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Screening for positive allosteric modulators of biological targets

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The benefit of using positive allosteric modulators of protein function in the therapy of human diseases is becoming more apparent. The advantage of positive allosteric modulators is that they can possess specificity and selectivity profiles as well as concentration-independent limits on activity that can significantly reduce off-target effects *in vivo*. However, many current screening paradigms are not designed to discover positive allosteric modulators, and modulators that are discovered serendipitously can be overlooked during the hit-picking process. The conditions needed to discover positive allosteric modulators in a HTS are reasonable and simple to implement, generally requiring consideration of the ligand concentration in a screen. Other considerations in the screening for positive allosteric modulators can be derived from the analysis of simple kinetic schemes that describe the interactions of ligands and modulators with different protein targets.

Allosteric modulation of protein function provides a unique approach for the treatment of human disease. The complex behavior of proteins can be regulated by allosteric interactions and targeted for drug-discovery efforts. Allosteric modulators can either increase (positive allostery) or decrease (negative allostery) the effect of a ligand on any given protein function. In addition, some allosteric modulators have no apparent effect on equilibrium ligand interactions with proteins (neutral allostery) and are identified by their effects on the microscopic rate constants k_{on} and k_{off} of the ligand [1,2]. Allosteric modulators of protein function that are marketed currently include cinacalcet, which is used for the treatment of secondary hyperthyroidism in dialysis patients, and members of the benzodiazepine family, which are used to treat anxiety and insomnia [3,4].

The varied effects of allosteric modulation *in vivo* highlight several advantages of using small-molecule allosteric modulators as therapeutic compounds. An increase in protein activity can be magnified by cooperative interaction between a ligand and a positive allosteric modulator. Moreover, unlike a small-molecule agonist, an allosteric modulator can be inactive in the absence of the endogenous agonist and active only when it is appropriate, such as when the agonist is present, thus limiting side-effects. Many proteins are grouped within families where sequence and/or subunit differences are apparent between cellular and tissue locations. However, because of similarities in function between members of a protein family, the localizing regions (e.g. transmembrane regions) and active sites are similar. Consequently, ligands that target the active site of one subtype usually also have affinity for the active sites of other subtypes. However, regions outside of conserved sequences can serve to confer specific, selective, regulatory control over subtype function. Thus, some allosteric modulators are selective for a specific subtype of protein, which confers either tissue- or subtype-specificity and selectivity despite the presence and activity of other members of the protein family [5]. Unlike competitive ligands, allosteric modulators achieve saturation beyond which increases in the concentration of the modulator have no additional effect on function. The allosteric effect is circumscribed by the extent of the cooperativity between the modulator and the endogenous ligand, which adds to the safety profile of a drug with respect to dosing regimes [6]. Finally, although much attention is focused on the ability of allosteric modulators to turn protein function on and off, another important aspect of allostery is the redirection of protein function to different mechanisms. Thus, therapeutic strategies should consider the various functions of a protein that depend on allosteric modulation.

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The role of allostery in protein function is complex, and small-molecule allosteric modulators have several effects that range from agonist activity to competitive antagonism, in addition to their allosteric effects [7,8]. In this review, allosteric modulation is restricted to the consideration of small-molecule compounds that increase protein activity in *in vitro* assays of protein function (positive allosteric modulators) and do not display other effects (e.g. agonism). For the purposes of this review, small-molecule, positive allosteric modulators, by their definition, bind to a site on a protein that is discrete from the substrate (or agonist) binding site and, by doing so, modify the interactions of the substrate (or agonist) with that binding site. Modulation is characterized as 'cooperativity' and equally describes the effect of a bound ligand on the interaction of an allosteric modulator with the allosteric site. Detailed investigations of the effects of modulators on protein function after the HTS stage are needed to prove positive allosteric mechanisms. In this review, it is presumed that such rigorous investigations are beyond the responsibility of most primary drug-screening groups (the goals of which include quality and speed) and, thus, are not presented. Several reviews present experimental formats that can be adopted to incorporate detailed characterizations of positive allostery into HTS formats [5,9,10].

For simplicity, a ligand that binds to an allosteric site is referred to as an allosteric modulator (A), and a ligand that binds to either a substrate-binding site or an agonist-binding site and initiates a function is referred to as a substrate (S) – even when simple binding (e.g. radioligand binding) is the only function measured. In the kinetic analyses of allosteric effects on protein function that are given below, equilibrium constants are presented as dissociation equilibrium constants. By definition, a cooperativity factor of >1 indicates positive cooperativity. Although not addressed in this review, negative allosteric modulators have cooperativity factors between 0 and 1, and neutral allosteric modulators have cooperativity factors equal to 1. The cooperativity factors presented in this review are relevant only to the kinetic schemes in which they are found. Although the symbol fonts assigned to cooperativity factors might be identical in different kinetic schemes, they might represent dramatically different effects on the interactions of ligands with a protein. The nature of a cooperativity factor depends on the context of the kinetic scheme of which it is a part.

It is important to note that the cooperativity between a ligand and a modulator can be unique to the ligand–modulator pair as well as to the target protein [2]. There is no guarantee that a given modulator will have the desired cooperative effect (or any cooperative effect) in the presence of either a different ligand or protein, or when measured against a different function of the same protein [5,11]. Therefore, it is advantageous to use a ligand that closely mimics the endogenous ligand for the protein in a HTS. Some allosteric modulators have affinity for several proteins. For example, amiloride (a diuretic drug) is an allosteric modulator with affinity for several cation-binding proteins [12], but analogs of amiloride are selective for members within a particular receptor family [13,14].

