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# Determining the Potency and Molecular Mechanism of Action of Insurmountable Antagonists

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## ABSTRACT

Insurmountable antagonism (maximal response to the agonist depressed) can result from a temporal inequilibrium involving a slow offset orthosteric antagonist or be the result of an allosteric modulation of the receptor. The former mechanism is operative when the antagonist, agonist, and receptors cannot come to proper equilibrium during the time allotted for collection of agonist response (hemi-equilibrium conditions). Allosteric effects (changes in the conformation of the receptor through binding of the allosteric modulator to a separate site) can preclude the agonist-induced production of response, leading to depression of maximal responses. In these cases, the effects on receptor affinity can be observed as well. The first premise of this article is that system-independent estimates of insurmountable antagonist potency can be made with no prior knowledge of molecular mechanism through the use of p$A_2$ (−log molar concentration of antagonist producing a 2-fold shift of the concentration response curve) measurements The relationship between the p$A_2$ and antagonist p$K_B$ (−log equilibrium dissociation constant of the antagonist-receptor complex) is described; the former is an extremely close approximation of the latter in most cases. The second premise is that specially designed experiments are required to differentiate orthosteric versus allosteric mechanisms; simply fitting of data to orthosteric or allosteric theoretical models can lead to ambiguous results. A strategy to determine whether the observed antagonism is orthosteric (agonist and antagonist competing for the same binding site on the receptor) or allosteric in nature is described that involves the detection of the hallmarks of allosteric response, namely saturation and probe dependence of effect.

Two major considerations in a drug discovery program for antagonists are the need for 1) system-independent estimates of potency and 2) knowledge of the molecular mechanism of action. The former enables systematic study of structure and activity and subsequent optimization of activity, whereas the latter allows prediction of the properties of the antagonist in the therapeutic situation. The major premise of this study is that knowledge of the mechanism of action of insurmountable antagonists is not required for the system-independent measure of antagonist potency. In fact, verisimilitude of data to specific theoretical models is an unreliable way to determine mechanism of action (vide infra). It will be proposed that specifically designed experiments are required to do so.

By definition, antagonists interfere with the ability of agonists to produce pharmacological response. The way they express this interference varies but generally involves changing the location parameter ($EC_{50}$; molar concentration pro-

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**ABBREVIATIONS:** RANTES, regulated on activation, normal T cell expressed and secreted (standard nomenclature for this chemokine is CCL5); FLIPR, fluorometric imaging plate reader; Darifenacin, (*S*)-2-{1-[2-(2,3-dihydrobenzofuran-5-yl)ethyl]-3-pyrrolidinyl}-2,2-diphenylacetamide hydrobromide; SR 48,968, (*S*)-*N*-methyl-*N*[4-(4-acetylamino-4-piperidino)-2-(3,4-dichlorophenyl) butyl]benzamide; FR173657, (*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-[2,4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminocarbonylmethyl] acrylamide; FR190997, 8-[2,6-dichloro-3-[*N*-[(*E*)-4-(*N*-methylcarbamoyl)cinnamidoacetyl]-*N*-methylamino]-benzyloxy]-2-methyl-4-(2-pyridylmethoxy)quinoline; SB203220, [*E-a*-[[2-butyl-1-(carboxy-1-naphalenyl)methyl-1*H*-imidazol-5-yl]-methylene]-2-thiophene-propanic acid]; AMD3100, 1,1′-[phenylenebis(methylene)]bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride; aplaviroc, 4-{[4-({(3*R*)-1-butyl-3-[(*R*)-cyclohexyl(hydroxy)methyl]-2,5-dioxo-1,4,9-triazaspiro-[5.5]undec-9-yl}methyl)phenyl]oxy}benzoic acid hydrochloride; Sch-C, (*Z*)-(4-bromophenyl){1′-[(2,4-dimethyl-1-oxido-3-pyridinyl)carbonyl]-4′-methyl-1,4′-bipiperidin-4-yl}methanone *O*-ethyloxime; vicriviroc, 4,6-dimethyl-5-{[4-methyl-4-((3*S*)-3-methyl-4-{(1*R*)-2-(methyloxy)-1-[4-(trifluoromethyl)phenyl]ethyl}-1-piperazinyl)-1-piperidinyl]carbonyl}pyrimidine; maraviroc, 4,4-difluoro-*N*-((1*S*)-3-{(3-*endo*)-3-[3-methyl-5-(1-methylethyl)-4*H*-1,2,4-triazol-4-yl]-8-azabicyclo[3.2.1]oct-8-yl}-1-phenylpropyl)cyclohexanecarboxamide; TAK779, *N*,*N*-dimethyl-*N*-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl]tetrahydro-2*H*-pyran-4-aminium chloride; DR, dose ratio; pilocarpine, (3*S*,4*R*)-3-ethyl-4- [(3-methylimidazol-4-yl) methyl]oxolan-2-one; arecoline, methyl 1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylate; HIV, human immunodeficiency virus.



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ducing 50% maximal response to the agonist) or the maximal asymptote (maximal response to the agonist) of the agonist concentration-response curve (or both). The influential pharmacologist, Sir John Gaddum, described a useful empirical classification of receptor antagonism in the form of surmountable and insurmountable antagonism (Gaddum, 1957). Thus, a surmountable antagonist produces dextral displacement (shifts to the right) of agonist concentration-response curves with no concomitant diminution of the maximal response to the agonist. The standard method of determining the potency of such antagonists is through Schild analysis (Arunlakshana and Schild, 1959) or with a Clark plot (Stone and Angus, 1976; Lew and Angus, 1996). A prerequisite to the correct use of these methods is the demonstration of parallel displacement of the agonist concentration-response curve, with no diminution of maximal response to the agonist. In contrast, insurmountable antagonists depress the maximal response, and the determination of potency depends on the model of antagonism used for data comparison.

The system-independent measurement of antagonist potency (as equilibrium dissociation constants of antagonist-receptor complexes denoted $K_B$) is the common currency of antagonist drug discovery. To correctly estimate the p$K_B$ of an antagonist, the molecular mechanism of action should be known, and the appropriate model should be used to analyze the data. However, in experimental pharmacology, the molecular mechanism of action often is not known making this approach untenable. It will be seen that the p$A_2$ (−log of the molar concentration of antagonist producing a 2-fold shift of the agonist concentration-response curve) is a useful empirical parameter and close approximation of the p$K_B$, even for insurmountable antagonists. With the exception of allosteric antagonists that block receptor signaling but increase the affinity of the receptor for the agonist, the resulting p$A_2$ is an upper limit for the p$K_B$ value, and the difference between the experimental p$A_2$ and true p$K_B$ will be small.

Although knowledge of the mechanism of action is not a strict prerequisite to reliable estimation of antagonist potency, it is important information with respect to the utility of the antagonist in therapeutic situations. This is because allosteric and orthosteric antagonists can have very different effects in the therapeutic environment (Kenakin 2004a,b). This article also outlines strategies for determining whether an antagonist produces insurmountable blockade via an orthosteric or allosteric mechanism.

## Materials and Methods

### Calcium Mobilization Studies

**Cell Culture.** U-2 OS (human osteosarcoma ATCC HTB-96; ATCC, Manassas, VA) cells were grown in Dulbecco's modified Eagle's medium/F-12 (Gibco 11039-021; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mM *l*-glutamine (Gibco 25030-081; Invitrogen) in T225 flasks. Cells were split twice weekly at a ratio of 1:5 to achieve 80 to 90% confluency on the day of transduction.

**BacMam Virus Generation.** Recombinant CCR5 BacMam baculovirus (GenBank accession number X91492) and the chimeric G-protein Gqi5 BacMam baculoviruses were generated according to established protocols.

**Transduction of U-2 OS Cells.** U-2 OS cells were removed from T225 cell culture flasks by washing twice with D-phosphate-buffered solution (Gibco 14190-144; Invitrogen) and applying cell dissociation solution (Sigma C-5914; Sigma-Aldrich, St. Louis, MO). Cells were resuspended in cell culture medium containing 5% CCR5 BacMam and 5% Gqi5 BacMam at a concentration of 3e5 cells/ml (50-μl volume). The expression of the chimeric G-protein Gqi5 allows the normally $G_I$-coupled CCR5 receptor to couple via $G_q$ effectors, such as phospholipase C. This solution was then plated into clear bottom 384-well microtiter plates (Costar 3712; Corning Life Sciences, Acton, MA). Cells were placed in an incubator at 37°C, 5% $CO_2$, 95% humidity for 24 h to allow for CCR5 and Gqi5 expression.

### FLIPR Functional $IC_{50}$ Studies

On the day of the assay, cells were removed from the incubator, and culture medium was removed and replaced with a calcium-sensitive dye (Calcium3 assay kit; Molecular Devices, Sunnyvale, CA) containing 2.5 μM probenecid (Sigma P8761; Sigma-Aldrich). The cells were allowed to dye load for 45 min at 37°C in the cell culture incubator and then allowed to equilibrate to room temperature for 15 min before assay. Compound plates were generated containing 3% dimethyl sulfoxide in loading dye buffer kept at room temperature. Test compounds were added to the cells at a 1:3 dilution, and calcium mobilization was measured using a FLIPR fluorescence imager (Molecular Devices). This first read was used to determine direct agonist activation of the CCR5 receptor by the test compounds. Plates were then returned to the 37°C incubator for 30 min and back at room temperature for 15 min to allow system to equilibrate. After the 45-min compound equilibration time, full concentration-response curves to RANTES were added to the cells at a 1:4 dilution, and calcium mobilization was measured using a FLIPR fluorescence imager.

