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In search of negative allosteric modulators of biological targets

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The value of using negative allosteric modulators of protein function in therapeutic treatment of human diseases is becoming more apparent. Many current screening paradigms, however, are not consciously designed to discover negative allosteric modulators, and those that are serendipitously discovered can be easily overlooked during the hit-picking process. The conditions necessary for the discovery of negative allosteric modulators in a high-throughput screen are quite reasonable and simple to implement, generally requiring a consideration of the ligand concentration in a screen. Other considerations in the screening for negative allosteric modulators can be derived from an analysis of simple kinetic schemes that describe the interactions of ligands and modulators with different protein targets.

Introduction

Allosteric modulators of protein function are ligands that modify protein responses to agonists, inhibitors and substrates by binding to sites (allosteric) other than active sites. Although multisubunit proteins are traditionally considered to be most amenable to allosteric modulation, recent observations show that monomeric proteins have binding sites that can serve to effect allosteric modulation [1,2]. In drug discovery, efforts are increasing to identify and develop positive, negative and neutral allosteric modulators of protein function. Upon binding, positive allosteric modulators increase protein function, negative allosteric modulators decrease protein function and neutral allosteric modulators have no observable effect on protein function. The advantages of using allosteric modulators as therapeutic drugs include concentration-independent limits on modulator function, efficacy only in the presence of the appropriate substrate and increased subtype specificity [3]. In this review, negative allosteric modulation will be reviewed with the intent to derive insights into screening for small molecule negative allosteric modulators. A similar treatment of positive allosteric modulators has been presented [4].

Primary screening

The role of allostery in protein function can be quite complicated and small molecule allosteric modulators can have a variety of effects, from agonist activity to competitive antagonist activity, in addition to their allosteric effects [5,6]. The treatment of allosteric modulation in this review will, however, be restricted to the discovery and dose–response characterization of those small molecule compounds that decrease protein activity in *in vitro* assays of protein function by means of negative allosteric modulation at a site distinct from the active (orthosteric) site. In a primary screen of protein function, negative allosteric modulators can cause a change in the observed signal of the assay in a manner similar to that of antagonists. Thus, primary screens for negative allosteric modulators are straightforward. Of some importance is the relative concentration of the substrate used in the screen because of the concentration-dependent effects the substrate can have on the relative activity of negative allosteric modulators and on an assay's robustness and sensitivity. Negative allosteric modulators cannot, however, be distinguished from other types of inhibitors from a simple scatter plot of % inhibition. Further characterization of the effects of negative allosteric modulators after the primary screen include, at a minimum, dose–response investigations in which the concentration of the modulator is varied under conditions identical to the primary screen. From the dose–response experiments, a measure of the potency of the negative allosteric modulator can be ascertained, usually designated as an IC_{50}. When there is no apparent modulation, it can be expected that a dose–response curve will be flat. It is crucial that the detailed investigations of modulator effects on protein function beyond the HTS stage are

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performed to positively prove negative allosteric mechanisms. In enzymology for example, inhibitors can be competitive, noncompetitive and uncompetitive as well as allosteric modulators. Although competitive inhibitors bind to the active sites of enzymes, non- and uncompetitive inhibitors bind to allosteric sites to inhibit function. These inhibitors differ, however, from true negative allosteric modulators in two fundamental ways:

- Enzyme species bound with non- and uncompetitive inhibitors are inactive, whereas modulator-bound enzymes can be active.
- There is no cooperativity between non- and uncompetitive inhibitor binding and substrate activity, whereas there is cooperativity between modulator binding and substrate activity.

In addition, artifacts, such as compound insolubility, can contribute to false identification of negative allosteric modulators. It is presumed in this review that such investigations are beyond the responsibility of most primary drug screening groups and, thus, will not be presented. There are several reviews that present experimental formats that can be adopted by HTS groups that desire to incorporate detailed characterizations of negative allostery into their screening formats [7–9].

Small molecule negative allosteric modulators, by definition, bind to a site discrete from the substrate/agonist-binding site on a protein and, upon doing so, modify the interactions and effects of the ligand with the protein. Such modulation is cooperative between the modulator and the ligand and equally describes the effect of the ligand itself on the interaction of the allosteric modulator with the protein. In the kinetic schemes presented, a small molecule that binds to an allosteric site on a protein will be referred to as an allosteric modulator, or 'A', and a ligand that binds to an substrate/agonist-binding site to initiate a function will be referred to as a substrate, or 'S', even for those circumstances in which simple binding is the only function being measured (Fig. 1). Cooperativity factors influence the interactions of both substrates and modulators with proteins (Greek characters in Fig. 1). Although the fonts assigned to cooperativity factors may be identical between the different kinetic schemes, the cooperativity factors may induce dramatically different effects on the interactions of substrates with a protein among the schemes. The nature of a cooperativity factor is dependent upon the context of the kinetic scheme of which it is a part. Thus, the cooperativity factors presented in this review are relevant only to the kinetic schemes in which they are found.

The cooperativity between a substrate and a modulator can be unique to that substrate/modulator pair, as well as to the target protein [10]. As a consequence, there is no guarantee that a given modulator will have the desired cooperative effect (much less, any cooperative effect) in the presence of a different substrate or protein, or even if measured against a different function of the same protein [7,11]. An interesting example of the unexpected nature of allosteric modulators is the interaction of the diastereomers of sadopine with dihydropyridine (DHP)-sensitive L-type Ca^{++} channels [12]. At 30°C, (−)-sadopine is a positive allosteric modulator of diltiazem binding to DHP receptors and (+)-sadopine is a negative allosteric modulator of diltiazem binding, while at 2°C, both diastereomers are negative allosteric modulators [12]. Some allosteric modulators have affinity for a variety of protein families, owing to interactions at conserved structures between the protein families. Although this nonselectivity might limit a modulator's attractiveness as a drug candidate, it is possible to take advantage of this promiscuity to leapfrog from one target modulator to another. For example, a library of positive allosteric modulators of $GABA_A$ receptors was screened for positive modulators of α7 nicotinic acetylcholine receptor function [13]. Chemical modification of a hit from the screen resulted in a modulator with selectivity for the α7 nicotinic acetylcholine receptor [13].

