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An Intuitive Look at the Relationship of K_i and IC_{50}: A More General Use for the Dixon Plot

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The design and synthesis of small-molecule enzyme inhibitors is of great interest in current research. Finding a potent inhibitor of an enzyme linked to a disease may lead to a multi-billion-dollar pharmaceutical product. To find such inhibitors, it is often necessary to quantitatively compare the inhibitory ability of a large number of target compounds. A recent review of protease inhibitors gives a perspective on the wide range and sheer number of reversible inhibitors of one class of enzymes *(1)*. Without a standard measurement of inhibition, comparing the inhibitor potencies of these molecules would be impossible.

Initially, it might seem easy to compare the potency of a set of inhibitors acting on the same target. In practice, it is not so straightforward. There is more than one way to report the inhibitory capacity of compounds, but two measurements are used predominately. The inhibition constant, K_i, denotes the equilibrium constant of the dissociation of the inhibitor-bound enzyme complex. A second number, the IC_{50} value, quantifies the concentration of inhibitor necessary to halve the reaction rate of an enzyme-catalyzed reaction observed under specified assay conditions.

Most biochemistry and enzymology textbooks treat the subject of enzyme inhibition with a fairly detailed description of the inhibition constant K_i, but few have any information about IC_{50}. Likewise, students taking biochemistry labs often determine the K_i for an inhibitor, but rarely determine an IC_{50}. In practice, though, IC_{50} values are the most commonly used assessment of enzyme–inhibitor interaction. It is therefore important for undergraduate and graduate students, as well as medicinal chemists in industry, to understand and appreciate the relationship of K_i and IC_{50}.

The Relationship of K_i and IC_{50}

The values of K_i and IC_{50} are often used to compare the relative potency of inhibitors. For K_i, smaller values denote tighter binding in most cases. For example, compound A with a K_i of 100 nM binds to an enzyme one thousand times tighter than compound B with a K_i of 100 μM. In other words, only 100 nM of A is necessary to bind the enzyme to the same degree as a 100 μM solution of B. Similarly, lower IC_{50} values suggest better inhibition. In a particular assay system, compound A, with an IC_{50} of 100 nM, requires one thousand times less inhibitor to reduce an enzyme activity in half than compound B, with an IC_{50} of 100 μM.

But what happens if we attempt to compare the potencies of inhibitors reported in the literature by different research groups? Let us look at a real example. The protease NS3 has been identified as a target for the treatment of hepatitis C *(1)*. Inhibitors of this enzyme have recently entered clinical trials. Three different groups have reported inhibitors, one with a K_i of 0.6 μM *(2)*, one with an IC_{50} value of 28 μM *(3)*, and another with an IC_{50} value of 6.4 μM *(4)*. How can we compare the potency of these inhibitors? Is a K_i of 0.6 μM ten times lower than an IC_{50} value of 6.4 μM? And what about IC_{50} values? Is an inhibitor with an IC_{50} of 6.4 μM necessarily more potent than one with an IC_{50} of 28 μM?

There is a difference between IC_{50} and K_i that is well illustrated by the way in which these values are determined. To find a K_i value for an inhibitor, one must determine rates of enzyme-catalyzed reactions while independently varying the concentration of substrate, [S], and the concentration of inhibitor, [I]. Specifically, the rate of an enzyme-catalyzed reaction is measured for a range of substrate concentrations against one concentration of inhibitor. This experiment is then repeated, typically five or six times, for different concentrations of inhibitor. These data, usually requiring 75–100 individual rate measurements if the experiment is done in triplicate, are then used to find K_i. Using these data, a variety of graphical methods may be used for the determination of the K_i values *(5)*. The values of IC_{50}, on the other hand, are obtained with much less effort. The IC_{50} value is determined at only one concentration of substrate over a range of inhibitor concentrations. Since determination of this value requires only about 15–20% as many data points, it is obvious why IC_{50} values are used when large numbers of compounds must be assayed. While K_i is a constant value for a given compound with an enzyme, an IC_{50} is a relative value, whose magnitude depends upon the concentration of substrate used in the assay. A compound will display five different IC_{50} values for the same enzyme if the five assays are performed employing different substrate concentrations.

This fact is described mathematically in the Cheng–Prusoff relationship *(6)*. For competitive inhibitors, which inhibit the enzyme-catalyzed reaction by binding to free enzyme, the relationship of IC_{50} to K_i is stated by eq 1,

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_M}\right) \qquad (1)$$

From this mathematical relationship, we can see that the IC_{50} value of a given competitive inhibitor is related to the K_i value of the inhibitor as a function of the substrate concentration, [S], used in the assay, and the Michaelis constant, K_M, of the substrate.

