Refining Cheng-Prusoff equation

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A generalisation of the Cheng-Prusoff formula relating affinity constant and the concentration of inhibitor giving 50% inhibition has been derived for the case when concentrations of ligands are not in great excess. It shows that methods ensuring precise solving of binding equations (including computer approaches and equations derived in the present work) can not be used if these "Cheng-Prusoff conditions" of ligands' excess are not observed. The trouble is that beyond these conditions analysis becomes unreliable: the error contained in estimations of necessary parameters appears abruptly multiplied in the calculated affinity constant.

1. Introduction

Determination of affinity constant of unlabeled ligand to a binder by measuring how its varying concentrations inhibit binding of labelled ligand (tracer) to the same binding sites is a commonest procedure in many fields of biochemistry, pharmacology and immunology. The simplest interpretation of this experiment consists in belief that the concentration of unlabeled ligand (inhibitor) which decreases binding of tracer by 50% may be taken for the measure of inhibitor's affinity constant.

The trivial rationale to think so is that if the inhibition curve is just a turned upside down binding curve of inhibitor-binder reaction, so its middle-point also may be taken as estimate for affinity constant.

Indeed, if tracer does not influence the equilibrium between inhibitor and binder then at the concentration of free inhibitor equal to its affinity dissociation constant binding sites are half-occupied by inhibitor and the remaining half binds an amount of tracer equal to one half from the tracer bound in the absence of inhibitor.

It is easy to find out that three pitfalls exist why this speculation may be erroneous:

1. Tracer may significantly shift equilibrium in inhibitor-binder reaction;
2. It is only total concentration of inhibitor that may be known in experiment, whereas its free concentration may significantly differ from total;
3. One half binding sites not necessarily binds two times lesser amount of tracer. If tracer is almost completely bound by binding sites in the absence of inhibitor, it may happen that half of binding sites may also bind tracer almost completely.

The currently standard procedure of dealing with these troubles is usually attributed to work [1]: avoiding second and third pitfalls by introducing a demand that only negligibly small fractions of ligands are bound by binding sites (i.e. low depletion) it is possible to derive the following equation describing disruption of equilibrium in inhibitor-binder reaction by tracer:

$$I_{50} = K_d \cdot (1 + \frac{T_0}{K_d^*}) \quad (ST << T_0;\ SI << I_{50}) \tag{1}$$

where I_{50} is the concentration of inhibitor giving 50% inhibition; K_d and K_d^* are dissociation affinity constants of inhibitor and tracer; S_0, T_0 and I_0 are total concentrations of binding sites, tracer and inhibitor; SI and ST - bound concentrations of inhibitor and tracer. It is worth to note that Cheng-Prusoff constraints should be valid only at the midpoint of inhibition curve (I_0=I_{50}) as it is this point for which eqn.(1) is written.

The necessity to keep bound concentrations small and uncertainty as to how strictly these Cheng-Prusoff constraints should be followed resulted in numerous attempts to develop more universal procedures for determination affinity constant from inhibition curve either by curve-fitting [e.g. 2] or by derivation of more general equations [e.g. 3]. Yet, these works unanimously omit investigation of robustness of using proposed procedures, i.e. the conditions under which the commonly appearing errors in necessary

estimations of binding parameters do not lead to fatal distortion of result were not determined.

More practically, it means that under certain conditions of experiment you may enter, for example, a 20% bigger number for the estimation of total concentration of binding sites into your favourite program (or equation) and to obtain an over 10-fold-higher calculated affinity constant. Or you may even receive a less-than-zero affinity constant, though, of course, computer programs would invite you to try to fabricate another value.

So, actually, it is possible to determine empirically whether you are in such unpleasant situation simply by varying input parameters and looking at the response in calculated affinity.

The present work presents an analytical investigation of this trouble deriving a direct upgrade of Cheng-Prusoff formula which remains sufficiently simple to understand how errors in input parameters translate into error in calculated affinity constant.