In a primary screen of protein function in the presence of a substrate, agonist or inverse agonist, positive allosteric modulators can change the observed signal of the assay. Thus, primary screens are relatively straightforward, provided that screens for positive allosteric modulators of protein function include either a substrate or an agonist (in this review, a positive allosteric modulator of receptor function is inactive in the absence of an agonist). Of importance is the relative concentration of substrates (for enzymes) and agonists (for receptors) because of the concentration-dependent effects of these reagents on the behavior of positive allosteric modulators in the screen. These effects are defined, in part, by the kinetic schemes that are developed for the interactions of ligands with proteins and are described by the equations that are derived from these kinetic schemes.

Further characterization of positive allosteric modulators after the primary screen include, at minimum, dose–response investigations in which the concentration of the modulator is varied under conditions that are identical to the primary screen. From these experiments, the ability of the positive allosteric modulator to affect receptor function can be ascertained, usually designated as EC_{50}. When no modulation of protein function is apparent, the dose–response curve is flat.

Enzymes

Two classes of enzyme that are screened routinely in drug discovery programs are single-substrate enzymes (such as proteases) and dual-substrate enzymes (such as kinases). The detailed reaction scheme for any particular enzyme can be complex when considering the regulatory aspects of the interactions between subunits, cofactors, coenzymes, allosteric modulators (or effectors) and substrates, in addition to aspects of enzyme structure (primary, secondary and tertiary) that influence binding affinities, catalytic mechanisms and rates. Thus, simple kinetic schemes are used to describe the modulation of enzyme activities, with the caveat that the enzyme systems adhere to the fundamental assumptions of a Michaelis–Menten format [15,16]. Although these simple schemes are, at best, approximations of the true kinetic mechanisms, they serve to demonstrate the effects of allosteric modulators on protein function [17,18].

Single-substrate enzyme assays

Scheme I describes the reaction of an enzyme with one substrate-binding site and one positive-allosteric-modulator-binding site where the circles represent the enzyme species (Figure 1). Note that because of schematic similarities, Schemes I and III are combined into a single scheme (Figure 1). In discussions of Scheme III (below), disregard the dashed arrows. Equilibrium is established between enzyme species, and the interaction of substrate with an unliganded enzyme is defined by K_S. The interaction of the substrate with modulator-bound enzyme is altered by the cooperativity factor α, and is defined by K_S/α. Thus, an increase in the value of α from unity (positive cooperativity) increases the affinity of the enzyme for the substrate because the overall value of K_S/α decreases. Similarly, the interaction of a positive allosteric modulator with an unliganded enzyme is defined by K_A. Because of the unique binding interactions between an enzyme and its ligands, the value of α is unique for each substrate–modulator pair for an enzyme. Thus, the interaction of a positive allosteric modulator with the substrate-bound enzyme is altered by the same cooperativity factor α and is defined by K_A/α. In the reaction scheme, each substrate-bound enzyme is catalytically active and leads to product at a specific rate (dashed arrows). In addition to affecting the affinity of the substrate for the enzyme (K-type modulation), the modulator might also affect the catalytic rate of the enzyme (V-type modulation). Thus, the catalytic rate for the

