Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 17, pp. 1947-1952, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00330-4

ESTIMATING K_I VALUES FOR TIGHT BINDING INHIBITORS FROM DOSE-RESPONSE PLOTS

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Abstract. A simple method is described by which the K_i value of tight binding inhibitors of enzymes can be calculated directly from the IC_{50} value determined from graphical analysis of dose-response curves. Experimental verification of this method is provided by study of 15 inhibitors of the enzyme stromelysis which span a broad range of K_i values.

Designing potent inhibitors of selected enzymes is a major goal of many industrial and academic laboratories. These efforts typically require high-throughput screening of large numbers of potential inhibitors in order to establish structure-activity relationships that drive the further design of new and better inhibitory compounds. The relative potency of inhibitors is normally quantified by comparing their dissociation constants (K_i) for the enzyme under study. In the case of tight binding inhibitors, where the K_i values approach the concentration of enzyme in solution, difficulties occur in determining the true dissociation constant for the inhibitor. In this letter we report the derivation of a simple method for determining the K_i value of a tight binding competitive inhibitor from dose-response curves of enzyme velocity as a function of total inhibitor concentration, and the experimental verification of this method for 15 competitive inhibitors of the enzyme stromelysis (MMP3; EC 3.4.24.17). During the preparation of this letter it was brought to our attention that the equation we have derived has previously been presented[1-3] in the literature. The value of this method for high throughput screening of tight binding enzyme inhibitors has not, however, been widely appreciated, nor has the method been adequately verified by experimental data. For these reasons we felt that it would be useful to the readership of this journal to reintroduce this derivation and illustrate its potential for high throughput screening of enzyme inhibitors.

Classically, the K_i value is determined from studies of the effect of inhibitor concentration on the activity of the enzyme under conditions of varying substrate concentration. This approach is time consuming and laborious, and hence is not conducive to high throughput screening efforts. A convenient alternative method is to construct a dose-response plot in which one plots the fractional activity of the enzyme in the presence of inhibitor as a function of inhibitor concentration at fixed concentrations of enzyme and substrate. These data can then be fit to equation 1 to yield an estimate of the IC_{50} value of the inhibitor.

$$\frac{v}{v_0} = \frac{1}{1+\left(\frac{[I]}{IC_{50}}\right)} \quad (1)$$

Here v and v_0 are the velocities of the enzyme catalyzed reaction in the presence and absence of inhibitor, respectively, [I] is the concentration of inhibitor present, and the IC_{50} is that concentration of inhibitor required to reduce the reaction velocity to one half that observed in the absence of inhibitor. The IC_{50} value determined in this way can then be converted to the inhibitor K_i by use of the equations of Cheng and Prusoff,[4] assuming that one knows the mode of inhibition (i.e., competitive, noncompetitive, uncompetitive, etc.) for that inhibitor. A great advantage of this procedure is that one can use a broad range of inhibitor concentrations for constructing the dose-response plots. This is particularly advantageous in high throughput screening where the appropriate range of inhibitor concentrations needed to effect near 50% inhibition varies widely from one inhibitor to another, and one does not have prior knowledge of what the appropriate concentration range might be for a particular inhibitor.

In a number of cases, efforts towards designing inhibitors have proved so successful that enzymologists are commonly screening compounds with dissociation constants that approach the concentration of total enzyme in the activity assay. Under these conditions one can no longer assume that the concentration of free inhibitor in solution is well approximated by the concentration of total inhibitor added, because depletion of the free inhibitor concentration by formation of enzyme-inhibitor complexes becomes significant. Hence, the classical treatments of enzyme inhibition data are no longer valid in these cases. For these situations, Henderson[5] has introduced an alternative method for determining the K_i value of tight binding inhibitors under steady state conditions, which makes use of equation 2:

$$\frac{[I]}{\left(1-\frac{v}{v_0}\right)} = \frac{v_0}{v} K_i \left(1+\frac{[S]}{K_m}\right) + [E_t] \tag{2}$$

