represent concentrations of free, inhibitor-bound, substrate-bound, and inactivated enzyme, respectively. [P], [S], and [I] represent concentrations of product, substrate, and inhibitor, respectively. K_m and K_I represent the binding affinity constants of substrate and inhibitor, respectively. k_{deg} and k_{inact} represent the first-order rate constants for the degradation of enzyme activity due to instability and inactivation, respectively. V_{max} represents the maximal velocity. v represents the velocity at a particular time and inhibitor concentration.

If $[E]_f$ represents the concentration of enzyme, free and bound, that is functionally catalytic, then the concentration of [EI] relative to $[E]_f$ is as follows:

$$\frac{[EI]}{[E]_f} = \frac{\frac{[E] \cdot [I]}{K_I}}{[E] + \frac{[E] \cdot [S]}{K_m} + \frac{[E] \cdot [I]}{K_I}} \quad (A1)$$

Simplifying:

$$[EI] = \frac{[E]_f \cdot [I]}{[I] + K_I \left(1 + \left(\frac{[S]}{K_m} \right) \right)} \quad (A2)$$

Based on the reaction scheme, the differential equation for $[E]_f$ is:

$$\frac{d[E]_f}{dt} = -[E]_f \cdot k_{deg} - [EI] \cdot k_{inact} \quad (A3)$$

Substituting Equation A2 into Equation A3:

$$\frac{d[E]_f}{dt} = -[E]_f \cdot k_{deg} - \frac{[E]_f \cdot [I]}{[I] + K_I \left(1 + \left(\frac{[S]}{K_m} \right) \right)} \cdot k_{inact} \quad (A4)$$

Integrating $[E]_f$ with respect to time:

$$[E]_f = [E]_f^{(0)} \cdot e^{-\left(k_{deg} + \frac{[I]}{[I] + K_I \left(1 + \left(\frac{[S]}{K_m} \right) \right)} \cdot k_{inact} \right) \cdot t} \quad (A5)$$

Expressing $[ES]$ in relation to $[E]_f$:

$$\frac{[ES]}{[E]_f} = \frac{\frac{[E] \cdot [S]}{K_m}}{[E] + \frac{[E] \cdot [S]}{K_m} + \frac{[E] \cdot [I]}{K_I}} \quad (A6)$$

Simplifying:

$$[ES] = \frac{[E]_f \cdot [S]}{[S] + K_m \left(1 + \frac{[I]}{K_I} \right)} \quad (A7)$$

Substituting A5 into Equation A7:

$$[ES] = \frac{[E]_f^{(0)} \cdot e^{-\left(k_{deg} + \frac{[I]}{[I] + K_I \left(1 + \left(\frac{[S]}{K_m} \right) \right)} \cdot k_{inact} \right) \cdot t} \cdot [S]}{[S] + K_m \left(1 + \frac{[I]}{K_I} \right)} \quad (A8)$$

Multiply both sides by k_{cat} :

$$v = \frac{[V]_{max}^{(0)} \cdot e^{-\left(k_{deg} + \frac{[I]}{[I] + K_I \left(1 + \left(\frac{[S]}{K_m} \right) \right)} \cdot k_{inact} \right) \cdot t} \cdot [S]}{[S] + K_m \left(1 + \frac{[I]}{K_I} \right)} \quad (A9)$$