ALLOSTERIC BINDING SITES ON CELL-SURFACE RECEPTORS: NOVEL TARGETS FOR DRUG DISCOVERY

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Cell-surface receptors are the targets for more than 60% of current drugs. Traditionally, optimizing the interaction of lead molecules with the binding site for the endogenous agonist (orthosteric site) has been viewed as the best means of achieving selectivity of action. However, recent developments have highlighted the fact that drugs can interact with binding sites on the receptor molecule that are distinct from the orthosteric site, known as allosteric sites. Allosteric modulators could offer several advantages over orthosteric ligands, including greater selectivity and saturability of their effect.

ORTHOSTERIC SITE
The endogenous agonist binding site on a receptor. This domain is also recognized by classic competitive antagonists and inverse agonists.

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Many avenues of drug discovery have long been driven by the concept of the cell-surface receptor as a selective target for chemotherapeutic agents, an idea that was first introduced by Paul Ehrlich just over a century ago[1]. Early impetus also came from John N. Langley's[2] proposal that receptors could receive extracellular chemical signals and transmit them to the cell, and that these extracellular signals could be mimicked or antagonized by specific pharmacological agents. Soon after, pharmacological characterization of cell-surface receptors led to an explosion in the number and types of drug available as medicines[3]. Today, drugs that target cell-surface receptors represent the largest proportion of medicines on the world market, with most of them mediating their therapeutic effects by acting at G-protein-coupled receptors (GPCRs). With the recent draft release of the human genome, the pre-eminence of cell-surface receptors as drug targets seems indisputable. Indeed, it is estimated that current medicinal agents that target GPCRs represent a knowledge base of only ~200 protein targets; a further 400 such gene products are predicted in the human genome, and this does not include splice variants. So, receptor-based drug discovery should continue to provide many more viable drug targets well into the new millennium.

The main principle underlying this drug discovery process has invariably been the optimization of lead molecules towards the classic agonist binding site on the receptor as a means for obtaining selectivity of action. Throughout this review, this site will be referred to as the ORTHOSTERIC SITE. Designing drugs to target orthosteric sites seems logical, given that the requisite high degree of complementarity between the endogenous hormone or neurotransmitter (agonist), on the one hand, and its binding pocket, on the other, ensures some specificity in activity. This principle also extends to drugs that mimic or inhibit the effects of the endogenous agonist through competitive interactions; the characteristic feature is that all ligands have spatial overlap for a common topographical domain on the receptor. In turn, this has led to the identification and classification of most receptor-targeting drugs as being agonists, neutral (competitive) antagonists or inverse agonists[4], the latter being defined as compounds that inhibit constitutive — that is, agonist-independent — receptor activity. However, recent developments in the field of cell-surface receptors, particularly GPCRs, highlight the fact that novel receptor-selective drugs need not be defined by the chemical space that encompasses traditional orthosteric ligands. From approximately the middle of the last decade, functional screening assays began to overtake radioligand binding assays as the high-throughput method of choice, and this has increased the variety of the biologically active compounds that are detected.

Whereas previously, a 'hit' would usually have been identified when there was an alteration in the binding of a radiolabelled tracer ligand in the presence of a test ligand, compounds are now being discovered that can modify receptor function even if they exert minimal effects on radioligand binding. The converse situation is also true; there are compounds that can exert a profound effect on the binding of some orthosteric ligands and have no effect on others, all at the same receptor. A likely mechanism for such effects has been well established in studies on enzymes and ligand-gated ion channels (LGICs), but has not been widely considered for ligands that act at GPCRs; namely, the phenomenon of allosterism.