The relationship of IC_{50} to K_i is more complex than this, though. Besides depending on [S], the relation of IC_{50} to K_i

also depends on the inhibitor's mode of inhibition. Cheng and Prusoff described the relationship of K_i and IC_{50} for uncompetitive inhibitors *(6),* which are inhibitors that bind only to the enzyme-substrate complex,

$$IC_{50} = K_i\left(1 + \frac{K_M}{[S]}\right) \qquad (2)$$

They also derived the equation relating IC_{50} to K_i for mixed inhibition.[2] Mixed inhibition can be pictured as intermediate between purely competitive inhibition and purely uncompetitive inhibition; these inhibitors bind to both free enzyme and enzyme–substrate complex, with inhibition constants K_{ie} and K_{ies}, respectively,

$$IC_{50} = \frac{[S] + K_M}{\left(\frac{[S]}{K_{ies}} + \frac{K_M}{K_{ie}}\right)} \qquad (3)$$

With all of these ambiguities, does this leave us with any way to compare IC_{50}'s and K_i's? Yes—as long as the mode of inhibition and the concentration of substrate used in the assay is reported, IC_{50} values may be theoretically converted into K_i values with the Cheng–Prusoff equations. Even if the concentration of substrate used in the assay is not provided, important information can still be obtained from these equations. In the case of competitive inhibition, IC_{50} values approximate K_i when the [S] used in the assay is much lower than K_M. According to eq 1, in the case where [S] is significantly smaller than K_M, $[S]/K_M$ approaches zero, and IC_{50} approaches K_i. Therefore, in the case of competitive inhibition, IC_{50} is always higher than K_i. Any reported IC_{50} value is an upper limit for the K_i of the compound.

As in competitive inhibition, IC_{50} values determined for uncompetitive inhibitors are always higher than K_i. One major difference exists, though. For uncompetitive inhibitors, IC_{50} values approximate K_i when the [S] used in the assay is much higher than K_M. At large values of [S], the ratio $K_M/[S]$ approaches zero, and IC_{50} approaches K_i (eq 2).

Mixed inhibition contains components of both competitive and uncompetitive inhibition, and therefore no simplification may be made for the general case. It is interesting to note though, that in a special case of mixed inhibition, referred to as simple mixed inhibition,[3] IC_{50} values do not change with [S]; in fact, IC_{50} is equal to K_i regardless of the concentration of substrate used in the assay (eq 3).

Many students will find these statements a bit abstract. Is there a more intuitive approach to understanding the relationship of IC_{50} and K_i in different modes of inhibition? A look at simplified kinetic mechanisms of inhibition may help. (More complex mechanisms may or may not be consistent with such treatment. Each case should be evaluated individually.)

Figure 1 depicts a kinetic scheme for competitive inhibition. In this simplified depiction, the enzyme–substrate complex, E·S, is transformed into product with a first-order rate constant. Thus, the rate of the reaction may be reduced in half (the definition of IC_{50}) when the concentration of E·S is cut in half relative to its concentration with no inhibitor present. In case A, IC_{50} is determined at low [S]. In this case, the enzyme exists in mostly the free form with little E·S present. The concentration of inhibitor needed to reduce [E·S] in half, the definition of IC_{50}, is equal to the amount needed to tie up half of the free enzyme, E—and this is the definition of K_i. At low [S], $IC_{50} \approx K_i$. In case B, with IC_{50} measured at high [S], the enzyme exists almost exclusively as E·S. A large excess of I is needed to even out the tug of war between the formation of E·I and E·S, thereby cutting the rate in half. At high [S], $IC_{50} > K_i$.

Uncompetitive inhibition is explored in Figure 2. In a similar treatment, case A describes the IC_{50} value determined at low [S]. In this scenario, there is little E·S present, so a large excess of inhibitor is needed to trap this complex (a second-order reaction) before it goes to product (a first-order reaction.) At low [S], IC_{50} is large. In case B, the high [S] used in the assay conditions forces the enzyme to exist in exclusively the E·S complex. In this situation, the amount of inhibitor needed to reduce [E·S] in half is the amount of inhibitor necessary to force half the [E·S] to be bound as E·S·I—the definition of K_i. High [S] means that the amount of inhibitor necessary to cut the rate in half—the IC_{50} value—is equal to K_i.

