Interpreting Steep Dose-Response Curves in Early Inhibitor Discovery

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Abstract: Many screening hits inhibit enzymes with steep dose-response curves, which are considered pathological. Three models might explain these curves: multisite binding, an inhibitor phase transition, or stoichiometric inhibition caused by a high enzyme to K_d ratio. Experiments with promiscuous aggregators, for which steep curves are common, suggest that these curves owe to stoichiometric inhibition, which predicts that IC_{50} should vary linearly with enzyme concentration. Most steep dose-response curves in screening may be due to this effect.

High-throughput screening dominates early lead discovery and is intensely studied.[1,2] Despite notable successes, the technique is plagued by artifactual hits.[3,4] One common indication of artifact among hits is a steep dose-response curve, often indicated by a high Hill coefficient.[5] For such compounds, inhibition rises much more quickly with concentration than one would expect. Thus, whereas a classical, single-site inhibitor increases from 10% to 90% inhibition over an 81-fold concentration range (Figure 1, red curve), compounds with steep dose-response curves can do the same over less than a 10-fold concentration range (Figure 1, black curve). In one public database of HTS dose-response curves, that from the NIH Chemical Genomics Center (NCGC) (http://pubchem.ncbi.nlm.nih.gov/), 21% of the inhibitors for the five enzymes reported have Hill coefficients above 1.5. Of course, care is warranted in interpreting Hill coefficients, or indeed IC_{50} and K_d values, directly out of screening, even in qHTS[6] campaigns. Still, taken at face value, this result suggests that over 1000 hits for these five enzymes bind, at micromolar concentrations or better, to multiple distinct sites on these five enzymes.

Despite their prevalence, the physical events underlying steep dose-response curves in enzyme assays are poorly understood (steep dose responses in cell-based assays can have many explanations,[7] and I will not consider them here). Three mechanisms may be considered. First, several inhibitor molecules may bind to one enzyme molecule; here, the slope of the dose-response curve will rise with number of inhibitor sites.[8] Second, the inhibitor may undergo a physical phase transition as its concentration is raised. Phase transitions like inhibitor precipitation or colloid formation have a sharp concentration dependence, and if this transition is coupled to inhibition, a steep dose-response curve will result. Finally, steep dose-response curves will occur in any enzyme−inhibitor pair when the enzyme concentration significantly exceeds the K_d value of the inhibitor. In this case, inhibition will appear stoichiometric, as illustrated below.

The first of these models, genuine multisite binding, may well apply to some inhibitors, especially those binding to oligomers. It seems unlikely, however, to explain the high percentage of screening hits with steep dose responses. The second phase-transition model is more plausible. Indeed, a major mechanism

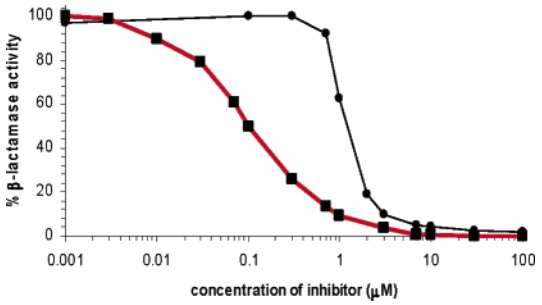

Figure 1. Dose−response curves of the transition-state analogue BZBTH2B (■, red curve) and the aggregator rottlerin (●, black curve), both β-lactamase inhibitors. Adapted from ref 9 and used with permission.

of artifactual inhibition is colloidal aggregation of the organic molecule followed by enzyme sequestration. These aggregates inhibit enzymes with little specificity, are widespread in screening libraries, and often have steep dose-response curves.[9,10] It is tempting to infer that these steep dose dependencies reflect critical aggregation concentrations. If true, the IC_{50} of an aggregator should depend largely on this critical concentration and should be relatively invariant with different enzymes. Instead, aggregates often have different IC_{50} values for different enzymes[11,12] and, more confounding still, increasing the concentration of a given enzyme increases the IC_{50} of an aggregating inhibitor. These properties are hard to reconcile with a phase transition model.

