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Kinetic determination of tight-binding impurities in enzyme inhibitors

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Abstract

A novel rate equation to characterize the dose-response behavior of a moderately potent ("classical") enzyme inhibitor contaminated with a very potent ("tight-binding") impurity is derived. Mathematical properties of the new rate equation show that, for such contaminated materials, experimentally observed I_{50} values are ambiguous. The four-parameter logistic equation, conventionally used to determine I_{50} values, cannot be used to detect the presence of tight-binding impurities in inhibitor samples. In contrast, fitting the newly derived rate equation to inhibitor dose- response curves can, in favorable cases, reveal whether the unknown material is chemically homogeneous or whether it is contaminated with a tight-binding impurity. The limitations of our method with respect to the detectable range of inhibition constants (both classical and tight-binding) were examined by using Monte-Carlo simulations. To test the new analytical procedure experimentally, we added a small amount (0.02 mole%) of a tight-binding impurity ($K_i = 0.065$ nM) to an otherwise weak inhibitor of human mast-cell tryptase ($K_i = 50.4$ μM). The resulting material was treated as "unknown." Our kinetic equation predicts that such adulterated material should show $I_{50} = 0.40$ μM, which was identical to the experimentally observed value. The best-fit value of the apparent inhibition constants for the tight-binding inhibitor was $K_i = (0.107 \pm 0.035)$ nM, close to the true value of 0.065 nM.
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Classical enzyme inhibitors are characterized by apparent inhibition constants (K_i) that are very much larger than the enzyme concentration ($[E]_0$) in a given assay. In contrast, tight-binding inhibitors are characterized by K_i values comparable in magnitude with $[E]_0$ or even much smaller ($K_i << [E]_0$). In this paper, we propose a new method of analyzing initial velocity data obtained with mixtures of classical and tight-binding inhibitors.

Our research has been motivated by anecdotal evidence that occasionally enzyme inhibitor samples encountered in screening for drug discovery are not chemically pure. For example, using analytical methods recently described in this journal [1,2], we have determined K_i values for hundreds of thousands of inhibitors. However, for certain inhibitors we found that the dose-response behavior deviated from the expected theoretical model. The question of whether this anomalous kinetic behavior could be explained by the presence of tight-binding impurities in inhibitors that are weakly potent, or even completely inactive arose.

To address this problem, we derived a rate equation that describes the dose-response kinetics of inhibitor mixtures consisting of one tight-binding inhibitor and one classical inhibitor. The mathematical properties of our equation show that the experimentally observed I_{50} values are ambiguous. Each observed value of I_{50} can arise in principle by infinitely many combinations of the classical inhibition constant, the tight-binding inhibition constant, and the molar fraction of tight-binding impurity. This is in contrast with chemically pure samples, for which Cha's equation [3] or the Cheng–Prusoff equation [4] give an unambiguous relationship between K_i and I_{50}.

The predictive power of the new rate equation was established in a series of Monte-Carlo simulations, using more than a million synthetic data sets that were similar

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in experimental design to those typically generated in the laboratory. We found that, under certain conditions, it is indeed possible to diagnose the presence of a tight-binding impurity in an otherwise moderately active or inactive sample. In most cases, the inhibition constant of the tight-binding contaminant could be determined reliably, but the "classical" inhibition constant of the weakly potent major component could not.

To verify our new kinetic method experimentally, we created an artificially impure inhibitor sample. A weakly potent inhibitor of human mast-cell tryptase (benzamidine, $K_i = 50\,\mu M$) was doped with a very small amount (0.02%) of the tight-binding inhibitor CRA-001390 ($K_i = 0.065\,nM$) [1]. The resulting "unknown" inhibitor had $I_{50} = 0.4\,\mu M$, exactly as predicted by our theoretical model.

The nonlinear least-squares fit to the commonly used four-parameter logistic equation gave no indication that the sample was not chemically homogeneous. In contrast, fitting the same experimental data to the mechanism-based Morrison equation [5,6] clearly revealed systematic discrepancies, suggesting the presence of a tight-binding impurity. Using our newly derived kinetic equation as the fitting model, we obtained a very satisfactory fit. The best-fit values were $K_i = (0.107 \pm 0.035)\,nM$ for the tight-binding inhibition constant and $(0.09 \pm 0.02)\%$ for the molar fraction of the tight-binding impurity. The apparent inhibition constant for benzamidine could not be determined.