Kinetic schemes and rate equations

Kinetic schemes and rate equations are presented for single- and dual-substrate enzyme reactions, and for simple ligand-binding assays and activated receptor assays (Fig. 1 and text body). A detailed discussion of these schemes and equations has been presented elsewhere [4]. Briefly, protein species are represented as circles that may have substrate (S), allosteric modulator (A), or both bound (Fig. 1). In Scheme IV, activated receptor species are represented as shaded circles. Dissociation equilibrium constants are presented for the interaction of substrates and modulators with each protein species. The dissociation equilibrium constants are modified by a cooperativity factor when either a substrate or a modulator interacts with a protein species that already has a ligand bound to it. Negative allosteric modulators have cooperativity factors with values between 0 and 1. Thus, the interaction of a negative allosteric modulator with a protein results in a decrease in the apparent affinity of the substrate for the protein (and vice versa). In contrast, positive allosteric modulators have cooperativity factors greater than 1 and increase the apparent affinity of the substrate for the protein. Neutral allosteric modulators have cooperativity factors equal to 1 and do not alter the apparent affinity of the substrate for the protein. Neutral allosteric modulators might be found by using a known modulator that binds to the same allosteric site and characterized by assays using the substrate. Additionally, neutral allosteric modulators are characterized by the investigations of the microscopic rate constants k_{on} and k_{off} (10). For enzymes, an additional cooperativity factor modifies the rate constant of product formation when a negative allosteric modulator is bound (χ in Scheme I and ε in Scheme II). Small molecule modulators that affect enzymatic rate constants are relatively rare and will not be addressed in this review. Rates of 'product' formation have been indicated in the kinetic schemes for activated receptors (Scheme IV). Because of the complexity of signal transduction mechanisms downstream of cell-surface receptor activation and the different cell-line-dependent contexts in which these mechanisms may exist (e.g. transfected HEK and CHO cell lines), inclusion of these rates in the subsequent rate equations has been omitted. It is important to note that the rate equations that are derived from the kinetic schemes presume Michaelis–Menten conditions for enzymes and equilibrium-binding conditions for cell-surface receptors. Thus, fitting these rate equations to real data from a reaction that may not have achieved steady state or equilibrium could result in erroneous values for the parameters being fit.

Common characteristics among models

There are characteristic behaviors of negative allosteric modulators that are shared among the different protein models investigated in this review. A distinguishing characteristic of negative allosteric modulators is the inhibition limit that may be reached in

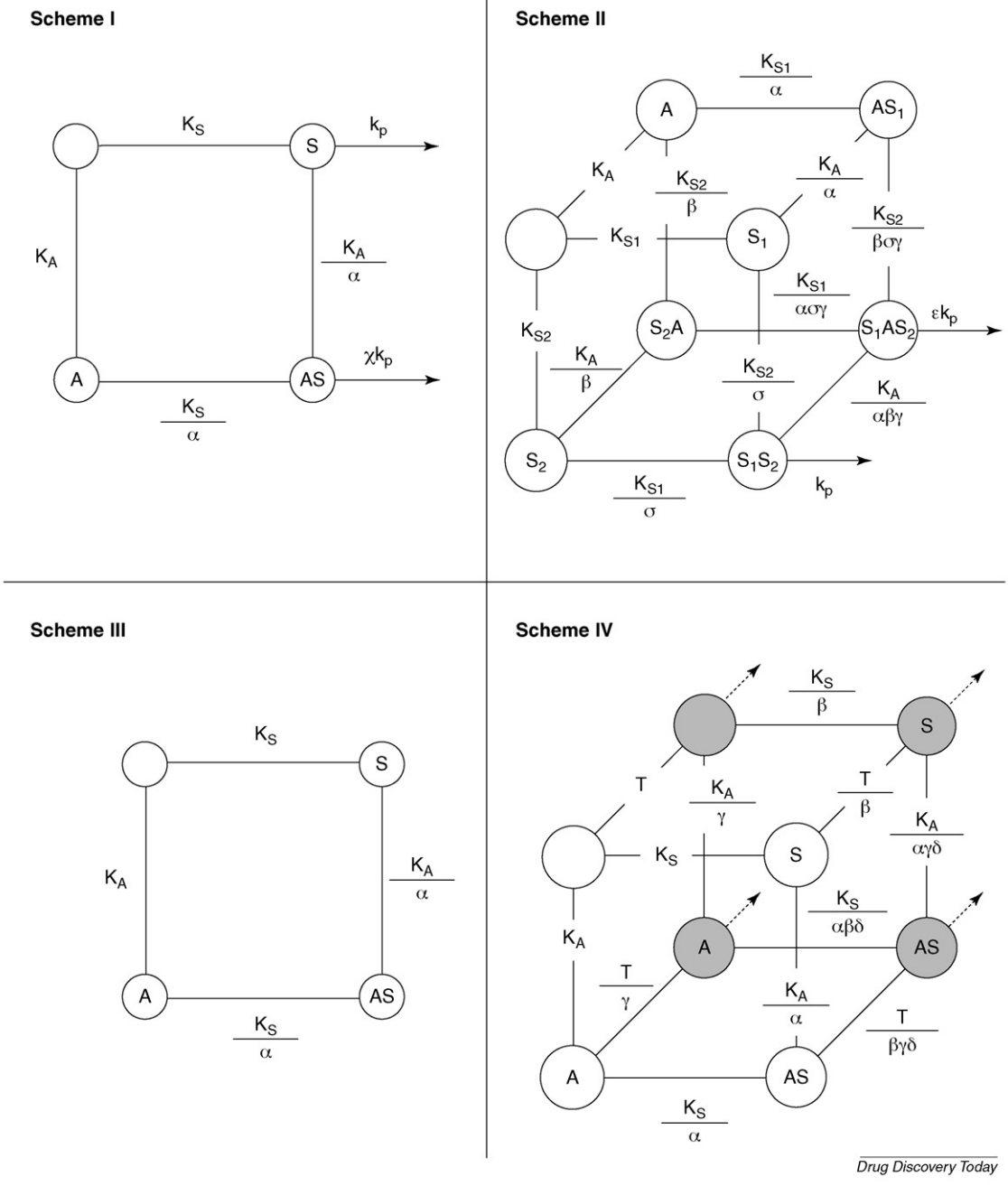

FIGURE 1

Kinetic schemes for the interactions of substrates, agonists and allosteric modulators with enzymes and cell-surface receptors. Dashed lines in Scheme IV indicate downstream receptor activity that is not accounted for in the derived equations (see text).