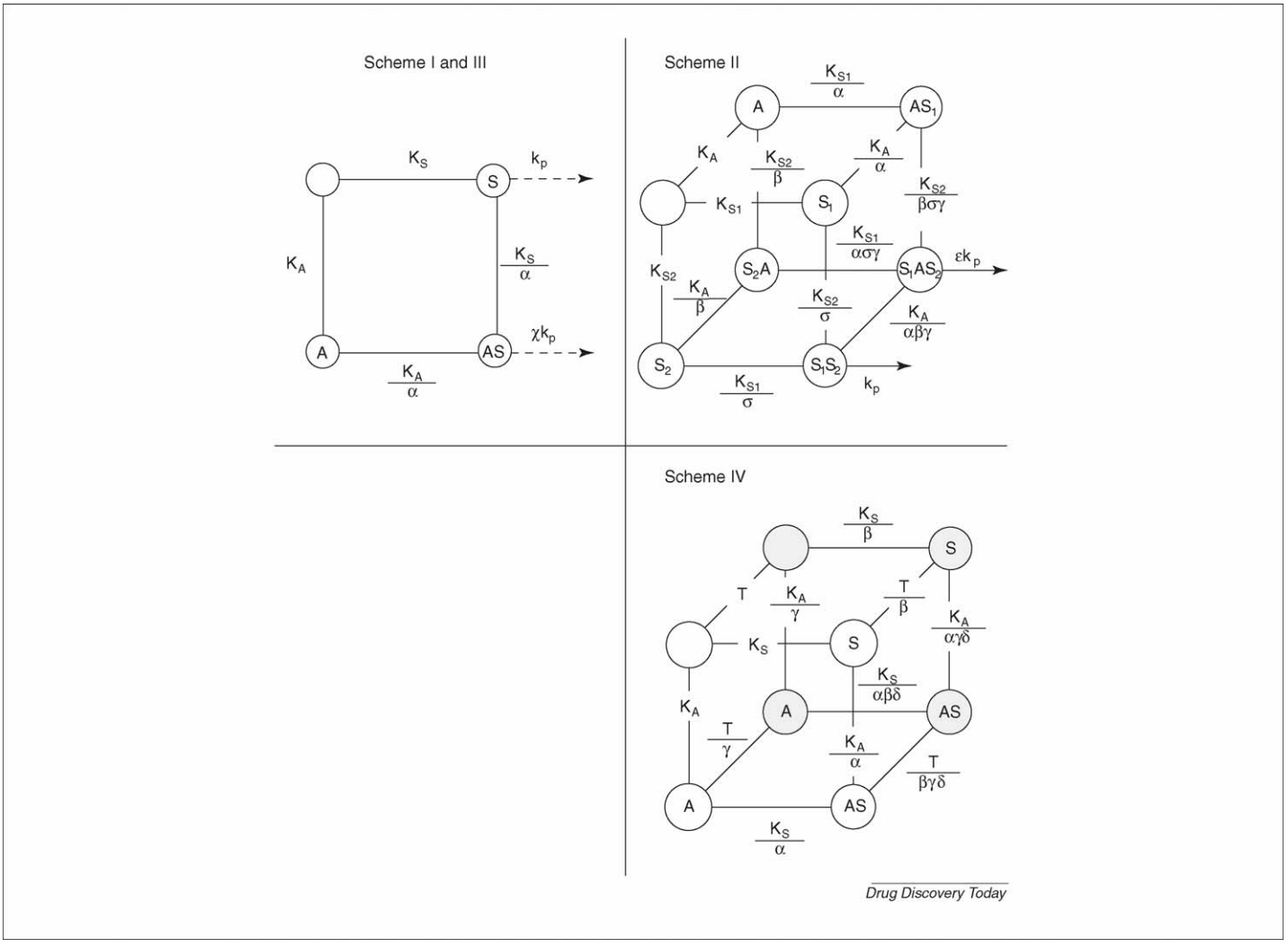

FIGURE 1

Kinetic schemes defining the interactions of substrates, agonists and allosteric modulators with enzymes and cell-surface receptors.

doubly liganded enzyme includes a cooperativity factor χ to describe the possible effect of the modulator on the catalytic rate of the enzyme [19]. Small-molecule V-type modulators of enzymes are relatively rare compared with K-type modulators, and so effects on catalytic rates are not considered here (χ is set to unity in all enzyme models). However, modulation of enzyme activity can include simultaneous effects on K_m and V_{max} (mixed-type modulators).

Algebraic treatment of Scheme I results in a velocity equation that includes the potential influence of a modulator on the activity of a single-substrate enzyme (Equation 1).

$$\frac{v}{V_{max}} = \frac{\frac{[S]}{K_S} + \frac{\alpha\chi[A][S]}{K_A K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{\alpha[A][S]}{K_A K_S}} \qquad \text{[Eqn 1]}$$

Several observations of this equation can be made based on the values of α, χ and [A]:

- When [A] = 0, the equation reduces to the Michaelis–Menten format.
- When α approaches 0, the equation reduces to a competitive inhibition format.
- When $\alpha = 1$ and χ approaches 0, the equation reduces to a non-competitive-inhibition format.
- When χ approaches 0 and either K_A or α (or both) are $\gg 1$, the equation reduces to an uncompetitive inhibition format (where $K_I = K_A/\alpha$).

Although Scheme I represents an approximation of the true mechanism of enzyme activity, these observations point out the general applicability of the model to the analysis of compound effects on protein function.

Dual-substrate enzyme assays

The interaction of a single allosteric modulator with an enzyme that contains two substrate-binding sites (such as a kinase) can be described by Scheme II (Figure 1). In this situation, it is presumed that the enzyme binds two substrates (S_1 and S_2) in random order, either with or without the allosteric modulator bound. Binding of both substrates is necessary to initiate enzyme function. With kinases it is common for the binding of the first substrate to have an allosteric effect (σ) on the binding of the second substrate, and the bound modulator can have allosteric effects (α and β) on the binding of each unique substrate. Because σ is an inherent, coop-

erative property between the two substrates in Scheme II, it is unaffected by the interaction of an allosteric modulator and is not addressed in this review. In addition to the cooperativity factors (σ, α and β) that influence the binding of ligands to the enzyme, an additional cooperativity factor γ describes the ability of the allosteric modulator to facilitate the efficacy by which the two bound substrates promote the enzyme toward a catalytic state. This is akin to the interaction of an essential cofactor such as Mg^{2+} with an enzyme that is already bound to two substrates [19]. Active enzyme complexes consist of species that have two substrate molecules bound. As with the single-substrate enzyme described in Scheme I (Figure 1), the bound allosteric modulator can also affect the catalytic rate of the enzyme (εk_p).

Algebraic treatment of Scheme II results in a velocity equation that includes the potential influence of a modulator on the activity of a dual-substrate enzyme (Equation 2).

$$\frac{v}{V_{max}} = \frac{\dfrac{\sigma[S_1][S_2]}{K_{S1}K_{S2}} + \dfrac{\alpha\beta\sigma\gamma[A][S_1][S_2]}{K_A K_{S1} K_{S2}}}{1 + \dfrac{[S_1]}{K_{S1}} + \dfrac{[S_2]}{K_{S2}} + \dfrac{\sigma[S_1][S_2]}{K_{S1}K_{S2}} + \dfrac{[A]}{K_A} + \dfrac{\alpha[A][S_1]}{K_A K_{S1}} + \dfrac{\beta[A][S_2]}{K_A K_{S2}} + \dfrac{\alpha\beta\sigma\gamma[A][S_1][S_2]}{K_A K_{S1} K_{S2}}} \quad \text{[Eqn 2]}$$

When [A] = 0, the rate equation reduces to that of a random Bi–Bi system, where two substrates must bind to initiate function but the order of binding is random [16]. When σ = 1 (no apparent cooperativity between S_1 and S_2) and the concentration of one of the substrates is saturating (e.g. $[S_1] \gg K_{S1}$) the equation reduces to that of Equation 1. It is evident from Scheme II that consideration of more complex (albeit reasonable) reaction schemes complicates the rate equations that are derived from them. For example, addition of a second allosteric binding site expands the scheme, bringing additional equilibrium constants, cooperativity factors and rate constants into the rate equation. Because such an addition complicates consideration of the scheme without adding much significance to the overall example, it is disregarded for this discussion.

