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Hill coefficients, dose–response curves and allosteric mechanisms

Heino Prinz

Abstract Hill coefficients (n_H) derived from four parameter logistic fits to dose–response curves were compared to calculated realistic reaction schemes and related to experimental data: (1) Hill coefficients may give information on the number of interacting sites but cannot distinguish between competitive, non-competitive or ortho-, iso-, or allosteric mechanisms. (2) For enzymatic dose–inhibition curves, Hill coefficients smaller than one do not indicate anticooperative binding but show that at least one ternary complex has enzymatic activity. (3) Hill coefficients different from one are proof for multiple ligand binding. The large variations of reported Hill coefficients corresponds to multiple allosteric binding, where induced conformational changes cause loss of the active conformation. Such a denaturation mechanism is in stark contrast to the desired specificity of drugs. The discussion is open.

Keywords Hill coefficients · Dose–response curves · Allosteric mechanisms · High throughput screening · Protein denaturation

Introduction

Over the last years, a large number of dose–response curves have been produced during screening campaigns [1]. Although these experiments usually had been designed simply for the determination of drug potency, some dose–response curves had been measured and analyzed according to a standardized protocol [2]. It is based on a four parameter

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logistic fit, "4PL" [2], which mathematically is identical to the Hill equation [3]. This function is a simplification, which must be confronted with realistic models of receptor activation and enzyme inhibition. The calculation of enzyme inhibition is different from the calculation of receptor activation, although both processes currently are analyzed with the same simplifications. Receptor activation is easier to calculate and therefore will be covered first.

The classical receptor concept [4] states that any physiologic response is a result of ligand binding, so that dose–response curves basically reflect binding curves.

$$L + R \rightleftarrows LR \rightarrow \text{response} \qquad (1)$$

When Θ is the fraction of bound ligand to maximal bound ligand or of response to maximal response, then Eq. 1 can be calculated with:

$$\Theta = [L]/(K_D + [L]) \qquad (2)$$

K_D is the equilibrium dissociation constant, which would be named EC_{50} (dose for 50% effect) in the case of classical dose-response curves or IC_{50} (50% inhibition) in the case of inhibition screens. In most cases, the real curves do not follow this simple hyperbolic function but show significant deviations. This was already observed in 1910 by Hill [3] for the binding of oxygen to hemoglobin. He used the following scheme:

$$n \times L + R \rightleftarrows L_n R \rightarrow \text{response} \qquad (3)$$
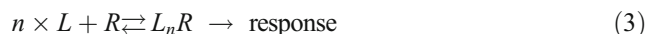
which leads to the corresponding "Hill equation":

$$\Theta = [L]^n/(K_D + [L]^n) \qquad (4)$$

The number of bound ligands n is called the Hill coefficient n_H. The dimension of the equilibrium dissociation constant K_D in this version of the Hill equation is M^n (molar to the n). At first glance, Eq. 3 looks simple and logical, but it is by no

means plausible [5, 6]. It only considers the fully occupied receptor L_nR and completely ignores all intermediate complexes such as LR, L_1R, L_2R, … Moreover, the application of the Hill equation to real dose–response (or binding) curves typically results in non-integer Hill coefficients and thus contradicts binding of n ligands (Eq. 3). The explanation for the binding of oxygen to hemoglobin has been given by Monod et al. [7], who proposed that the four subunits of hemoglobin exist in two different conformations with markedly different affinities for O_2. Allosteric interactions between those subunits would then ensure that a conformational change of one subunit leads to conformational changes of the others. This concept dramatically reduced the probability of the intermediate complexes LR, L_1R, L_2R, … and thus would justify Eq. 3 as a first approximation to their plausible model.

The Hill equation (Eq. 4) has proven to be practicable. Today, it is applied as a "4 parameter logistic function" (4PL) with K_Dn (non-integer n) instead of K_D for the analysis of dose–response curves [1]. On should keep in mind, however, that logistic functions were developed for the calculation of population growth (as a function of time) and that application to drug binding (as a function of concentration) never has been justified. Logistic functions are so simple to calculate that they often are included in routine analysis software and thus may be regarded as a standard for screening studies. Realistic reaction schemes require numerical solutions of non-linear equations, which have not yet been established in the field.

Complicated binding patterns, in particular those expressing a Hill coefficient larger than one, sometimes are taken as an indication for allostery, since allosteric interactions between protein subunits had been used for the analysis of O_2 binding [7]. A different type of allosteric interactions, namely those between substrate and other ("allosteric") protein binding sites, had been identified in crystal structures of known inhibitors [8]. This eventually led to a much broader definition of allostery [9, 10], where allosteric interactions are seen as conformational changes of a protein elicited by one compound, which then affect the binding of or the functional response to another compound. This definition is semantically correct [10], but it is not surprising [11], and indeed an intrinsic property of proteins [12], that a ligand that binds one site should affect its conformation elsewhere. When ligand binding leads to inhibition, this definition of allostery corresponds to ligand induced protein denaturation.