a dose–response experiment, even as modulator concentration continues to increase (Fig. 2a). This limit depends, in part, on the concentrations of substrate and modulator in the assay, the value of the cooperativity factor of the modulator and the effect of modulator binding on protein structure. Changing the substrate concentration changes the maximal inhibition that can be achieved for any given modulator (Fig. 2b). As might be expected, the choice of substrate concentration influences the discovery of negative allosteric modulators with differing cooperativity values and potencies. Generally, the higher a substrate concentration is in a screen, the less probable it is that a negative allosteric modulator with a weaker potency or with weaker cooperativity will be discovered. Of course, if the substrate concentration is too low, the signal-to-background ratio can be insufficient for a robust screen. The value of the cooperativity factor also contributes to the inhibition limit that is reached in an assay. When the cooperativity value of a negative allosteric modulator approaches zero, assay inhibition approaches 100% (Fig. 2a). The effect of modu-

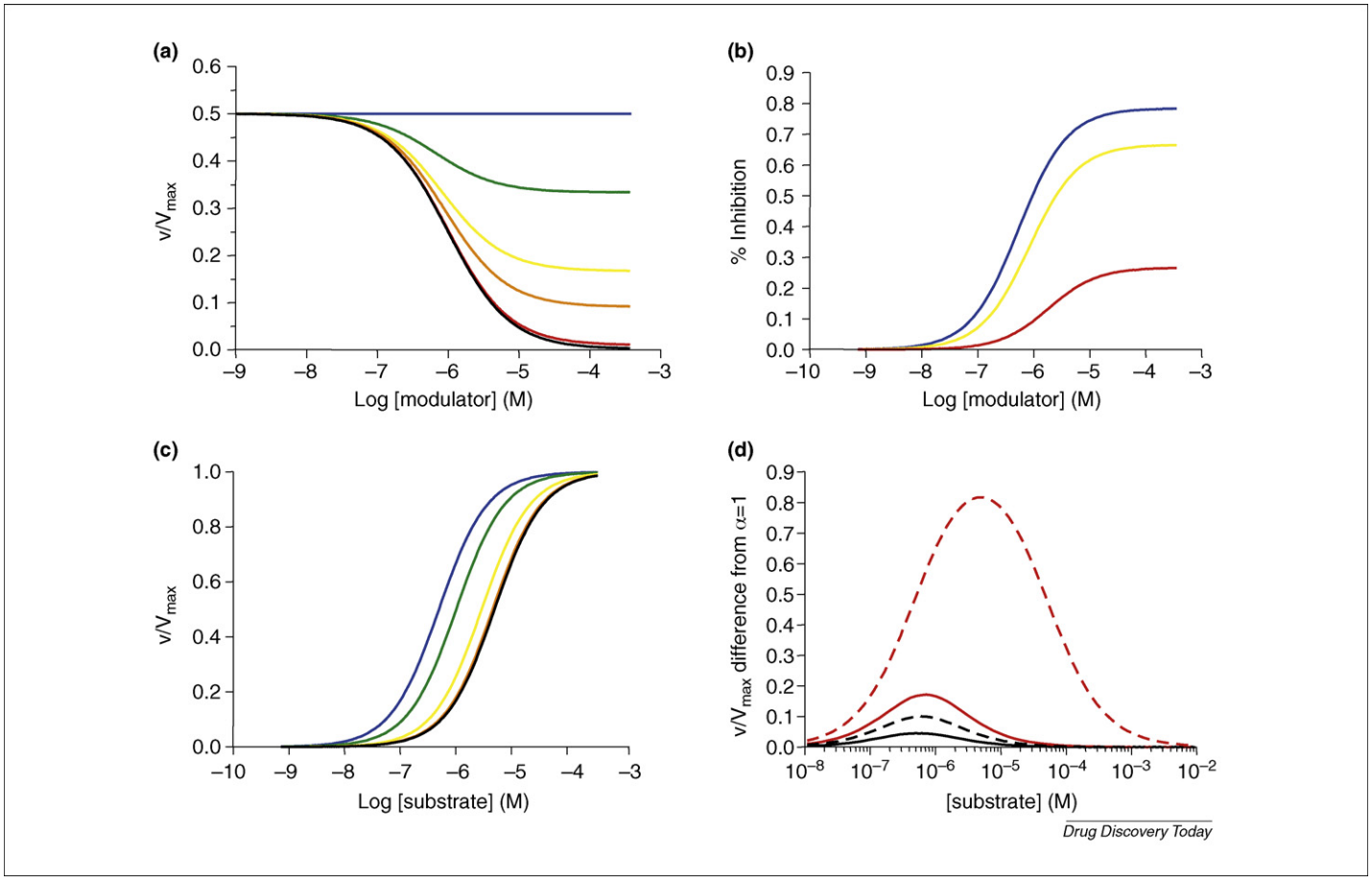

FIGURE 2

Dose–response curves to allosteric modulator for a single-substrate enzyme. Modulator concentrations are as indicated. $\chi = 1$. **(a)** [S] = K_S = K_A = 500 nM. Values of α are 1 (blue), 0.5 (green), 0.2 (yellow), 0.1 (orange), 0.01 (red) and 0.002 (black). **(b)** Value of $\alpha = 0.2$; $K_S = K_A$ = 500 nM. Values of [S] are 50 nM (blue), 500 nM (yellow) and 5 μM (red). **(c)** Dose–response curves to substrate in the presence of a modulator for a single-substrate enzyme. Substrate concentrations are as indicated. $K_S = K_A$ = 500 nM; $\alpha = 0.01$; $\chi = 1$. Values of [A] are 0 (blue), 660 nM (green), 6 μM (yellow), 53 μM (orange), 480 μM (red) and 4.3 mM (black). **(d)** Signal difference plot generated from substrate dose response curves to modulators of differing α values. $K_S = K_A$ = 500 nM. [A] = 500 nM (solid lines), $\alpha = 0.667$ (black), $\alpha = 0.0002$ (red). [A] = 50 μM (dotted lines), $\alpha = 0.667$ (black), $\alpha = 0.0002$ (red).