The concentration of substrate in the primary screen affects the ability to discover modulators with different cooperativities. These conditions are discussed later. In addition, to reduce the number of false positives it is important that the screen includes saturating concentrations of all essential cofactors with which the therapeutic paradigm is not concerned (e.g. analogs and mimics of the essential cofactors).

Modeling the effects of allosteric modulators on enzymes

An interesting observation, which is common to all models investigated, comes from the dose–response curves of a single-substrate enzyme bound to a positive allosteric modulator. At sufficiently high concentrations of positive allosteric modulator in a dose–response assay, the enzyme velocity reaches a limiting value that depends on the extent of the cooperativity (i.e. at any given value of α, an increase in [A] results in no further increases in enzyme velocity) (Figure 2). This indicates saturation of the allosteric site by the modulator. As the value of the cooperativity factor continues to increase (positive cooperativity), the enzyme velocity approaches, but does not exceed, the expected maximum velocity of the assay (e.g. V_{max} for Equation 1, but the concentration of the positive allosteric modulator that is required to achieve this maximum continues to decrease (Figure 2). The EC_{50} for any given α-type modulator and substrate concentration can be determined from a dose–response curve and represents the concentration of positive allosteric modulator that is needed to increase the enzyme velocity to half the observed maximum.

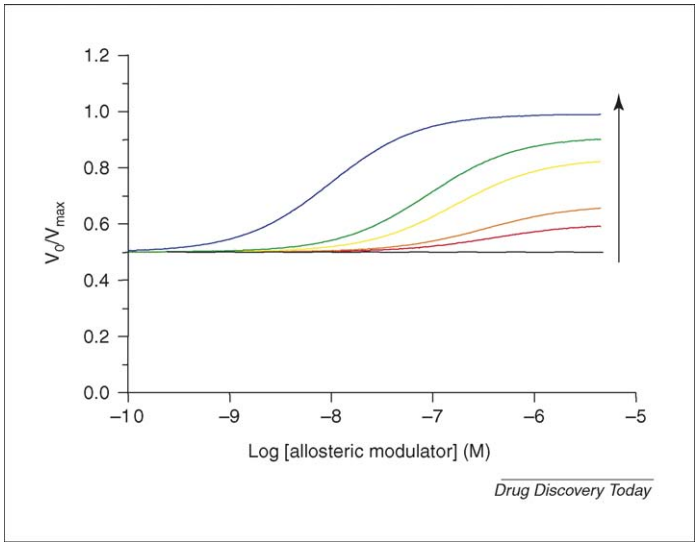

FIGURE 2

Dose–response curves to allosteric modulator for a single-substrate enzyme. The arrow indicates the direction of change. $[S] = K_S = K_A = 5 \times 10^{-7}$ M; χ = 1. Values of α are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue).

A dual-substrate enzyme screen requires dose–response curves that include saturating concentrations of each substrate, in turn, to determine the specificity of a modulator for the appropriate substrate (this is difficult with assays that utilize radioactive ATP). For example, other than increasing basal enzyme activity, saturating concentrations of S_2 in a dose–response assay has no qualitative effect on an allosteric modulator of S_1 because a positive modulator of S_1 increases the maximum possible velocity of the enzyme in the assay to V_{max} in the presence of saturating concentrations of S_2 (Figure 3a,b). However, increasing the concentration of S_1 in the presence of the modulator increases the basal enzymatic rate but not the maximum possible velocity because the concentration of S_2 is limiting and is unaffected by the allosteric modulator (Figure 3c). Thus, the effect of a modulator on enzyme velocity becomes more difficult to detect as the concentration of S_1 increases because the basal velocity approaches the maximum possible velocity for the assay conditions (Figure 3a,c).

In the case of kinases, there is an additional cooperativity factor γ (Scheme II, Figure 1). At relatively low values of γ, there is no qualitative difference in the effect on enzyme velocity compared with the effects of an α-type modulator. However, at higher values of γ enzyme velocity exceeds the expected maximum possible velocity (e.g. $v_0/V_{max} = 0.5$ when each $[S] = K_S$) and approaches V_{max} (Figure 3d). As with α-type modulators, an increase in γ reduces the concentration of the positive allosteric modulator that is needed to achieve the maximum velocity (Figure 3d).

As described above, the initial identification of a compound as a possible allosteric modulator of enzyme activity relies partly on the characteristic limiting effect of high concentrations of the compound on velocity. A compound with high cooperativity is