Methods

Phosphatase assays (6) were performed by means of automated systems consisting of Tecan EvoWare robots and Genios Pro MTP readers. Reaction velocity was determined from the slope of the absorbance change of the substrate (*p*-nitrophenyl phosphate) at 405 nm and related to control without enzyme for 0% activity and without inhibitor for 100% activity. All reaction mixtures contained 1 mM 1,4-dithio-D,L-threitol added on the day of the experiment from 100 mM stock and 0.0125% (*v/v*) of the detergent NP-40 (Calbiochem 492015).

For model calculations, three equations follow from the summation of total enzyme, total substrate, and total inhibitor concentration, respectively, including all complexes that appear in the respective reaction schemes. The complexes were calculated from the free concentrations and the respective equilibrium dissociation constants, e.g., $I_2E = I \times I \times E/(K_D1 \times K_D2)$. All calculations were done with the program Matlab R2008a and its optimization toolbox (Mathworks.com). The routine fsolve was employed for solving the set of non-linear equations, and lsqcurvefit was used for multi-parameter nonlinear fits. Curve fitting was performed with single original dose–response curves and not with average values because quality control had shown that the dilution factor in the dilution series was more accurate than the initial dispense steps.
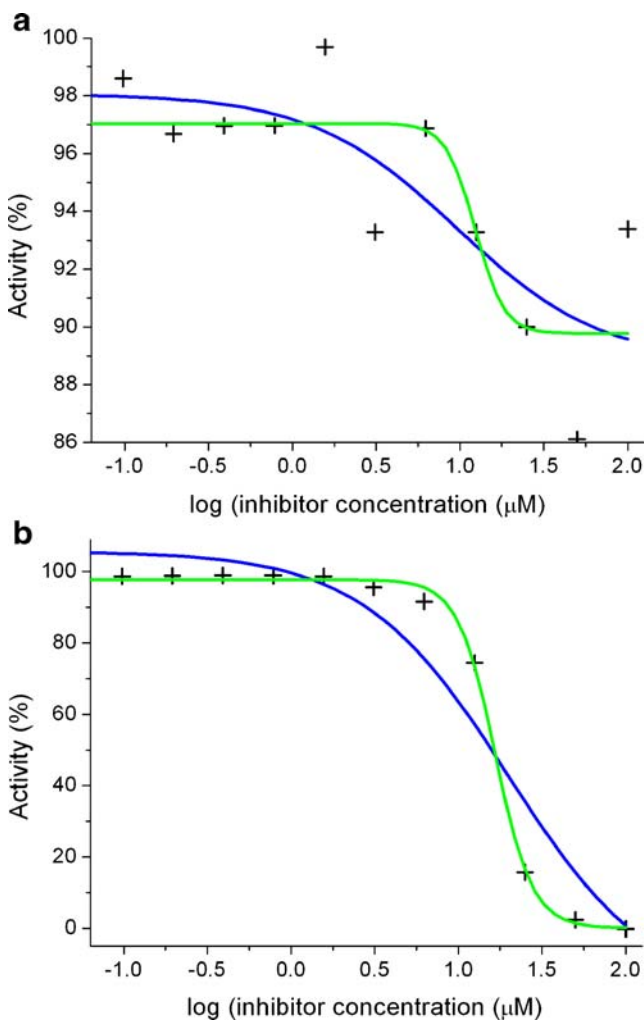
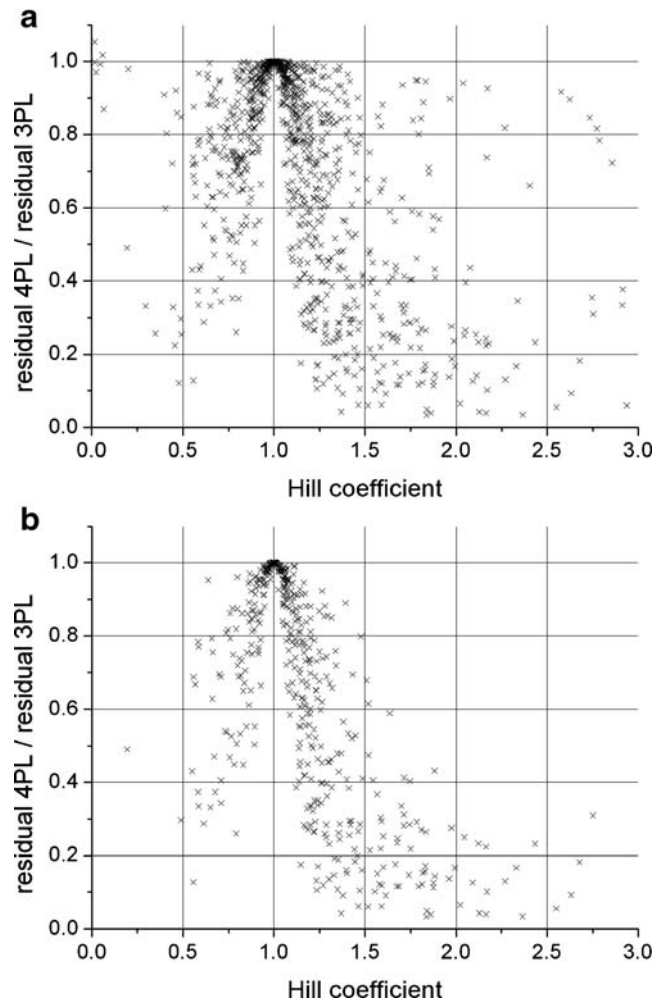## Results

