

# Assessing Receptor Affinity for Inverse Agonists: Schild and Cheng-Prusoff Methods Revisited

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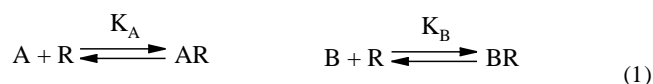
**Abstract:** Classical methods for the estimation of antagonist affinity constants were developed under the assumption of one unique state for the receptor. The finding of receptor constitutive activity, which implies that at least two (one active and the other inactive) receptor states coexist at equilibrium, extended the concept of antagonism by distinguishing between neutral antagonists and inverse agonists. To account for the complexity introduced in the concept of antagonism, classical Schild and Cheng-Prusoff methods have been revisited within the two-state model of agonism. The resulting equations match the classical expressions for neutral antagonists but not for inverse agonists. It is suggested a revision of current routine procedures for antagonist affinity estimation.

## INTRODUCTION – G PROTEIN-COUPLED RECEPTORS AND COMPETITIVE ANTAGONISM

G protein-coupled receptors (GPCRs) constitute the largest family of cell-surface receptors, with nearly 2000 members identified thus far. They regulate the function of most cells in the body, and account currently for about 1% of the genes present in a mammalian genome. These receptors respond to a wide variety of structurally diverse ligands, ranging from small molecules, such as biogenic amines, nucleotides and ions, to lipids, peptides, proteins, and even light. Ligands (agonists and antagonists) acting on GPCRs are commonly used in drug therapy of numerous diseases, including cardiovascular and mental disorders, retinal degeneration, cancer, and AIDS. It is estimated that these receptors are targets for approximately 50% of clinically used drugs [1].

Antagonism refers to the inhibitory effect exerted by a ligand (antagonist) to receptor physiological function. As receptor function is driven by agonists, antagonist effect is usually measured relative to agonist action. Different classes of antagonists can be considered depending on whether the antagonist binds to the same (orthosteric) or to a different (allosteric) receptor site as that used by the agonist. In this paper, we will limit our discussion to competitive antagonists. Competitive reversible antagonism is defined [2] as the condition in which the agonist and antagonist bind reversibly to the same recognition sites on the receptor and, thus, compete for them when they are present concomitantly.

Correct classification of antagonists is necessary both in basic and clinical pharmacology and, to this end, accurate measurement of antagonist potency is fundamental. Traditionally, all the methods developed to quantify antagonist potency were based on the original work by Gaddum [3], where the following equilibria were considered:



R is the free receptor, A the agonist, B the antagonist, and  $K_A$  and  $K_B$  the equilibrium dissociation constants for the AR and BR ligand-receptor complexes, respectively. The fractional receptor occupancy by the agonist in the presence of the antagonist is given by:

$$\frac{[AR]}{[R_0]} = \frac{[A]}{[A] + K_A \left(1 + \frac{[B]}{K_B}\right)} \quad (2)$$

where  $[R_0] = [R] + [AR] + [BR]$  is the total receptor concentration. Equation 2 establishes that the fractional receptor occupancy by the agonist decreases as greater is either the concentration of the antagonist or the affinity of the latter for the receptor.

The concentration of agonist producing half occupation of total receptor concentration in the presence of the antagonist,  $A_{50}$  (Equation 3), can be used as an index of the effect of the antagonist on agonist binding.

$$A_{50} = K_A \left(1 + \frac{[B]}{K_B}\right) \quad (3)$$

## CLASSICAL METHODS FOR ESTIMATING ANTAGONIST AFFINITY: SCHILD AND CHENG-PRUSOFF

From Equation 2, Arunlakshana and Schild [4] developed a quantitative model of competitive antagonism (Equation 4) by using the dose ratio (dr) concept, that is the ratio of concentrations of agonist in the presence ( $[A']$ ) and in the absence ( $[A]$ ) of antagonist which yield equal responses. It is assumed that if two responses are equal the same occurs for the corresponding receptor occupation. This approximation overcomes the problem of not knowing the relationship between binding and response [5].

$$\frac{[A']}{[A]} = dr = \frac{[B]}{K_B} + 1 \quad (4)$$

Equation 4 can be used for the estimation of the antagonist dissociation constant, which is normally done after a logarithmic transformation.