Our new method of kinetic analysis should be of interest in all biochemical laboratories where large numbers of enzyme inhibitors are evaluated with the goal of extracting apparent inhibition constants or I_{50} values from dose-response curves. Many such laboratories are involved in secondary screening of enzyme inhibitors as potential therapeutics.

Methods

Materials

Tos-Gly-Pro-Lys-pNA (Sigma, St. Louis, MO) and benzamidine (Sigma-Aldrich, Milwaukee, WI) were purchased from the indicated commercial sources. Mast-cell tryptase was purified from the immortalized human mast-cell line, HMC-1, as previously reported [7]. CRA-001390 was synthesized at Celera Genomics (formerly Axys Pharmaceuticals compound APC-1390) as described [8].

Experimental

Kinetic measurements were performed in a total reaction volume of $100\,\mu L$ in 96-well U-bottomed microtiter plates (Falcon). Measurements of substrate hydrolysis were made using a SpectraMax 250 kinetic plate reader (Molecular Devices, Sunnyvale, CA). Tryptase (50 pM active site) was combined with inhibitor at varying concentrations in a buffer containing 50 mM Tris (pH 8.2), 150 mM NaCl, 0.05% Tween 20, 10% dimethyl sulphoxide and $50\,\mu g/ml$ heparin for 1 h at room temperature. Control reactions in the absence of inhibitor were performed in replicates of eight. Reactions were initiated by the addition of substrate (0.5 mM Tos-Gly-Pro-Lys-pNA) and the rate of substrate hydrolysis was measured by monitoring the change in absorbance at 405 nm over 10 min. Initial velocities were determined by linear regression of progress curves.

Computational

I_{50} values were determined by nonlinear regression of percentage inhibition data by using the four-parameter logistic equation (1), where p ("percentage inhibition") is the relative decrease in enzyme activity due to the inhibitor concentration $[I]_0$. The regression analysis was performed by the commercial software packages Prism (GraphPad) and SigmaPlot (SPSS)

$$p = p_{\min} + \frac{p_{\max} - p_{\min}}{1 + ([I]_0/I_{50})^n}. \qquad (1)$$

Initial velocity data were analyzed by nonlinear regression using a modification of the Marquardt algorithm [9]. The following equations have been employed as theoretical models. Eq. (2) applies to a pure tight-binding or classical inhibitor. Eq. (3) applies to a single tight-binding or classical inhibitor present as an impurity (molar fraction α) in a completely inactive bulk material

$$v = v_0 \frac{[E]_0 - [I]_0 - K_T + \sqrt{([E]_0 - [I]_0 - K_T)^2 + 4[E]_0 K_T}}{2[E]_0}, \qquad (2)$$

$$v = v_0 \frac{[E]_0 - \alpha[I]_0 - K_T + \sqrt{([E]_0 - \alpha[I]_0 - K_T)^2 + 4[E]_0 K_T}}{2[E]_0}. \qquad (3)$$

The kinetics of a tight-binding inhibitor (apparent inhibition constant K_T, molar fraction α) mixed with a classical inhibitor (apparent inhibition constant K_C) is described by Eq. (4), where the auxiliary variable β is defined by Eq. (5). We have derived Eq. (4) by using the algebraic method of Segel [10, p. 22] based on the rapid-equilibrium approximation

$$v = v_0 \frac{[E]_0 - \alpha[I]_0 - \beta K_T + \sqrt{([E]_0 - \alpha[I]_0 - \beta K_T)^2 + 4[E]_0 \beta K_T}}{2\beta[E]_0}, \qquad (4)$$

$$\beta = 1 + (1 - \alpha)[I]_0/K_C. \qquad (5)$$

The dependence of I_{50} on inhibition constants for a mixture of a classical and a tight-binding inhibitor is described by Eq. (6). When the molar fraction of the tight-binding inhibitor is set to unity ($\alpha = 1$, representing a pure tight-binding inhibitor), Eq. (6) turns into Eq. (7) according to Cha [3]. On the other hand, when α is set to zero (representing a pure classical inhibitor), we obtain the Cheng–Prusoff equation, $I_{50} = K_C$, as a special case for classical inhibitors [4]

$$I_{50} = \frac{[E]_0/2 + K_T}{([E]_0/2 + K_T)(1 - \alpha)/K_C + \alpha}, \tag{6}$$

$$I_{50} = [E]_0/2 + K_T. \tag{7}$$

Under special circumstances, both apparent inhibition constants K_C and K_T and the enzyme concentration $[E]_0$ are known, and the task is to compute the molar fraction of tight-binding impurity from the observed value of I_{50}, using Eq. (8)