Statistical errors and systematic variations

The main obstacle in quantitative data analysis is the signal/noise ratio of the data and the complexity of the models. As a general rule, an increasing number of fitting parameters will increase the goodness of any fit. A four-parameter fit to Eq. 4 simply employs one additional parameter as compared to the more plausible 1:1 competition and therefore will appear superior to most computers. A daunting example is shown in Fig. 1a, where the enzyme activity was only marginally decreased with the addition of inhibitor. Automated data processing gave preference for the four-parameter fit (green line) and resulted in a Hill coefficient of 5. Such analysis is not warranted by the quality of the data, where even the three-parameter fit to Eq. 2 (blue line) was not justified because the fitted minimal value was well outside the range of confidence.

At least in our hands, a full dose–response curve is sufficient for the determination of a Hill coefficient, as shown in Fig. 1b. For a large data set and for automated data processing, one has to find a method to discriminate between reliable and non-reliable experiments. The following procedure may be useful: As a first precaution, one should exclude all data, where the resulting IC_{50} value is outside the measured concentration range and where the fitted maximum

and minimum are outside the error range of the control experiments. This precaution alone will exclude the experiment in Fig. 1a. Then, one may compare the goodness of a four-parameter fit (Eq. 4) to a three-parameter fit (Eq. 2). The quotient of the respective residual sum of squares (res4/res3) varies with the Hill coefficient as shown in Fig. 2. For $n_H=1$, the residuals are expected to be identical for identical curves, and the quotient must be 1. For Hill coefficients that differed from one outside the experimental error range, the quotient res4/res3 decreased. Figure 2a shows the distribution of res4/res3 for a set of experiments as a function of Hill coefficients, and Fig. 2b shows the same set of experi-

Fig. 2 Quality control for the significance of the obtained Hill coefficients before (a) and after (b) the removal of outliers. The residual sum of squares of four-parameter logistic fits (residual 4PL) was related to the residual sum of squares of 1:1 competitive binding (residual 3PL). This quotient was plotted vs. the Hill coefficient obtained from 4PL

Fig. 1 High throughput fits for dose–activity curves. The enzymatic activity was related to the activity in the absence of inhibitor (100%). The background was measured from the signal in the absence of enzyme. The data were obtained from a twofold dilution series. *Blue line* competitive 1:1 inhibition ($n_H=1$), *green line* four-parameter logistic fit. **a** Inhibition of the phosphatase MPTPA by compound 5738, $n_H=4.99$. **b** Inhibition of the phosphatase PTP1b by compound 9873, $n_H=3.89$

ments after the outliers were removed with the above procedure. The quotient res4/res3 was 0.88 for the experiment shown in Fig. 1a and 0.034 for the experiment shown in Fig. 1b.

Dose–response curves for agonists and receptors

The receptor concept originally was developed from a pharmacological analysis of the nicotinic acetylcholine receptor [4]. Its dose–response curve typically gave Hill coefficients larger than one and smaller than two, but direct binding curves to the isolated nicotinic receptor from *Electrophorus electricus* [13] showed anticooperative or independent binding to two sites with a Hill coefficient smaller than one. This seeming discrepancy can be explained when two ligands are required for the

opening of an ion channel, such as depicted in reaction Eq. 5:

$$R \rightleftharpoons RL \rightleftharpoons LRL \rightleftharpoons LR$$

(5)
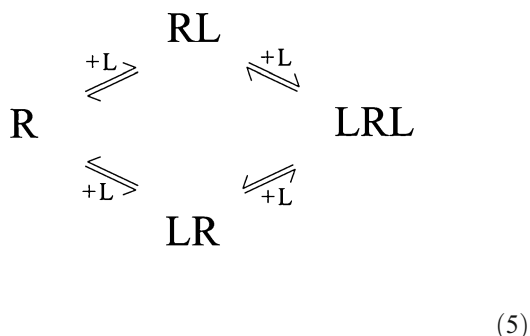
The binding curve would be calculated as the sum of bound ligand $RL+LR+2*$ LRL, but the response would be proportional to LRL. This is illustrated in Fig. 3, where binding to independent sites (blue line) may lead to a response curve (red line) with Hill coefficients larger than 1. In order to observe this variation of the Hill coefficient, the quantity log $[\Theta/(1-\Theta)]$ can be plotted versus log $[L]$. This representation is called a Hill plot. The rational for this plot follows from a modification of Eq. 4

$$\log(\Theta/(1-\Theta)) = n \times \log[L] - \log K_D$$

(6)

where log $[\Theta/(1-\Theta)]$ should be a linear function of the log of the ligand concentration. For stepwise binding
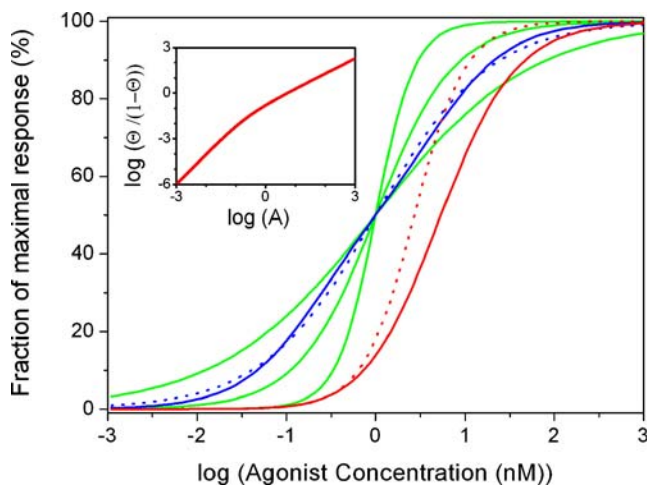

Fig. 3 Theoretical dose–response curves according to reaction Eq. 5 and two independent binding sites for the ligand L. The affinities are assumed to be 0.2 and 5 nM, respectively. All curves are normalized to 1. *Green lines* Hill Eq. 4 with Hill coefficients 0.5, 1, and 2. The *solid blue line* is the binding curve. The *dashed blue line* is a corresponding four-parameter fit with a Hill coefficient of 0.69. The *red line* corresponds to the response following LRL in Eq. 5. The *dashed red line* results from a four-parameter fit of the first 40 (of 100) data points with a Hill coefficient of 1.52

schemes, the Hill plot is by no means linear. When the response is caused by the fully occupied receptor in Eq. 5, it will result in a curve with an initial slope equal to the number of ligands required for the activation, leveling off toward a slope of 1 for higher ligand concentrations (insert in Fig. 3).

Figure 3 already illustrates some important points: (1) Dose–response curves with Hill coefficients other than one may result from receptors with more than one binding site; (2) the shape of the dose–response curve depends on the complex, which triggers the response; and (3) the highest possible experimentally determined Hill coefficient is equal to or smaller than the number of binding sites involved in facilitating that response.

States and sites

Allosteric mechanisms are concerned with different conformational states of a protein. These states may exist in the absence of ligand [7] or may be induced by its binding [14]. In all cases, the simplest reaction schemes concerning conformational states R and $R*$ of the receptor would be

$$\begin{array}{ccc} R & \xrightleftharpoons{+L} & RL \\ \updownarrow & & \updownarrow \\ R* & \xrightleftharpoons[+L]{} & R*L \end{array}$$
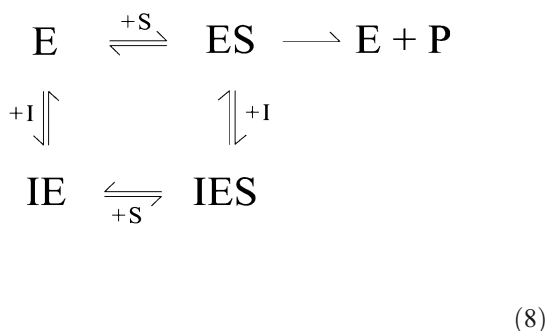
(7)
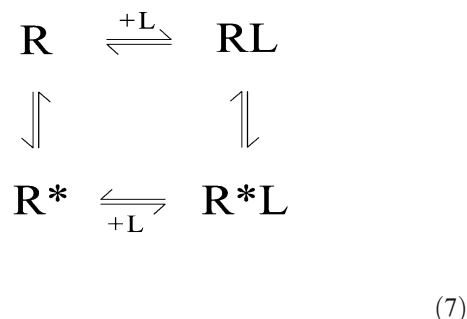
For kinetic experiments, this scheme generally should lead to biphasic reactions, even if the probability of $R*$ in the absence of ligand might be extremely low (induced fit). For equilibrium experiments like dose–response curves, free R and $R*$ or bound LR and $LR*$ will each be in equilibrium for any given ligand concentration. The sums of free and bound receptor populations will vary with the ligand concentration just as shown in Eqs. 1 and 2 with its Hill coefficient of one. This can be generalized: Under equilibrium condition, all conformational states with the same ligand occupancy (R and $R*$, LR and $LR*$, L_2R and L_2R*, L_3R, and L_3R*, respectively) will be in equilibrium, independent of the ligand concentration. Therefore, the concentration dependence of receptor occupancy (the underlying principle of dose–response curves) will only depend on the number of binding sites and not on conformational states. An allosteric mechanism, whereby the binding of one ligand to one allosteric site will elicit a