$$\log(dr - 1) = \log[B] - \log K_B \quad (5)$$

It is commonly accepted that linearity of  $\log(dr-1)$  on  $\log [B]$  with a slope of unity is an indication of competitive antagonism [2]. Under this premise, the antagonist dissociation constant is obtained from the intercept of Equation 5 by linear regression (Schild regression). To provide a more general expression of Equation 5 by allowing a slope different from unity, the equilibria from Equation 1 are often extended to the case that the ligand-receptor complexes are formed by  $n$  molecules of the agonist A ( $A_nR$ ) and  $m$  molecules of the antagonist B ( $B_mR$ ). The resulting Schild equation is [2]:

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$$\log(dr^n - 1) = m \log[B] - \log K_B \quad (6)$$

Equation 6 is correct if it is used empirically. However, a mechanistic interpretation for  $n$  and  $m$  greater than one is unreliable. The former equation has been obtained by assuming that the receptor contains multiple binding sites. However, only two ligand-receptor complexes,  $A_nR$  and  $B_mR$ , with the response arising from the formation of  $A_nR$ , are considered. Stoichiometric combinations as  $A_xR$ ,  $B_yR$ , or  $A_xB_yR$ , with  $x < n$  and  $y < m$ , are not included in the model. This is a simplification that can lead to misleading conclusions.

The Cheng-Prusoff equation [6] was originally derived to calculate the dissociation constant ( $K_i$ ) for an inhibitor of an enzymatic reaction (Equation 7).

$$K_i = \frac{I_{50}}{1 + \frac{[S]}{K_m}} \quad (7)$$

where  $I_{50}$  is the inhibitor concentration for which the reaction rate in the presence of the inhibitor is half of the reaction rate in the absence of the inhibitor, for a given substrate concentration ( $[S]$ ) and Michaelis constant ( $K_m$ ).

The Cheng-Prusoff equation can be translated directly from the enzymatic to the pharmacological scenario by making the analogy between inhibitor and antagonist. From Equation 1, the dissociation constant of the antagonist B is:

$$K_B = \frac{B_{50}}{1 + \frac{[A]}{K_A}} \quad (8)$$

where  $B_{50}$  is the antagonist concentration required to reduce the receptor occupancy by the agonist to half the value of that in the absence of the antagonist, for a given agonist concentration ( $[A]$ ) and agonist dissociation constant  $K_A$ . Equation 8 is correct for binding but not for functional studies. For the latter case, it is necessary to include a function for the transduction of receptor occupancy by the agonist into response. If Equation 9 is used as the transducer function [7],

$$\frac{E}{E_m} = \frac{[AR]}{K_E + [AR]} \quad (9)$$

the resulting Cheng-Prusoff equation is [8]:

$$K_B = \frac{I_{50}}{1 + \frac{[A]}{EC_{50}}} \quad (10)$$

where  $EC_{50}$  is the agonist concentration that elicits half maximum response in the absence of the antagonist and  $I_{50}$  is the antagonist concentration that reduces the agonist effect to half the value for a given  $[A]$  concentration. For clarity, we make the distinction between ( $A_{50}$ ,  $B_{50}$ ) and ( $EC_{50}$ ,  $I_{50}$ ) to differentiate between occupation and functional data, respectively.

If the transducer function is

$$\frac{E}{E_m} = \frac{[AR]^p}{K_E^p + [AR]^p} \quad (11)$$

then, the resulting Cheng-Prusoff equation under the assumption that the operational efficacy is large ( $\tau = \frac{[R_0]}{K_E} \gg 1$ ) renders [9]:

$$K_B = \frac{I_{50}}{\left(2 + \left(\frac{[A]}{EC_{50}}\right)^n\right)^{\frac{1}{n}} - 1} \quad (12)$$

Equation 12 is the proper form of the Cheng-Prusoff equation when we are dealing with steeper ( $n > 1$ ) or flatter ( $n < 1$ ) agonist concentration-effect curves. It is worthwhile to note that this equation has been derived by assuming a high efficiency of the signal transduction machinery. The latter assumption leads in turn to symmetric logistic concentration-effect curves (see [10] for a review on the analysis of the (a)symmetry of concentration-effect curves).

A number of empirical variants of Schild and Cheng-Prusoff methods can be found in the literature [11-17]. However, our aim was to apply these techniques under a mechanistic framework whose limits can encompass the GPCR system.