$$\alpha = \frac{K_C/I_{50} + 1}{K_C/([E]_0/2 + K_T) - 1}. \tag{8}$$

Results

I_{50} for inhibitor mixtures

For chemically homogeneous classical inhibitors, I_{50} is identical to K_C by definition (Cheng–Prusoff equation [4]). For homogeneous tight-binding inhibitors, the plot of I_{50} vs. K_T also is a straight line with unit slope, but this time with nonzero intercept equal to $[E]_0/2$ (Cha's equation (7), [3]). In contrast, the I_{50} for inhibitor mixtures depends on the apparent inhibition constants as a rectangular hyperbola, defined by Eq. (6).

It is obvious from Eq. (6) that any given value of I_{50} can be produced by infinitely many combinations of K_T, K_C, and α. This result suggests that any statistical method of analyzing dose-response curves that is based merely on determining I_{50} values (for example, fitting dose-response curves to the four-parameter logistic equation Eq. (1)) will produce ambiguous results. In contrast, nonlinear regression of dose-response data using the mechanism-based equation (4) offers at least the possibility of distinguishing between chemically homogeneous and heterogeneous samples.

A major concern is that Eq. (4) contains four adjustable parameters (K_C, K_T, α, and v_0). This large number of adjustable parameters can cause a large degree of mutual dependency (coupling or correlation) among adjustable parameters. An extremely high degree of correlation among parameters could prohibit the accurate determination of all parameter values from real-world experimental data. A standard approach to

investigating the possibility of such parameter correlation is to apply Monte-Carlo simulation techniques.

Monte-Carlo simulations

A tight-binding impurity in an inhibitor preparation can be detected kinetically if and when the parameters K_C, K_T, and α can be determined simultaneously from dose-response data. To ascertain which (if any) combinations of K_C, K_T, and α can be so determined, we chose a typical experimental design and a typical measurement error (e.g., 2 or 3%) for a series of Monte-Carlo simulations. The experimental design included nine inhibitor concentrations, starting from the maximum $[I]_0 = 1.0\,\mu M$, seven twofold serial dilutions (0.5, 0.25, 0.125...μM), and the negative control ($[I]_0 = 0$).

Using this layout, we employed Eq. (4) to simulate 10,000 dose-response curves for each particular combination of K_C, K_T, and α. "Experimental" data sets were generated by taking the theoretical data set and superimposing on it normally distributed pseudo-random noise with standard deviation equal to 1, 2, or 3% of the simulated velocity. Eq. (4) was then fit to the synthetic data sets to obtain least-squares values of adjustable parameters K_C, K_T, and α. The purpose of this analysis is to compare these best-fit parameters with their simulated "true" values.

A representative example is shown in Fig. 1. The dashed curve represents a theoretical dose-response curve simulated with $[E]_0 = 1.0\,nM$, $K_C = 0.1\,\mu M$, $K_T = 0.1\,nM$, and $\alpha = 1.0\%$. The data points (open circles) were generated by superimposing pseudo-random error with standard deviation equal to 3% of the simulated velocity. The least-squares fit of the simulated data (solid curve) yielded $K_C = 0.065\,\mu M$, $K_T = 0.041\,nM$, and $\alpha = 0.6\%$. Clearly, the 3% pseudo-random noise significantly distorted the values of the fitting parameters. In some simulations the best-fit values of model parameters K_C, K_T, and α were much closer to their true values, but in other simulations the best-fit values were even more distorted.