Allosteric ligands interact with binding sites on the receptor molecule that are topographically distinct from the classic orthosteric site. Hence, the structural features that determine the binding of orthosteric agonists, competitive antagonists and inverse agonists are different from those of ligands that bind to ALLOSTERIC SITES. Allosteric drugs are able to modulate receptor activity through conformational changes in the receptor protein that are transmitted from the allosteric site to the orthosteric site and/or directly to effector coupling sites. As already shown for some LGICs, allosteric modulators have several advantages over orthosteric ligands as potential therapeutic agents (see below), and evidence is now emerging that many GPCRs also have allosteric binding sites. These sites are therefore attractive drug targets, and the drug discovery process now has the capability and, importantly, the capacity, to screen for allosteric modulators in addition to orthosteric ligands. This review provides a brief discussion of allosteric sites on GPCRs and LGICs, the advantages of allosteric modulators as therapeutic agents, and the approaches that are required to successfully detect and quantify ALLOSTERIC INTERACTIONS in drug discovery programmes.

A model of allosteric interactions

Standard orthosteric drug–receptor theory, which forms the basis for the quantitative comparison of the drug affinity values that are derived in most drug discovery programmes, is based on the law of mass action and some simplifying assumptions[5]. In essence, orthosteric (that is, competitive) interactions are characterized by mutually exclusive binding between different ligands for a common site on the receptor. The standard model for this interaction is shown in FIG. 1a, together with a simulation that depicts the classic behaviour predicted by the model for the effect of increasing the concentration of an orthosteric ligand, B, on the occupancy of another orthosteric ligand, A. In this model, the quantitative correspondence between ligand concentration and receptor occupancy is adequately characterized by a single ligand–receptor affinity value, which is expressed as an equilibrium association constant (for example, K_a and K_b in FIG. 1), or its reciprocal, the equilibrium dissociation constant. By contrast, allosteric models are not as simple because they must incorporate the ability of the receptor to present different binding sites to different ligands. At this point, two important distinctions are required. For the purpose of this review, an allosteric