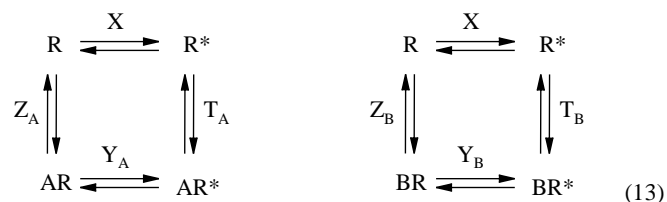
### CONSTITUTIVE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS SUGGESTS A REVISION OF THE METHODS FOR AFFINITY CONSTANT ESTIMATION

Gaddum [3], Schild [4] and Cheng-Prusoff [6] methods were developed under the assumption of one unique state (R) for the receptor and, consequently, one unique class of competitive antagonists. However, a pioneering work [18] on  $\delta$  opioid receptors, showing that competitive antagonists can have negative efficacy, and data [19, 20] from discrete mutations of receptor sequence indicated that GPCRs are constitutively active and can yield functional response in the absence of agonist. This spontaneous receptor activity has been observed over the last decade in a wide variety of GPCR systems [21, 22]; basically, it implies that at least two, one active ( $R^*$ ) and the other inactive (R), receptor states coexist at equilibrium. This finding changed the concept of antagonism. The classic definition of competitive antagonists as those substances lacking intrinsic activity and which preclude the binding of agonists to receptors by competing for the same recognition sites became out of date, and the terms negative antagonist/inverse agonist and neutral antagonist appeared. Inverse agonists are those ligands that bind preferably to the R state decreasing the level of constitutive (basal) response whereas neutral antagonists show the same affinity for both R and  $R^*$  states keeping the basal response unchanged (see [23-26] for a discussion on the mechanisms of inverse agonists at GPCRs and [27] for a historical review).

To properly characterize antagonist function on GPCRs, it seems logical that a change in the interpretation of antagonist behavior should be accompanied by a revision of the methods of binding constant calculation. The aim of the present study was to reformulate the aforementioned methods for the estimation of antagonist affinity under the simplest method that accounts for receptor constitutive activity: the two-state model of agonism.

### Competitive Binding Under the Two-State Model of Agonism

The two-state model of agonism was originally developed for ion channels [28, 29] and later applied to receptors [30-32]; a detailed description of the two-state model can be found in ref. [33]. Equation 13 resumes the two-state model for two ligands, A and B, competing for the receptor species.



R and R\* are the inactive and active free receptor, respectively, and AR, AR\*, BR and BR\* the ligand-bound species. The signal transduction mechanism associated to GPCRs includes three main components: the receptors, the G proteins, and the effectors. The latter are activated by a complex mechanism which involves the GDP/GTP exchange and the dissociation of the  $\alpha$  and  $\beta\gamma$  subunits of the heterotrimeric G protein after receptor activation. For simplicity, the G protein component has not been included explicitly in the chemical equilibria. Instead, it is assumed that physiological response arises from R\* conformation without distinguishing between free (R\*) or complexed (AR\* and BR\*) receptor species. The equilibrium constants are defined as:

$$X = \frac{[R^*]}{[R]}; \quad Z_L = \frac{[L][R]}{[LR]}; \quad T_L = \frac{[L][R^*]}{[LR^*]}; \quad Y_L = \frac{[LR^*]}{[LR]}$$

where L denotes either A or B. For the purpose of the present study, we suppose that A is a positive agonist ( $T_A < Z_A$ ) and B is either a neutral antagonist ( $T_B = Z_B$ ) or an inverse agonist ( $T_B > Z_B$ ).

To avoid the empirical decision of assigning a mathematical function to the transduction of receptor occupation into response, the fraction of receptors in the active form ( $f_{R^*}$ ) will be considered as the fractional functional response. This quantity is defined as:

$$f_{R^*} = \frac{[R^*] + [AR^*] + [BR^*]}{[R_0]} = \frac{T_A + [A] + \frac{T_A}{T_B} [B]}{aT_A + b_A [A] + \frac{T_A}{T_B} b_B [B]} \quad (14)$$

where  $[R_0] = [R] + [R^*] + [AR] + [AR^*] + [BR] + [BR^*]$ ,  
 $a = 1 + \frac{1}{X}$ ,  $b_A = 1 + \frac{T_A}{XZ_A}$ , and  $b_B = 1 + \frac{T_B}{XZ_B}$ .