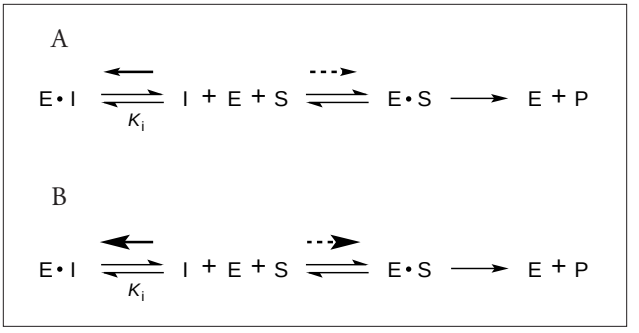

Figure 1. Competitive inhibition, A: IC_{50} determined at low [S], B: IC_{50} determined at high [S].

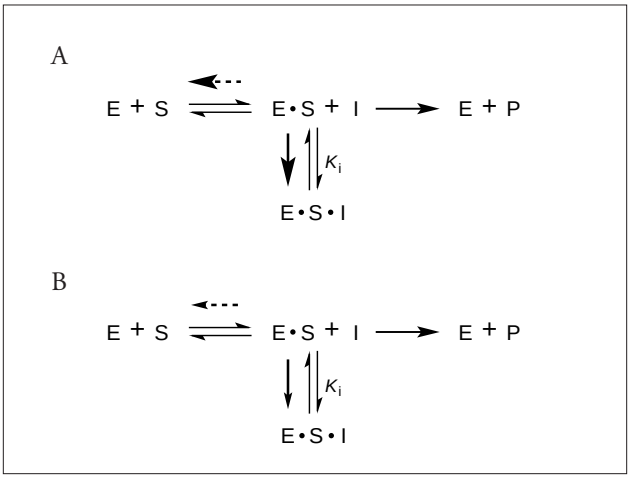

Figure 2. Uncompetitive inhibition, A: IC_{50} determined at low [S], B: IC_{50} determined at high [S].

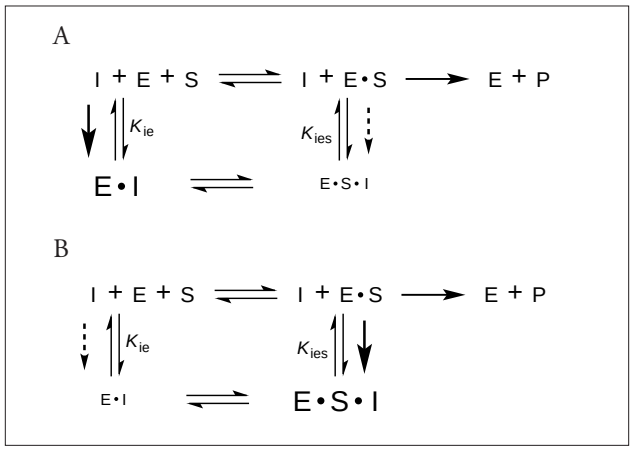

Figure 3. Mixed inhibition, A: IC_{50} determined at low [S], B: IC_{50} determined at high [S].

Simple-mixed inhibition may be pictured as a balance between competitive and uncompetitive inhibition (Figure 3). In the case where [S] is low, the [E·S] is low. In such a case, the inhibitor acts as a competitive inhibitor, and $IC_{50} \approx K_i$ as noted above. In cases where the [S] is high, the [E·S] is high. At high [S], the inhibitor behaves primarily as an uncompetitive inhibitor, and as in the case of uncompetitive inhibitors, IC_{50} approximates K_i. Thus, the IC_{50} value approximates the K_i value at all ranges of [S] for simple mixed inhibition.

Determination of IC_{50} Values

The Cheng–Prusoff equation allows for conversion of IC_{50} values to K_i values if the mechanism of inhibition and the [S] used in the assay is known. Of course, the underlying assumption is that the determination of the IC_{50} value is accurate. But exactly how are these numbers determined? The data are first plotted as the percent of enzymatic activity remaining (or as percent of activity inhibited) as a function of added inhibitor, giving a nonlinear curve (Figure 4). The concentration of inhibitor necessary to reduce the percent activity to 50% is then obtained. Most reports neglect to describe how this is done. The most mathematically accurate way to do this is to use a computer to perform a nonlinear regression, but in practice, this is often not done. Some investigators eyeball the graph to get an approximate value. Others plot percent activity as a function of log [I] and then use logistic regression programs to determine the inflection (Figure 5). Newer mathematical models based on this approach are still being proposed *(7)*. At times, however, logistic analysis is not accurate. This problem has been recognized, and a linear plot has been proposed for the determination of IC_{50} values of competitive inhibitors *(8)*. This plot has not gained wide acceptance from biochemists, most likely because it is more suited to pharmacology.