## Results

### Mechanism of Receptor Antagonism: Orthosteric and Allosteric Mechanisms

Two distinct molecular mechanisms of receptor antagonism can produce depression of agonist concentration-response curves: orthosteric and allosteric binding. The former describes the interaction of the antagonist with the agonist binding site to preclude or retard agonist binding to the receptor. The latter involves the binding to a unique antagonist binding site on the receptor (different from that used by the agonist), with alteration of agonist behavior in the protein by means of a change in the receptor conformation. The first molecular mechanism to be considered is the orthosteric binding of the antagonist to the agonist binding site; a critical aspect to this process is the relative kinetics of interaction of the antagonist and agonist with the receptor.

### Kinetics of Receptor Antagonism

**Antagonist Equilibration and Determination of Irreversible Blockade.** It is useful to define two types of temporal approach to equilibrium, namely equilibration kinetics and re-equilibration kinetics. The equilibration kinetics that involves the binding of the antagonist to the receptor and attainment of antagonist-receptor occupancy commensurate with the $K_B$ (equilibrium dissociation constant of the antagonist-receptor complex) and concentration of antagonist in the receptor compartment. Within this context, an irreversible (a chemical bond forms between the antagonist and the receptor protein) or pseudo-irreversible (no chemical bond is formed but the binding is so tight that no dissociation of the antagonist occurs) antagonist refers to one that, once bound, does not appreciably dissociate from the receptor within a reasonable amount of time (as set by the limits of the exper-

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iment). A corollary to this is the fact that the onset of antagonism is also very slow, to the point that it is not possible to produce a steady state with partial antagonism of receptors without removing the antagonist from the receptor compartment. An example of this is the use of receptor alkylating agents, such as $\beta$-haloalkylamines (Moran et al., 1969). If left to "equilibrate" with receptors, these types of agents continually alkylate and inactivate receptors until complete receptor inactivation occurs or the alkylating agent is removed from the receptor compartment, either by washing with drug-free medium or reaction of the reactive alkylating species with nonspecific proteins or water (Cook et al., 1980). Thus, in the continuous presence of such antagonists, a steady-state submaximal inhibition of response cannot be obtained. Irreversible and pseudo-irreversible antagonism are relevant to this article only insofar as experiments need to be done to determine that they are not the mechanism of action of the insurmountable blockade. Only after this is established can the quantification of the potency of the antagonist producing insurmountable blockade be initiated.

**Re-Equilibration Kinetics.** In typical pharmacological experiments, the receptor preparation is pre-equilibrated with the antagonist to ensure correct receptor occupancy according to the concentration and the $K_B$. Under ideal circumstances, when agonist is added to determine the extent of antagonism, the receptors should re-equilibrate with the antagonist and agonist in the receptor compartment according to their respective concentrations and equilibrium dissociation constants (mass action) (see Fig. 1A). For this to occur, a suitable time period must be allowed for determination of response; i.e., there must be sufficient time for the antagonist to dissociate from the receptors and the agonist to bind to unbound receptors. However, in practice, antagonists often have a slow rate of offset, and the window for re-equilibration may be shorter than required for true equilibrium to be attained. The extent of re-equilibration of an agonist, antagonist, and receptor population determines the shape of the agonist concentration-response curve to the agonist in the presence of the antagonist. The expression for the extent of antagonist-receptor occupancy that occurs in the presence of agonist was derived by Paton and Rang (1965). The expression, derived in Appendix I, describes response as follows (see Fig. 1B):

$$\text{Response}_A = \frac{[A]/K_A(1-(\vartheta(1-e^{-k_2\Phi t})+\rho_B e^{-k_2\Phi t}))\tau E_{max}}{[A]/K_A((1-(\vartheta(1-e^{-k_2\Phi t})+\rho_B e^{-k_2\Phi t}))\tau+1)+1} \quad (1)$$

where

$$\vartheta = [B]/K_B/([B]/K_B + [A]/K_A + 1) \quad (2)$$

$$\rho_B = [B]/K_B/([B]/K_B + 1) \quad (3)$$

$$\Phi = ([B]/K_B + [A]/K_A + 1)/([A]/K_A + 1) \quad (4)$$

$E_{max}$ is the maximal response capability of the system, $\tau$ describes the efficacy of the agonist and sensitivity of the system to agonist stimulation (see Appendix I), $t$ is time, and $k_2$ is the rate of offset of the antagonist from the receptor.

Two kinetic extremes of eq. 1 define surmountable (com-

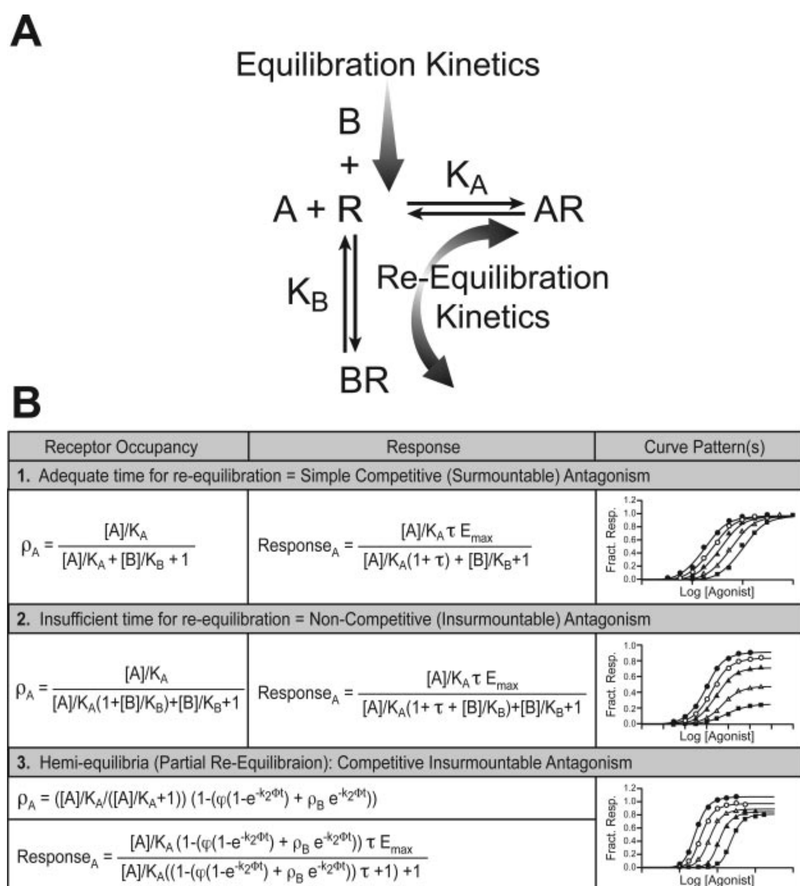


**Fig. 1.** A, schematic diagram depicting two types of antagonist kinetics. Equilibration kinetics refers to the process of the antagonist binding to the receptors and coming to equilibrium according to the $K_B$ and the concentration. Re-equilibration kinetics refers to the binding of added agonist to a preparation pre-equilibrated with antagonist and the process of the agonist and antagonist achieving equilibrium with the receptor population according to their respective equilibrium dissociation constants and relative concentrations within the time allowed for measurement of agonist response. B, summary of kinetic extremes for orthosteric antagonism whereby the agonist and antagonist do (panel 1), do not (panel 2), or partially (panel 3) equilibrate according to mass action kinetics in the time allotted for observation of agonist response. Equations are derived in Appendix I.

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petitive) and insurmountable (noncompetitive) antagonism. Thus, if sufficient time is allowed for re-equilibration of agonist, antagonist, and receptors, then $t \gg k_2^{-1}$, $e^{-k_2\Phi t} \rightarrow 0$, and eq. 1 reduces to the Gaddum (1937) equation for simple competitive antagonism. Under these circumstances, the antagonism is surmountable; the equation for agonist response in the presence of antagonist is given by eq. 5 (see Fig. 1B),

$$\text{Response}_{\text{A}} = \frac{[\text{A}]/K_{\text{A}}\ \tau\, E_{\max}}{[\text{A}]/K_{\text{A}}(1+\tau) + [\text{B}]/K_{\text{B}} + 1} \qquad (5)$$

The other kinetic extreme is where there is no time for re-equilibration and the antagonist is essentially irreversible with respect to the agonist as it occupies receptors during the response-gathering phase of the experiment. Under these circumstances, $t \ll k_2^{-1}$, $e^{-k_2\Phi t} \rightarrow 1$, and eq. 1 reduces to the equation for noncompetitive antagonism defined by Gaddum et al. (1955). The antagonism is insurmountable (if there is no receptor reserve to the agonist vide infra) and given by eq. 6 (see Fig. 1B),

$$\text{Response}_{\text{A}} = \frac{[\text{A}]/K_{\text{A}}\ \tau\, E_{\max}}{[\text{A}]/K_{\text{A}}(1+\tau+[\text{B}]/K_{\text{B}}) + [\text{B}]/K_{\text{B}} + 1} \qquad (6)$$