Equation 14 can be used to analyze the dependence of  $f_{R^*}$  on [A] in the presence of B. Equation 14 is a Hill equation with a slope parameter of unity. The corresponding  $f_{R^*}/[A]$  curve is characterized by:

$$\text{lower asymptote} = \frac{T_B + [B]}{aT_B + b_B [B]}$$

$$\text{upper asymptote} = \frac{1}{b_A}$$

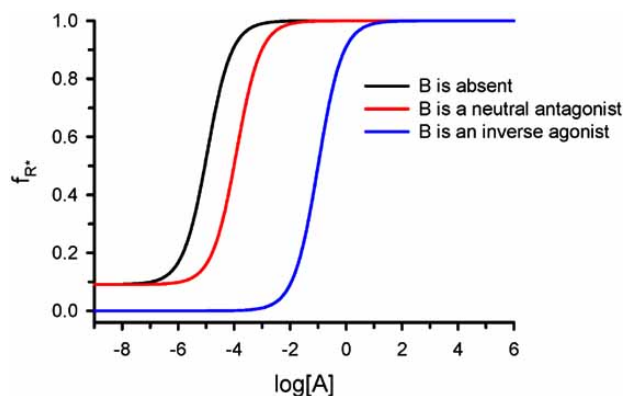
$$EC_{50} = \frac{a(b_A - a) + \frac{[B]}{T_B} \left( ab_A + b_A b_B - 2ab_B + \frac{b_B [B]}{T_B} (b_A - b_B) \right)}{\frac{b_A}{T_A} \left( b_A - a + (b_A - b_B) \frac{[B]}{T_B} \right)} \quad (15)$$

The lower and the upper asymptotes are the  $f_{R^*}$  values for  $[A]=0$  and  $[A] \rightarrow \infty$ , respectively and  $EC_{50}$  is the concentration of A for which

$$f_{R^*} = \text{lower asymptote} + \frac{1}{2} (\text{upper asymptote} - \text{lower asymptote}).$$

It should be noted that when B is absent, the lower asymptote is equal to  $\frac{1}{a}$  and  $EC_{50} = \frac{aT_A}{b_A}$  [33, 34]. In the particular case of B being a neutral antagonist,  $T_B = Z_B$  and  $b_B = a$ . Accordingly,  $EC_{50}$  from Equation 15 reduces to  $EC_{50} = \frac{aT_A}{b_A} \left( 1 + \frac{[B]}{T_B} \right)$ . It is worth noting the equivalence between this expression and the correspond-

ing one for  $A_{50}$  from the Gaddum equation (eq. 3). In addition, the lower asymptote is equal to  $1/a$ , i.e. the basal response.



**Fig. (1).** Simulation of the fractional response of a full agonist A in the absence (black) and in the presence of a neutral antagonist (red) and an inverse agonist (blue) ligand B, when present. The dissociation constants for the active and inactive receptor species (Equation 13) are: A, full agonist ( $T_A = 10^{-6}$ ;  $Z_A = 1$ ); B, neutral antagonist ( $T_B = 10^{-6}$ ,  $Z_B = 10^{-6}$ ); and B, inverse agonist ( $T_B = 10^{-6}$ ,  $Z_B = 10^{-9}$ ). The ratio for free active receptor and inactive receptor concentrations is  $X = [R^*]/[R] = 10^{-1}$ . See text for a comparative analysis of concentration-effect curves.

Figure (1) depicts the variation of  $f_{R^*}$  on  $\log[A]$  for B either absent or present. The ligand A has been considered as a full agonist ( $T_A \ll Z_A$ ), whereas two situations have been considered for B when present: neutral antagonist ( $T_B = Z_B$ ) and inverse agonist ( $T_B > Z_B$ ). The  $X = \frac{[R^*]}{[R]} = 10^{-1}$  value allows for the detection of

basal response (both A and B are absent). In all the situations the upper asymptote is 1, which is in agreement with A being a full agonist. The lower asymptotes for B both absent and present as a neutral antagonist match the basal response. As expected, the lower asymptote is lower than the basal response when B is an inverse agonist. A shift to the right of the curve relative to that in the absence of B is observed for B being both a neutral antagonist and an inverse agonist.