where [S] and $[E_t]$ are the total concentrations of substrate and enzyme, respectively, and K_m and K_i are the Michaelis constant for the substrate and the dissociation constant for the inhibitor, respectively. Thus if one were to plot $[I]/(1-v/v_0)$ as a function of v_0/v, equation 2 would predict a linear relationship between these parameters, with slope equal to $K_i(1 + [S]/K_m)$ and y-intercept equal to $[E_t]$. In principal, then, one could determine the K_i value of a tight binding competitive inhibitor by measuring the diminution of enzyme activity with varying concentrations of inhibitor at a fixed substrate concentration, and application of equation 2. Two problems arise in the use of this strategy for high throughput screening. The first is that, despite the linearity predicted by equation 2, one finds that the experimental Henderson plots are only linear over a finite range of v/v_0 values. We have determined that when the value of v/v_0 is less than 0.2 or greater than 0.9, one observes significant deviation from the predicted linearity (data not shown) which severely limits the precision of ones estimation of K_i. As illustrated in Figure 1, if one restricts the data to those points for which v/v_0 falls between 0.2 and 0.9, the fit to equation 2 is very good. The difficulty with this approach for high throughput screening is that one does not

know *a priori* what concentrations of inhibitor will yield v/v_0 values in the usable range. In favorable circumstances one may obtain enough usable data points for construction of a Henderson plot, but this will be largely fortuitous. Even in these cases, one will be forced to disregard a potentially large number of data points that fall outside of the usable range. A second, albeit less severe, problem with the use of Henderson plots for high throughput screening is the increased number of data manipulations that must be performed before these plots can be constructed. For these reasons we sought a means by which the full range of experimental data points might be used in estimating the K_i values for a large and diverse group of inhibitors, that required a minimum of complex data manipulations.

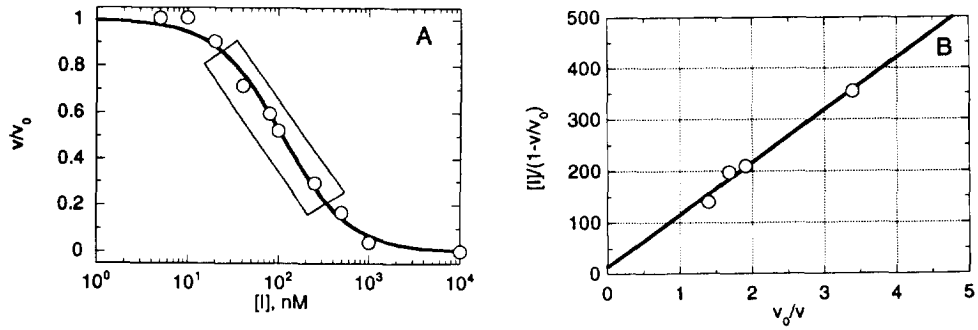

Figure 1. **(A)** Dose-response plot for a typical competitive inhibitor of human recombinant MMP3. The solid line drawn through the data represents the non-linear least squares best fit to equation 1. The rectangle encloses that region of the dose-response curve for which data can be used in construction of Henderson plots. **(B)** Henderson plot of selected data from **(A)**. The line drawn through the data represents the linear least squares fit to equation 2. See text for further details.

Reconsidering equation 2 in light of our definition of IC_{50} (*vide supra*), we find that the K_i value of a tight binding competitive inhibitor can be determined directly from the IC_{50} value of the inhibitor in situations where $[E_t]$, [S], and K_m have been determined precisely. Recall that when [I] = IC_{50}, the value of v/v_0 is, by definition, 0.5. Of course, it follows that under these conditions the value of v_0/v must be equal to 2.0. Using this information we may recast equation 2 as follows:

$$K_i = \frac{\left(IC_{50} - \frac{[E_t]}{2}\right)}{\left(1 + \frac{[S]}{K_m}\right)} \quad (3)$$

Thus, one could test the effects of an inhibitor on enzyme activity over a broad range of inhibitor concentrations, as in Figure 1A, and use all of the data to determine the IC_{50} value by use of equation 1. Knowing the values of $[E_t]$, [S], and K_m one could then directly convert the measured IC_{50} value into the K_i value for the inhibitor by use of equation 3. This method is very simple to use, and allows one to use the full range of data obtained from a broad screening of inhibitor concentrations. A danger in use of this method is that it relies very heavily on the precision of ones determinations of IC_{50} and $[E_t]$. Using a large number (e.g., 10) and broad range of inhibitor concentrations (e.g., several orders of magnitude) ensures that good estimates of IC_{50} can be obtained by application of equation 1 to experimental data. The value of $[E_t]$ must reflect the total concentration of enzyme active sites capable of catalysis, and not merely the total protein concentration. This is best determined by titration of an enzyme stock solution with a tight binding inhibitor under conditions where the enzyme and inhibitor concentrations are both much greater than the K_i. Various spectroscopic methods have been used to follow the course of such titrations, based on changes in signal from the enzyme or the inhibitor upon complex formation. For our studies (see below) we followed the titration by the effect of the inhibitor on the total enzymatic activity of our stock solution. An additional limitation of the current method is that the IC_{50} values obtained are only valid under equilibrium conditions. Some tight binding inhibitors show slow binding kinetics and thus display a time dependence of their inhibitory potencies (these are referred to as slow, tight binding inhibitors). For such inhibitors a prolonged preincubation of the enzyme with the inhibitor must proceed initiation of the reaction with substrate to ensure that true equilibrium is established. Often, however, it is mechanistically informative to follow the kinetics of inhibition for time dependent inhibitors. The present method would not be particularly useful for these types of studies.