The dependence of aggregating inhibitors on enzyme concentration has always been perplexing, since increasing the concentration of enzymes present at nanomolar concentrations should not significantly affect the free concentration of a micromolar inhibitor.[11] In fact, there are conditions where classical enzymology would predict such perplexing behavior, and that is when the concentration of the enzyme is much higher than the K_d of the inhibitor (mechanism 3). In this case, one may increase an inhibitor well past its K_d value without detectably affecting enzyme activity. Only when the inhibitor concentration approaches that of the enzyme will inhibition mount, and since its concentration is by now well above its K_d, it will do so rapidly. If one raises the enzyme concentration still further in this zone, the apparent IC_{50} of the inhibitor will also rise. This is the effect we have observed with aggregating inhibitors. In this stoichiometric model, inhibition depends on the enzyme to K_d ratio as follows:[13]

$$\frac{[I]}{K_d} = \frac{\text{inh }\%}{100 - \text{inh }\%} + \frac{[(\text{inh }\%)/100][E]}{K_d} \qquad (1)$$

where [I] is the inhibitor concentration, inh % is the percent inhibition, and [E] is the enzyme concentration.

Consider a nanomolar inhibitor, for simplicity a noncompetitive one. When the enzyme concentration is much lower than the K_d, the second term in eq 1 becomes negligible and behavior is dominated by the first term. Thus, 10% inhibition will occur at $^1/_9$ of the K_d (111 pM). Inhibition of 50% will occur at the K_d value of 1 nM, and 90% inhibition will occur at 9 nM, giving a classic dose-response curve (Figure 2, red curve). If the enzyme concentration is raised past the K_d value of the inhibitor, as is often necessary for potent inhibitors, the slope of the inhibition curve will begin to change. At 10 nM enzyme, $10K_d$,

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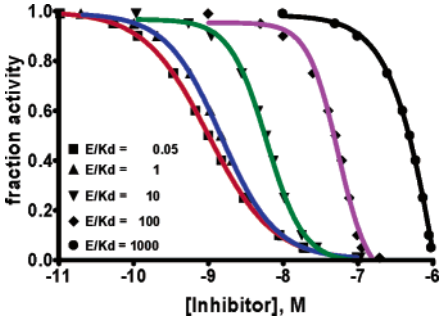

Figure 2. Dose-response curves predicted by the stoichiometric model for a 1 nM inhibitor measured at increasing enzyme concentrations: 50 pM enzyme (red, $0.05K_d$), 1 nM enzyme (blue, K_d), 10 nM enzyme (green, $10K_d$), 100 nM enzyme (purple, $100K_d$), 1 μM enzyme (black, $1000K_d$). All curves were from GraphPad Prism using a sigmoidal dose-response curve model with variable slope. A general version of this figure may be found in ref 13.

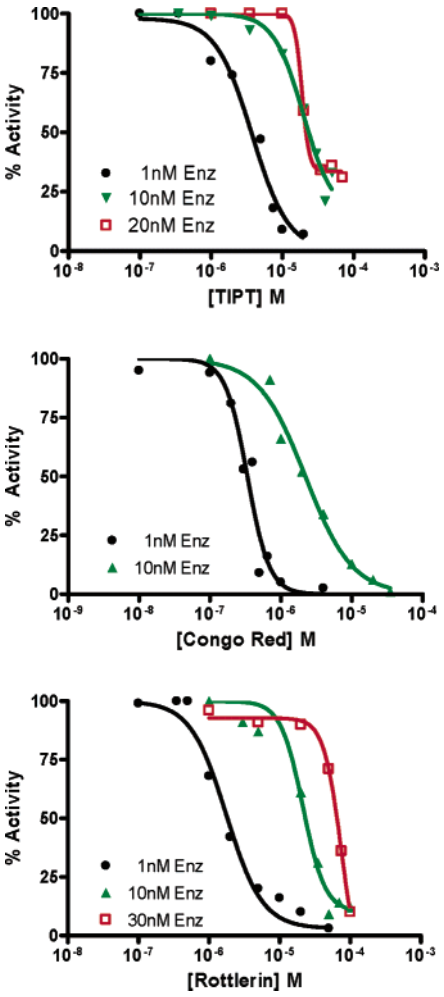

Figure 3. Dose-response curves of the promiscuous aggregators tetraiodophenolphthalein (TIPT), congo red, and rottlerin at 1 nM (black curves), 10 nM (green curves), and 20 nM (for tetraiodophenolphthalein, red curve) or 30 nM (for rottlerin, red curve) concentrations of β-lactamase.

the second term in eq 1 can no longer be ignored. Here, inhibition only rises to 10% when the inhibitor concentration is over the K_d value (1.1 nM). Inhibition then increases to 90% by 18 nM. This dose response is thus steeper, and the IC_{50} value is 6 nM, no longer reflecting the true K_d of 1 nM (Figure 3, green curve). As one adds more enzyme, the first term in eq 1 becomes negligible. The curves sharpen, and the IC_{50} becomes

Table 1. Dependence of Inhibition and Hill Slopes on Enzyme Concentration for Three Aggregators