Equation 6 is important for understanding the effects of insurmountable antagonists on observed maximal response because of the phenomenon of agonist-receptor reserve. The receptor reserve relates to the number of receptors that need be activated in the preparation to attain maximal response. The magnitude of the receptor reserve is dependent on the receptor density ($[\text{R}_t]$), the efficiency of the receptor coupling in the tissue (magnitude of $K_{\text{E}}$), and also the intrinsic efficacy of the agonist (also incorporated in $K_{\text{E}}$). The magnitude of $\tau$ can be used to simulate different effective receptor reserves for agonists. Therefore, the effect of noncompetitive receptor blockade for systems of high-receptor reserve and no receptor reserve can be calculated with eq. 6. It is important to consider receptor reserve in the description of noncompetitive blockade and hemi-equilibria, because it can dictate what is observed, namely dextral displacement of the concentration-response curve, with or without depression of the maximal response. Figure 2A shows the effect of a slow offset antagonist (yielding insurmountable blockade) in a tissue with essentially no receptor reserve ($\tau$ = 1). It should be stressed that this may due to the tissue (low-receptor density and/or poor efficiency of receptor coupling) or the agonist (low efficacy). It can be seen that concentration-response curves with depressed maxima are produced by all concentrations of antagonist. This is consistent with the fact that, in the absence of receptor reserve, all receptors are required for production of maximal response; thus, if the antagonist eliminates any portion of these receptors, this will be reflected in the magnitude of the maximal response. In contrast, this same antagonist produces different effects in a system with a high-receptor reserve ($\tau$ = 100) (see Fig. 2B). In this system, the maximal response can be attained by activation of only a small portion of the receptor population (either through high-receptor density, efficient receptor coupling, or high-agonist efficacy; i.e., a different agonist is used). Under these circumstances, noncompetitive antagonism will produce a substantial dextral displacement of the concentration-response curves before depression of the maximal response ensues (see Fig. 2B). These effects are demonstrated experimentally with the baculovirus expression system for the chemokine CCR5 receptor; this technology allows the level of receptor expression to be controlled by the quantity of baculovirus used to transduce the cells with receptor. It can be seen in Fig. 2C that a low level of CCR5 expression (0.1% baculovirus) produces a system where a 3 nM concentration of the insurmountable CCR5 antagonist aplaviroc (Watson et al., 2005) completely suppresses the response to the chemokine RANTES. In contrast, a higher level of receptor expression (produced by transduction with 5% baculovirus; note larger ordinates values and $\text{pEC}_{50}$) produces a system where 3 nM aplaviroc does not completely suppress the response to RANTES.

**Agonist/Antagonist Hemi-Equilibria.** Between the kinetic extremes of $t \gg k_2^{-1}$ and $t \ll k_2^{-1}$ lie conditions where

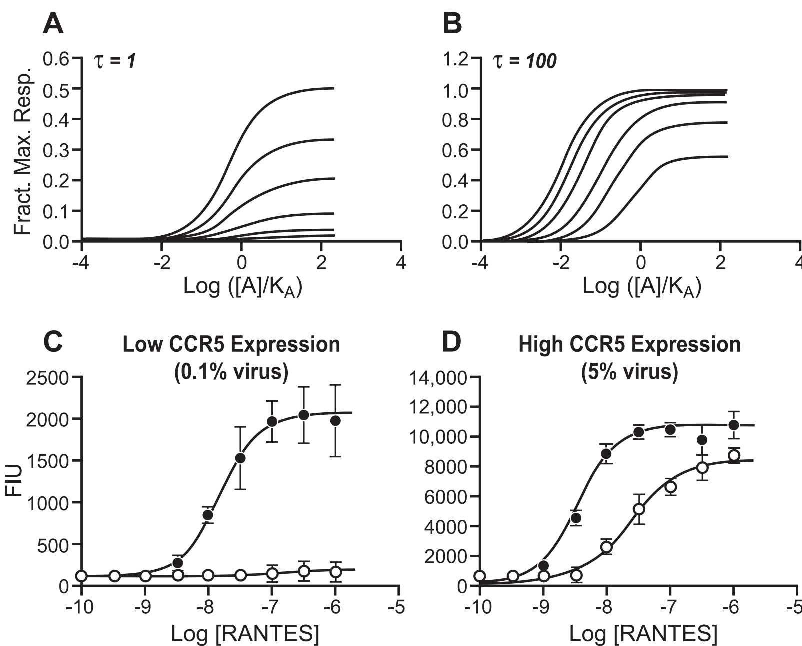


**Fig. 2.** Effect of a purely noncompetitive antagonist (no re-equilibration) on response. A and B, ordinates are fractional receptor occupancy. C and D, fluorescence intensity units for calcium fluorescence. Abscissae are logarithms of agonist concentration as a fraction of $K_{\text{A}}$ (A and B) and molar concentrations of RANTES (C and D). A, system with no receptor reserve ($\tau$ = 1). Curves are control (furthest to the left) and responses in the presence of $[\text{B}]/K_{\text{B}}$ = 1, 3, 10, 30 and 100. B, same conditions as in A except in a system with a high receptor reserve ($\tau$ = 100). C, low (0.1%) baculovirus transduction of cDNA for human CCR5 receptor into U-2 OS cells. Responses to the CCR5 agonist RANTES in the absence (filled circles) and presence (open circles) of the receptor modulator aplaviroc (3 nM). D, same experiment as shown in C except in cells transduced with a higher concentration of CCR5 cDNA baculovirus (5%) yielding a higher expression level of CCR5 receptors. Bars represent S.E.M.

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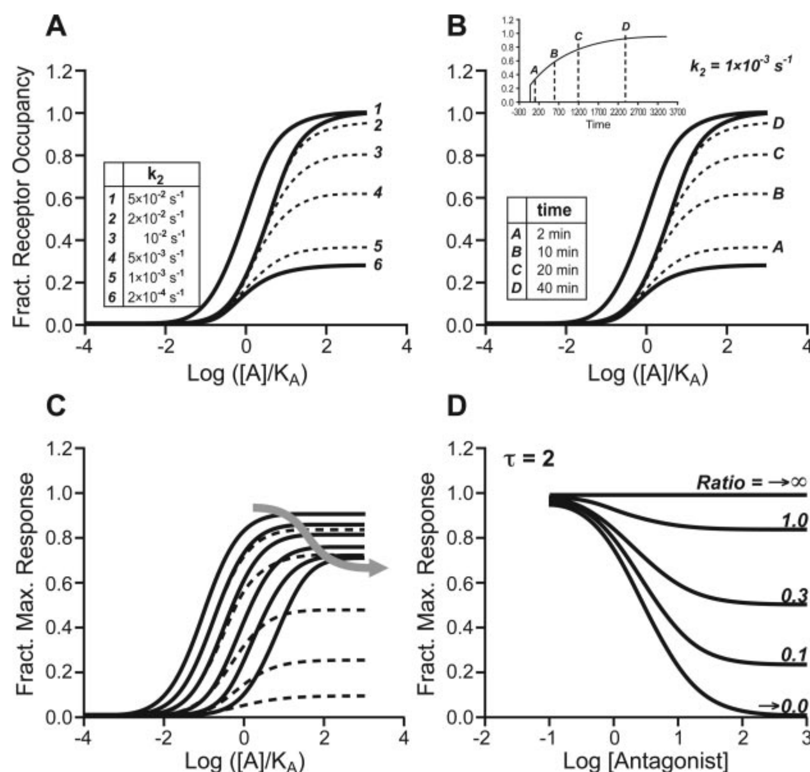


**Fig. 3.** Hemi equilibrium kinetics. A, concentration response curves to a fast acting agonist in the absence of antagonist (curve furthest to the left) and in the presence of antagonists with various rates of offset ($k_2$ values shown in key) at a concentration of $[B]/K_B = 3$. The time for measurement of response is 2 min. With $k_2 = 2 \times 10^{-4}\ s^{-1}$, essentially no re-equilibration takes place upon addition of agonist (pure noncompetitive antagonism). With increased rates of offset, increasing re-equilibration occurs. With $k_2 \geq 5 \times 10^{-2}\ s^{-1}$, essentially complete re-equilibration occurs and simple competitive antagonism with no depression of maximal response is observed. B, increasing times for measurement of response for a slow-acting orthosteric antagonist ($k_2 = 1 \times 10^{-3}\ s^{-1}$) for $[B]/K_B = 3$. Inset shows the kinetics of response production by a concentration of the agonist producing maximal response ($[A]/K_A = 100$). It can be seen that a rapid initial increase in response (due to occupation of unoccupied receptors) is followed by a slower phase where the agonist and antagonist re-equilibrate with the receptor population. If only 2 min is allowed for measurement of response, a severely depressed concentration-response curve results. With increasing equilibration times, the maximal increase until (at 40 min) simple competition with minimal depression of the maximal response is observed. C, hemi-equilibrium states among antagonist, agonist, and receptors. A, response calculated with eq. 1 showing a hemi-equilibrium condition resulting in a depressed maximal response to the agonist that reaches a plateau. For this simulation, $k_2 = 5 \times 10^{-5}\ s^{-1}$, $\tau = 10$, $t = 90$ min. Curves show antagonist concentrations as follows: 0 = control curve furthest to the left; $[B]/K_B$ = 1, 3, 10, 30, and 100. Dotted lines show expected purely noncompetitive behavior of the same antagonist (no re-equilibration). Such an effect would be observed for response times of ≤200 s. D, maximal response to an agonist in the presence of orthosteric antagonists of differing rates of offset for a system of $\tau = 2$. Numbers next to the curves represent ratios of time for response collection and rate of offset ($k_2 \times t$). A value → 0 represents a case where no re-equilibration occurs, whereas a value of → ∞ represents completely competitive behavior (no diminution of maximum). The intervening curves show various states of hemi-equilibria; the limiting ordinate values represent the level of the plateau of maximal responses; i.e., no amount of antagonist will depress the agonist maximal response below this level.

the antagonist and agonist partially re-equilibrate with receptors. The degree to which this occurs depends upon the rate of offset of the antagonist (denoted $k_2$) and the length of time allowed for re-equilibration. In cases where the time is insufficient for re-equilibration, a selectively lower agonist occupancy will be attained at higher concentrations of agonist requiring greater receptor occupancy; i.e., higher concentrations of agonist will not achieve proper occupancy. This was described by Paton and Rang (1965) as a hemi-equilibrium condition. An example of a range of agonist-receptor occupancies, in the presence of a range of antagonists of different $k_2$ values, is shown in Fig. 3A. A feature of hemi-equilibria is that it is dependent on the amount of time allowed for re-equilibration (i.e., the time window allowed for collection of agonist response in the presence of the antagonist). If sufficient time is allowed, even a very slowly dissociating antagonist will eventually re-equilibrate with the agonist to produce simple competitive kinetics. Figure 3B shows the effect of increasing the window of time to collect agonist response in the presence of a slow offset antagonist.