conformational change of the protein, therefore, must show a dose–response curve with a Hill coefficient of one. Only if more than one ligand is involved, either by allosteric interactions of protein subunits or by the binding to more than one site, can a Hill coefficient other than one be envisioned.

Dose–activity curves for inhibitors

The physiological response of a drug may not only be caused by the action of drugs on a receptor but also by the inhibition of an enzyme. In terms of classical pharmacology, the enzyme–substrate complex might be regarded as a receptor, where the binding of a drug leads to inhibition (the response). The underlying dose–activity curves for enzymes are more difficult to calculate than dose–response curves for receptors, since they involve an additional unknown, namely, the substrate concentration. In the simples case, the following reaction scheme has to be calculated:

$$
\begin{array}{ccc}
E & \overset{+S}{\rightleftharpoons} & ES \longrightarrow E+P \\
{\scriptstyle +I}\Updownarrow & & \Updownarrow{\scriptstyle +I} \\
IE & \underset{+S}{\rightleftharpoons} & IES
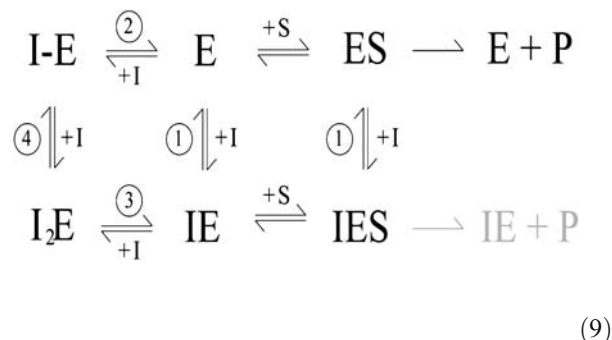\end{array}
$$

$$(8)$$

For competitive inhibitors, Eq. 8 can be simplified, since direct competition excludes the ternary complex *IES*.

The reaction velocity of enzymatic catalysis usually is observed either by means of the product formation or substrate depletion. Under the common steady state conditions of enzyme reactions, this velocity is proportional to the enzyme–substrate complex *ES*. Therefore, the calculation of a dose–activity curve can be reduced to the calculation of binding equilibria of the complexes *ES*, *IS*, and *IES* as a function of the inhibitor concentration *I*. For the reaction Eq. 8, the dose–activity curve has a Hill coefficient of one when calculated from the equilibrium complex *ES* as a function of *I*. This is true for all combinations of equilibrium constants. It is independent of the existence of the ternary complex *IES* or whether or not this complex may express residual enzymatic activity. Even if one would add additional conformational states of the enzyme, the Hill coefficient could not differ from one.

Only when more than one inhibitor molecule can bind to the enzyme, Hill coefficients other than one may be

obtained. In the simplest case, two inhibitor molecules may be considered to bind to one enzyme molecule, as described in Eq. 9:

$$
\begin{array}{ccc}
I\text{-}E & \overset{②}{\underset{+I}{\rightleftharpoons}} & E & \overset{+S}{\rightleftharpoons} & ES \longrightarrow E+P \\
④\!\!\downarrow{\scriptstyle +I} & & ①\!\!\downarrow{\scriptstyle +I} & & ①\!\!\downarrow{\scriptstyle +I} \\
I_2E & \overset{③}{\underset{+I}{\rightleftharpoons}} & IE & \overset{+S}{\rightleftharpoons} & IES \longrightarrow IE+P
\end{array}
$$

$$(9)$$

Of these complexes, the ternary complexes I_2E and *IES* are decisive for the shape of the dose–response curve: If the binding affinity for the second inhibitor is larger than for the first one, a steeper slope in the dose–activity curve will be observed as compared with reaction Eq. 9. This corresponds to an increase in the Hill coefficient, as expected from this cooperative binding. If the complex *IES* should retain enzymatic activity, (reaction indicated in gray from *IES* to *IE+P* in Eq. 9, then a different mechanism would be implied. The inhibitor might bind to independent sites, either as *I–E* competing with the substrate or as *IE* to a site independent from the substrate binding site.