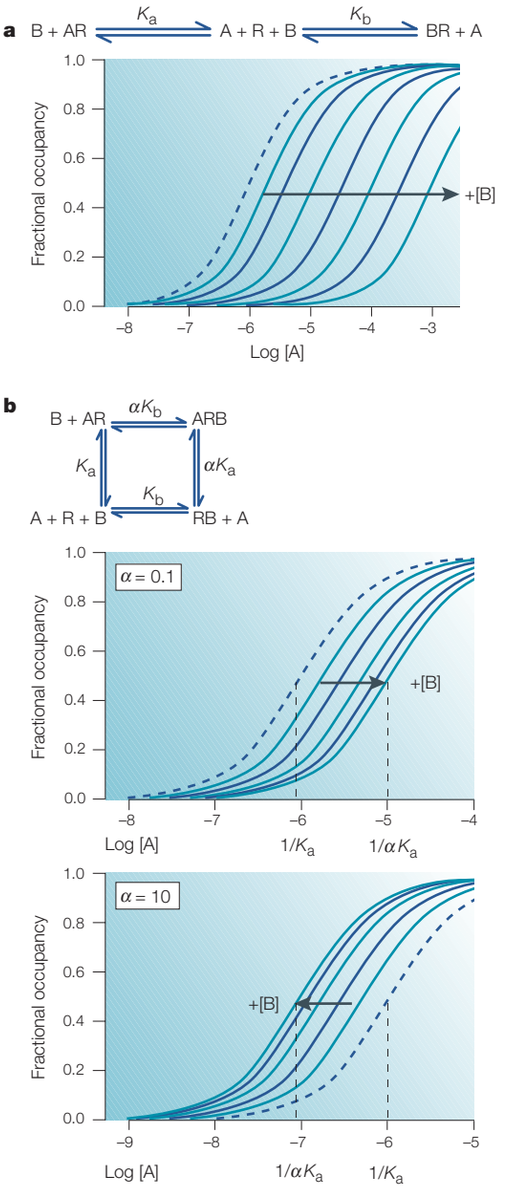


Figure 1 | **Models of orthosteric and allosteric binding.** **a** | Orthosteric competition. Ligands A and B compete for binding to the same site on the receptor, R, according to their relative concentrations and affinities, the latter are denoted by the equilibrium association constants, K_a and K_b, respectively. Also shown are simulations that are based on the model for the effects of B on the occupancy of A. Competitive antagonism predicts progressive dextral displacement of the occupancy of one orthosteric ligand in the presence of increasing concentrations of another. **b** | The ternary complex model (TCM) of allosteric interaction. In this model, the allosteric modulator, B, binds to a site that is topographically distinct from the orthosteric site that is used by ligand A. This modifies the orthosteric ligand affinity to a limit that is determined by the cooperativity factor, α, which characterizes the strength of the interaction between allosteric and orthosteric sites. The simulations show the effects of B on A when ligand affinity is either enhanced ($\alpha = 10$) or diminished ($\alpha = 0.1$) by a factor of 10. Note the limit in the predicted occupancy shifts for ligand A as the concentration of modulator B is progressively increased; irrespective of the concentration of the modulator, the dissociation constant of A cannot exceed $1/\alpha K_a$. [A], concentration of A; [B], concentration of B.

ALLOSTERIC SITE
A modulatory binding site on a receptor that is topographically distinct from the agonist binding site.

ALLOSTERIC INTERACTION
An interaction between two topographically distinct binding sites on the same receptor complex.

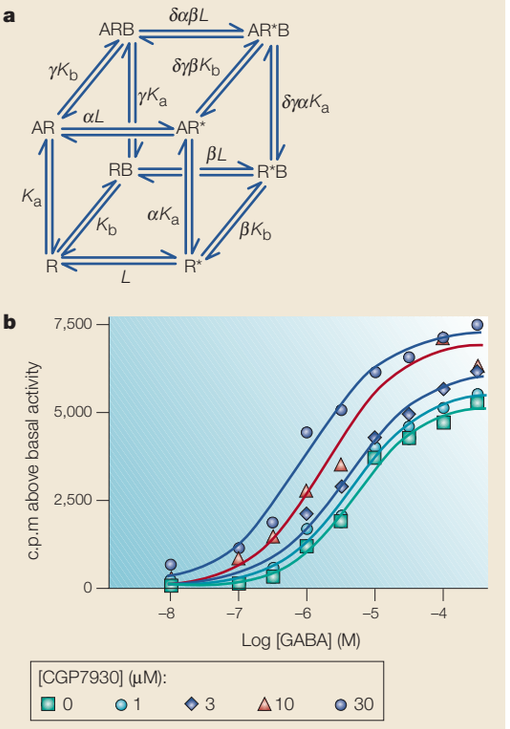
interaction is defined as an interaction that occurs between two (or more) topographically distinct binding sites on the same receptor complex, whereas an ALLOSTERIC TRANSITION is defined as the global isomerization of a receptor protein from one conformation to another; this isomerization can be ligand driven, or it can occur in the absence of ligand due to random thermal fluctuations[6–8]. The distinction between these two terms is necessary because the concepts that they embody have been used to derive different receptor models that have each been called 'allosteric' (BOX 1), leading to some confusion within the field[9].

Box 1 | **Historical development of allosteric receptor models**

The term 'allosteric', from the Greek meaning 'other site', was introduced in a seminal series of enzymology papers by Monod and colleagues[63–65], who identified several characteristics that are associated with classic allosteric proteins, including: an oligomeric structure; multiple ligand binding sites; multiple conformational equilibria in the absence of ligand; and the stabilization of specific protein conformations by ligands. Interestingly, these characteristics were already known to be associated with ligand-gated ion channels (LGICs), and so the first allosteric receptor models — that is, those that dealt explicitly with receptor isomerization between different conformational states[66,67] — actually pre-dated the allosteric enzyme models of Monod and colleagues. An important property of these models is the prediction of receptor activity in the absence of ligand as a consequence of the isomerization process; that is, constitutive receptor activity[68–71]. These models are now more commonly referred to as 'two-state' or 'multi-state' models.