There are numerous combinations of $k_2$ values and time periods where a characteristic plateau effect of depressed maxima can be seen. Therefore, unlike true noncompetitive blockade, where successively higher concentrations of antagonist produce successively greater depressions of maxima, hemi-equilibrium can be attained where the maximal response is depressed to a new common level below that of the control concentration-response curve (see Fig. 3C). Such submaximal plateaus of maximal agonist response cannot be accommodated within the model for true noncompetitive blockade as described by eq. 6 where it can be seen from an inspection of this equation that, as $[B] \rightarrow \infty$, the maximal response to the agonist diminishes to zero. In contrast, conditions of hemi-equilibria can be shown to predict plateaus of maximal response to the agonist that are depressed below control but never reach zero response. This can be seen by examining the effects of antagonists in regions where the maximal response to the agonist is observed. Thus, substituting a ratio of 1000:1 of agonist to antagonist concentrations (to insure agonist maximal response), it can be seen

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that limiting values of $\vartheta \rightarrow 0$ and $\Phi \rightarrow 1$ can be attained. Substituting into eq. 1 and imposing the condition of agonist maximal response ([A]/$K_A$ = 1000 [B]/$K_B$), it can be seen that the maximal response to that agonist in the presence of a saturating concentration of antagonist ([B] $\rightarrow \infty$) as a fraction of the control maximal response is given by eq. 7.

$$\text{Agonist maximal response} = \frac{(1 - e^{-k_2 t})(\tau + 1)}{(1 - e^{-k_2 t})\tau + 1} \quad (7)$$

The important aspect of eq. 7 is the fact that it has maximal asymptote values that are <1 (depressed maximum) at low values of $k_2$, $\tau$, and short periods of response collection time $t$ but that the maximal response to the agonist will not be depressed to zero. The relationship between rate of offset and time for collection of response (as the product $k_2 \times$ t) and the maximal response to agonists for antagonists demonstrating hemi-equilibria is shown in Fig. 3D. In general, hemi-equilibria may not always produce such effects, but if such effects are seen, they are indicative of a hemi-equilibrium system. The equations for agonist-receptor occupancy and response for kinetic extremes (simple competitive and pseudo-irreversible) as well as hemi-equilibria derived in Appendix are summarized in Fig. 1B.

**Allosteric Effects.** Another molecular mechanism that can result in insurmountable antagonist effects is allosteric modulation. In this case, the antagonist binds to its own site on the receptor to cause a change in conformation of the receptor that results in a change in its behavior toward the agonist; i.e., the affinity and/or the efficacy of the agonist can be altered by an allosteric interaction. This condition can be modeled by a combination of the Ehlert allosteric model (1988) and the operational model (Black and Leff, 1983); the resulting model (derived in Appendix II) is shown in Fig. 4A (Kenakin, 2005). The response to an agonist in the presence of an allosteric modulator is given by eq. 8 (Appendix II),

$$\text{Response} = \frac{[A]/K_A \tau(1 + \alpha\xi[B]/K_B)E_{max}}{[A]/K_A(1 + \alpha[B]/K_B + \tau(1 + \alpha\xi[B]/K_B)) + [B]/K_B + 1} \quad (8)$$

where $K_A$ and $K_B$ are the equilibrium dissociation constants of the agonist and antagonist-receptor complexes respectively, $\alpha$ is the ratio of affinities of the receptor in the absence and presence of the modulator, $E_{max}$ is the maximal response of the system, and $\xi$ is the ratio of efficacies of the agonist in the presence and absence of the modulator. It can be seen that, if the modulator completely prevents receptor activation by the agonist ($\xi = 0$) and also if it has no effects on the affinity of the agonist for the receptor ($\alpha = 1$), eq. 8 reduces to the equation for insurmountable antagonism first published by Gaddum et al. (1955) [written for the operational model (eq. 6)]. It should be noted that more extensive and explicit treatments of allosteric effects on receptor activation can be found in Hall (2000) and Ehlert (2005) and that the model described here is a minimal model for quantification of antagonist effect and not determination of molecular mechanism of allosteric receptor blockade of activation.

An interesting feature of the allosteric model shown in Fig. 4A is that it can accommodate conditions whereby the effect of the allosteric modulator on the agonist concentration-curve is the production of a new nonzero maximal asymptote for the agonist response. There is no a priori reason for an allosteric modulation to only reduce the signaling capability of a receptor; it could also increase it. Figure 4 show patterns of antagonist profiles for allosteric modulators that have different effects on receptor signaling (value of $\xi$) and agonist affinity (varying $\alpha$). Figure 4B shows a modulator that increases receptor signaling (agonist efficacy) but decreases agonist affinity. Figure 4C shows the effects of decreasing both signaling and affinity but where the signaling capacity of the receptor is not completely blocked by the modulator

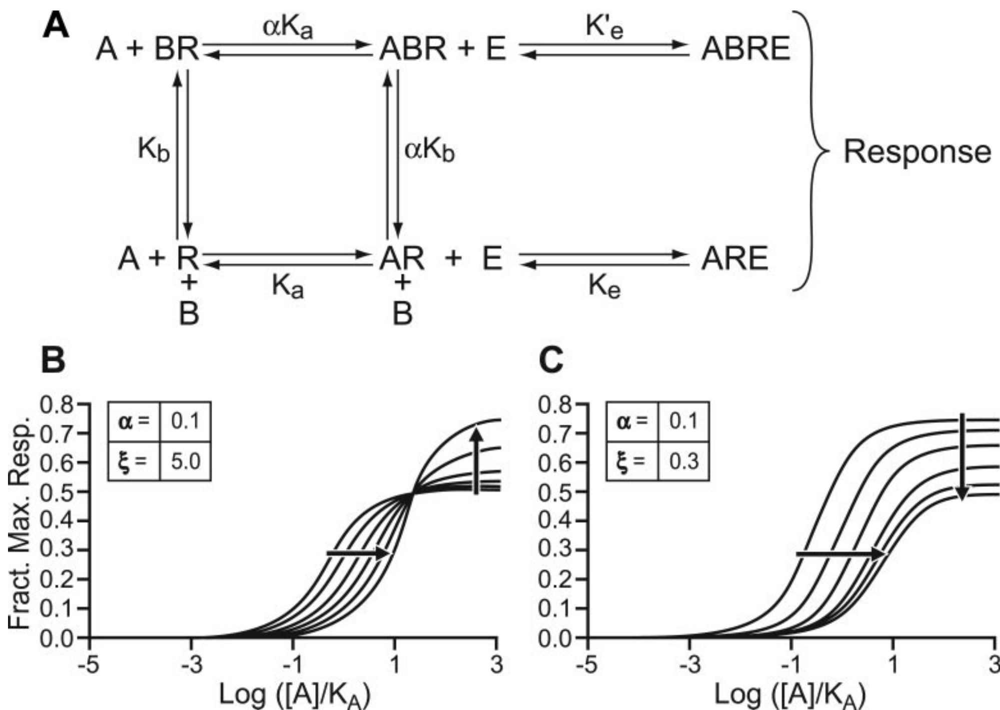


**Fig. 4.** Model of allosteric receptor function. A, combination of the Ehlert model and operational model for receptor function. In this case, the allosterically modified agonist-bound receptor may still signal and interact with the response elements of the cell with an equilibrium association constant $K_e'$; in the absence of allosteric modulation, the activated receptor interacts with response elements with constant $K_e$ (see Kenakin, 2005). Divergence of allosteric effect on binding (affinity) and function (efficacy). B, response according to eq. 8 for modulator that increases agonist efficacy ($\xi$ = 5) but decreases agonist affinity ($\alpha$ = 0.1). Curves shown for control and [B]/$K_B$ = 3, 10, 30, 100, and 300. C, responses according to eq. 8 for a modulator that decreases both affinity ($\alpha$ = 0.1) and efficacy ($\xi$ = 0.3). Note how a limiting amount of blockade occurs beyond which the agonist response is resistant to further inhibition.

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($\xi \neq 0$). It is important to experimentally determine whether the insurmountable antagonist is able to completely suppress agonist response. If a residual agonist response resists blockade by the antagonist, assuming it is due to a specific receptor effect of the agonist, this indicates that the antagonist is allosteric in nature and that $\xi \neq 0$ in eq. 8.