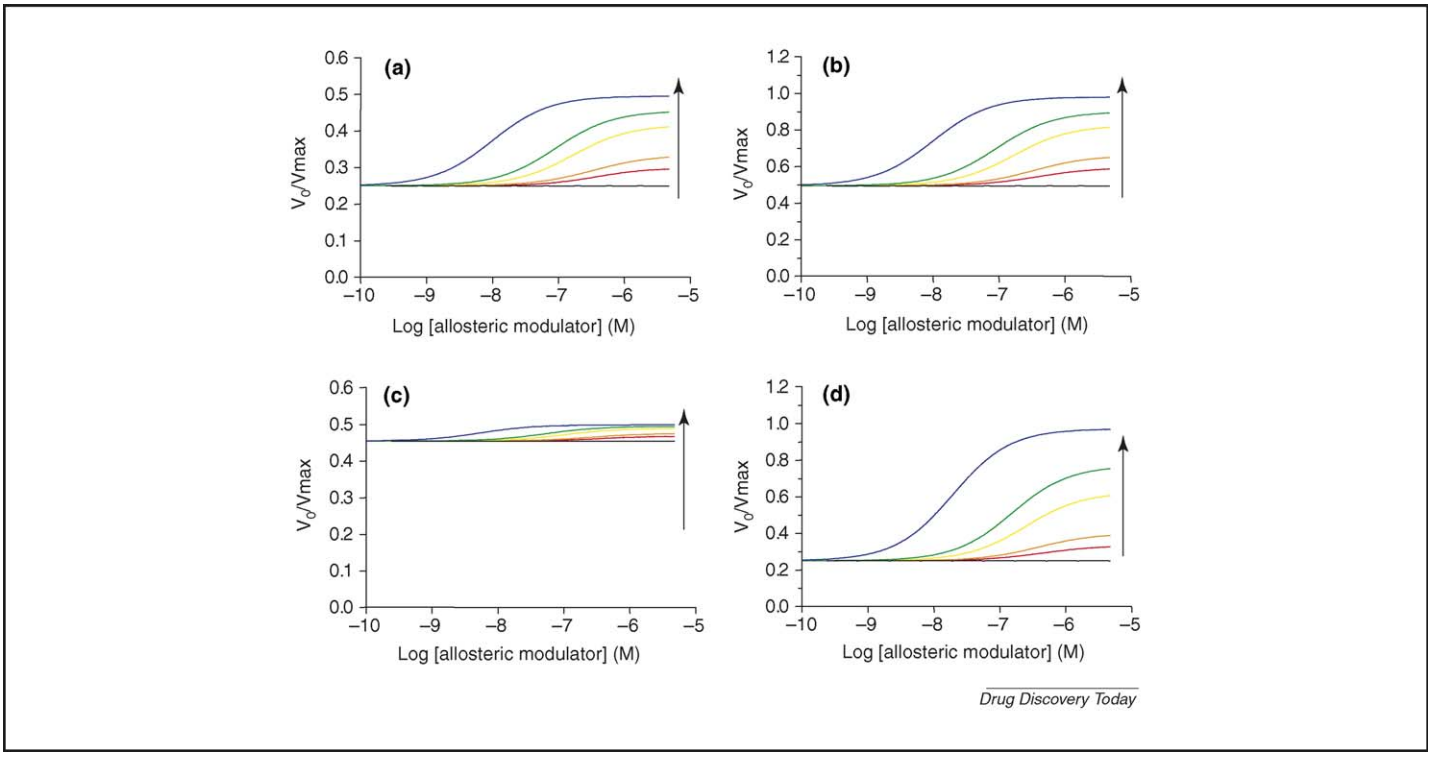

FIGURE 3

Dose–response curves to allosteric modulator for a dual-substrate enzyme. The arrow indicates the direction of change. Values of α are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue); β, 1; σ, 1. **(a)** Equimolar concentrations of S_1 and S_2. $[S_1] = [S_2] = K_S = K_A = 5 \times 10^{-7}$ M; γ = 1. **(b)** Saturating concentration of S_2. $[S_2] = 5 \times 10^{-5}$ M; $[S_1] = K_S = K_A = 5 \times 10^{-7}$ M; γ = 1. **(c)** Ten-fold excess of S_1. $[S_1] = 5 \times 10^{-6}$ M; $[S_2] = K_S = K_A = 5 \times 10^{-7}$ M; γ = 1. **(d)** Values of γ are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). $[S_1] = [S_2] = K_S = K_A = 5 \times 10^{-7}$ M; α = 1.

identified readily from a dose–response analysis, especially at low substrate concentrations where such allosteric modulators can cause large changes in enzyme velocity (Figure 3a). However, the HTS selection criteria (maximum velocity cut-off), the robustness of the assay format (Z-factor), the sensitivity of the detection method, and non-specific effects of the compound can combine to frustrate discovery of compounds with low cooperativity values. Although it is impossible to predict the range of cooperativity values of hits, the substrate concentration can be selected to impose a lower threshold on the cooperativity values of compounds selected in a screen. A concentration of $[S] \approx K_S$ is optimal for the discovery of compounds with lower cooperativity. When tested at $[S] \approx K_S$, compounds with low cooperativity display a maximum difference from the controls. This observation is more apparent in a dose–response investigation of an enzyme substrate (Figure 4a). As the concentration of the substrate decreases in a screen, the ability to detect differences in the effect of a modulator decreases for compounds with low cooperativities and increases for compounds with higher cooperativities (Figure 4b).

Cell-surface receptors

Simple ligand-binding assays

Screening assays for modulators of cell-surface receptors encompass simple binding formats (radioligand binding) and whole-cell functional formats (Ca^{2+} influx), where the effect of substrate binding to a single site is measured. In simple binding formats, a scheme that includes the interactions of both radioligand and allosteric modulator is referred to as the ternary complex model (Scheme III, Figure 1) [20]. In the consideration of Scheme III, disregard the presence of the dashed arrows. Algebraic treatment of Scheme III results in a binding equation that includes the influence of a modulator on substrate binding where fractional binding (Y/Y_o) is determined (Equation 3).

$$\frac{Y}{Y_0} = \frac{\frac{[S]}{K_S} + \frac{\alpha[A][S]}{K_A K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{\alpha[A][S]}{K_A K_S}} \qquad \text{[Eqn 3]}$$

This particular formulation of Equation 3 is presented so that it is identical to that of Equation 1 (absent a catalytic rate). Indeed, the formal methodology for deriving rate and binding equations in enzyme kinetics and receptor pharmacology, respectively, are equivalent [21].