lator binding on protein structure can also influence the extent of inhibition. For example, imatinib mesylate (Gleevec®), initially characterized as a competitive inhibitor of ATP for the Bcr-Abl tyrosine kinase, actually binds to an allosteric site and results in an inactive conformation of the protein [1,14]. Unlike in the case for non- and uncompetitive inhibitors, the inactive form of the kinase cannot bind ATP and the modulator thus appears to be a competitive inhibitor under some assay conditions. Conversely, it is important to note that some antagonists may appear to behave like negative allosteric modulators under certain assay conditions. For example, a compound may reach its solubility limit before completely inhibiting the assay, thus reaching a limiting inhibition level. In receptor assays, subpopulations of receptors may exist that have little affinity for the antagonist and yet still respond to the substrate.

Modeling the effects of negative allosteric modulators on enzymes

Single-substrate enzyme assays

The kinetic scheme for single-substrate enzyme activity includes the cooperativity factor α that affects the interactions of both substrate and modulator with the enzyme (Scheme I). Eqn (1) is derived from a consideration of Scheme I.

$$\frac{v}{V_{max}} = \frac{([S]/K_S) + (\alpha\chi[A][S]/K_A K_S)}{1 + ([S]/K_S) + ([A]/K_A) + (\alpha[A][S]/K_A K_S)} \quad (1)$$

In dose–response curves to α-type modulators, decreasing the value of α increases the extent of inhibition with little effect on the IC_{50} value (Fig. 2a). The extent of enzyme inhibition reaches a limit and no longer changes, even as the concentration of the modulator continues to increase (Fig. 2a). Under the conditions of the modeling presented here, a cooperativity value of 0.01 results in 100% enzyme inhibition and further reductions in the value of α do not result in further changes in potency (Fig. 2a). The limit of α-type modulator inhibition is also apparent when performing a dose–response curve against the substrate in the presence of the negative allosteric modulator. When the cooperativity value is kept constant and the concentration of the modulator is increased, dose–response curves to the substrate no longer shift rightward but reach a limiting position and overlay each other (Fig. 2c). A similar phenomenon is observed with decreasing values of α at a constant modulator concentration. In response to a true competitive inhi-

bitor, the dose–response curves would continue a rightward shift with increasing inhibitor potency or concentration. In another contrast to uncompetitive and noncompetitive inhibitors, increasing substrate concentration overcomes negative allosteric inhibition of a single-substrate enzyme (Fig. 2b,c).

The implication of substrate concentration in a screen for negative allosteric modulators of a single-substrate enzyme is that if the substrate concentration is either too high or too low, it can be too difficult to detect inhibition (Fig. 2c). It is clear that the higher the concentration of test compound, in the assay, the greater the chance to observe assay inhibition. Because it is impossible to predict the cooperativity values of modulators that might be discovered in a screen, it is only the substrate and compound concentration in the screen (beyond reagent and reaction conditions) that can be optimized for finding modulators. The effect of increasing substrate concentration on enzyme activity in the presence of modulators can be assessed to provide insight into optimal substrate concentrations. The data for substrate dose–response curves generated by compounds with decreasing α values can be subtracted from the curve for α = 1 and plotted against increasing substrate concentration (Fig. 2d). At low modulator concentration (0.5 μM), the optimal substrate concentration needed to discover modulators with α values between 0.667 and 0.0002 is similar and is approximately its K_M. If the modulator concentration is increased 100-fold to 50 μM, then the optimal substrate concentrations needed to discover modulators of different α values differ (Fig. 2d). For modulators with an α value of 0.0002, the optimal substrate concentration would be ten times its K_M. Given that strong modulators provide a relatively large signal difference even when [S] = K_M, assays can be run with substrate at its K_M to discover modulators with the highest variety of cooperativity values (Fig. 2d). Similar analyses can be performed for models of cell-surface receptors although considerations of substrate efficacy and receptor state need to be included. The magnitudes of the signal differences between different conditions depend upon the other parameters specified in the model and do not necessarily reflect what may actually be observed in practice owing to signal strength and variation, and assay conditions and robustness. General guidelines for selecting substrate concentrations for various modulators are provided for both enzymes and receptors (Table 1). It is important to note that in those assays that utilize a fluorescent or radiolabeled substrate at concentrations well below K_M or K_D, it may be difficult to discover any modulators at all.

Dual-substrate enzyme assays

The kinetic scheme for dual-substrate enzymes includes cooperative effects that are not present in the scheme for single-substrate enzymes (Fig. 1, Scheme II). Eqn (2) is derived from a consideration of Scheme II.

$$\frac{v}{V_{max}} = \frac{(\sigma[S_1][S_2]/K_{S1}K_{S2}) + (\alpha\beta\sigma\gamma\varepsilon[A][S_1][S_2]/K_AK_{S1}K_{S2})}{1 + ([S_1]/K_{S1}) + ([S_2]/K_{S2}) + (\sigma[S_1][S_2]/K_{S1}K_{S2}) + ([A]/K_A) + (\alpha[A][S_1]/K_AK_{S1}) + (\beta[A][S_2]/K_AK_{S2}) + (\alpha\beta\sigma\gamma[A][S_1][S_2]/K_AK_{S1}K_{S2})} \quad (2)$$

In Scheme II, modulators can separately influence the interaction of each substrate with the enzyme (α and β) and can also influence the efficacy with which the two substrates promote the enzyme to an active form (γ). Cooperativity between substrates (σ) can occur in dual-substrate enzymes where the binding of one substrate influences the affinity of a second substrate [15]. The cooperativity between the binding of different substrates is referred to as heterotropic cooperativity and can be positive or negative [16]. Allosteric proteins can also have several identical sites that can bind the same substrate, for example hemoglobin, and cooperativity between these sites is called homotropic cooperativity [16]. Because σ is an inherent cooperative property between substrates acting at orthosteric sites, it will not be addressed in this review. The rate equation for the dual-substrate enzyme reaction is formulated on a random Bi-Bi reaction scheme where two substrates must bind to initiate enzyme function but the order of binding is random [17].