By far the more experimentally observed form of allosteric antagonism shows complete blockade of the response ($\xi = 0$) leading to the following equation for description of insurmountable allosteric blockade:

$$\text{Response} = \frac{[A]/K_A\, \tau E_{\max}}{[A]/K_A(1 + \tau + \alpha[B]/K_B) + [B]/K_B + 1} \quad (9)$$

It can be seen that both orthosteric (slow offset antagonists and hemi-equilibrium conditions; eq. 6) and allosteric antagonists (eq. 9) can produce insurmountable antagonism. It might be assumed that fitting experimental data to the various models for these effects could be used to both estimate and measure antagonist affinity and determine mechanism of action. However, in practical terms, there is such a variety of parameters in the models that data fitting can lead to ambiguous results; i.e., more than one model may fit the data very well. For example, Fig. 5 shows that a theoretical dataset for insurmountable antagonism fit to either an orthosteric model or allosteric model. The data were designed purposely to fit to both models with minimal differences in sum of squares; i.e., a computer would choose either model depending on very slight variations of two data points. This underscores the unreliability of fitting data to these equations with confidence that the outcome will correctly unveil molecular mechanism of action and potency of the antagonist. In view of this shortcoming, the premise of this paper will be that the system-independent measure of antagonist potency is needed. Furthermore, it will be proposed that determination of the molecular mechanism of action of the antagonist should be done with separate lines of experiments. The first to be considered is the determination of antagonist potency.

**Measuring Potency of Insurmountable Antagonists.** Theoretical examination of the various models for orthosteric and allosteric insurmountable antagonism shows that, if concomitant dextral displacement is observed with maximal response depression due to the antagonist, then the experimentally determined $pA_2$ for antagonism is a close approximation of the $pK_B$ for both orthosteric and allosteric antagonists. In cases where re-equilibration between agonist and antagonist is allowed to occur, the familiar simple competitive antagonism, typified by dextral displacement of agonist concentration-response curves with no depression of maxima, is observed, and the dextral displacement to the right of the concentration response curve of 2 (dose ratio DR = 2) is produced by a concentration of competitive antagonist equal to the $K_B$. The $pA_2$ is obtained by adding the value of log(DR-1) to the negative logarithm of the molar concentration of antagonist [$pA_2$ = log(DR-1) − log[B]]. The −log of the molar concentration of antagonist producing a DR = 2 is referred to as the $pA_2$; it is an empirical estimate of the $pK_B$. In orthosteric systems, the relationship between the $pA_2$ of an insurmountable antagonist and the true $pK_B$ can be shown to be (see Appendix III)

$$pA_2 = pK_B + \log(1 + 2[A]/K_A) \quad (10)$$

where [A] is the concentration of agonist on the control concentration-response curve that yields the level of response at which the DR is calculated (as a fraction of the equilibrium dissociation constant of the agonist-receptor complex, $K_A$). From this equation, it can be seen that the $pA_2$ is an accurate estimate of the $pK_B$ at low levels of agonist-receptor occupancy ($[A]/K_A \rightarrow 0$). This relationship indicates that, if agonist-receptor occupancy is substantial (i.e., substantial value for $[A]/K_A$, low $\tau$), the observed $pA_2$ will overestimate the true affinity of the antagonist. The key to determining the significance of this effect is the magnitude of the $[A]/K_A$ term. For systems with a large receptor reserve (high-receptor density, efficient receptor coupling, and high-agonist efficacy), $[A]/K_A \ll EC_{50}$ for response. The operative levels of $[A]/K_A$ for the concentration-response curve are dependent upon the efficacy of the agonist, receptor density, and efficiency of the receptor coupling; specifically the magnitude of $\tau$. Equation 10 can be rewritten in terms of the control concentration-response curve $EC_{50}$ (concentration of agonist producing 50% maximal response). This puts into perspective the error inherent in the $pA_2$ measurement as an estimate of the $pK_B$ for insurmountable antagonists. Under these circumstances, eq. 10 becomes (see Appendix III for derivation)

$$pA_2 = pK_B + \log(1 + (2[A]/EC_{50}(1 + \tau))) \quad (11)$$

DR values are calculated in regions of the agonist concentration-response curve where $[A] < EC_{50}$, and in most cases, $\tau >$

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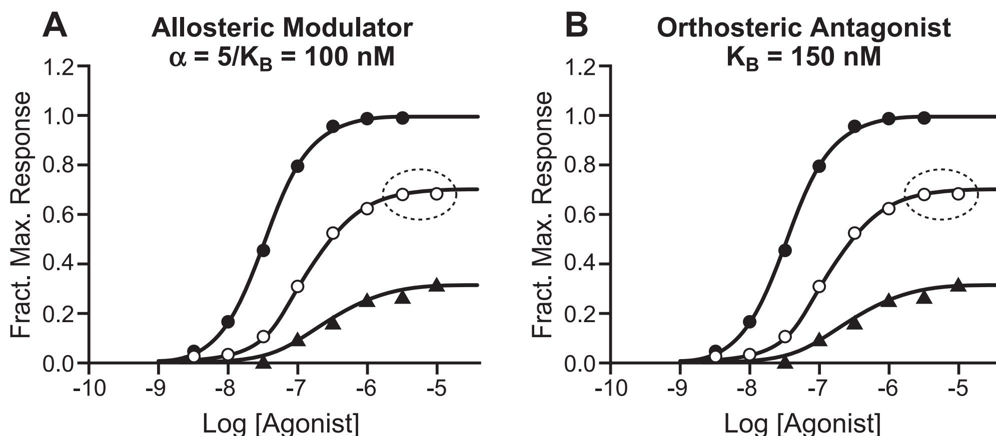


**Fig. 5.** Simulation demonstrating the fallacy of relying on goodness of fit to determine mechanism of action. Simulation data fit to a model of allosteric insurmountable antagonism (A) (eq. 9 and an orthosteric model (B) (eq. 6 of insurmountable antagonism. The only difference between these datasets is the value of the circled points (0.67 value changed to 0.65).

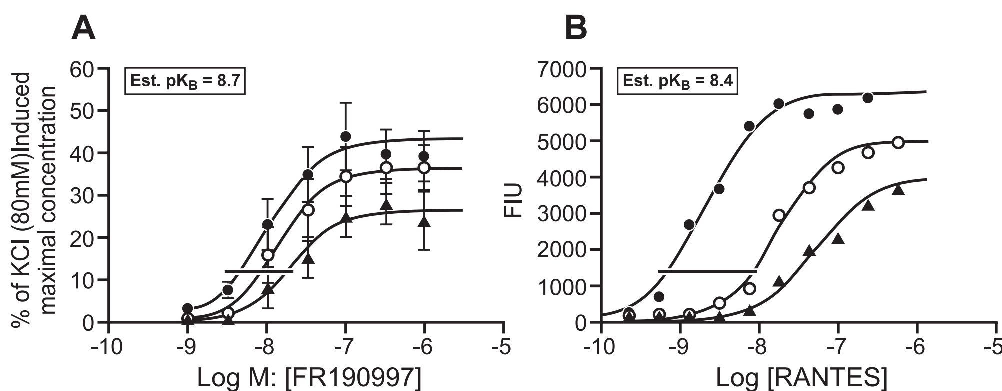


**Fig. 6.** Estimated $pA_2$ values from experimentally observed insurmountable antagonism. A, blockade of bradykinin $B_2$-receptor mediated responses to the agonist FR190997 by the insurmountable antagonist FR173657 in guinea pig ileum. Responses in the absence (filled circles) and presence of 1 (open circles) and 3 nM FR173657 (filled triangles). Curves are redrawn from Meini et al. (2000). B, blockade of CCR5 receptor-mediated responses to the chemokine agonist RANTES by the allosteric modulator SCh-C in U-2 OS cells. Responses in the absence (filled circles) and presence of 17.6 (open circles) and 26.3 nM Sch-C (filled triangles).

TABLE 1

Correspondence of $pA_2$ estimates from published literature and independent measures of antagonist potency for orthosteric and allosteric insurmountable antagonists

| Antagonist | Receptor | $pA_2$[a] | Corrected $pK_B$[b] | Indep. $pK_i$[e] | Reference |
|---|---|---|---|---|---|
| Orthosteric | | | | | |
| FR173657 | G. P. Bradykinin $B_2$ | 9.0 | 8.7 | Binding 8.7 ± 0.01 | Meini et al., 2000 |
| SR 48,968 | G. P. $NK_2$ Receptor | 10.6 | 10.55 | Gaddum 10.84 + 0.19 | Kerr et al., 2000 |
| Darifenacin | Canine Muscarinic m3 | 9.1 | 9.0 | Binding 8.8 | Choppin and Eglen, 2001 |
| Allosteric ($\alpha \neq 1$) | | | | | |
| Aplaviroc ($n$ = 3) | Human CCR5 | 8.65 + 0.37 | $\alpha \approx 0.8$ $pK_B$ = 8.4[c] | Binding 8.6 ± 0.22 | Watson et al., 2005 |
| | Human CCR5 | 8.7 ± 0.32 | $\alpha \approx 0.8$ $pK_B$ = 8.45[c] | | Watson et al., 2005 |
| Sch-C ($n$ = 4) | Human CCR5 | 7.9 + 0.4 | $\alpha \ll 1$ no corr.[d] | Binding 8.2 ± 0.22 | Watson et al., 2005 |
| | Human CCR5 | 8.0 ± 0.3 | $\alpha \ll 1$ no corr.[d] | | Watson et al., 2005 |
| Vicriviroc ($n$ = 5) | Human CCR5 | 8.3 ± 0.25 | $\alpha \ll 1$ no corr.[d] | Binding 8.4 ± 0.2 | Watson et al., 2005 |
| Maraviroc ($n$ = 4) | Human CCR5 | 8.7 + 0.2 | $\alpha \ll 1$ no corr.[d] | Binding 8.7 ± 0.2 | Watson et al., 2005 |
| TAK779 ($n$ = 4) | Human CCR5 | 7.9 ± 0.2 | $\alpha \ll 1$ no corr.[d] | Binding 7.8 ± 0.18 | Watson et al., 2005 |

[a] Dose-ratios at a 25% maximal response level were used to calculate $pA_2$ values with the equation $pA_2 = -\log[B] + \log(DR\text{-}1)$ where [B] is the concentration of antagonist. Where two estimates of $pA_2$ are shown, two separate concentrations of antagonist were used to make the estimate.