2. Theory

Competition of inhibitor and tracer for the single type of binding sites is described by the set of equations:

$$K_d^* = \frac{(S_0 - SI - ST)\cdot(T_0 - ST)}{ST} \tag{2a}$$

$$K_d = \frac{(S_0 - SI - ST)\cdot(S_0 - SI)}{SI} \tag{2b}$$

In the absence of inhibitor ($I_0 = 0$ and $SI = 0$) it is easy to derive from (2a) that:

$$T_0 = B_{max}\cdot\left(1 + \frac{K_d^*}{(S_0 - B_{max})}\right) \tag{3}$$

where B_{max} is maximal signal on inhibition curve (i.e. ST in the absence of inhibitor).

At the midpoint of inhibition curve by definition $ST = B_{max}/2$ and $I_0 = I_{50}$, so eqns. (2a) and (2b) are written as:

$$K_d^* = \frac{(S_0 - SI - B_{max}/2)\cdot(T_0 - B_{max}/2)}{B_{max}/2} \tag{4a}$$

$$K_d = \frac{(S_0 - SI - B_{max}/2)\cdot(I_{50} - SI)}{SI} \tag{4b}$$

substituting (3) to (4a) leads to:

$$SI = S_0 - B_{max}/2 - \frac{K_d^*\cdot(S_0 - B_{max})}{S_0 - B_{max} + 2\cdot K_d^*} \tag{5}$$

eliminating SI in (4b) with (5) leads to:

$$I_{50} = \left(S_0 - \frac{B_{max}}{2} - \frac{K_d^*\cdot(S_0 - B_{max})}{S_0 - B_{max} + 2\cdot K_d^*}\right)\cdot\left(1 + \frac{K_d\cdot(S_0 - B_{max} + 2\cdot K_d^*)}{K_d^*\cdot(S_0 - B_{max})}\right) \tag{6}$$

Upon introduction of a new variable: $f = (S_0 - B_{max})/S_0$ (i.e. f is the fraction of free binding sites in the absence of inhibitor) rearrangements result in the following precise formula for I_{50}:

$$I_{50} = \left(1 + (1+f)\cdot\frac{K_d}{K_d^*}\right)\cdot\frac{S_0}{2} + \frac{K_d}{f} + f\cdot\frac{f\cdot S_0}{f\cdot S_0 + 2\cdot K_d^*}\cdot\frac{S_0}{2} \tag{7}$$

It may be shown that the third term in eqn.(7) equals to the difference between SI and $S_0/2$ and, therefore, it describes the third pitfall listed in introduction. Rather surprisingly, it has next to no practical significance as it is always is smaller (and usually much smaller) than the first term. Indeed, third term has two multipliers for $S_0/2$ both less than unity, whereas first term has one multiplier which is bigger than unity.

These two terms become comparable in a rare situation when tracer is almost completely bound in the absence of inhibitor still occupying only a small fraction of binding sites. So, the third term may be neglected, yet its appearance may be improved by introducing a new variable $t=(T_0-B_{max})/T_0$, (i.e. t is the fraction of free tracer in the absence of inhibitor). With it eqn.(7) alters into:

$$I_{50}=\left(1+(1+f)\cdot\frac{K_d}{K_d^*}+f\cdot\frac{1-t}{1+t}\right)\cdot\frac{S_0}{2}+\frac{K_d}{f} \quad (8)$$

If tracer and inhibitor are homologous ligands it may be usually taken for granted that $K_d<K_d^*$. In this case the value of multiplier for $S_0/2$ lies between 1 and 4. In view of large uncertainty in S_0 discussed further, this multiplier may be taken equal to 2, so the simplified version of eqn.(8) is:

$$I_{50}\approx S_0+\frac{K_d}{f} \qquad (K_d\leq K_d^*) \quad (9)$$

To relate eqn.(8) to Cheng-Prusoff formula it may be noted that under Cheng-Prusoff condition $ST<<T_0$ the unity within the brackets in eqn.(3) disappears; upon substituting it to the eqn.(1) a remarkably short equivalent form of Cheng-Prusoff equation is obtained:

$$I_{50}\approx\frac{K_d}{f} \qquad (ST<<T_0; SI<<I_{50}) \quad (10)$$

Naturally, eqn.(8) transforms into eqn.(10) either when the concentration of binding sites S_0 is made small or when the amount of added tracer T_0 is made big (corresponding to small f). Both ways, obviously, ensure approaching the Cheng-Prusoff conditions.