By contrast, the study of G-protein-coupled receptor (GPCR) behaviour remained largely operational until the last two decades of the twentieth century because of the relative lack of detailed tools with which to analyse drug actions at these receptors at the molecular level. However, the proposal that receptors could translocate within the cell membrane and associate with other membrane-bound proteins[72] led to the second major development in allosteric receptor theory, the ternary complex model (TCM) of De Lean and colleagues[73]. The earliest applications of the TCM were to the allosteric interaction between agonist binding and G-protein coupling[73–75], but the model was equally applicable to mechanisms that involved the simultaneous binding of two ligands to one receptor[13,76], a phenomenon that was first shown for the muscarinic-acetylcholine family of receptors[76–78], but was subsequently shown for other GPCRs (TABLE 1). One commonly observed difference between the TCM as a model for receptor–G-protein interactions, on the one hand, and for receptor–modulator interactions, on the other, is that the former situation can lead to shallow binding curves if G-protein amount is limiting, whereas the latter does not, because allosteric modulators are invariably present in vast excess relative to the concentration of receptor. The mechanisms are otherwise, however, formally identical.

Finally, the conclusive demonstration of constitutive receptor activity at a GPCR[79] led to the realization that both the TCM and the multi-state models of receptor action reflect two sides of the same coin. So, current allosteric receptor models for both LGICs and GPCRs have combined features of both the TCM and multi-state models[10,11,80,81] to accommodate allosteric modulator effects on both orthosteric ligand affinity and the ability of the receptor to isomerize between active and inactive states. An allosteric two-state model[10,11] is shown in panel **a** for the simplest scenario of two different receptor conformations, one active (R*) and one inactive (R), each containing two topographically distinct binding sites. This model therefore describes allosteric modulator effects on efficacy in addition to affinity. Such extensions of the simpler allosteric receptor models are required to accommodate newer data that are accumulating from functional receptor assays, such as the effects of the allosteric enhancer CGP7930 (2,6-di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-ropyl)-phenol) on the agonist-mediated signalling of the $GABA_B$-receptor (γ-aminobutyric acid B receptor) heterodimer. Panel **b** shows the effects of CGP7930 on GABA-mediated [^{35}S]GTPγS binding in Chinese hamster ovary (CHO) cell membranes that are expressing the human $GABA_B$-receptor heterodimer. Note that the modulator causes not only an increase in the potency of the endogenous agonist, GABA, but also a concentration-dependent enhancement of the maximal receptor-mediated response — a clear example of allosteric modulation of ligand–receptor efficacy. This finding cannot be reconciled with the simple TCM. c.p.m, counts per minute; [CGP7930], concentration of CGP1930 (μM); [GABA], concentration of GABA (M); K_a, equilibrium association constant of A; K_b, equilibrium association constant of B; L, receptor isomerization constant; α, β, δ, γ, ligand–receptor cooperativity factors. Redrawn with permission from REF. 43 © (2001) American Society for Pharmacology and Experimental Therapeutics (ASPET).



ALLOSTERIC TRANSITION
The isomerization of a receptor protein between multiple conformational states.