	1 nM AmpC	10 nM AmpC	20 nM AmpC	30 nM AmpC
		Tetraiodophenolphthalein		
IC_{50}, μM	3.8	20	19	NM[a]
std error	±0.6	±5	±1	
Hill slope	−1.7	−2.1	−8.6	NM[aa]
		Congo Red		
IC_{50}, μM	0.34	2.2	NM[a]	NM[a]
std error	±0.03	±0.6		
Hill slope	−2.8	−1.3	NM[a]	NM[a]
		Rottlerin		
IC_{50}, μM	1.8	21	NM[a]	76
std error	±0.22	±1.7		±22
Hill slope	−1.71	−2.77	NM[a]	−3.441

[a] NM, not measured.

linearly dependent on enzyme concentration. Thus, at 100 nM enzyme, 100-fold the K_d, 10% inhibition is reached at 10 nM inhibitor, the IC_{50} increases to 51 nM, and the dose response goes from 10% to 90% inhibition over a 9-fold change in concentration (Figure 2, purple curve). In this "zone" of inhibition,[13] the measured IC_{50} reflects nothing of the true K_d but only the concentration of the enzyme.

The consequences for curve shape and enzyme concentration of this stoichiometric model was first discussed by Avram Goldstein in 1943,[13] though the form of eq 1 dates from earlier work.[14] Goldstein was not concerned with aggregation, of course, but rather with the behavior of potent inhibitors. His model is general and predicts the behavior of all inhibitors, potentially including aggregates active in this zone. When the K_d is much lower than the enzyme concentration, IC_{50} depends linearly on enzyme concentration and the steepness of the dose-response curves will also increase with enzyme added, up to some maximum.[13] Such behavior differs from what one would predict from multisite inhibition or from a phase transition.

To test this stoichiometric model, I investigated the behavior of three established promiscuous aggregators, tetraiodophenolphthalein, congo red, and rottlerin, with the model enzyme AmpC β-lactamase. As in previous studies, all three inhibitors were used as supplied by the manufacturer.[9,11] β-Lactamase is a good enzyme to test this hypothesis, since it is active at low concentrations and has substrates that are hydrolyzed more or less rapidly, allowing one to use both low and high enzyme concentrations without exhausting the substrate.

All assays were performed in 50 mM Tris, pH 7.2; all inhibitors were tested at 1 and 10 nM β-lactamase, with rottlerin further tested at 30 nM enzyme and tetraiodophenolphthalein at 20 nM enzyme. Rottlerin and tetraiodophenolphthalein were made up in DMSO and diluted into reaction buffer; all reactions were controlled for the presence of DMSO. Mixtures of congo red that were homogeneous to the eye could be made up directly in buffer. AmpC β-lactamase was overexpressed and purified and its concentration determined spectrophotometrically, as described.[15] In reactions at 1 nM enzyme, 200 nM nitrocefin was used as a substrate. For higher enzyme concentrations, a slower substrate, the C3′ methyl ester of cephalothin bearing the penicillin-G side chain, was used at 100 μM.[11,12] Hydrolysis was monitored at 262 nm for the cephalothin analogue and at 482 nm for nitrocefin. In all assays, substrate was added last and enzyme was incubated for 5 min in the presence of inhibitor at its final concentration.

All three inhibitors had steep dose-response curves at 1 nM β-lactamase (Figure 3), with nominal IC_{50} values in the low micromolar or, for congo red, high nanomolar range and Hill coefficients well above 1 (Table 1). These nominal IC_{50} values

reflect the total amount of organic material added to the buffer; the actual concentration of the inhibiting species, the colloidal aggregate, is at least several orders of magnitude lower than the nominal concentration. Increasing the enzyme concentration 10-fold, to 10 nM, increased the IC_{50} values for all inhibitors (Table 1). For tetraiodophenolphthalein and congo red, potency decreased by five to 6-fold, slightly less than the maximum predicted, whereas for rottlerin it decreased 12-fold, slightly more than predicted. When enzyme concentration was increased to 30 nM, the IC_{50} of rottlerin increased by slightly more than 3-fold again. Increasing the enzyme concentration 2-fold further to 20 nM did not increase the apparent IC_{50} of tetraiodophenolphthalein, but the meaning of this is clouded by its precipitation at around 30 μM, just slightly above its IC_{50} at 20 nM enzyme. At lower concentrations of inhibitor, where it has not precipitated, the 20 nM enzyme curve is right-shifted compared to the 10 nM enzyme curve, as expected.