The dose–response of a dual-substrate enzyme to changes in the values of α, β, or γ is generally similar to that of the response of a single-substrate enzyme to changes in its cooperativity factor α, namely assay inhibition reaches a limit that can be less than 100% that does not change with increasing modulator concentration. For either α- or β-type modulators the specificity of the modulator for one substrate or the other can be determined by performing the dose–response experiment against the modulator in the presence of saturating concentrations of each substrate in turn. Increases in one substrate's concentration can overcome modulator inhibition of that substrate while increases in the second substrate cannot (Fig. 3a). Increasing the concentration of the second substrate simply increases the signal of the assay, the maximum of which is limited by the concentration (and modulation) of the first substrate. For some screening paradigms, it may be desirable to find antagonists of only a single substrate. Thus, including saturating concentrations of one substrate generally does not compro-

TABLE 1

General guide for optimal substrate concentrations when screening for different types of negative allosteric modulators.

	Enzyme	
	α	***γ***
Single substrate	K_S	NA
Double substrate	K_S	20× K_{Sx} when $[S_1] = [S_2]$ K_{S1} when $K_{S1}=[S_1]\ll[S_2]$

	Receptor		
	α	***γ***	***δ***
Binding	K_S	K_S	K_S
Activity	K_S	10× K_S	10× K_S

For cell-surface receptors, the optimal substrate concentrations were calculated with a substrate efficacy (β) of 100 and $T = 100$ (high proportion of resting state receptors).

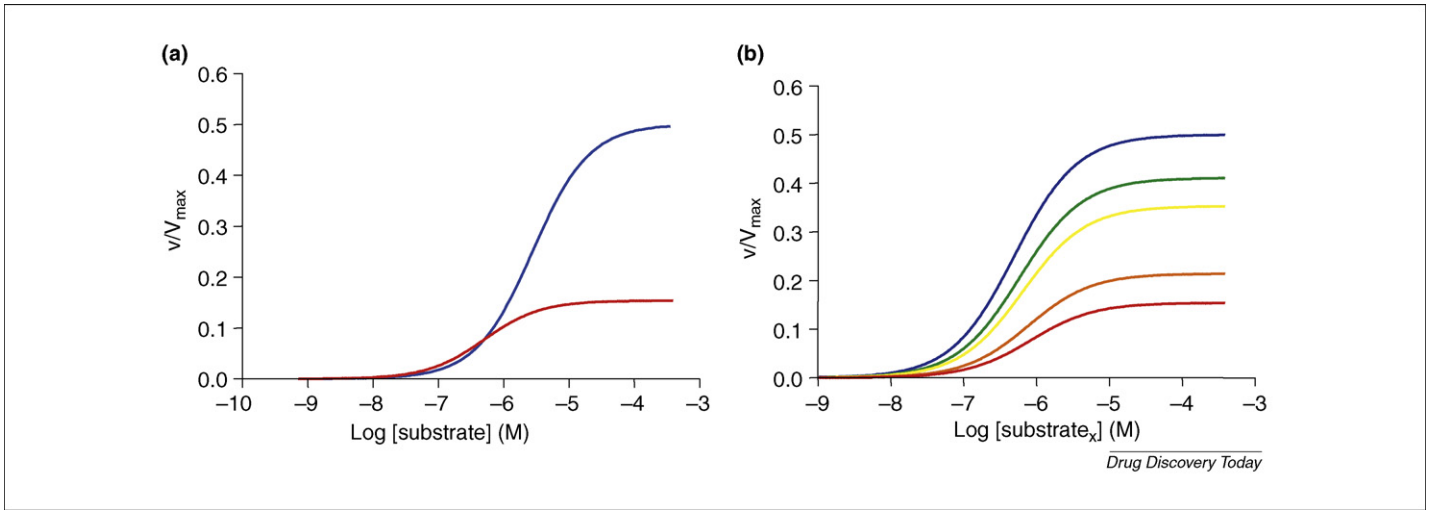

FIGURE 3

Modulator effects on dual-substrate enzyme. **(a)** Effect of increasing substrate concentration on substrate-specific (S_1) inhibition by an α-type modulator. $K_S = K_A = 500$ nM; $\alpha = 0.1$. $\beta = \gamma = \delta = \chi = 1$. Substrate concentrations are as indicated. S_1 (blue), S_2 (red). **(b)** Effect of increasing either substrate concentration (S_1 or S_2) on inhibition by a γ-type modulator. $K_S = K_A = 500$ nM; $\alpha = \beta = \delta = \chi = 1$. Values of γ are 1 (blue), 0.67 (green), 0.5 (yellow), 0.2 (orange) and 0.1 (red).

mise discovery of negative allosteric modulators of the other substrate.

For γ, negative allosteric modulators reduce the ability of the substrates to promote the enzyme to an active state. This is similar to the effect of substituting divalent metals in some enzyme assays where substitution results in suboptimal activity [18]. Unlike that of α or β, increasing the concentration of either substrate cannot overcome enzyme inhibition in the presence of a γ-type modulator because both substrates' ability to promote enzyme activity is affected (Fig. 3b). However, for weaker γ-type modulators, sufficient concentrations of either substrate can boost activity to within the error measurements of total expected enzyme function and could be overlooked in less robust assays.