We wanted to develop a new, and more biochemically intuitive, linear method for IC_{50} determination. Although nonlinear regression analysis of these data is the most mathematically sound means to obtain quantitative values of this type, in practice, many enzymologists still use linear plots in determining kinetic data. More importantly for the student, a linear technique analogous to others taught in introductory enzyme kinetics (Lineweaver–Burk, Eadie–Hofstee, etc.) might facilitate greater understanding.

The derivation of the Michaelis–Menton equation for competitive inhibition (eq 4) relates the rate of reaction (V_0) to the concentration of inhibitor, [I] *(9)*

$$V_o = \frac{V_{max}[S]}{[S] + K_m\left(1 + \frac{[I]}{K_i}\right)} \quad (4)$$

Equation 4 may then be rearranged in a manner analogous to the Lineweaver–Burk treatment to yield

$$\frac{1}{V_o} = \frac{[S] + K_M}{V_{max}[S]} + \frac{K_M}{V_{max}[S]K_i}[I] \quad (5)$$

Because the reaction rate is inversely proportional to inhibitor concentration for competitive inhibition (eq 5), a linear plot may be obtained by plotting the inverse of the relative rates against inhibitor concentration at a constant [S]. Setting the value of $1/V_o$ to zero,

$$0 = \frac{[S] + K_M}{V_{max}[S]} + \frac{K_M}{V_{max}[S]K_i}[I] \quad (6)$$

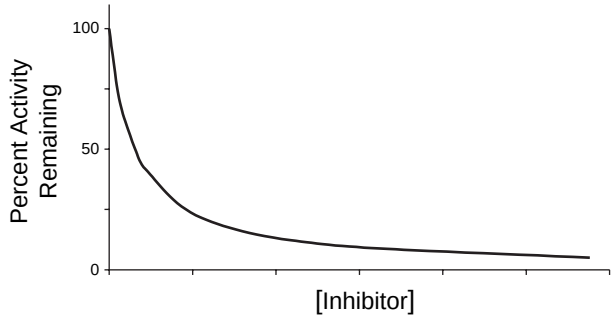

Figure 4. IC_{50} determination: plotting the percent of enzymatic activity remaining as a function of inhibitor concentration.

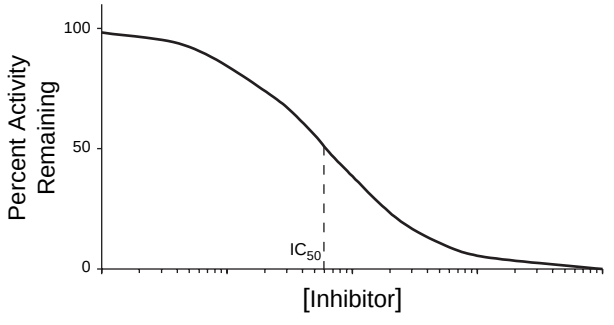

Figure 5. IC_{50} determination: plotting percent of enzymatic activity remaining as a function of inhibitor concentration in a log scale.

Figure 6. The Dixon plot for a competitive inhibitor: K_i may be determined from the intersection of data obtained at two substrate concentrations, S_A and S_B.

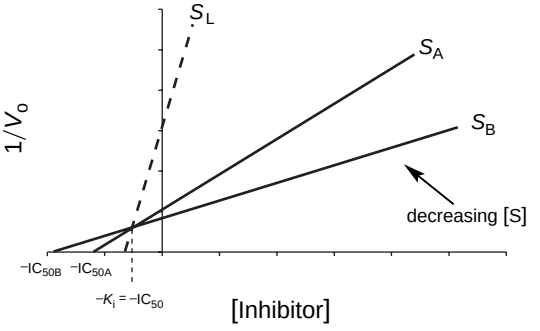

Figure 7. The Dixon plot illustrated as a series of IC_{50} determinations. For competitive inhibition, IC_{50} approaches K_i when assays are performed at low concentration of substrate (S_L).

and solving for [I] (the *x*-intercept)

$$-\left(\frac{[S] + K_M}{K_M}\right)K_i = [I] = x\text{-intercept} \qquad (7)$$

The *x*-intercept of eq 5 (derived in eq 7) is identical to the value of $-IC_{50}$ according to Cheng–Prusoff relationship (eq 1)! Similar treatments of uncompetitive and mixed inhibition reveal a similar result. (See supplemental material, section A.[W]) While this treatment has not been used as a graphical means of determining IC_{50}, this is not the first time this graphical technique has been employed. This is the basis for the well-known Dixon plot.