A summary of 30,000 representative regression analyses for three different combinations of K_C, K_T, and α (10,000 data sets for each combination) is shown in Fig. 2. Figs. 2A and B use the same values of model parameters ($K_C = 0.1\,\mu M$, $K_T = 0.1\,nM$, and $\alpha = 1.0\%$), only the simulated experimental error was different. A slight increase in experimental error, from 1% in Fig. 2A to 2% in Fig. 2B, causes a significant increase in the uncertainty of the inhibition constants K_C and K_T. This is illustrated by the widening of the histograms. Additionally, in many of the Monte-Carlo simulations the classical inhibition constant K_C could not be determined at all. The solid black rectangles represent all regression analyses where the best-fit value of K_C was several orders of magnitude larger than the simulated value,
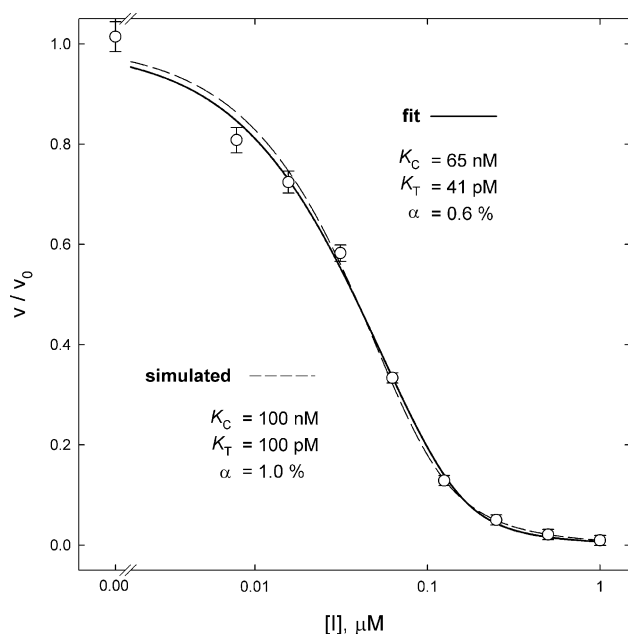
Fig. 1. A typical simulation/regression sequence from a Monte-Carlo study of Eq. (4), using $[E]_0 = 1.0$ nM. Synthetic data points were generated at $[I]_0 = 1, 0.5, 0.25, \ldots, 0.0078125$, and 0 nM. Pseudo-random error with standard deviation of 3% relative to the generated value was superimposed on the ideal data, and the resulting data set was fit to Eq. (4). Ten thousand such simulation/regression runs were performed for each combination of inhibition constants K_T, K_C, and the molar fraction of tight-binding impurity, α.

corresponding to effectively no inhibition. In these cases the 99% bulk material behaved as completely inactive, and only the tight-binding constant K_T could be determined at all. These results suggest that the measurements of initial velocities must be very accurate (experimental error approximately 1%) to determine the tight-binding inhibition constants for impurities.

The relative magnitude of classical versus tight-binding inhibition constants had even greater effect on the possibility of determining simultaneously the values of K_C, K_T, and α in Monte-Carlo simulations. This is illustrated in Fig. 2C, where both K_C and K_T have 10 times greater values compared to those in Fig. 2A. The uncertainties of both K_C and K_T in Fig. 2C ($K_C = 1000 \times [E]_0$, $K_T = [E]_0$) are much larger than those in Fig. 2A ($K_C = 100 \times [E]_0$, $K_T = [E]_0/10$). These results suggest that simultaneous determination of both apparent inhibition constants (K_C and K_T) will be possible only in a restricted range of their values.

To investigate this range, we have performed 1302 Monte-Carlo simulations, each with 10,000 dose-response data sets. The apparent inhibition constant K_C had 21 different values and the apparent inhibition constant K_T had 62 different values ($21 \times 62 = 1302$). K_C was varied from 0.1 to 8.6736 µM, stepping logarithmically by a factor of 1.25, whereas K_T was varied from 0.001 to 815.66 nM, again stepping logarithmically

by the same factor. For each of the 1302 combinations of inhibition constant values (assuming $[E]_0 = 1.0$ nM and standard error of measurement 1%) we assessed the spread of best-fit values, as illustrated for three such Monte-Carlo runs in Fig. 2. The conclusions can be summarized as follows.

The classical inhibition constant K_C could be determined from dose-response curves only if two conditions were met simultaneously: (i) the true value of K_C was lower than approximately $500 \times [E]_0$ and (ii) the true value of K_T was lower than approximately $0.5 \times [E]_0$. Outside of this range, the best-fit values of K_C were many orders of magnitude larger than the true values, suggesting that the classical inhibitor had virtually zero activity. Thus, for most combinations of K_C and K_T, the inhibitor sample behaved as though it were composed of a completely inactive (ballast) material contaminated with a tight-binding impurity. In other words, for most combinations of K_C and K_T, Eq. (3) was the most suitable fitting model.

On the other hand, the tight-binding inhibition constant K_T (and the molar fraction of impurity α) could be reliably determined regardless of the true value of K_C, but only if K_T was in the range between $0.005 \times [E]_0$ and $0.5 \times [E]_0$. These results suggest that the detection of a tight-binding impurity in an inhibitor sample will be most reliable when the enzyme concentration is at least comparable in magnitude with K_T or higher ($[E]_0 \geqslant K_T$). However, extremely high enzyme concentrations relative to K_T ($[E]_0 > 200 \times K_T$) again are not useful.