Table 1 | **Selected allosteric modulators of cell-surface receptors**

Receptor	Allosteric modulator(s)	References
Ligand-gated ion channels		
$GABA_A$ receptor	Benzodiazepines; β-carbolines; barbiturates; steroids (allopreganolone, pregnan-2-one); picrotoxin; ethanol; general anaesthetics	24,25,83–89
$GABA_C$ receptor	Picrotoxin, zinc	83
Glycine receptor	Picrotoxin; zinc; glutathione	83
Nicotinic acetylcholine receptors	Quinacrine; phencyclidine; local anaesthetics; various alkaloids (physostigmine, gallanthamine, codeine); 5-HT; ivermectin	21,23,90–97
NMDA receptors	Glycine; polyamines (spermine, spermidine); ifenprodil; ethanol; zinc; histamine; arachidonic acid	98–102
Purine P2X receptor	d-tubocurarine; cibacron blue	103
G-protein-coupled receptors		
Adenosine A_1	PD81723; PD117975	60,104–107
Adenosine A_{2A}	Amilorides	42
Adenosine A_3	VU5455; VU8504	108
α_1-Adrenoceptor	Amilorides; benzodiazepines	109,110
α_{2A},α_{2B}-Adrenoceptor	Amiloride	16,59,111,112
α_{2D}-Adrenoceptor	Agmatine	113
β_2-Adrenoceptor	Zinc	114
Calcium-sensing receptor	NPS467; NPS568; L-amino acids	115,116
Chemokine* CXCR3	IP-10; I-TAC	117
Chemokine* CCR5, CXCR4	Trichosanthin	118
Chemokine* CCR1, CCR3	UCB35625	119
Dopamine D_1	Zinc	120
Dopamine D_2	Amilorides; zinc	121,122
Endothelin ET_A	Aspirin; sodium salicylate	123,124
$GABA_B$	CGP7930; CGP13501	43
Glutamate $mGlu_1$	CPCCOEt; Ro67-7476; Ro01-6128; BAY36-7620	38,44,50
Glutamate $mGlu_5$	MPEP	37,125
Muscarinic M_1–M_5	Gallamine; alcuronium; brucine	14,76,78,126
Neurokinin NK_1	Heparin	127
Purine $P2Y_1$	2,2′-pyridylsatogen tosylate	128,129
Serotonin $5\text{-}HT_{1B/1D}$	5-HT moduline	130,131
Serotonin $5\text{-}HT_{2A}$	Oleamide	51
Serotonin $5\text{-}HT_7$	Oleamide	51,52

* Chemokine receptors are named according to the number and spacing of conserved cysteines: CC, Cys-Cys; CXC, Cys-Xaa-Cys.
CPCCOEt, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester; GABA, γ-aminobutyric acid;
5-HT, 5-hydroxytryptamine; NMDA, *N*-methyl D-aspartate; mGlu, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)-pyridine.

Mechanistically, the two terms reflect different aspects of the same phenomenon — namely, the ability of a protein to adopt multiple conformations that differ in their biological binding/signalling properties, and the ability of ligands to selectively enrich subsets of these conformations from within the global conformational ensemble that is available to the protein. Although receptor models have been developed to encompass both of these properties (BOX 1), the starting point for most drug discovery programmes aimed at detecting small-molecule modulators acting through extracellular ligand binding sites that are distinct from the orthosteric site remains a relatively simple model that explicitly incorporates cross-interactions between different sites.

Shown in FIG. 1b is the ternary complex model (TCM), the simplest mechanism that describes an allosteric interaction between two ligands, A and B, each binding concomitantly to topographically distinct binding sites on the same receptor protein. In this model, ligand A binds to the orthosteric site with an affinity of K_a, whereas ligand B binds to an allosteric site with an affinity of K_b. The symbol α is often termed the 'cooperativity factor', and quantifies the magnitude by which the affinity of one ligand is changed by the other ligand when both are bound to the receptor to form the ternary complex, ARB. The ability of an allosteric modulator to cause such a change in orthosteric ligand affinity relates to the perturbation that the modulator induces in receptor conformation. As the equilibrium binding of any

ligand–receptor complex depends on the ratio of ligand association and dissociation rates[5], then the simplest mechanism that underlies allosteric interactions in the TCM is an alteration in orthosteric ligand association and/or dissociation. This aspect of allosteric interactions forms the basis of specific assays that are designed to detect and/or validate allosteric modulators (see below).