Modeling the effects of negative allosteric modulators on receptors

Simple ligand-binding assays

The kinetic scheme and resulting inhibition curves for simple receptor ligand-binding assays resemble those of a single-substrate enzyme minus the kinetic rate constants and is known as the ternary complex model (Fig. 1, Scheme III) [19]. Scheme III and Eqn (3) can account for modulator binding to both ion channel and G-protein coupled receptors. Eqn (3) is derived from a consideration of Scheme III where fractional binding (Y/Y_0) replaces enzyme velocity as a measure of function.

$$\frac{Y}{Y_0} = \frac{([S]/K_S) + (\alpha[A][S]/K_A K_S)}{1 + ([S]/K_S) + ([A]/K_A) + (\alpha[A][S]/K_A K_S)} \quad (3)$$

Within the context of the model, Scheme III can account for substrate and modulator binding to any protein in which the functional measurement is ligand binding. As with the case of single-substrate enzymes, decreasing the value of α in a dose–response curve to a modulator increases the extent of inhibition. The extent of receptor inhibition reaches a limit and no longer changes as the concentration of modulator continues to increase. As in enzyme assays, α-type modulators with very low co-operativity values can result in 100% inhibition under certain assay conditions [2,20]. Again, increasing the substrate concentration can overcome the inhibitory effects of a negative modulator.

Activated receptor assays

In assays of cell-surface receptors, theoretical treatment of receptor activity can include the concept of an activated receptor species that promotes initiation of biological function. Consideration of an activated state for receptor function is included in the formulation of the binding and activation scheme for cell-surface receptors, and such activated receptor species are represented as shaded circles in Scheme IV. Scheme IV and Eqns (4a) and (4b) can account for modulator binding to both ion channel and G-protein coupled receptors. Other receptor states can be included, such as desensitized receptor states, but such an addition complicates consideration of the scheme without adding much significance to the overall example.

Scheme IV is referred to as the cubic ternary complex model [21]. In Scheme IV, the transition between a receptor in its resting and activated states is described by the state transition constant T. When the value of T decreases, the population of activated receptors increases. Ligands can have different affinities for activated receptors and those differences are reflected in the factors β and γ that affect substrate and modulator affinities, respectively (Fig. 1; Scheme IV). The apparent value of T can be influenced upon binding of substrate or modulator to the receptor, again reflected by the factors β and γ (Scheme IV). The value of β is a measure of the efficacy of the substrate for receptor activation. Thus, when $\beta > 1$, the substrate is an agonist and when the value of β increases from 1, the population of activated receptors increases. In models of constitutively active receptors (where activated receptor species are predominant), an inverse agonist has β values between 0 and 1. Models of Scheme IV where the value of β is varied are better understood in the absence of modulator, in which case the models are simply ones of receptor agonism. In a manner analogous to

that of dual-substrate enzymes, there is an additional cooperativity factor δ that describes the ability of an allosteric modulator to alter the efficacy of a substrate to activate receptor function.

It is important to describe a possible behavior of some receptor modulators that have implications for drug development efforts. A compound may bind to an allosteric site on a cell-surface receptor and not influence the affinity of a ligand for the active site of that receptor. By binding to the allosteric site, however, that same compound may influence other measurable receptor functions. For example, repertaxin is an allosteric modulator of CXCR1 and CXCR2 chemokine receptors that reduces migration of polymorphonuclear (PMN) cells *in vitro* [22]. Despite its measurable effect on *in vitro* PMN cell migration, repertaxin does not compete with radiolabeled CXCL8 for the receptor in radioligand-binding assays [22]. Such modulators are increasingly being noted (11). Modulators discovered in an *in vitro* migration assay might not progress through secondary development if a radioligand-binding assay is employed for the effort. Thus, it is crucial that the development efforts utilize an assay format that is similar to the primary screen.

Algebraic treatment of Scheme IV results in two equations, one that describes fraction of receptors with bound substrate regardless of activation state, and one that describes the fraction of receptors in an active state regardless of the ligands bound (Eqns (4a) and (4b), respectively) [23]. The fractional receptor form for each equation is defined as Y/Y_0.

$$\frac{Y}{Y_0} = \frac{\begin{array}{c}([S]/K_S) + (\alpha[A][S]/K_AK_S) \\ + (\beta[S]/TK_S) + (\alpha\beta\gamma\delta[A][S]/TK_AK_S)\end{array}}{\begin{array}{l}1 + ([S]/K_S) + ([A]/K_A) + (\alpha[A][S]/K_AK_S) \\ \quad + (1/T) + (\beta[S]/TK_S) + (\gamma[A]/TK_A) \\ \quad + (\alpha\beta\gamma\delta[A][S]/TK_AK_S)\end{array}} \quad (4a)$$

$$\frac{Y}{Y_0} = \frac{(1/T) + (\beta[S]/TK_S) + (\gamma[A]/TK_A) + (\alpha\beta\gamma\delta[A][S]/TK_AK_S)}{\begin{array}{l}1 + ([S]/K_S) + ([A]/K_A) + (\alpha[A][S]/K_AK_S) + (1/T) \\ \quad + (\beta[S]/TK_S) + (\gamma[A]/TK_A) + (\alpha\beta\gamma\delta[A][S]/TK_AK_S)\end{array}} \quad (4b)$$

Increases in the values of α and δ in Eqn (4a) have results qualitatively similar to those when varying α in Eqn (3). At a certain modulator concentration, further increases in concentra-