Experimental example

To test our method based on Eq. (4), we have contaminated a weak mast-cell tryptase inhibitor with a tight-binding impurity. Benzamidine ($K_C = 50.4$ µM) was doped with a tryptase inhibitor CRA-001390 ($K_T = 0.065$ nM, [1]) at the molar ratio 5000:1 (0.02% impurity). For both pure and contaminated benzamidine, initial reaction velocities were measured at eight different inhibitor concentrations, plus negative control ($[I]_0 = 0$). The enzyme concentration was 0.05 nM. Using Eq. (6), we can predict the I_{50} for this mixture of inhibitors to be approximately 0.4 µM.

The initial velocities were analyzed by two different methods. The first method consisted of nonlinear regression using the conventional four-parameter logistic equation (1). The results are summarized graphically in Fig. 3. The best least-squares fit values of I_{50} were (50.4 ± 3.3) µM for pure benzamidine and (0.40 ± 0.05) µM for benzamidine contaminated with CRA-001390. Dose-response curves for both pure benzamidine and contaminated benzamidine fit to Eq. (1) very well, as is seen from the fact that the experimental data points (open circles in Fig. 3) deviate very little from the best-fit curves. Thus, the results of fit to Eq. (1) do not indicate
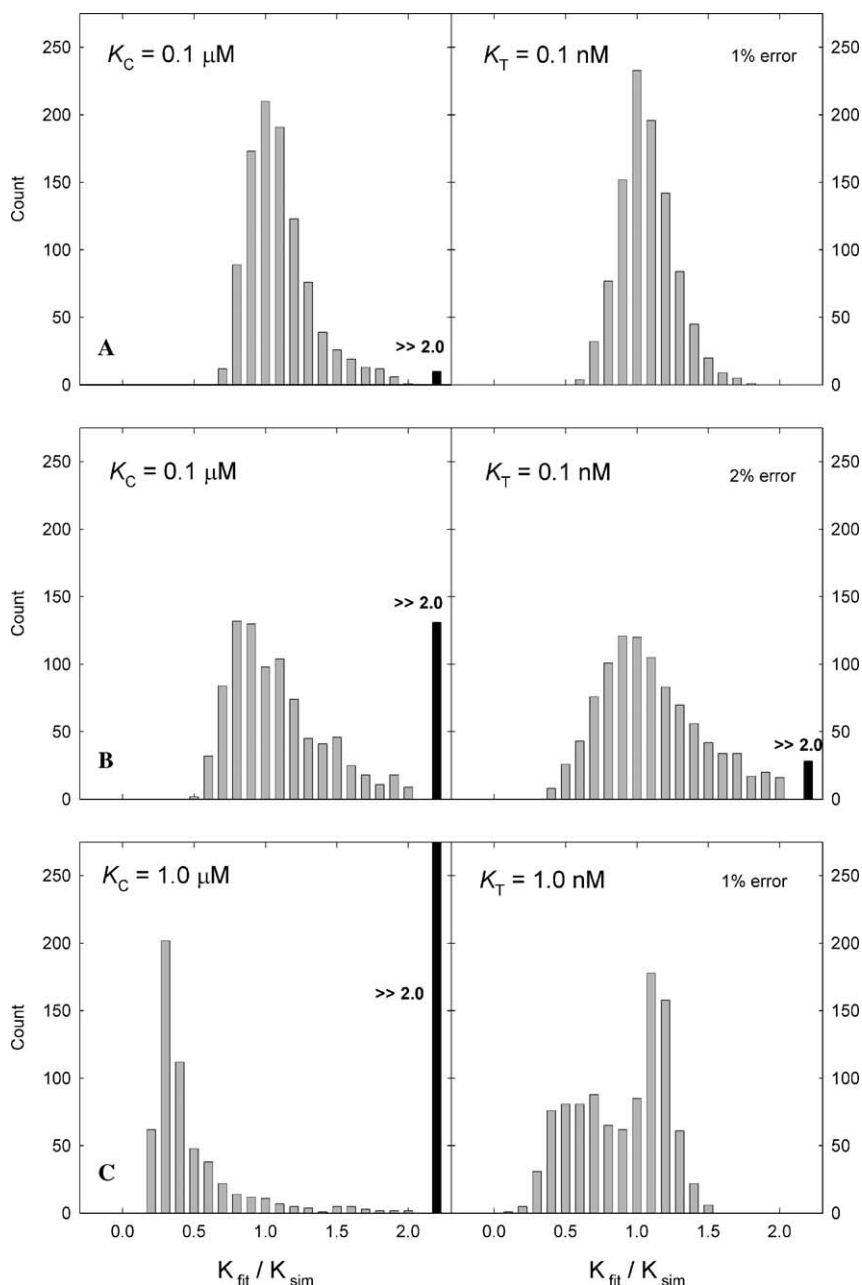
Fig. 2. Summary of three typical Monte-Carlo simulations, all conducted at $[E]_0 = 1.0$ nM and with the molar fraction of impurity $\alpha = 1\%$. (A) Simulated values were $K_C = 0.1\,\mu$M, $K_T = 0.1$ nM, with the pseudo-random error 1%. (B) Same as A, except for pseudo-random error 2%. (C) Simulated values $K_C = 1.0\,\mu$M, $K_T = 1.0$ nM, with the pseudo-random error 1%. For explanation see text. In total, 1302 such Monte-Carlo simulations were performed at 1302 different combinations of K_T, K_C and α (approximately 1.3 million synthetic data sets).