[b] Correction according to eq. 11 for orthosteric antagonists and eq. 13 for allosteric antagonists.

[c] Estimated maximal $[A]/K_A = 0.5$; correction $1 + 2\ \alpha[A]/K_A = 1.8$

[d] Modulator completely blocks binding of RANTES (Watson et al 2005) at $K_d$ concentration indicating that $\alpha < 0.05$ for this receptor probe. Under these circumstances, correction factor $\ll 1$.

[e] An independent estimate of the $pK_B$ is available either from binding or, in the case of SR 48,968, a functional analysis by the method of Gaddum et al. (1955).

1. Assuming a maximal limit of $[A] = EC_{50}$ and $\tau < 1$, the maximal limit for the correction factor for $pA_2$ to $pK_B$ is 3, with a more probable value <2. A sample of an experimentally derived $pA_2$ value from published insurmountable antagonism data for an orthosteric antagonist is shown in Fig. 6A, with calculation from more examples in the literature given in Table 1A.

A similar derivation can be carried out for allosteric insurmountable antagonists. For allosteric modulators (see Appendix IV),

$$pK_B = pA_2 - \log(1 + 2\alpha[A]/K_A) \qquad (12)$$

with a scaling to the $EC_{50}$ for the agonist concentration curve, eq. 12 becomes (see Appendix IV)

$$pA_2 = pK_B + \log(1 + (2\alpha[A]/EC_{50}(1 + \tau))) \qquad (13)$$

For allosteric modulators that decrease the affinity of the receptor for the antagonist ($\alpha < 1$), this effect actually decreases the error between the observed $pA_2$ and the true $pK_B$ and thus improves the method. In contrast, it can be seen that, if the allosteric modulator increases the affinity of the receptor for the agonist ($\alpha > 1$), the error produced by the insurmountable nature of the blockade may become substantial. On the other hand, if a dextral displacement is observed experimentally, then the value of $\alpha$ most likely will not interfere with the estimate of the $pK_B$ by the $pA_2$ method. A sample of experimentally derived $pA_2$ value from published insurmountable antagonism data for allosteric antagonists is shown in Fig. 6B, with data for other antagonists given in Table 1B. A summary of the use of the $pA_2$ for $pK_B$ estimates for all types of insurmountable antagonism is given in Fig. 7.

### Detecting Allosteric Antagonism: Permissive Antagonism

The foregoing discussion illustrates how the potency of an insurmountable antagonist can be estimated with little regard for the molecular mechanism. However, there are practical reasons for determining whether a given insurmountable antagonist produces its effects through an orthosteric or an allosteric mechanism (see *Discussion*). There are two features of allosteric modulators that can be used for identification of this molecular mechanism of action; these are probe dependence and saturability. As can be seen from Fig. 4, there is a species [ABR] that is the receptor bound to both the agonist and the antagonist. There is no reason a priori for this species not to be able to signal; i.e., the antagonist may modify the affinity of the receptor for the agonist, but the agonist binding site is not occluded and it can still bind and potentially signal. However, the quality and quantity of signal may change because the allosteric antagonist produces an allosteric change in conformation. The allosteric effect on affinity is quantified by the magnitude of the cooperativity constant $\alpha$, and this is specific to the particular agonist cobinding to the receptor. Thus, an allosteric antagonist may reduce the affinity of the receptor for one agonist and not

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| Type of Antagonism | $pA_2$ = | Correction Factor | Curve Pattern(s) |
|---|---|---|---|
| Competitive Surmountable | $pK_B$ | None but Schild regression must be linear with unit slope | |
| Hemi-Equilibria | $pK_B + \psi Log(1+2[A]/K_A)$ $\psi <<1$ | Very slight overestimation to no correction | |
| Orthosteric Insurmountable | $pK_B + Log(1+2[A]/K_A)$ | Slight overestimation (maximal error ≈2) | |
| Allosteric Insurmountable | $pK_B + Log(1+2\alpha[A]/K_A)$ | Very slight overestimation (for modulators with $\alpha$ <1) | |

**Fig. 7.** Summary of the relationship between the $pA_2$ for various mechanisms of antagonism and the $pK_B$.

change (or even increase) the affinity to another. In essence, the antagonist becomes permissive with respect to the effects it may allow certain receptor probes (i.e., agonists or radioligands) to have (Kenakin, 2005). For example, the m2 muscarinic receptor allosteric modulator eburnamonine produces a maximal 30-fold antagonism of the agonist pilocarpine, no effect on the agonist arcaidine propargyl ester, and a 15-fold maximal potentiation of the agonist arecoline (Jakubic et al., 1997). The agonist-specific nature of these effects is quantified by the magnitude of $\alpha$ for each receptor probe (pilocarpine $\alpha = 0.033$, arcaidine propargyl ester $\alpha = 1$, arecoline $\alpha = 15$). This is in stark contrast to orthosteric antagonists that block all agonists and receptor probes equally. This then becomes an important differentiator of allosteric (over orthosteric) antagonism.

These effects also illustrate a second important feature of allosteric modulators, namely saturation of effect. This occurs as a direct result of the fact that allosteric modulators bind to their own site on the receptor. At concentrations of $[B]/K_B > 100$, the binding sites for the modulator essentially are saturated, and thus no further allosteric effect can result. This can lead to the production of characteristic patterns of antagonism typified by saturable maximal effects. For example, the allosteric muscarinic receptor modulator alcuronium produces a maximal 100-fold displacement of acetylcholine concentration-response curves in rat heart (Christopoulos, 2000). This is in contrast to simple competitive effects that produce theoretically limitless dextral displacement of agonist concentration-response curves. In practical terms, the concepts of probe dependence and saturability can be used to detect possible allosterism by studying the effects of allosteric modulators over the largest concentration range possible (detect saturability) and with as many different receptor probes (agonists or radioligand) as possible (detect probe dependence).

## Discussion

There are well characterized tools and methods for the system-independent determination of both the affinity and mechanism of action of antagonists. However, a prerequisite to the effective use of these tools is knowledge that the antagonist binds either to the agonist binding site (orthosteric to the agonist) or an allosteric binding site (separate site on the receptor affecting the agonist through a receptor conformational change). In early stage drug discovery, it is seldom that such mechanistic details are known; thus, the process of antagonist potency determination and elucidation of molecular mechanism of action is data-driven. This article describes, for insurmountable antagonists, operational meth-

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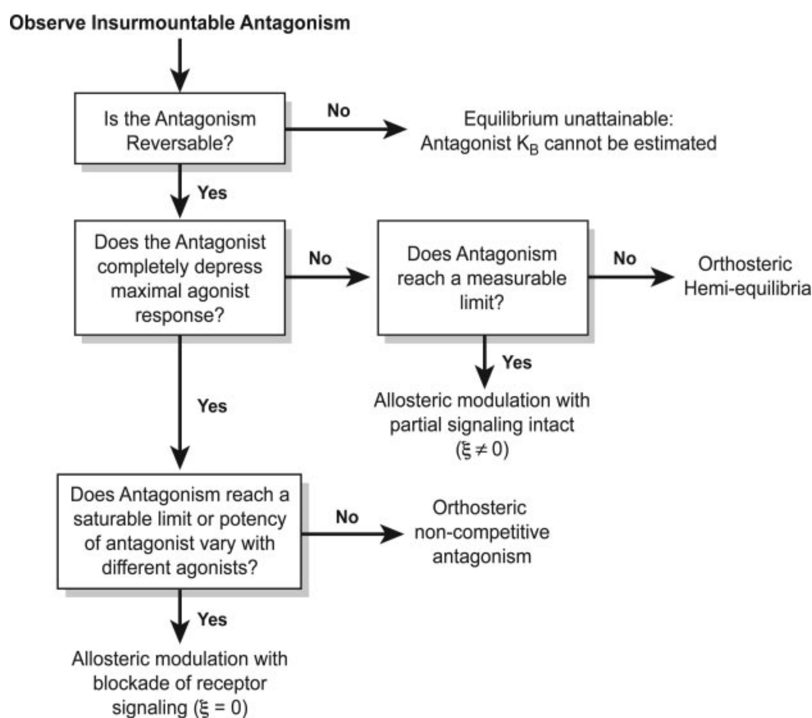


**Fig. 8.** Schematic diagram showing the logic for determining the potency of various insurmountable antagonists.

ods to determine 1) the system-independent affinity of the antagonist for the receptor and 2) whether or not the antagonism is orthosteric or allosteric in nature.

Although insurmountable antagonism can be the result of orthosteric or allosteric mechanisms, some general conclusions can be drawn from an analysis of the models describing both mechanisms of action; these are outlined schematically in Fig. 8. A prerequisite to these analyses is the condition that the antagonism is not irreversible or pseudo-irreversible, with respect to equilibrium of the antagonist with the receptor. Therefore, it first must be shown that the antagonism is reversible by washing with drug-free medium.