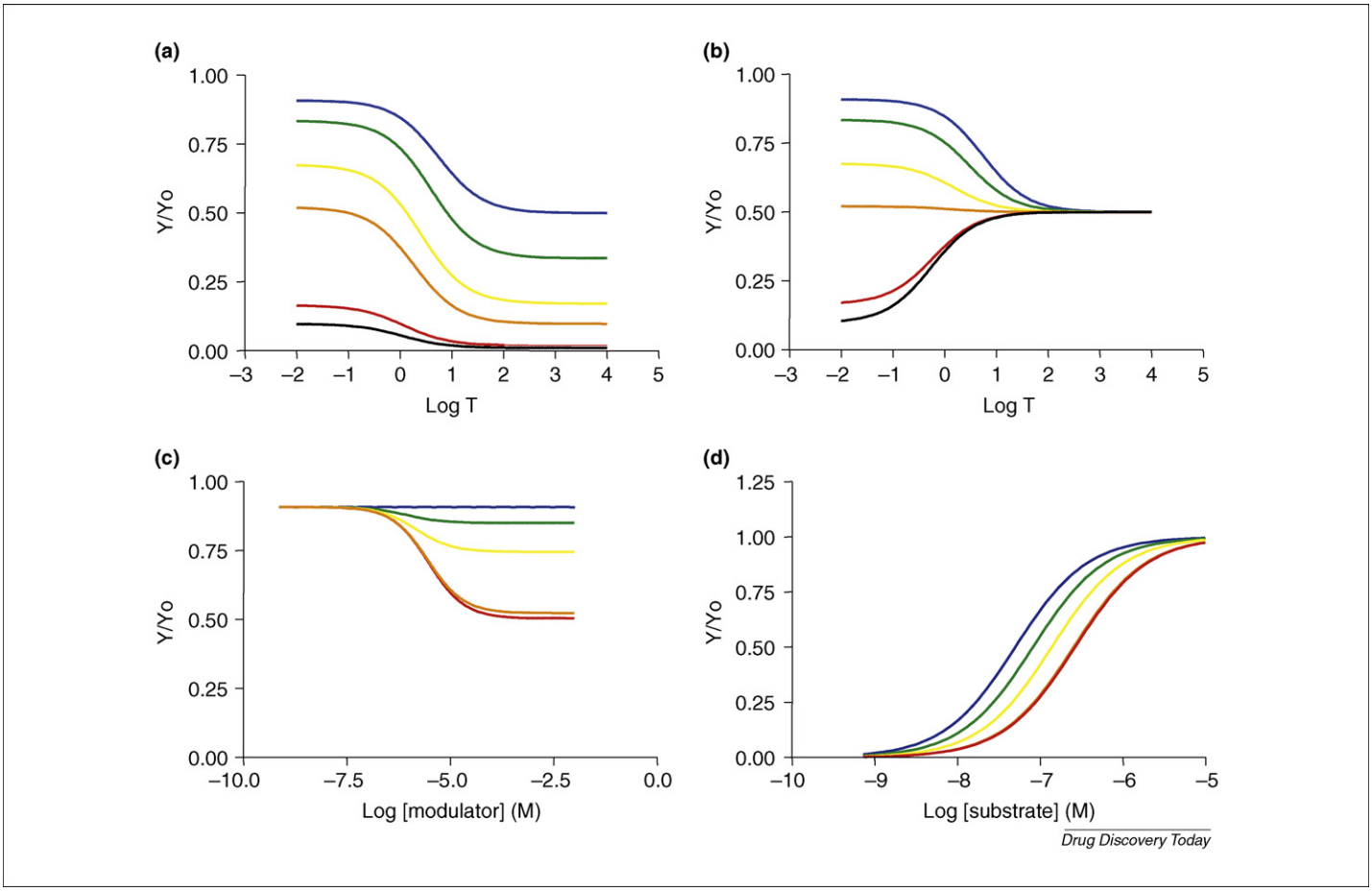

FIGURE 4

Binding assays of activated receptors. **(a)** Effect of *T* on α-type modulation of receptor binding. $K_S = K_A = 500$ nM; [S] = 500 nM; [A] = 500 μM; γ = δ = 1; β = 10. Values of *T* are as indicated. Values of α are 1 (blue), 0.5 (green), 0.2 (yellow), 0.1 (orange), 0.01 (red) and 0.001 (black). **(b)** Effect of *T* on δ-type modulation of receptor binding. $K_S = K_A = 500$ nM; [S] = 500 nM; [A] = 50 μM; α = γ = 1. β = 10. Values of *T* are as indicated. Values of δ are 1 (blue), 0.5 (green), 0.2 (yellow), 0.1 (orange), 0.01 (red) and 0.001 (black). **(c)** Effect of γ-type modulator on receptor binding. $K_S = K_A = 500$ nM; [S] = 500 nM; α = δ = 1. β = 100; *T* = 10. Concentrations of [A] are as indicated. Values of α are 1 (blue), 0.5 (green), 0.2 (yellow), 0.01 (orange) and 0.002 (red). **(d)** Effect of [S] on γ-type modulation of receptor binding. $K_S = K_A = 500$ nM; [A] = 5 μM; α = δ = 1; β = 100; *T* = 10. Concentrations of [S] are as indicated. Values of γ are 1 (blue), 0.5 (green), 0.2 (yellow), 0.01 (orange) and 0.0002 (red).