in any way that the dose-response curve B in Fig. 3 is generated by a chemically heterogeneous material.

The second method of statistical analysis consisted of nonlinear regression of the same initial velocity data using (in turn) Eqs. (2)–(4), instead of the four-parameter logistic equation (1). The results are graphically summarized in Fig. 4.

Least-squares fit of dose-response data for contaminated benzamidine to Eq. (2), describing a chemically homogeneous tight-binding or classical inhibitor, is

represented by the dashed curve in Fig. 4. It is important to note a pronounced lack of fit. Indeed, within the experimental errors represented by the error bars shown in Fig. 4, only two of nine experimental data points overlap with the best-fit curve.

Least-squares fit of the same data to Eq. (4), describing a chemically heterogeneous mixture of a classical inhibitor contaminated by a tight-binding impurity, is represented by solid curve in Fig. 4. The best-fit values and formal standard errors of adjustable parameters
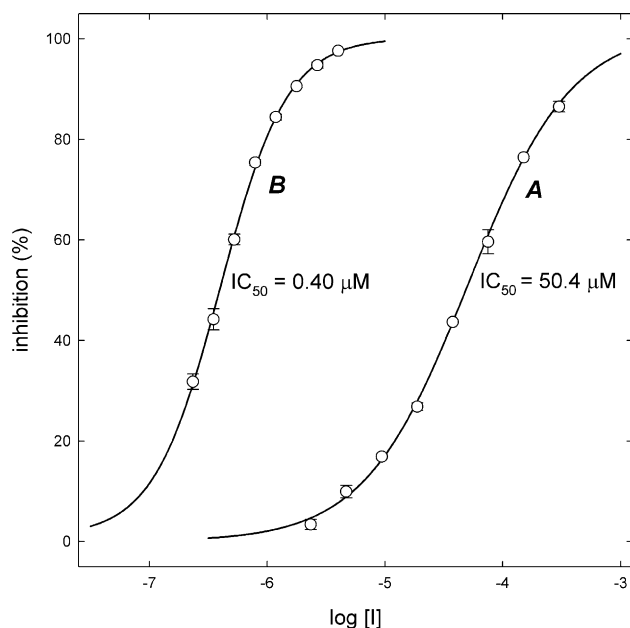
Fig. 3. Dose-response curves for the inhibition of human mast-cell tryptase by (A) pure benzamidine ($K_C = I_{50} = 50\,\mu M$) or (B) benzamidine doped with 0.02% of the tight-binding inhibitor CRA-001390 ($K_T = 0.065\,nM$). Data points are averages from triplicate measurements of initial velocities. Percentage inhibition values (p) were fit to the four-parameter logistic Eq. (1) to obtain I_{50} values. Eq. (6) predicts for "adulterated" benzamidine $I_{50} = 0.40\,\mu M$.
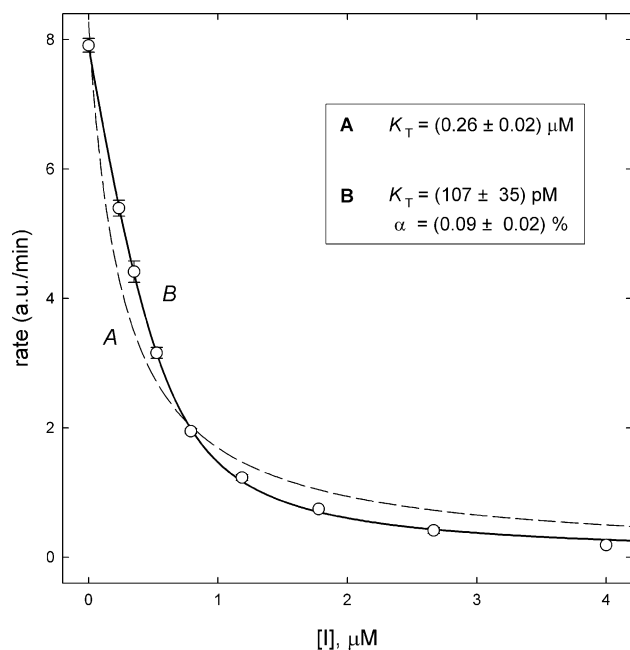