Other mechanisms of insurmountable effects on agonist concentration-response curves also must be excluded. For example, if the agonist produces release of an endogenous agonist in the system, then competitive antagonists produce depression of the maximal response (Black et al., 1980). Furthermore, other nonspecific mechanisms for depression of agonist maximal response, such as toxic cellular effects or receptor effects such as desensitization, must be excluded from consideration. After it has been determined that irreversible antagonism is not the reason for the insurmountable effects, certain key questions can be asked to delineate the molecular mechanism of the ligand. A major observation is to determine whether the blockade is complete, i.e., if high concentrations of antagonist can completely eliminate the agonist response. If this is not the case, then either an allosteric effect, such as a partial inhibition of receptor signaling to the agonist, is produced by the modulator or a hemi-equilibrium is producing a partial shortfall in the equilibration among agonist, antagonist, and receptors (see Fig. 8). These latter two possibilities can be differentiated by the fact that saturation of an allosteric binding would cease to affect both the location parameter of the curves as well as the maxima; i.e., at some point, there would be no further shift or depression of the curves. In the case of hemi-equilibria, dextral displacement can continue much like competitive antagonism. For example, the AT1 antagonist SB203220 [*E*-*a*-[[2-butyl-1-(carboxy-1-naphalenyl)methyl-1*H*-imidazol-5-yl]-methylene]-2-thiophene-propanic acid] produces a marked depression maximal angiotensin response that reaches a plateau that remains constant through three orders of magnitude of the antagonist (Aiyar et al., 1995). Desensitization of the receptor by the agonist also has been shown to lead to depression of maximal responses. A plateau depression of the maximal response due to hemi-equilibria has been described by Lew et al. (2000) when desensitization to the agonist is operative.

In general, antagonism becomes detectable (i.e., a 2-fold shift to the right of the agonist concentration-response curve) when the antagonist is present in the receptor compartment at concentrations causing approximately 50% receptor occupancy (by definition, the concentration defined as the equilibrium dissociation constant of the antagonist-receptor complex). Thus, the $pA_2$ is formally the $pK_B$ for simple competitive antagonists and, parenthetically, also approximates the $pK_B$ for insurmountable antagonists if dextral displacement of the curve is produced concurrently with depression of maximal response. The error incurred due to the insurmountable aspect of the antagonism is minimal (overestimation by a factor proportional to the agonist-receptor occupancy) as described by eq. 11 for orthosteric antagonism and eq. 13 for allosteric antagonism. A practical limitation of relying on dextral displacement of agonist concentration-response curves for antagonist potency estimates is encountered if there is little measurable shift in the concentration-response curve with antagonism. If this occurs, it indicates either that there is little receptor reserve in the system or that the antagonist allosterically blocks signaling and increases the affinity of the receptor for the agonist. Under these circumstances, a $pA_2$ cannot be measured, leading to a requirement for an alternative approach, and estimation of

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antagonist potency can be achieved through measurement of an $IC_{50}$. The only case where the $IC_{50}$ will differ significantly from the $K_B$ is for allosteric antagonists that enhance the affinity of the receptor for the agonist ($\alpha > 1$). This effect can be detected with experiments that examine the $IC_{50}$ with different concentrations of agonist. If $\alpha > 1$ for a given modulator, then the potency of the modulator will increase as it blocks increasing concentrations of agonist. Although this can be detected most readily in an $IC_{50}$ mode, it can be masked by the presence of a receptor reserve in the system. With systems of high-receptor reserve, the $IC_{50}$ values for a modulator will tend to be increased with increasing agonist concentration. Therefore, it is difficult to detect this effect in systems of high-receptor reserve unless a range of antagonists is used and differences in the $IC_{50}$ behavior with varying agonist concentration can be observed.

Determination of molecular mechanism of antagonism can be an important step in the drug development process as allosteric antagonists can have properties very different from orthosteric molecules (Kenakin, 2004a,b). Knowing the molecular mechanism of the antagonism thus can prepare subsequent study of the drug candidate, because allosteric mechanisms can lead to complex relationships among different receptor probes in the therapeutic environment. For example, the allosteric effects of CCR5 chemokine receptor-mediated HIV-1 inhibitor molecules, such as aplaviroc, can be dependent on the nature of the chemokine (Maeda et al., 2004; Watson et al., 2005). In the case of chemokine-induced chemotaxis effects where there is a redundancy in agonists for various receptors, such probe dependence may be highly relevant to therapy. In addition, allosteric antagonists offer a completely different paradigm for receptor antagonism through the property of permissive signal processing. For example, whereas an orthosteric antagonist of the chemokine receptor CXCR4 would have the therapeutic property of blocking HIV X4 tropic virus to prevent AIDS (Feng et al., 1996), it also would block the beneficial functioning of the receptor as it mediates responses to the natural agonist stromal-derived factor 1. Blockade of this latter property can lead to developmentally lethal effects (specifically producing hematopoiesis and developmental defects in the cerebellum, heart, and gastrointestinal tract) (Nagasaw et al., 1996; Tachibana et al., 1998; Zou et al., 1998). The permissive separation of antagonism has been demonstrated for this receptor by the selective antagonism of stromal-derived factor 1$\alpha$ by the antibody T140 and receptor antagonist AMD3100; responses to the peptide fragments RSVM and ASLW are unaffected (Sachpatzidis et al., 2003). The therapeutically relevant preservation of natural function with concomitant elimination of pathological function is a unique feature of allosteric antagonists. Therefore, knowledge of such potential is highly relevant to the drug discovery process.

In the case of surmountable antagonism, a useful method is to test as wide a range of antagonist concentrations as possible to detect possible nonlinearity of the Schild regression at higher antagonist concentrations. This is utilizing the concept that allosteric effects are saturable (i.e., a limiting value for the antagonism will be found resulting in a curvilinear portion in the Schild regression) (Christopoulos and Kenakin, 2002). In the case of insurmountable antagonism, successive comparison of the data to models of orthosteric and allosteric insurmountable blockade, under varying circumstances, can be a useful approach, but it should be cautioned that equations with multiple parameters can fit data ambiguously. In these cases, more than one model may fit the data equally well. For this reason, goodness of fit alone is a poor indicator of mechanism of action (various combinations of $\alpha$ and $\tau$ may yield comparable fits as shown in Fig. 5).

The most generally applicable method of determining possible allosterism is to test for probe dependence. Whereas orthosteric effects are immune to changes in agonist (the $pK_B$ for antagonism of responses to all agonists should be the same), allosteric effects may not be. Thus, changes both in the potency and linearity of the Schild regression may be encountered with different agonists for allosteric modulators. It should be recognized that the determination of allosterism through observation of probe dependence and saturability is a one-way process; i.e., it will detect these effects if the reagents and conditions are correct but may not if the wrong agonist and/or conditions are used. Therefore, lack of observation of probe dependence and/or saturability is not conclusive evidence of orthosteric effect. If no probe dependence or saturability is detected, it may be that the wrong agonists are being used and/or the $\alpha$ values for these agonists are very low.

In summary, data-driven determination of antagonist potency and mechanism of action is a common process in drug discovery. Analyses of surmountable antagonist effects are straightforward (Schild or Clark plot analysis). Insurmountable effects can result either from orthosteric temporal effects or allosterism, and in these cases, circumspection is needed in determining the molecular mechanism of antagonism. This article outlines a strategy to do so and also outlines the theoretical basis for measuring antagonist potency in a mechanism-independent manner (with a $pA_2$). The therapeutic implications of determining orthosteric versus allosteric antagonism also are discussed.

# Appendix

## Appendix I: Derivation for Response to an Agonist as a Temporal Function of Re-equilibration among Antagonist, Agonist, and Receptors

It is useful to define the following relationships.

$$\vartheta = [B]/K_B/([B]/K_B + [A]/K_A + 1) \quad (14)$$

is the receptor occupancy by the antagonist in the presence of the antagonist

$$\rho_B = [B]/K_B/([B]/K_B + 1) \quad (15)$$

which is the antagonist-receptor occupancy.

$$\Phi = ([B]/K_B + [A]/K_A + 1)/([A]/K_A + 1) \quad (16)$$

is the receptor occupancy by the agonist in the presence of the agonist. For these equations, $K_B = k_2/k_1$, [A] is the agonist, [B] is the antagonist, $k_1$ is the rate of onset, $k_2$ is the rate of offset of the antagonist from the receptor, and $K_A$ is the equilibrium dissociation constant of the agonist-receptor complex. From Paton and Rang (1965), the occupancy by an antagonist [B] with time in the presence of a concentration of agonist [A] is given by eq. 17.

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$$\rho_{AB} = \vartheta(1 - e^{-k_2\Phi t}) + \rho_B e^{-k_2\Phi t} \quad (17)$$

It is assumed that a fast-acting agonist will equilibrate with the free receptors (given by $1 - \rho_{AB}$) immediately and then re-equilibrate with the antagonist-bound receptors according to eq. 17 over time. The agonist-receptor occupancy curve is then given by eq. 18.

$$\rho_A = ([A]/K_A/([A]/K_A + 1))(1 - (\vartheta(1 - e^{-k_2\Phi t}) + \rho_B e^{-k_2\Phi t})) \quad (18)$$

When agonist pharmacological response is monitored, the effects of agonist efficacy, efficiency of receptor coupling, and receptor density also must be considered. The most effective way to deal with these issues is to express pharmacological response in terms of the operational model of receptor function as described by Black and Leff (1983). The operational model makes minimal assumptions about the biochemical reactions that convert agonist stimulation of receptor into cellular response. Thus, response is considered to be a generic hyperbolic function of the agonist-receptor complex (denoted [AR]) binding to elements of the cellular stimulus-response cascade (Black and Leff, 1983),

$$\text{Response} = E_{max}[AR]/([AR] + K_E) \quad (19)$$

where $E_{max}$ is the maximal response-producing capability of the system and $K_E$ is the equilibrium dissociation constant for the general function relating [AR] to cellular response. This term encompasses the efficacy of the agonist and the efficiency of receptor coupling in the cell. The concentration of agonist-bound receptors [AR] is given by $\rho_A[R_t]$, where $\rho_A$ is the agonist receptor-occupancy by mass action binding and $[R_t]$ is the receptor density. Therefore, for all calculations of response for the various models, the operational model will be used in the form

$$\text{Response} = \rho_A[R_t]E_{max}/(\rho_A[R_t] + K_E) \quad (20)$$

where $\rho_A$ will be the fractional receptor occupancy in the presence of the antagonist. In accordance with the operational model, a term $\tau$ is introduced ($\tau = [R_t]/K_E$), representing the receptor density on the surface of the cell, the ability of the cell to convert stimulus into response, and the intrinsic efficacy of the agonist (Black and Leff, 1983) leading to a metameter of eq. 20 as eq. 21.