Two observations of Equation 3 can be made based on consideration of the values of α and [A]:

- When [A] = 0, the equation reduces to a simple binding format.
- When α = 0, the equation reduces to a competitive inhibition format.

Activated receptor assays

In assays of cell-surface receptors, the theoretical treatment of receptor activity includes the concept of an activated receptor species that promotes the initiation of biological function. Consideration of this activated state for receptor function can be included in a formulation of the binding and activation scheme for cell-surface receptors, and such activated receptor species are

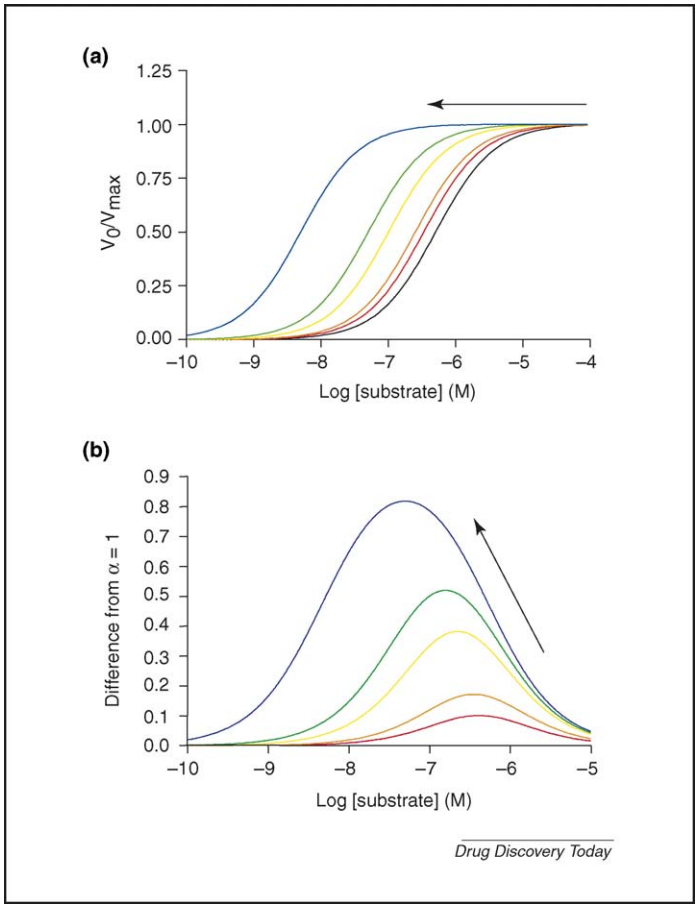

FIGURE 4

Dose–response curves to substrate for a single-substrate enzyme. The arrow indicates the direction of change. **(a)** Effect of α on substrate dose–response curves to a single-substrate enzyme. $\chi = 1$. Values of α are 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). **(b)** Difference between substrate dose–response curves as the value of α increases from unity. The curve for $\alpha = 1$ is subtracted from subsequent curves where $\alpha > 1$ across substrate concentration. These differences are plotted as a function of the substrate concentration. Difference between $\alpha = 1.5$ (red), 2 (orange), 5 (yellow), 10 (green),100 (blue) and unity.

represented as shaded circles in Scheme IV (Figure 1). This scheme is referred to as the cubic ternary complex model [22]. In Scheme IV, the transition between a receptor in its resting and activated states is described by the state transition constant *T*. Ligands can have different affinities for activated receptor species and these differences are reflected in the factors β and γ, which affect substrate and modulator affinities, respectively (Scheme IV). Although *T* is concentration-independent, its apparent value can be influenced by binding of either substrate or modulator to the receptor in its resting state, which, again, is reflected by the factors β and γ, respectively (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. In models of constitutively active receptors (where activated receptor species are predominant) an inverse agonist has a β value between 0 and 1. Models of Scheme IV in which the value of β varies are better understood in the absence of modulator, in which case the models are of receptor agonism. Models of Scheme IV in which the value of γ varies address circumstances in which the modulator has agonist activity and are not addressed here. The discovery of allosteric agonists in a screen is common and can complicate studies of receptor allosterism. Theoretical models of allosteric agonism are addressed elsewhere [23]. As with dual-substrate enzymes there is an additional cooperativity factor, δ, which describes the ability of an allosteric modulator to alter the efficacy of a substrate for activation of a receptor.

Unlike the enzyme models presented above, Scheme IV lacks catalytic rates that describe the rates of 'product' formation from activated receptor species. Because of the complexity of the signal-transduction mechanisms that are downstream of cell-surface-receptor activation and the different cell-line-dependent contexts in which these transduction mechanisms might exist (e.g. transfected HEK and CHO cell lines), catalytic rates are not included in the scheme. However, such complexities point out an interesting phenomenon and raise an instructive observation in screening for allosteric modulators of receptor function. Modulators discovered in a whole-cell, functional-receptor screen might not be active in a secondary radiolabel-binding format [11]. This is because the modulator might affect one receptor function (e.g. phosphoinositide hydrolysis) but not a different receptor function (e.g. ligand binding). Thus, the primary screening format must be relevant to the therapeutic paradigm. Hit-to-lead efforts, therefore, must employ a screening format that is similar to the primary screen. Development efforts on promising allosteric modulators can be abandoned because hit-to-lead groups prefer alternative secondary screening formats.