Fig. 4. Nonlinear least-squares fit of inhibition data for human mast-cell tryptase ($[E]_0 = 0.05\,nM$). The inhibitor was benzamidine doped with the tight-binding inhibitor CRA-001390. Curve A is the best-least-squares fit to Eq. (2) for a *pure* tight-binding or classical inhibitor. Note the pronounced lack of fit (dashed curve misses most of the data points). Curve B is the best least squares fit to Eq. (4) for a *mixture* of a classical inhibitor contaminated with a tight-binding inhibitor. The best-fit values were molar fraction $\alpha = (0.09 \pm 0.02)$%, apparent inhibition constant $K_T = (107 \pm 35)$ pM. The actual values for inhibitor CRA-001390 were $\alpha = 0.02$%, $K_T = 65$ pM [1].

were $v_0 = (7.90 \pm 0.31)$ a.u./min, $\alpha = (0.087 \pm 0.015)$%, $K_C = (1.9 \pm 49.8)$ M, and $K_T = (0.107 \pm 0.035)$ nM. The best-fit value of the apparent inhibition constant for benzamidine is not only extremely large (38,000 times larger than the true value $K_C = 50\,\mu M$), but also is accompanied by a large formal standard error. These results mean that the inhibition constant for benzamidine cannot be determined at all from the available data and that the sample behaves effectively as a completely inactive material containing a small amount of a tight-binding impurity. Accordingly, we repeated the regression analysis using Eq. (3) instead of Eq. (4). The results were identical for the relevant model parameters: $v_0 = (7.90 \pm 0.31)$ a.u./min, $\alpha = (0.087 \pm 0.015)$%, and $K_T = (0.107 \pm 0.035)$ nM. These results suggest that the inhibitor is not chemically pure. The regression analysis accurately diagnosed that the adulterated sample contains less than 100 ppm of a tight-binding impurity with K_i approximately 100 pM (true values $\alpha = 20$ ppm, $K_i = 65$ pM).

Discussion

We have recently described methods for kinetic analysis that are suitable for fully automatic determination of inhibition constants and I_{50}s [1,2]. The underlying theoretical model for dose-response curves is the Eq. (2) for "tight-binding" inhibition [5,6], which is also applicable to weakly active or "loose-binding" inhibitors. These methods have been used successfully in our laboratories to analyze hundreds of thousands of inhibitor samples. However, occasionally we observe that a certain dose-response curve does not fit Eq. (2) very well.

There are many possible explanations for such deviations between the experimental data and the given theoretical model. In this paper, we focused on one explanation that seems reasonable based on the complex realities of drug research. Indeed, de novo enzyme inhibitors are often products of multistep chemical syntheses and purification methods whose efficiency is necessarily finite. Such syntheses almost always yield a preparation containing a single inhibitory species. However, is it possible that an occasional anomaly in dose-response behavior could be caused by the presence of tight-binding impurities in otherwise weakly potent or even entirely inactive material?

To answer this question, we applied two different methods of analyzing inhibitor dose-response data. The first method was traditional, based on the four-parameter logistic equation. A systematic survey of biochemical literature on the evaluation of enzyme inhibitors reveals that, year after year (e.g., [11–13]), I_{50} remains a favorite measure of inhibitory potency. Similarly, the four-parameter logistic equation remains

a favorite mathematical model for inhibitor dose-response curves.