3. Discussion

As it may be seen from eqn.(9) the error in the calculated affinity constant is defined by the error contained in the difference I_{50}-S_o (the multiplier for So presented in exact equation (8) is omitted for simplicity).

As it was shown above, S_o is actually a correction to Cheng-Prusoff approximation, and the trouble with its subtracting is that when it becomes comparable to I_{50} (i.e. when Cheng-Prusoff constraints are violated and the precise solution gives significantly - at least 2-3-fold - different result in comparison with Cheng-Prusoff approximation) then the difference I_{50}-So becomes several times smaller than both I_{50} and S_o. Yet the absolute errors in this difference is roughly speaking a sum of absolute errors in S_o and in I_{50}. And as difference is several times smaller, relative error (coefficient of variation) in it becomes several times bigger.

Everything dealt with errors is a traditionally “difficult to follow” theme in biochemistry. So, for clarity let’s consider a numerical example. Let's put I_{50} equal to unity (arbitrary units) with 20% error (confidence interval 0.8-1.2) and S_o equal to 0.75 also with 20% error (interval 0.60-0.90). With these values correct value of I_{50}-S_o is 0.25. The Cheng-Prusoff approximation neglects S_o and provides as estimation 1.0; precise solution might give 0.25 (i.e. 4-fold correcting Cheng-Prusoff estimation), yet the difference I_{50}-S_o is 4-fold smaller than I_{50}, hence the relative error in it (and in affinity constant) is over 4-fold bigger than that in I_{50} - i.e. over 80% (too much). Taking boundary numbers from the confidence interval it is easy to see that, for example 0.8-0.90 will give less-than-zero calculated affinity; something like 0.8-0.799 will give a thousand-fold error in affinity constant and so on.

Therefore under this extent of violating Cheng-Prusoff conditions the 20% error is too big for reliable determination of affinity constant. Alas, significantly less than 2-3-fold correcting Cheng-Prusoff approximation is of little practical importance and, on the other side, accuracy of estimates for I_{50} and S_o significantly better than 20% seems unrealistic.

So, beyond Cheng-Prusoff conditions errors in data (which are typically not small) appear abruptly multiplied in the estimation provided by precise solving binding equations. And, practically, such estimation is not usable.

This conclusion equally applies to computer approaches. There is absolutely no magic in curve-fitting: it is just another way for precise solving binding equations. It may be said that computer programs are just a more convenient and objective method to find the concentration I_{50}. These programs really do it better than any graphical method; more importantly, they also can filter out non-specific binding and detect possible heterogeneity in binding sites. Yet the statements that precise solving equations may be used practically beyond Cheng-Prusoff conditions is equally erroneous both in analitical and in computerised upgrades of Cheng-Prusoff procedure.

With these regards it is, perhaps, reasonable to consider the first term in eqn.(8) as a convenient marker of closeness the system to Cheng-Prusoff conditions rather than a usable correcting term. In other words, the most practical way to interpret inhibition curve is to calculate I_{50} both with and without correcting term in eqn.(8); if the difference is less than approximately 2-fold, it is reasonable to take mean value as a final estimation. If difference is bigger, it is recommendable to conduct another experiment under conditions closer meeting Cheng-Prusoff constraints.

As concerning writing Cheng-Prusoff equation in the form of eqn.(10), it applies to an important immunological problem of determination "true" affinity constant [e.g. 4] from the variation of inhibition experiment where the antigen immobilised on solid phase and unlabeled antigen in solution compete for antibodies in solution; after completing this reaction the amount of antibodies bound to solid phase antigen is determined by adding antiidiotypic labelled antibodies. In this case, obviously, the solid phase antigen plays the part of tracer in all equations derived above, and the trouble is that its concentration is not known. Therefore, values T_0 and K_d^* necessary for eqn.(1) are not available, whereas fraction f in equations (8) or (10) may be easily estimated in the following experiment: after incubating antibodies with solid phase antigen the unbound fraction must be added again into another well with solid phase antigen and the rest of procedure must be completed in both wells; obviously, the ratio of signal in second well to the signal in first well directly provides estimation for f. So, this variation of inhibition experiment may be consistently analysed following usual Cheng-Prusoff procedure.

References

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