To test the reliability of the current method, we have studied the effects of 15 competitive inhibitors on the activity of the matrix metalloproteinase, stromelysin (MMP3). For this study, purified recombinant human MMP3[6] was used at a final concentration of 10 nM in each assay. The activity of the enzyme was measured using a fluorescence assay that utilized the fluorescent peptide described by Knight et al.[7] We determined the K_m of this peptide for MMP3 to be 8.2 μM; the final concentration of substrate used in each assay was 10 μM. Figure 1A illustrates a typical dose-response curve for one of the inhibitors used here. The solid line drawn through the data represents the non-linear least squares best fit of the data to equation 1, which allows us to determine the IC_{50} value for the inhibitor as described above. The excellent fit of the experimental data to equation 1, seen in this Figure, is typical of the quality of the data for all 15 inhibitors tested here. The region of the dose-response curve that is enclosed by the rectangle in Figure 1A represents the limited region over which we find good agreement

between the data and equation 2, when the data are analyzed by Henderson plots. Figure 1B illustrates the Henderson plot constructed with the usable data from Figure 1A. For the 15 inhibitors studied, we have constructed both dose-response and Henderson plots as seen in Figures 1A and 1B, and determined the K_i values for these inhibitors from these plots using equations 2 and 3. Figure 2 illustrates the agreement between

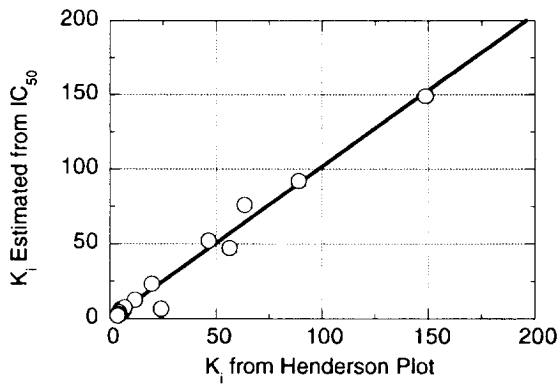

Figure 2. Correlation of K_i values (reported in nanomolar units) estimated from IC_{50} values by equation 3 (y-axis) and those determined from Henderson plots according to equation 2 (x-axis) for 15 competitive inhibitors of MMP3 . The solid line drawn through the data represents the linear least squares best fit.

these K_i estimates. The 15 inhibitors tested here span a broad range of potencies, with K_i values from ca. 2 to 150 nM. If both methods of determining inhibitor potency yielded exactly identical values of K_i one would expect the data in Figure 2 to be fit by a straight line with slope of 1.00 and y-intercept equal to zero. The line drawn through the data in Figure 2 represents the linear least squares best fit of the data, and has slope and y-intercept values of 1.02 and -0.8 respectively. These values are remarkably close to the expected values for perfect agreement between the two methods.

The data presented here suggest that our simple method for determining K_i values for tight binding inhibitors from dose-response plots provides estimates of inhibitor potency that are comparable to those obtained by Henderson analysis. This simple method can very conveniently be used in high throughput screening of large numbers of potential inhibitors, as long as one can obtain reasonably precise estimations of $[E_t]$, [S]. and K_m. While we have illustrated the use of this method for competitive inhibitors, equation 3 can be adapted easily for use with other inhibitor types as well.

Acknowledgments

We thank Randine Dowling for help in data analysis, June Davis for helpful suggestions, and Stuart Rosenfeld, Jodie Duke and Milton C. Hillman, Jr. for providing the recombinant MMP3 used in this study.

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(*Received in USA* 1 *June* 1995; *accepted* 20 *July* 1995)