It is important to note that, unlike radioligand-binding assays, whole-cell functional assays are not necessarily performed under equilibrium conditions. Equilibration of interacting species is assumed in the construction of the kinetic schemes and in the derivation of the associated equations. Therefore, application of the derived equations (which presume the establishment of equilibrium) to data from whole-cell functional experiments (which might not be in equilibrium) can result in significant quantitative differences between the calculated value and the true value of a cooperative factor.

Algebraic treatment of Scheme IV results in two equations, one that describes fraction of receptors with bound radioligand and one that describes the fraction of receptors in an active state. The fractional receptor form for each equation is defined as Y/Y_o [23]. The equation for fractional binding includes, in the numerator, only receptor species that have bound radioligand, regardless of activation state (Equation 4a).

$$\frac{Y}{Y_0} = \frac{\frac{[S]}{K_S} + \frac{\alpha[A][S]}{K_A K_S} + \frac{\beta[S]}{TK_S} + \frac{\alpha\beta\gamma\delta[A][S]}{TK_A K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{\alpha[A][S]}{K_A K_S} + \frac{1}{T} + \frac{\beta[S]}{TK_S} + \frac{\gamma[A]}{TK_A} + \frac{\alpha\beta\gamma\delta[A][S]}{TK_A K_S}} \quad \text{[Eqn 4a]}$$

In the absence of a transition of the receptor to an activated state (i.e. T approaches ∞), Equation 4a reduces to Equation 3. The equation for the fraction of receptors in an active state includes, in the numerator, only activated receptor species, regardless of ligands bound (Equation 4b).

$$\frac{Y}{Y_0} = \frac{\frac{1}{T} + \frac{\beta[S]}{TK_S} + \frac{\gamma[A]}{TK_A} + \frac{\alpha\beta\gamma\delta[A][S]}{TK_A K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{\alpha[A][S]}{K_A K_S} + \frac{1}{T} + \frac{\beta[S]}{TK_S} + \frac{\gamma[A]}{TK_A} + \frac{\alpha\beta\gamma\delta[A][S]}{TK_A K_S}} \quad \text{[Eqn 4b]}$$