Figure (2) depicts the variation of  $f_{R^*}$  on  $\log[B]$  at a fixed [A] concentration, say  $EC_{50}$  in the absence of B. The upper asymptote is equal to  $\frac{1}{2} \left( \frac{1}{a} + \frac{1}{b_A} \right)$  whereas the lower asymptote is equal to  $\frac{1}{b_B} = \frac{1}{1 + \frac{T_B}{XZ_B}}$ . The lower asymptote depends on the antagonist

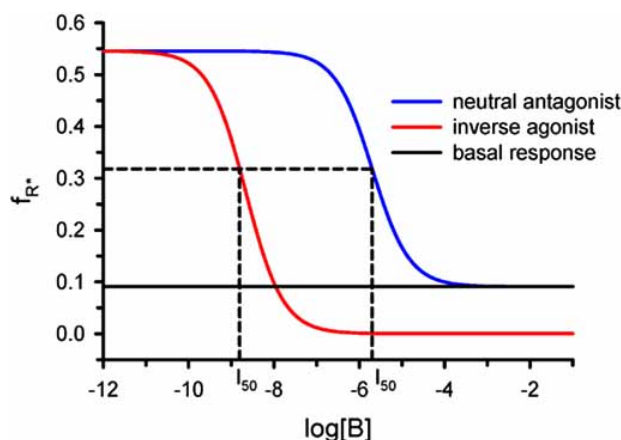
profile of ligand B. For a neutral antagonist, the lower asymptote is equal to  $\frac{1}{a}$  (basal response) whereas for an inverse agonist, the

lower asymptote depends on the  $T_B/Z_B$  ratio and on the X value. With the values used in the simulation, the lower asymptote for the inverse agonist is equal to  $10^{-4}$ . A shift to the left is shown for the inverse agonist relative to the neutral antagonist. The shift can be quantified by the  $I_{50}$  parameter defined as the concentration of B that reduces the effect of A to half its value. This constitutes the basis of the Cheng-Prusoff method (see below).

### 1. The Schild Regression for Inverse Agonists

A discussion of Schild method for inverse agonists by making use of the ternary [35] and extended ternary complex model [20]

can be found in recent works [36, 37]. Alternatively, we have used the two-state model in the present study to gain more simplicity in



**Fig. (2).** Simulation of the concentration-inhibition curves for a neutral antagonist (blue) and an inverse agonist (red) ligand B in the presence of a fixed concentration of a full agonist A. The agonist concentration is chosen as the  $EC_{50}$  when the agonist is acting alone. The dissociation constants for the active and inactive receptor species (Equation 13) are: A, full agonist ( $T_A=10^{-6}$ ;  $Z_A=1$ ); B, neutral antagonist ( $T_B=10^{-6}$ ;  $Z_B=10^{-6}$ ); B, inverse agonist ( $T_B=10^{-6}$ ;  $Z_B=10^{-9}$ ). The ratio for free active receptor and inactive receptor concentrations, which is  $X=[R^*]/[R]=10^{-1}$ , determines the basal response (black, solid line). The location of the curves along the X-axis is determined by the  $I_{50}$  parameter (see Cheng-Prusoff section).

the resulting equations and facilitate the analysis. It is worth noting that a number of simulations for the Schild regression analysis under the two-state model were shown in ref [33]; however, an explicit equation was not provided. Remarkably, an application of the two-state model to a system, outside of GPCR scenario but with properties resembling those of inverse agonism, can be found in a pioneer work [32] by David Colquhoun. In this paper, ionic conductance changes produced in postsynaptic membranes by transmitters analogues were analyzed under a model in which the channel was assumed to exist in two states, shut (R) or open ( $R^*$ ). Amongst others, the case of drugs which favor the R conformation and thus tend to close the channels, a concept analogous to inverse agonism, was considered. The Schild equation given in this study [32] is coincident with the one derived below (Equation 19).

Following the Schild procedure,  $[A]$  and  $[A']$  are the agonist concentrations producing equal responses in the absence and in the presence of an antagonist B, respectively. From Equation (14) we have:

$$f_{R^*} = \frac{T_A + [A]}{aT_A + b_A[A]} \quad (16)$$

$$f_{R^*} = \frac{T_A + [A'] + \frac{T_A}{T_B}[B]}{aT_A + b_A[A'] + \frac{T_A}{T_B}b_B[B]} \quad (17)$$

Combining equations (16) and (17) leads to:

$$\frac{[A']}{[A]} = dr = 1 + \frac{[B]}{T_B} \left( \frac{1}{b_A - a} \left( b_A - b_B + \frac{T_A}{[A]}(a - b_B) \right) \right) \quad (18)$$