In this paper, we have demonstrated one potential pitfall of this traditional approach. In particular, the four-parameter logistic equation cannot discriminate between a chemically homogeneous inhibitor (classical or tight-binding) and a heterogeneous sample consisting of bulk material that has (a) a moderate or no inhibitory potency and (b) a highly potent impurity. Indeed both experimental dose-response curves in Fig. 3 (curve A for a chemically pure inhibitor and curve B for an inhibitor mixture) fit the available data extremely well.

Because the fit to the four-parameter logistic Eq. (1) is very good in both cases, there is no "red flag" that would alert the investigator clearly to the presence of a tight-binding impurity. Indeed, curve B in Fig. 3 appears as just another half-micromolar inhibitor, instead of an essentially inactive material containing a small amount of compound with $K_i = 65$ pM.

The only possible giveaway is the Hill slope parameter n in Eq. (1). For the contaminated benzamidine (curve B in Fig. 3) we obtained $n = (1.53 \pm 0.14)$, whereas for pure benzamidine (curve A) the slope parameter was $n = (0.98 \pm 0.07)$. However, in practice the Hill slope parameter was found not sufficiently reliable to reveal conclusively the presence of tight-binding impurities. Even for experimental data that are affected by small measurement error (less than 1%), for nearly optimally designed experiments and for kinetically pure inhibitors, the Hill slope parameter varied widely between 0.7 and 1.5 simply because of random errors. This was especially true when both p_{max} and p_{min} in Eq. (1) were treated as adjustable parameters. Although these observations are merely anecdotal in the scope of this report, a more detailed examination of the Hill slope parameter n as an indicator of anomalous inhibitor behavior will be presented elsewhere.

A more successful approach to correctly diagnosing the presence of tight-binding impurities in enzyme inhibitors is based on our newly derived rate Eq. (4), used in conjunction with Eq. (2). Our approach is easily amenable to automation in a high-throughput setting. Such automated analysis of inhibitor dose-response curves consists of three separate steps. In the first step, the data are analyzed by using Eq. (2), which assumes that the inhibitor preparation is chemically pure. In the second step, the same data are fit to Eq. (4), which assumes the presence of a tight-binding impurity. In the third step, we apply a standard model-discrimination analysis based on the F statistic [14].

According to our method of analysis, an inhibitor sample is flagged as possibly containing a tight-binding impurity if two conditions are satisfied simultaneously. The first condition is that the traditional Eq. (2) gives a poor fit based on the residual sum of squared deviations. The second condition is that the calculated F statistic suggests a statistically significant improvement in the goodness of fit, going from the simple Eq. (2) to the more complex Eq. (4). When both conditions are satisfied, the experimentally determined values of I_{50} and K_i should not be trusted, and the sample should be carefully scrutinized for trace amounts of tight-binding impurities.

The approach described in this paper, as in any other analytical methodology, has distinct limitations and requirements. Our mathematical model does not take into account the detailed mechanism of inhibition (competitive vs. noncompetitive) and operates only on apparent inhibition constants. Another restriction arises from the assumed composition of the inhibitor mixture. In the hypothetical case of two tight-binding inhibitors present as a mixture, it would be necessary to solve a cubic equation [15]. However, we have purposely restricted ourselves to the less general case, where only one inhibitor is tight-binding. Therefore, our theoretical model arises more simply, as solution to a quadratic equation.

A more important limitation of our analytical method lies in the condition that the inhibition constants of the impurity and the bulk material must be significantly different from each other. Only then can both inhibition constants be determined reliably at the same time, as is illustrated by Monte-Carlo simulations. Our experimental example illustrates the opposite scenario, where simultaneous determination of K_C and K_T was not possible. Furthermore, the impurity's inhibition constant must fall into a certain specific range (several orders of magnitude wide) relative to the enzyme concentration used in the assay. The model-discrimination method described here requires that the initial velocities are measured very accurately and reproducibly (less than 2% experimental error) and that the experiment is nearly optimally designed with regard to the inhibitor concentrations chosen. Finally, in fitting dose-response data to Eq. (4), we assumed that the enzyme concentration $[E]_0$ can be reasonably treated as a fixed parameter. Our previous paper [2] describes in detail the appropriate statistical treatment of those experimental situations where the assumption of constant $[E]_0$ does not hold.

We hope that other researchers active in the kinetic analysis of enzyme inhibitors, especially those evaluating the inhibitory potency in the drug discovery process, will find good use for our new analytical method and the underlying mathematical model. In our experience, abandoning the traditional exclusive reliance on I_{50}—a step taken in these laboratories several years ago—certainly did enhance the information content of a massive data stream flowing from various inhibitor screening projects.

Acknowledgment

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