All these considerations have to be substantiated with experimental data, but data fitting can be ambiguous whenever too many parameters are required. Equation 9 implies four distinct parameters, namely K_I1, K_I2 and K_I3 for the equilibrium dissociation constants of inhibitor binding and the unknown activity of the ternary complex IES. Typically, the equilibrium constant for substrate binding as well as the concentrations for substrate and inhibitor are known. The equilibrium dissociation constant K_I4 follows from $K_I4 = K_I1 \times K_I3/K_I2$ and thus need not be fitted. The K_D value for the substrate S follows from the experimentally determined K_m value and also need not be varied in the fitting algorithm. However, just as in four-parameter fits, the amplitude and the background of the signal have to be varied in order to adjust the theoretical curves to experimental variations. This gives a total of six parameters (three equilibrium dissociation constants for the inhibitor, one factor for IES activity, and max and min) required for a fit to Eq. 9 as opposed to the four parameters required for the four-parameter logistic fit.

There are three logical restrictions that may be applied to reaction Eq. 9 in order to reduce the number of fitting parameters:

1. The inhibitor binding sites may be independent. This translates to $K_I3=K_I2$.

2. The inhibitor binding sites may be equivalent. This translates to $K_I2 = K_I1$.
3. The ternary complex *IES* may be fully inactive.
4. The ternary complex *IES* may be fully active.

When these restrictions are combined, the four following combinations will each lead to two inhibition equilibrium constants and either 1 or 0 for the activity factor, therefore to a total of four fitting parameters: 1 and 3, 1 and 4, 2 and 3 and 2 and 4. Table 1 lists the parameters derived with these restrictions together with the corresponding residual sum of squares. The blue lines in Fig. 4 represent 1:1 competition, which is the same as a four-parameter fit with the Hill coefficient of 1. The green lines represent a four-parameter logistic fit, and the dashed red lines represent the optimal fit to reaction Eq. 9 with the four parameters indicated (bold) in Table 1. Inspection of Table 1 leads to the following conclusions: (1) Dose–activity curves with a Hill coefficient smaller than one could only be obtained assuming activity of the ternary complex IES; (2) curves with large Hill coefficients could not be fitted with the assumption of independent inhibitor binding sites.

The data shown in Fig. 4 were, of course, not the only data that had been used for curve fitting. Dose–response curves with Hill coefficients larger than or equal to two could not be analyzed with Eq. 9, since the number of inhibitor binding sites had to be larger than the Hill coefficient. The symmetrical 4PL curves (green lines in Fig. 1b and 4b) are slightly different from experimental data (Fig. 1b) or the stepwise inhibition scheme (red dashed line

in Fig. 4b). However, this difference is small, and the small decrease in activity observed at low inhibitor concentrations (Fig. 1b) may well be obscured by experimental variations.

Discussion

All the above calculations have shown that dose–response curves for receptor agonists and enzyme inhibitors alike should always give a Hill coefficient of one if only one binding site on the receptor or enzyme is occupied. This statement also holds for specific allosteric mechanisms, whereby the binding to one allosteric site may elicit conformational changes of the protein. Such changes might lead to an increase in activity or to inhibition, but the Hill coefficient of the corresponding dose–response curve would always be one. Only if more than one drug binds, either as a set of agonists to a receptor or ligands to a set of subunits or as inhibitors to an enzyme, Hill coefficients other than one might be observed.

The experimental evidence concerning dose–response curves with Hill coefficients other than one is overwhelming: To the best of our knowledge, most researchers working in the field of high throughput screening consistently find that the shape of their dose–response curves and, hence, the Hill coefficient depends on the ligand employed. Inspection of the NIH database [15] confirms this anecdotal evidence. All dose–response experiments documented there were evaluated using the standard four-parameter logistic function and are represented in a comparable format. The data are public.