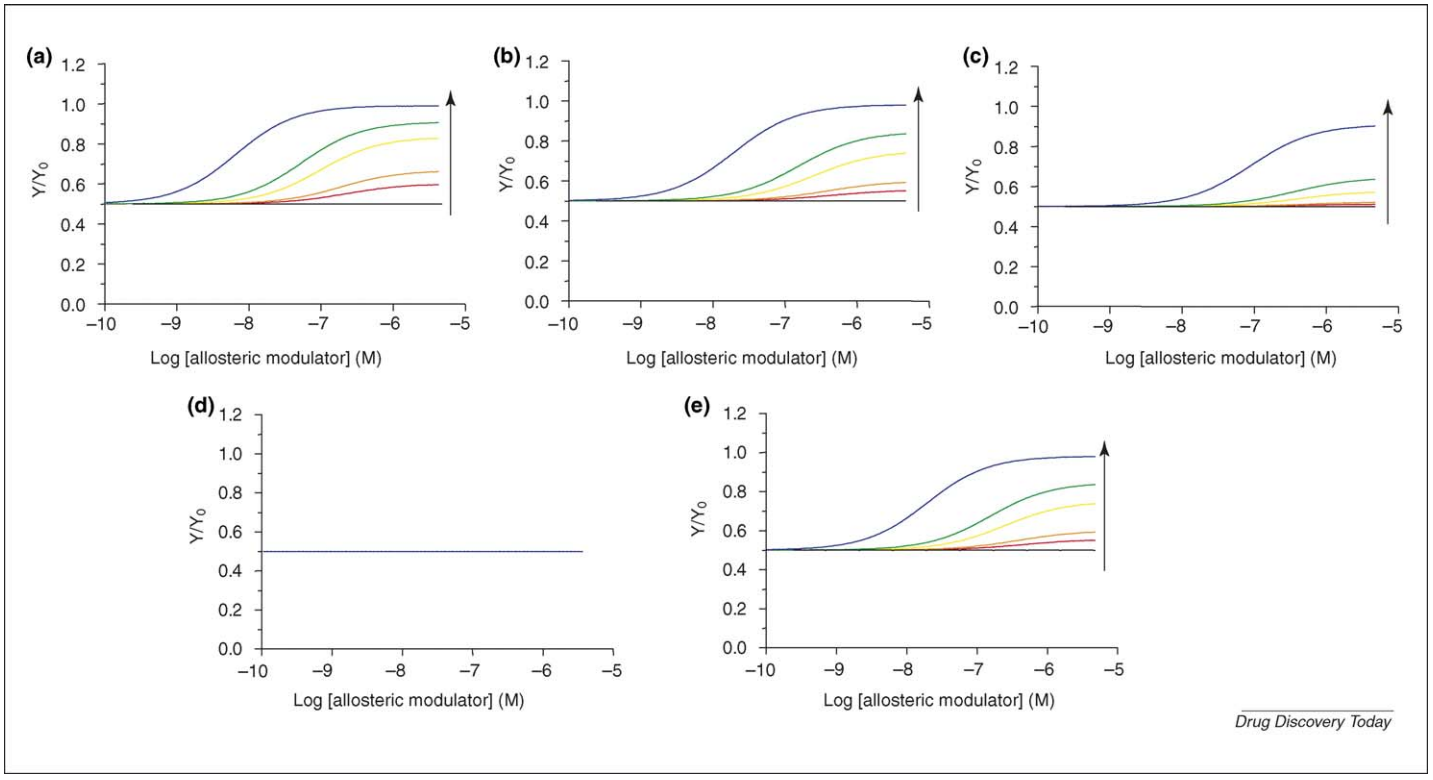

FIGURE 5

Dose–response curves to allosteric modulator of a G-protein-coupled receptor (GPCR). The arrow indicates the direction of change. **(a)** Effects of α on fractional binding of radiolabel. Data are generated using Equation 3. $[S] = K_S = K_A = 5 \times 10^{-7}$ M. Values of α are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). **(b)** Effects of δ on fractional binding of radiolabel. Data were generated using Equation 4a. $T = 1$; $\beta = 1$; $\gamma = 1$; $[S] = K_S = K_A = 5 \times 10^{-7}$ M. Values of δ are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). **(c)** Effect of T on fractional binding of radiolabel. Data were generated using Equation 4a. $T = 10$; $\beta = 1$; $\gamma = 1$; $[S] = K_S = K_A = 5 \times 10^{-7}$ M. Values of δ are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). **(d)** Effects of α on receptor activity. Data are generated using Equation 4b. $T = 1$; $\beta = 1$; $\gamma = 1$; $\delta = 1$; $[S] = K_S = K_A = 5 \times 10^{-7}$ M. Values of α are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue). Note that all curves are flat because α has no effect on agonist efficacy. **(e)** Effects of δ on receptor activity. Data were generated using Equation 4b. $T = 1$; $\alpha = 1$; $\beta = 1$; $\gamma = 1$; $[S] = K_S = K_A = 5 \times 10^{-7}$ M. Values of δ are: 1 (black), 1.5 (red), 2 (orange), 5 (yellow), 10 (green) and 100 (blue).

Modeling the effects of allosteric modulators on receptors

As the value of α increases in a model of Equation 3, the fractional binding of the substrate (agonist) reaches a limiting value at a high concentration of allosteric modulator, which depends on the extent of the cooperativity (Figure 5a). As the value of the cooperativity factor increases further, the fractional binding of the agonist approaches the maximum for the assay ($Y/Y_o = 1.0$) and the concentration of allosteric modulator required to achieve this maximum decreases (Figure 5a). The EC_{50} for any given α-type modulator and agonist can be determined from a dose–response curve and represents the concentration of allosteric modulator needed to increase fractional binding to half the observed maximum.

As with single-substrate enzymes, initial identification of a compound as a possible allosteric modulator of radioligand binding relies partly on the characteristic limiting effect of cooperativity on fractional occupation at high concentrations of the compound. A compound with high cooperativity is identified readily from a dose–response analysis, especially at low substrate concentrations where such allosteric modulators can effect a large change in fractional occupation. As with enzyme assays, the conditions and parameters of the experiment can combine to impede discovery of compounds with low cooperativity values. In radioligand binding assays, usually $[S] \ll K_S$ and so such formats are less likely to discover modulators with low α values.

The results of increasing the values of α and δ in Equations 4a and 4b are qualitatively similar to those of varying α in Equation 3. At any given value of either α or δ, increasing the concentration of allosteric modulator does not result in further increases in fractional occupation and the concentration of allosteric modulator required to achieve this maximum continues to decrease. In Equation 4a, the factor δ describes the influence of a modulator on the efficacy of a substrate to activate the receptor and has a relationship with T that α does not. In radioligand binding when the receptor can assume an activated state, a δ-type modulator is distinct from an α-type modulator because the magnitude of the influence of a δ-type modulator on fractional binding depends on the value of T. As T increases (and the population of activated receptors decreases), the effect of δ on fractional occupation becomes less pronounced for values of δ closer to unity (compare Figure 5b with Figure 5c). By contrast, the value of T has no effect on α-type modulation of receptor function (data not shown).

In assays that measure receptor activity, such as Ca^{2+} influx, activity reaches a limiting value at high concentrations of allosteric modulator, which depends on the extent of the cooperativity.

As the value of the cooperativity factor continues to increase, receptor activity approaches a maximum value and the concentration of allosteric modulator required to achieve this maximum continues to decrease (data not shown). The EC_{50} for any given α-type or δ-type modulator and substrate concentration is determined from a dose–response curve and represents the concentration of allosteric modulator needed to increase fractional binding to half the observed maximum. Again, the substrate concentration used in the assay imposes limits on the magnitude of the cooperativity factor for modulators identified in a screen: as substrate concentration decreases, the ability to detect differences in activity increases for modulators with higher cooperativity values.

There is a crucial difference between α-type and δ-type modulators of receptor function presented in Scheme IV (Figure 1). An α-type modulator affects the affinity of the agonist and, thus, it cannot stimulate receptor activity in the presence of a neutral agonist ($\beta = 1$) regardless of its cooperativity value (Figure 5d). However, δ-type modulators influence the efficacy of an agonist and can increase the receptor response to maximum levels (i.e. $Y/Y_o = 1.0$), even in the presence of a weak or neutral agonist (Figure 5e) [23,24].

Conclusions

Allosteric modulators of several proteins have been discovered using a variety of screening formats, sometimes serendipitously [25]. Several allosteric modulators are either in clinical trials or on the market [6,26,27]. The kinetic schemes outlined above demonstrate that although the initial identification of a potential positive allosteric modulator might be straightforward, characterization of the specific mechanism of the modulation can be complex. The simplified schemes do not account for more complicated behaviors of proteins (e.g. enzyme hysteresis, non-equilibrium conditions and receptor internalization), which 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 the identification of allosteric modulators in a screen. Allosteric modulators have rich and diverse effects on protein function that might be advantageous as potential therapeutics. However, such traits do not diminish the challenges of hit-to-lead investigations that accompany the development of any drug.

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