$$\text{Response} = \rho_A\,\tau\,E_{max}/(\rho_A\tau + 1) \quad (21)$$

In terms of agonist response, $\rho_A$ from eq. 18 is substituted into eq. 21 to yield

$$\text{Response} = \frac{[A]/K_A(1 - (\vartheta(1 - e^{-k_2\Phi t}) + \rho_B e^{-k_2\Phi t}))\tau E_{max}}{[A/K_A((1 - (\vartheta(1 - e^{-k_2\Phi t}) + \rho_B e^{-k_2\Phi t}))\tau + 1) + 1} \quad (22)$$

## Appendix II: Allosteric Model for Functional Receptor Effects

The basic assumption is that the allosteric modulator [B] binds to its own site on the receptor and causes modification of the pattern of interaction with an agonist; this can be in the form of a change in the affinity of the receptor for the agonist (denoted by the term $\alpha$) and/or the efficacy of the agonist-receptor complex in initiating response (as determined in terms of the operational model as the binding of the agonist-receptor complex to the stimulus-response apparatus of the cell with an overall operational equilibrium association constant denoted $K_e$). Once the modulator is bound, the operational affinity of the agonist-receptor complex for the stimulus-response machinery is defined by $K_e'$ (see Fig. 4). The quantities of [AR] and [ABR] complex are calculated from the Ehlert model of allosteric interaction (1988). The ability of the agonist to induce system response in the absence of modulator is defined by $\tau$ (where $\tau = [R_t] \times K_e$); in the presence of modulator, it is defined as $\tau'$ ($[R_t] \times K_e'$). A term $\xi$ is defined, which is the ratio of $\tau$ values in the presence and absence of modulator ($\xi = \tau'/\tau$).

The equilibrium species are (refer to Fig. 4) as follows:

$$[AR] = [ABR]/\alpha[B]K_b \quad (23)$$

$$[BR] = [ABR]/\alpha[A]K_a \quad (24)$$

$$[R] = [ABR]/\alpha[B]K_b[B]K_b \quad (25)$$

According to the operational model, the response-producing species activate the response elements of the cell according to eq. 26,

$$\text{Response} = \frac{[AR]/K_E + [ABR]/K_E'}{[AR]/K_E + [ABR]/K_E' + 1} \quad (26)$$

where $K_E = K_e^{-1}$ and $K_E' = K'^{-1}_e$. The amount of any receptor species is given by the fractional amount of receptor multiplied by the total receptor number; thus, eq. 26 can be rewritten as follows:

$$\text{Response} = \frac{\rho_{AR}[R_t]/K_E + \rho_{ABR}[R_t]/K_E'}{\rho_{AR}[R_t]/K_E + \rho_{ABR}[R_t]/K_E' + 1} \quad (27)$$

where $\rho_{AR}$ is the fraction of receptor in the [AR] form given by eq. 28,

$$\rho_{AR} = [A]/K_A/([A]/K_A(1 + \alpha[B]/K_B) + [B]/K_B + 1) \quad (28)$$

and $\rho_{ABR}$ is the fraction of receptor in the [ABR] form given by eq. 29.

$$\rho_{ABR} = \alpha[A]/K_A[B]/K_B/([A]/K_A(1 + \alpha[B]/K_B) + [B]/K_B + 1) \quad (29)$$

Substituting eqs. 28 and 29 into 27 and defining $\tau$ as $[R_t]/K_E$ and $t'$ as $[R_t]/K_E'$, eq. 27 can be rewritten as follows.

$$\text{Response} = \frac{[A]/K_A(\tau + \alpha\tau'[B]/K_B)E_{max}}{[A]/K_A(1 + \alpha[B]/K_B + (\tau + \alpha\tau'[B]/K_B)) + [B]/K_B + 1} \quad (30)$$

Finally, defining $\xi$ as the ratio of $\tau$ values for the agonist-bound receptor when it is and is not bound to modulator $\xi = \tau'/\tau$, eq. 30 becomes eq. 31.

$$\text{Response} = \frac{[A]/K_A\tau(1 + \alpha\xi[B]/K_B)E_{max}}{[A]/K_A(1 + \alpha[B]/K_B + \tau(1 + \alpha\xi[B]/K_B)) + [B]/K_B + 1} \quad (31)$$

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## Appendix III: Relationship of p$A_2$ and p$K_B$ for Insurmountable Orthosteric Antagonism

For simple competitive antagonism with adequate time for agonist-antagonist re-equilibration (surmountable antagonism), $\rho_B$ is given by $[B]/K_B/([B]/K_B + [A]/K_A + 1)$ to yield the well known Gaddum equation for simple competitive antagonism for agonist-receptor occupancy in the presence of the antagonist denoted $\rho_{AB}$ $([A]/K_A)/([A]/K_A + [B]/K_A + 1)$ (Gaddum, 1937). Under these circumstances, the equation for response to the agonist in the presence of the simple competitive antagonist becomes

$$\text{Response} = \frac{[A]/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau) + [B]/K_B + 1} \quad (32)$$

A relationship for equiactive agonist concentrations in the absence and presence of antagonist to yield a dose ratio of 2 ($[B] = 10^{-pA_2}$) can be made to calculate the ratio of this empirical concentration (p$A_2$) to the true $K_B$ value.

$$\frac{2[A]/K_A\ \tau E_{max}}{2[A]/K_A(1 + \tau) + [10^{-pA_2}]/K_B + 1} = \frac{[A/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau) + 1} \quad (33)$$

It can be seen through simplifying this relationship that

$$10^{-pA_2} = K_B \quad (34)$$

as expected from the Schild equation (i.e., $pA_2 = pK_B$) of unit slope.

This same procedure can be done to equate the empirical p$A_2$ to p$K_B$ for a completely noncompetitive antagonist in which the agonist and antagonist do not re-equilibrate due to kinetics. Under these circumstances, the equation for antagonist occupancy is given by mass action, and agonist-receptor occupancy in the presence of antagonist ($\rho_{AB}$) is given by the eq. 1 with no time for agonist, antagonist, and receptor re-equilibration (eq. 6) for noncompetitive receptor blockade.

$$\text{Response} = \frac{[A]/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau + [B]/K_B) + [B]/K_B + 1} \quad (35)$$

The relationship between equiactive concentrations with a dose ratio of 2 in the presence and absence of antagonist is given by eq. 36.

$$\frac{2[A]/K_A\ \tau E_{max}}{2[A]/K_A(1 + \tau + [10^{-pA_2}]/K_B) + [10^{-pA_2}]/K_B + 1} = \frac{[A/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau) + 1} \quad (36)$$

Simplification of this relationship yields an equation relating p$A_2$ and $K_B$ as follows:

$$10^{-pA_2} = K_B/(1 + 2[A]/K_A) \quad (37)$$

$$pK_B = pA_2 - \log(1 + 2[A]/K_A) \quad (38)$$

The magnitude of the correction term $(1 + 2[A]/K_A)$ can be scaled to the system by relating this to the $EC_{50}$ (molar concentration of agonist producing 50% maximal response to that agonist) of the control agonist concentration-response curve. The equation for response in terms of the operational model is (Black and Leff, 1983) shown in eq. 39.

$$\text{Response} = \frac{[A]/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau) + 1} \quad (39)$$

It can be seen from this equation that the $EC_{50}$ concentration is given by $EC_{50} = K_A/(1 + \tau)$; therefore, any value of $[A]/K_A$ can be expressed with the relation $[A]/K_A = [A]/[EC_{50}(1 + \tau)]$. Under these circumstances, eq. 38 becomes eq. 40.

$$pK_B = pA_2 - \log(1 + (2[A]/EC_{50}(1 + \tau))) \quad (40)$$

## Appendix IV: Relationship of p$A_2$ and p$K_B$ for Insurmountable Allosteric Antagonism

The counterpart of eq. 36 for allosteric systems is

$$\frac{2[A]/K_A\ \tau E_{max}}{2[A]/K_A(1 + \tau + \alpha[10^{-pA_2}]/K_B) + [10^{-pA_2}]/K_B + 1} = \frac{[A]/K_A\ \tau E_{max}}{[A]/K_A(1 + \tau) + 1} \quad (41)$$

The equation for the relationship between the p$A_2$ and the $K_B$ of an allosteric modulator that produces insurmountable antagonism then becomes the following:

$$10^{-pA_2} = K_B/(1 + 2\alpha[A]/K_A) \quad (42)$$

$$pK_B = pA_2 - \log(1 + 2\alpha[A]/K_A) \quad (43)$$

in terms of functional responses expressed as multiples of the $EC_{50}$.

$$pK_B = pA_2 - \log(1 + (2\alpha[A]/EC_{50}(1 + \tau))) \quad (44)$$

For allosteric modulators that decrease the affinity of the receptor for the antagonist ($\alpha < 1$), this effect actually decreases the error between the observed p$A_2$ and the true p$K_B$ and thus improves the method. In contrast, it can be seen that, if the allosteric modulator increases the affinity of the receptor for the agonist ($\alpha > 1$), the error produced by the insurmountable nature of the blockade may become substantial.

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