Consistent with the predictions of the model, the slope of the IC_{50} curves increased with enzyme concentration for both rottlerin and tetraiodophenolphthalein, though it decreased for congo red. The steepness of the curves can be measured by their Hill coefficients, which for the former inhibitors reach values of -3.4 and -8.6 at the highest enzyme concentrations (Table 1). Hill coefficients depend on the parameters used in a fit, and one should be cautious about overinterpreting these numbers. Indeed, the larger-than-expected increases in IC_{50} values for rottlerin may be attributed to the difficulties in accurate curve fitting with such steep dose responses and to their sensitivity to small changes in enzyme concentration. Still, it is clear that the slopes of the curves increase steadily with enzyme concentration for these two inhibitors (Figure 3).

Intriguingly, the dose-response curves for tetraiodophenolphthalein and rottlerin appear to saturate below full inhibition at high enzyme concentrations. For tetraiodophenolphthalein, this happens between 30 and 40 μM, and for rottlerin it is around 80 μM. At these concentrations, both inhibitors begin to precipitate, as indicated by changes in their UV−vis spectra at these concentrations and, certainly for tetraiodophenolphthalein, by visible flocculation. Apparently, these molecules do not inhibit as precipitants. Promiscuous aggregators thus seem to be subject to at least two equilibria: at a certain concentration, they transition from a soluble form to a colloidal aggregate, but as concentration is raised further, they precipitate. It is only the intermediate, colloidal aggregate that appears to be inhibitory (see also ref 16).

These results are consistent with the stoichiometric inhibition model of Goldstein and inconsistent with the multisite or phase transition models. The IC_{50} values of all inhibitors depend almost linearly on enzyme concentration. For both rottlerin and tetraiodophenolphthalein the dose-response curves become steeper with enzyme concentration, also consistent with theory. The correspondence between theory and experiment is imperfect; the IC_{50} of tetraiodophenolphthalein does not increase in going from 10 to 20 nM enzyme nor does the dose-response curve of congo red sharpen with enzyme concentration. These discrepancies may be attributed to difficulties with measurements under sensitive dose-response conditions and to inhibitor precipitation.

Allowing for these discrepancies, this model has several implications. It predicts that enzyme inhibitors with steep dose-response curves have K_d values below the enzyme concentration. It is worth emphasizing that this prediction is general and is not restricted to promiscuous inhibitors. Indeed, Goldstein's "zone inhibition" model (eq 1 and Figure 2) was derived for classical inhibitors whose K_d values were simply much lower than the enzyme concentration.[13] Multisite binding or phase transitions cannot be completely discounted, but they are implausible for most screening hits. This will be true of potent, well-behaved inhibitors as well as covalent modifiers and aggregating inhibitors. An advantage of the stoichiometric model is that it may be easily tested by increasing enzyme concentration; a linear or nearly linear increase in IC_{50} with enzyme concentration supports this mechanism.

By the same logic, a steep dose-response curve does not itself mean that an inhibitor is artifactual; as in Goldstein's original analysis,[13] it could simply be very potent. This will be more likely when enzyme concentrations are high, and the implied K_d values are thus also high. Conversely, low enzyme concentrations imply low K_d values, and these are rarely credible for primary screening hits. In such cases, steep dose-response curves may indicate covalent or aggregation-based activity. Covalent inhibitors are more likely at lower concentrations, near those of the enzyme itself, whereas aggregates will manifest at higher apparent concentrations, where the colloidal particles form.

Aggregator dose-response curves can level out at less than 100% inhibition at high enzyme concentrations, owing to inhibitor precipitation. This supports earlier inferences that colloidal aggregation differs from precipitation,[16] with inhibition a property of only the former. Intriguingly, many screening hits also have dose-response curves that saturate at less than 100%.[6] Although there are classical reasons why this might be so, such behavior might imply that such screening hits pass through a phase transition, such as precipitation, before enzyme saturation.

A final implication of this study is that the K_d values of colloidal aggregates can be low indeed. On the basis of the lowest concentration of enzyme used here, 1 nM, and an enzyme to K_d ratio of at least 10, the K_d for all three inhibitors can be no greater than 100 pM. The molecular meaning of such low K_d values remains uncertain, though it might suggest extensive interaction between enzyme and aggregate. What it does imply is that promiscuous aggregation would be an even greater problem were it not for the use of most enzymes at concentrations well above these subnanomolar K_d values. Whereas one response to promiscuous inhibitors has been to lower the concentrations of organic molecules in screens, for many promiscuous inhibitors one could achieve the same effect by raising enzyme concentrations. Certainly the opposite strategy, moving to ever lower concentrations of enzyme, should be approached with care, as it could reveal previous promiscuous inhibitors fortuitously hidden by a high ratio of enzyme to K_d.

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