By using the logarithm transformation:

$$\log(dr - 1) = \log[B] - \log T_B + \log C_{Schild} \quad (19)$$

where  $C_{Schild} = \frac{1}{b_A - a} \left( b_A - b_B + \frac{T_A}{[A]}(a - b_B) \right)$ . Comparison

of Equation 19 with Equation 5 indicates that regression of  $\log(dr - 1)$  on  $\log[B]$  maintains the linearity and the slope (unity) of the classical Schild expression. The intercept, however, is now more complex. Remarkably, this complexity is reduced for the particular case of B being a neutral antagonist. For this class of ligands  $Z_B = T_B$ , which implies  $b_B = 1 + \frac{T_B}{XZ_B} = 1 + \frac{1}{X} = a$ . Consequently,  $\log C_{Schild} = 0$  and then Equation 20, which matches the classical Schild expression, is obtained

$$\log(dr - 1) = \log[B] - \log T_B \quad (20)$$

The intercept provides the value of  $T_B=Z_B$ , which can be assigned to the commonly used term  $K_B$ .

In the case of B being an inverse agonist, the use of the intercept of the Schild regression for affinity constant estimation needs further analysis. It is worth noting that  $C_{Schild}$  depends on  $[A]$ . However, the influence of the agonist concentration term decreases as the agonist concentration increases with a limit value of

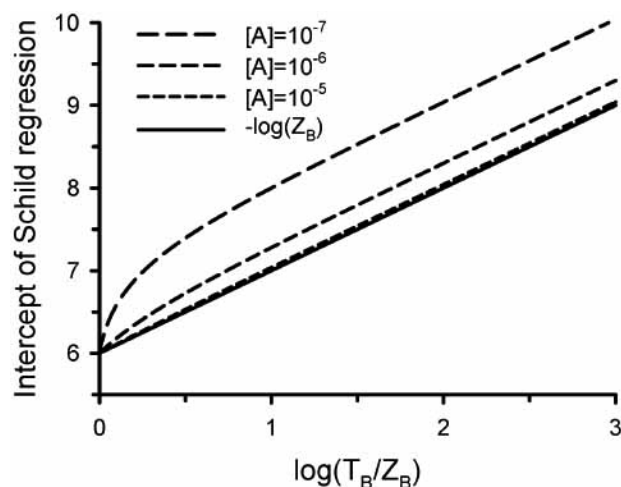
$C_{Schild} = \frac{1}{b_A - a} (b_A - b_B)$ . In addition, if A is a full agonist

then  $\frac{T_A}{Z_A} \ll 1$  and  $\frac{T_A}{Z_A} \ll \frac{T_B}{Z_B}$ ; and, accordingly,

$C_{Schild} \approx \frac{T_B}{Z_B}$ . In the latter case, Equation 19 can be rewritten as

$$\log(dr - 1) \approx \log[B] - \log T_B + \log \frac{T_B}{Z_B} = \log[B] - \log Z_B \quad (21)$$

Thus, the intercept of the Schild regression for an inverse agonist B approximates the dissociation constant of this ligand for the inactive receptor species, when A is a full agonist and  $[A]$  is large enough. To avoid subjectivism, the  $[A_{50}]$  value can be taken as a reference.



**Fig. (3).** Simulation of the intercept of the Schild regression (Equation 19) for  $[A]=10^{-7}$  (long dashed),  $[A]=10^{-6}$  (medium dashed) and  $[A]=10^{-5}$  (short dashed) as the ratio  $T_B/Z_B$  varies. Simulated lines are compared with the reference  $-\log(Z_B)$  function (solid line). Values of  $Z_A=1$ ,  $T_A=10^{-6}$ ,  $T_B=10^{-6}$ , and  $X=[R^*]/[R]=10^{-1}$  are kept fixed. Apparent  $pK_B$  values approximate true  $pZ_B$  values as  $[A]$  increases, see text for discussion.