Table 1 Parameters used for fitting the experiments in Fig. 4 to reaction scheme 10

Model	1:1 Inhib	4PL	1&3	1&4	2&3	2&4
Figure 4a						
IC$_{50}$ (μM)	1.99	1.44				
n$_H$	1.0	0.56				
K$_I$1 (μM)			105.7	3.74	3.64	**0.45**
K$_I$2 (μM)			1.69	1.22		
K$_I$3 (μM)					2703	**2.63**
Max (%)	99.4	120.3	79.2	86.8	79.2	**96.6**
Min (%)	20.2	8.2	20.2	16.01.09	20.2	**16.1**
Residuals	*88.5*	*11.3*	*88.9*	*31.6*	*88.9*	***13.1***
Figure 4b						
IC$_{50}$ (μM)	7.9	6.94				
n$_H$	1.0	1.82				
K$_I$1 (μM)			10.6	4.04	**55.2**	53.7
K$_I$2 (μM)			4689	4823		
K$_I$3 (μM)					**0.93**	0.73
Max (%)	105.6	99.9	106.5	108,4	**100.4**	100.7
Min (%)	-14.25	-0.6	-2.9	-8.4	**-0.34**	-1.34
Residuals	*323.9*	*39.2*	*105.3*	*116.8*	***28.5***	*38.5*

The conditions 1 and 3, 1 and 4, 2 and 3, 3 and 4 translate to $K_D3 = K_D1$ and inactive *IES*, $K_D3 = K_D1$ and active IES, $K_D2 = K_D1$ and inactive *IES*, $K_D2 = K_D1$ and active *IES*, respectively
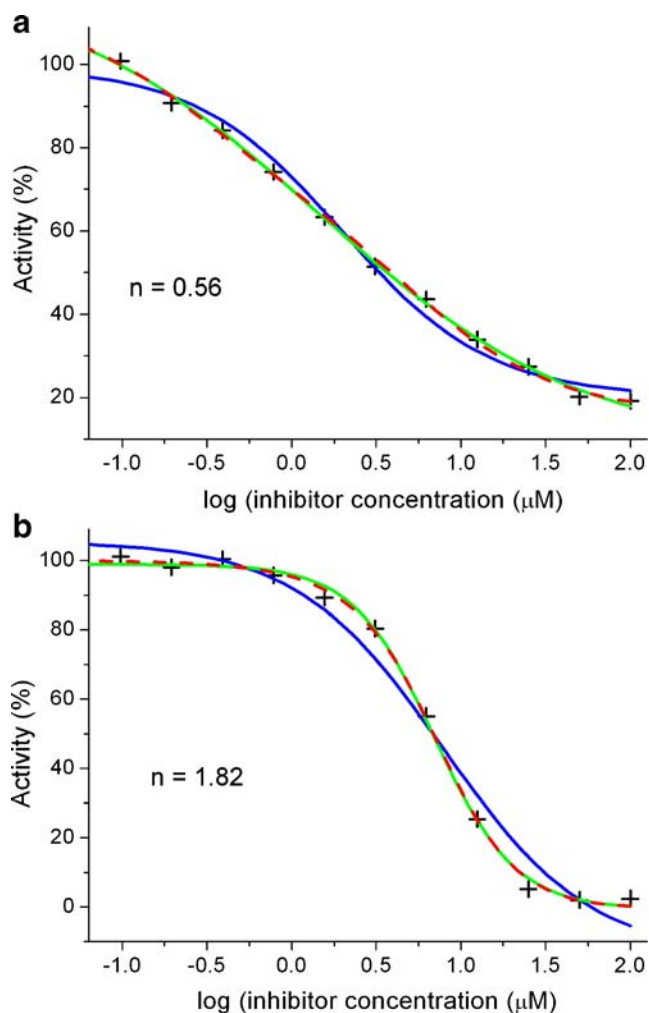
Fig. 4 Dose–activity curves with low (**a**) and high (**b**) Hill coefficients. The enzymatic activity of the phosphatase MPTPB was related to the activity in the absence of inhibitor (100%). The background was measured from the signal in the absence of enzyme. The data (+) were obtained from a dilution series (by a factor of 2). *Blue line* Data fit to competitive 1:1 inhibition ($n_H=1$), *green line* four-parameter logistic fit. *Dashed red line* Data fit to reaction Eq. 9 with parameters (highlighted in *bold letters*) listed in Table 1. **a** Compound 6555, $n_H=0.56$, optimal fit with the conditions 2 and 4. **b** Compound 40763, $n_H=1.82$, optimal fit with the conditions 2 and 3

Inspecting the different experiments shows that the distribution of the Hill coefficients depends on the assay and the target. Some assays can be analyzed in the framework of hyperbolic binding to one site, since their average Hill coefficient is one within their error margin. Most of the assays published in the NIH database give average Hill coefficients larger than one and non-Gaussian broad distributions. For example, the three assays with the largest number of dose–response curves (as of April 5th 2009) within the NIH databank [15] gave average Hill coefficients of 3.2, 1.5, and 2.4. The first of these (NIH identifier, AID 1458) was a cellular assay with a large statistical error of the

obtained Hill coefficient (±1.4). The second assay (AID 1460) [16] had measured tau aggregation and gave ±0.7 as a standard deviation of the Hill coefficient. The third assay (AID 893) is a classical enzyme inhibition assay for HSD14B4 activity. The histograms of these last two assays are shown in Fig. 5. Clearly, the distributions are different, but most observed Hill coefficients are significantly different from one, as indicated in red in Fig. 5. For HSD14B4 inhibition, less than 1% shows a Hill coefficient of one. Note that for distinct molecular mechanisms, one would expect one Gaussian distribution (or more) around one maximum (or more), reflecting the respective number of binding sites.