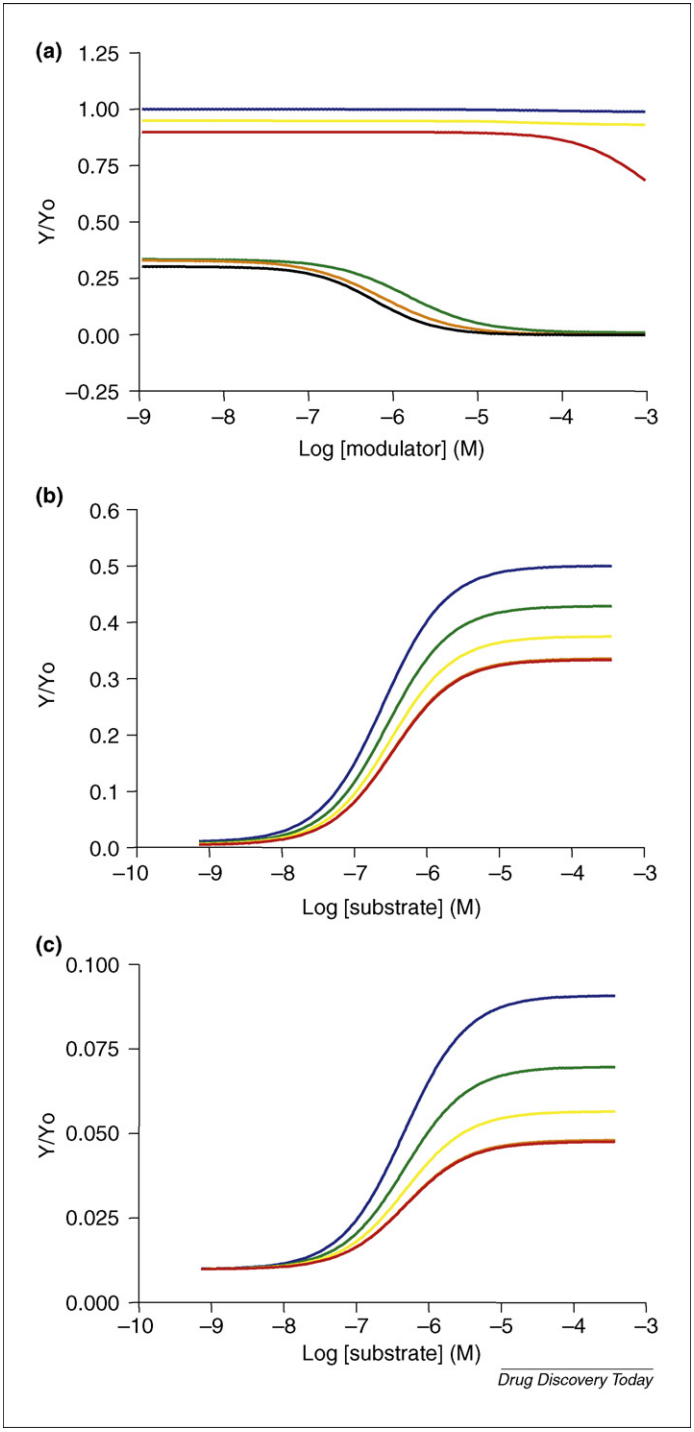

FIGURE 5

Function assays of activated receptors. **(a)** Effect of *T* on the modulation of receptor activity. $K_S = K_A = 500$ nM; [S] = 500 nM. β = 100. Values of [A] are as indicated. Values of *T*, α, δ and γ are α = 0.0002 and *T* = 0.01 (blue), α = 0.0002 and *T* = 100 (green), δ = 0.0002 and *T* = 0.01 (yellow), δ = 0.0002 and *T* = 100 (orange), γ = 0.0002 and *T* = 0.01 (red), γ = 0.002 and *T* = 100 (black). Lines have been offset for clarity. **(b)** Effect of [S] on γ-type modulation of receptor activity. $K_S = K_A = 500$ nM; [A] = 5 nM; Concentrations of S are as indicated. α = δ = 1. *b* = 100. *T* = 100. Values of γ are 1 (blue), 0.5 (green), 0 2 (yellow), 0.01 (orange), 0 0002 (red). **(c)** Effect of [S] on δ-type modulation of receptor activity. $K_S = K_A = 500$ nM; [A] = 5 nM; Concentrations of S are as indicated. α = γ = 1. *b* = 100. *T* = 100. Values of δ are 1 (blue), 0.5 (green), 0 2 (yellow), 0.01 (orange), 0 0002 (red).

tion do not result in further decreases in fractional occupation and the extent of receptor inhibition reaches a limit and no longer changes when the concentration of modulator increases. Additionally, the inhibitory effects of both modulator types can be overcome with higher concentrations of substrate. In Scheme IV, the factor δ describes the influence of a modulator on the efficacy of a substrate to activate the receptor. In binding assays (Eqn (4a)), a δ-type modulator is distinct from an α-type modulator in its dependency on the value of *T*. At low cooperativity values ($\ll 1$), it is possible for an α-type modulator to almost completely inhibit substrate binding regardless of the value of *T* whereas a δ-type modulator can completely inhibit substrate binding only when the value of *T* is less than 1 (Fig. 4a,b). This suggests that the receptor state in an assay (resting or active) will affect which type of modulator will probably be found in a particular screen. In Scheme IV, γ-type negative allosteric modulators behave like inverse agonists, favoring formation of resting receptor states. In models of Eqn (4a), γ-type modulators have much less of an effect on receptor binding than other modulator types and do not generally reach 100% inhibition in a binding assay (Fig. 4c). It is less probable that γ-type modulators will be found in binding assays, compared to activity assays, especially if picking thresholds are to conservative. As might be expected, increasing concentrations of substrate can overcome γ-type inhibition in this assay format (Fig. 4d).

In models of Eqn (4b), the value of *T* plays a much more important role for negative allosteric modulators. The three modulator classes α, γ and δ can inhibit receptor function to 100% only when $T > 1$ (Fig. 5a; Lines have been offset for clarity). Regardless of the value of *T*, the extent of modulator inhibition in dose–response curves can plateau at less than 100% inhibition (depending on the cooperativity value) and remain unchanged with increasing modulator concentration. Additional differences between the modulators emerge when dose–response curves to a substrate is performed. For α-type modulators, increasing the concentration of substrate can overcome inhibition regardless of the value of *T*. For γ- and δ-modulators, increasing the concentration of substrate cannot overcome inhibition when $T > 1$ (Fig. 5b,c). It is probable that γ-modulators may have been discovered for $GABA_A$ and mGluR7 receptors [24,25]. Detailed investigations are required to confirm the mechanism of inhibition and to distinguish between γ- and δ-modulators. For example, γ-type modulators are expected to affect the relative proportion of resting and open state receptors [26].

Conclusion

Allosteric modulators have been discovered for a variety of proteins and in a variety of screening formats, sometimes serendipitously. Several allosteric modulators are either in clinical trials or on the market [26]. The kinetic schemes outlined above demonstrate that, although initial identification of a potential negative allosteric modulator may be straightforward, the characterization of the specific mechanism of the modulation can become quite complex. The simplified schemes described above do not account for more complicated behaviors of proteins (enzyme hysteresis, nonequilibrium conditions and receptor desensitization) or compound artifacts (insolubility) that can contribute to the difficulty in recognizing true modulators. Finally, the quality and conditions

of the screening assay are important factors in the discovery of positive allosteric modulators. Assay noise and/or arbitrary maximum activity cut-offs can preclude allosteric modulators from being picked in a screen. As rich and diverse as allosteric modulator effects are on protein function and despite the advantages that allosteric modulators may provide as therapeutics, the challenges of hit-to-lead investigations that accompany the development of any drug remain.

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