Figure (3) illustrates quantitatively these results by representing the intercept of Schild regression given by Equation 19

$(-\log T_B + \log C_{Schild})$  as a function of  $\log \frac{T_B}{Z_B}$ , with  $T_B=10^{-6}$  and  $X=10^{-1}$ , for three  $[A]$  values ( $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ ). We see that the intercept of Schild regression is equal to  $-\log T_B = -\log Z_B$  for neutral antagonists  $\left(\frac{T_B}{Z_B} = 1\right)$  independently of  $[A]$  and it is approxi-

mately equal to  $-\log Z_B$  for inverse agonists  $\left(\frac{T_B}{Z_B} > 1\right)$  for  $[A]=10^{-5}$  (a value close to  $[A_{50}]=1.1 \times 10^{-5}$ ). For lower  $[A]$  values ( $10^{-6}$  and  $10^{-7}$ ) the intercept is greater than  $-\log Z_B$ . In particular, for  $\log \frac{T_B}{Z_B} = 2$ , which implies a true  $pZ_B=8$  value, the estimates from the intercept are 9.04, 8.30, and 8.04 for  $\log[A]$  equal to -7, -6, and -5, respectively.

**2. The Cheng-Prusoff Equation for Inverse Agonists**

$I_{50}$  is the concentration of the antagonist B that reduces the effect of the agonist A to half its value. If  $f_{R^*}$  (Equation 16) is the effect of A in the absence of B, then in the presence of  $I_{50}$  the effect is:

$$f_{R^*}' = \text{basal response} + \frac{1}{2}(f_{R^*} - \text{basal response}) = \frac{1}{a} + \frac{1}{2} \left( f_{R^*} - \frac{1}{a} \right) = \frac{T_A + [A] + \frac{T_A}{T_B} I_{50}}{aT_A + b_A[A] + \frac{T_A}{T_B} b_B I_{50}} \tag{21}$$

By combining equations (21) and (16), the following equation is obtained:

$$I_{50} = T_B \left( 1 + \frac{[A]}{A_{50}} \right) \frac{(a - b_A) a [A]}{2(b_B - a) T_A a + (b_B(a + b_A) - 2ab_A) [A]} \tag{22}$$

where  $A_{50}=aT_A/b_A$  is the concentration of the agonist A producing half maximum response in the absence of B.

If B is a neutral antagonist then  $b_B=a$  and

$$I_{50} = T_B \left( 1 + \frac{[A]}{A_{50}} \right) \tag{23}$$

which matches the classical Cheng-Prusoff equation. By using this equation the dissociation constant  $T_B=Z_B$  is correctly estimated.

In general, we can write:

$$\log \left( 1 + \frac{[A]}{A_{50}} \right) = \log I_{50} - \log T_B + \log C_{Cheng-Prusoff} \tag{24}$$

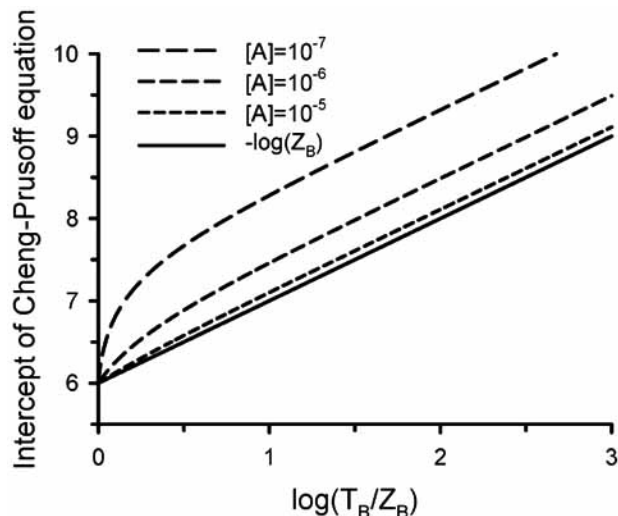
where

$$C_{Cheng-Prusoff} = \frac{2(b_B - a) T_A a + (b_B(a + b_A) - 2ab_A) [A]}{(a - b_A) a [A]}$$

We proceed in analogy with Schild regression and call the  $(-\log T_B + \log C_{Cheng-Prusoff})$  quantity the intercept of the Cheng-Prusoff equation, which, as it occurs for the Schild regression, depends on  $[A]$ . In the case of B being an inverse agonist, the  $C_{Cheng-Prusoff}$  term tends to  $\frac{T_B}{Z_B} \left( \frac{1+a}{a} \right)$  as  $[A]$  increases if A is a full agonist,  $\frac{T_A}{Z_A} \ll 1$ , and B is a full inverse agonist,  $\frac{T_B}{Z_B} \gg 1$ . In this case, Equation 24 can be rewritten as

$$\log \left( 1 + \frac{[A]}{A_{50}} \right) \approx \log I_{50} - \log Z_B + \log \left( \frac{1+a}{a} \right) \tag{25}$$

In contrast to Schild regression, a term  $\left( \log \left( \frac{1+a}{a} \right) \right)$  dependent on the basal response affects the estimation of the dissociation constant of the ligand to the inactive receptor species. Remarkably, the influence of this term increases as the basal response,  $1/a$ , increases.