General allosteric mechanisms [8–12, 17] imply that allosteric inhibitors bind to one specific site on a protein.
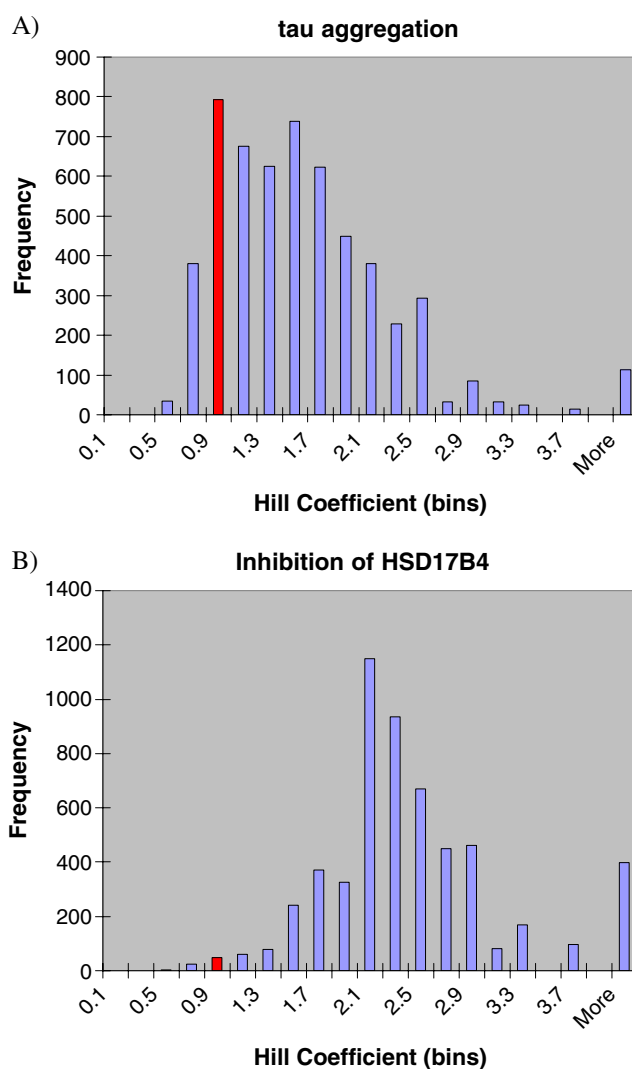
Fig. 5 Histograms obtained from the variation of Hill coefficients derived from four-parameter logistic fits. **a** Results from 5703 dose response curves (AID 1460) of tau inhibition. **b** Inhibition of HSD17B4, hydroxysteroid (17-beta) dehydrogenase 4 (AID 893) n_H values calculated from 5650 dose–response curves

Their binding leads to a conformational change, which in turn leads to enzyme inhibition. In many cases, specific allosteric sites have been identified by crystal structures of enzymes with inhibitors. The evidence for allosteric or conformational interactions is overwhelming, but why should a newly synthesized compound fit to an enzyme and elicit a specific mechanism, which is not observed for endogenous ligands? Could it be that allosteric inhibition simply is the loss of the active conformation of the protein? Binding to any conformationally sensitive location of a protein would lead to denaturation and loss of activity. This would be a plausible allosteric effect, since any change of the active conformation is likely to lead to partial loss of active structure and to inactivation. Moreover, loss of activity is well documented for compounds breaking secondary or tertiary protein structure such as ionic detergents or protons or denaturing organic solvents or chaotropic ions. Following this argument, desired allosteric inhibition (with Hill coefficient of one) would be one special case of a general allosteric denaturing process involving allosteric binding sites and Hill coefficients increasing with the number of sites. Denaturation by allosteric interactions can explain the broad non-Gaussian distribution of Hill coefficients (Fig. 5).

Another plausible explanation for varying Hill coefficients lies in the concept of transient binding patches [6]. This concept considers the dynamic nature of the binding process itself and postulates stepwise substrate recognition. Ligands, which would fulfill some requirements of first recognition, could then competitively inhibit substrate binding without the requirement of 1:1 stoichiometry. Both concepts consider dynamic proteins and multiple binding interactions. This contradicts most molecular models for drug development with specific interactions of one compound to one defined site on a protein. Had there ever been a successful drug developed from a compound which had shown a Hill coefficient different from 1?

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