It should be noted that another important property of allosteric interactions is that they are reciprocal in nature; whatever A does to B, B does to A. In contrast to orthosteric interactions, therefore, allosteric interactions of this type are characterized by two affinity constants, together with the cooperativity factor, the latter defining the degree of allosteric interaction. In the TCM shown in FIG. 1b, values of $\alpha > 1$ result in increased affinity (positive cooperativity), whereas values of $\alpha < 1$ denote a decrease in affinity (negative cooperativity). For very high degrees of negative cooperativity ($\alpha \ll 1$), the interaction might seem indistinguishable from competitive (mutually exclusive) binding. Interestingly, it is also possible for the affinity of either ligand to remain unaltered at the occupied receptor; α is then equal to 1, and the interaction is characterized by 'neutral' cooperativity. Another property of the TCM is that the magnitude and direction of the allosteric effect between the two sites (that is, the α-value) depends on the chemical nature of A and B; different pairs of ligands that interact at the same orthosteric and allosteric sites of a given receptor can still have markedly different types of cooperativity. This is an important consideration for drug discovery programmes that target allosteric sites (see below).

In practice, the TCM is also the model with the minimum number of parameters that are required to define allosteric drug properties that can accurately be determined experimentally. Although more recent receptor models have been developed to more faithfully reflect the full theoretical spectrum of allosteric effects[10,11], they are defined by more parameters than can routinely be determined in assays that are designed predominantly for drug-screening purposes. By contrast, the TCM has already proved to be remarkably robust in quantifying the behaviour of several allosteric modulators at both LGICs and GPCRs[12–16]. TABLE 1 lists a range of ligands that have been shown to act at allosteric sites that are distinct from those that are recognized by the endogenous agonist, on both LGICs and GPCRs. Although the list of receptors might seem rather diverse, allosteric interactions share several common features that are related to the consequences of cooperativity. Specifically, in the TCM, the effects of allosteric modulators on the fractional receptor occupancy by the orthosteric ligand A (ρ_A) can be described by the following expression[13,14,17,18]:

$$\rho_A = \frac{[A]}{[A] + K_{App}} \quad (1)$$

with K_{App} being given by EQN 2:

$$K_{App} = K_A \frac{\left(1 + {}^{[B]}/_{K_B}\right)}{\left(1 + {}^{\alpha[B]}/_{K_B}\right)} \quad (2)$$

In these equations, K_A and K_B denote the equilibrium dissociation constants of A and B (that is, $1/K_a$ and $1/K_b$ from the model in FIG. 1) respectively, at the free receptor. If the interaction between A and B is positively COOPERATIVE ($\alpha > 1$), then $K_{App} < K_A$, and the binding curve of ligand A at the modulator-occupied receptor will be shifted to the left relative to the binding curve of A at the free receptor. By contrast, negative cooperativity between A and B ($\alpha < 1$) will cause a rightward displacement of the binding curve for A (that is, $K_{App} > K_A$). FIG. 1b illustrates these relationships for the binding of an orthosteric ligand in the presence of increasing concentrations of an allosteric modulator with an α-value of either 0.1 (negative cooperativity) or 10 (positive cooperativity). This figure also illustrates an important aspect of allosteric interactions — namely, that these types of interaction approach a limit, the extent of which is governed by the magnitude of α. The closer the value of α is to 1, the more readily the limit is approached with increasing concentrations of modulator. This property of a limit to an allosteric interaction is particularly important with respect to the therapeutic advantages that are offered by allosteric modulators, as well as the types of assay that can be optimized to screen for this behaviour.

COOPERATIVE BINDING
The binding of two or more molecules of the same ligand to a receptor complex. Sometimes used in a less strict sense to describe the concomitant binding of more than one molecule of any chemical type to a receptor complex.