**Fig. (4).** Simulation of the intercept of the Cheng-Prusoff equation (Equation 24) for  $[A]=10^{-7}$  (long dashed),  $[A]=10^{-6}$  (medium dashed) and  $[A]=10^{-5}$  (short dashed) as the ratio  $T_B/Z_B$  varies. Simulated lines are compared with the reference  $-\log(Z_B)$  function (solid line). Values of  $Z_A=1$ ,  $T_A=10^{-6}$ ,  $T_B=10^{-6}$ , and  $X=[R^*]/[R]=10^{-1}$  are kept fixed. Apparent  $pK_B$  values approximate true  $pZ_B$  values as  $[A]$  increases. Errors in  $pK_B$  estimates are greater in Cheng-Prusoff than in Schild analysis (compare Fig. 3 and Fig. 4). See text for discussion.

Figure (4) is constructed in a similar fashion to Fig. (3). The intercept of the Cheng-Prusoff equation (the term  $-\log T_B + \log C_{Cheng-Prusoff}$  from Equation 24) varies with  $\log \frac{T_B}{Z_B}$ , with  $T_B=10^{-6}$  and  $X=10^{-1}$ , for three  $[A]$  values ( $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ ). We see that the intercept of the Cheng-Prusoff equation is equal to  $-\log T_B = -\log Z_B$  for neutral antagonists  $\left(\frac{T_B}{Z_B} = 1\right)$  and this result is independent of  $[A]$ . On the contrary, the intercept of the Cheng-Prusoff equation tends to  $-\log Z_B + \log \left( \frac{1+a}{a} \right)$  for inverse agonists  $\left(\frac{T_B}{Z_B} \gg 1\right)$  as  $[A]$  increases. In particular, for  $\log \frac{T_B}{Z_B} = 2$ , which implies a true  $pZ_B=8$  value, the estimates from the intercept are 9.32, 8.49, and 8.11 for  $\log[A]$  equal to -7, -6, and -5, respectively. It is worth noting that the errors in the estimates are greater than in the Schild analysis. In addition, the limit of the calculated intercept is equal to  $-\log 10^{-8} + \log \left( \frac{1+1+10}{1+10} \right) = 8.04$  as  $[A]$  increases. With a higher level of receptor constitutive activity ( $X=1$ ) the limit of the intercept and the corresponding error would be greater (8.18 for a true  $pZ_B=8$  value).

## CONCLUSIONS

The Schild regression and the Cheng-Prusoff methods have been revisited within the two-state model of agonism considering that the antagonist can be neutral or negative (inverse agonist). The resulting Schild and Cheng-Prusoff equations match the classical expressions for neutral but not for negative antagonists. In the latter case, a term depending on the concentration of the added agonist appears. The errors in affinity estimation under the Cheng-Prusoff method are greater than under the Schild analysis. Interestingly, when the agonist is a full agonist, the errors in antagonist affinity estimation under both methods are lower as greater are the concentrations of the agonist. The estimates of the antagonist-receptor dissociation constant for an inverse agonist tend to the true constant corresponding to the inactive receptor state as the agonist concentration increases, if the Schild but not the Cheng-Prusoff equation is used. In the latter case, a term, which is dependent on the basal response, appears; the importance of this extra term increases as the level of receptor constitutive activity increases. In view of the above properties, concentrations of the agonist equal to or preferably higher than its EC<sub>50</sub> should be used.

The therapeutic relevance of inverse agonism is becoming more important as much information is gained with this type of ligands [38]. Many drugs previously thought to act as neutral antagonists have now been classified as inverse agonists [26], making necessary further investigation of the effects that their negative efficacy can have [39]. Because accurate measurement of antagonist potency is crucial for drug discovery, it may be concluded that routine procedures for antagonist affinity estimation on GPCRs should be revised on the light of the current knowledge of receptor theory [40]. Furthermore, the increasing complexity of pharmacological research gives our consideration a broader scope. In this regard, and in dealing with the functional selectivity phenomenon, a similar conclusion, that is the necessity for revising classic concepts in quantitative pharmacology to incorporate new findings, was provided recently [41].

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