In 1953, Dixon proposed a graphical means for determining a K_i value requiring much less data than the conventional method *(10).* Rather than acquiring reaction rates for multiple [S] for a series of [I], his plot allowed for the determination of K_i by testing the effect of a range of added inhibitors at only two [S].

For competitive inhibitors, two lines determined at two different [S] intersect at the value of $-K_i$ (Figure 6). A similar treatment is possible for simple mixed inhibition (see supplemental material[W]), though Dixon pointed out that such a graphical method does not work with uncompetitive inhibition.

This new analysis of the Dixon plot explains why K_i may be determined in this way and reveals its more broad applicability to IC_{50}, rather than K_i, determination. The Dixon plot is simply a series of IC_{50} determinations at a variety of [S]. Inhibition data for a range of [I] at a constant [S] may be plotted as a Dixon plot to give the IC_{50} value at that [S] as the opposite value of the *x*-intercept. Thus, an important aspect of the Dixon plot has not been utilized. Although a Dixon plot may provide a good estimate of K_i values for competitive and simple mixed inhibition, it also provides accurate IC_{50} values for competitive, uncompetitive, and mixed inhibition.

An intuitive analysis of the Dixon plot may be used to understand why the Dixon plot is suitable for the determination of IC_{50} values as well as K_i. For competitive inhibition, Dixon observed that K_i could be determined by the point of intersection of two lines determined at two concentrations of substrate (Figure 6). By thinking of the Dixon plot as a series of IC_{50} determinations, we see that as [S] gets infinitely low, the *x*-intercept of its line approaches the value of K_i observed by Dixon (Figure 7). Cheng and Prusoff suggested this same fact mathematically: as [S] approaches zero, IC_{50} is equal to K_i. Both uncompetitive and mixed inhibition may be treated in a similar manner (supplemental material, section B[W]).

A summary of the relationship of IC_{50} to K_i and the use of the Dixon plot in determining IC_{50} values are provided in Table 1. Students must understand that the potency of different inhibitors of the same enzyme may not be compared by directly comparing IC_{50} and K_i values. Only with knowledge of both the mode of inhibition and the assay conditions employed may some comparison may be made. Furthermore,

Table 1. Summary of the Relationship of IC_{50} and K_i

Relationship	Competitive Inhibition	Uncompetitive Inhibition	Mixed Inhibition	Simple Mixed Inhibition
How is K_i related to IC_{50}?	$IC_{50} > K_i$	$IC_{50} > K_i$	Not Applicable	$IC_{50} = K_i$
$IC_{50} \approx K_i$.	When [S] ≈ 0	When [S] ≈ ∞	Varies	At any [S]
Can K_i be determined from a Dixon plot?	Yes	No	No	Yes
Can IC_{50} be determined from a Dixon plot?	Yes	Yes	Yes	Yes

IC_{50} values may be determined from a Dixon plot even if the mode of inhibition is unknown. We believe biochemists will prefer this linear method for IC_{50} determination to currently accepted practices. Lastly, we suggest that manuscripts that report IC_{50}'s must always report the substrate concentration at which they were determined, especially if the paper has no experimental protocols reported. One way to do this would be to add the concentration of substrate in the same units as the IC_{50} in a parenthetical subscript. For example, $IC_{50} = 5\ \mu M_{10}$ would indicate an IC_{50} of 5 μM determined at a substrate concentration of 10 μM.

Acknowledgments

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[W]Supplemental Material

Linear plot for IC_{50} determination for uncompetitive and mixed inhibition is shown in supplemental material, section A. Intuitive analysis of a Dixon plot for simple mixed and uncompetitive inhibition is shown in supplemental material, section B. A practice problem for students is presented in supplemental material, section C. All supplemental material is available in this issue of *JCE Online*.

Notes

1. Author's current address is: Department of Chemistry, Mount Union College, Alliance, OH 44601.
2. Mixed inhibition is often called noncompetitive inhibition in textbooks, but this nomenclature usually results in confusion for the student. Mixed inhibition is more descriptive, because it may be thought of as a mix of competitive and uncompetitive inhibition.
3. Simple mixed inhibition is the special case of mixed inhibition when the inhibitor binds equally well to the free enzyme, E, and the enzyme–substrate complex, E·S. In this case, the inhibition constants K_{ie} and K_{ies} are equal.

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