Advantages of allosteric modulators

There are at least three general advantages to using allosteric modulators for producing or modifying physiological responses in comparison to standard orthosteric drugs. The first advantage is that the effect of allosteric modulators is saturable; once the allosteric sites are completely occupied, no further allosteric effect is observed[19]. By contrast, classic orthosteric (competitive) antagonism can theoretically be infinite, as it depends only on the relative concentrations of the competing species. Saturability of allosteric effects applies to both positive and negative allosteric modulators, and is therefore useful for drug candidates that are aimed at either enhancing or antagonizing receptor-mediated effects; the modulators can be given in relatively high doses without fear of overstimulating or overinhibiting the system. So, there is a 'ceiling' to the effects of an allosteric modulator that is retained even with excessive doses. Indeed, safety in overdosage is an acknowledged clinical advantage of the benzodiazepines as a class of positive $GABA_A$-receptor (γ-aminobutyric acid A receptor) modulators, compared with other ligands that act directly at the GABA binding site or the ion channel itself.

A second advantage of allosteric ligands, especially positive modulators, relates to their ability to selectively tune responses only in tissues in which the endogenous agonist exerts its physiological effects[20]. Normal neurohumoral signalling involves the pulsatile release of hormones and variations in the activity of nerves that release neurotransmitters; an allosteric modulator would be expected to exert its effects only when endogenous agonist is present. If nerve activity is reduced, the modulator would have minimal effects, despite its continued presence in the receptor compartment. This is not possible with orthosteric agonists, which will continuously modify receptor function as long as they

Box 2 | **Endogenous allosteric modulators**

By definition, the orthosteric binding site on a receptor comprises amino acids that form contacts with the endogenous agonist for that receptor; this site has therefore specifically evolved to interact with an endogenous hormone or neurotransmitter. By contrast, allosteric binding sites need not satisfy this criterion, and might simply represent accessory domains that normally have structural roles. However, as listed in TABLE 1, some ions, including sodium, calcium and zinc, have been found to modulate the activities of certain ligand-gated ion channels (LGICs) and G-protein-coupled receptors through allosteric mechanisms. Other endogenous modulators of cell-surface receptors include L-amino acids, glycine, amidated lipids such as oleamide, peptides such as 5-hydroxytryptamine (5-HT) moduline, the eicosanoid arachidonic acid and various neuroactive steroids. Even classic neurotransmitters, such as 5-HT and histamine, have been found to modulate the nicotinic acetylcholine and *N*-methyl D-aspartate (NMDA) LGICs, respectively (see TABLE 1 for references). Interestingly, one report has found that human eosinophil major basic protein might have a role in muscarinic M_2-receptor dysfunction of the airways by means of an allosteric effect on receptor function[82]. So, although allosteric binding sites on receptors are attractive therapeutic targets for synthetic ligands, some of them can also influence receptor function under normal physiological, or perhaps pathophysiological, conditions through interaction with endogenous modulators.

are present. So, allosteric modulators can process both spatial and temporal information gained from the physiology of the system to produce optimum effect.

The third advantage of allosteric drugs is the potential for greater receptor subtype selectivity, which can be achieved by either (or both) of two mechanisms. The first such mechanism relates to the location of the allosteric site. Data from mutagenesis experiments on both LGICs and GPCRs are now beginning to reveal amino acids that contribute to binding domains that are structurally distinct from those that comprise the orthosteric binding site. There is even evidence to indicate that endogenously produced ligands might act at some of these allosteric sites (BOX 2). In the case of LGICs, this should not be surprising, as these receptors have several features in common with classic allosteric enzymes, including an oligomeric quaternary structure that is invariably associated with multiple topographically distinct but interacting domains[21] that are targeted by various endogenous and synthetic ligands. For example, the $GABA_A$ LGIC, similar to the nicotinic acetylcholine LGIC, is a pentamer that comprises various combinations of distinct α-, β-, γ-, δ-, ε-, θ- and ρ-subunits[21–23]. The ability of benzodiazepine ligands to modulate GABA-mediated channel activity relies heavily on the presence of a γ_2-subunit to form an allosteric binding interface with specific α-subunits; $GABA_A$ pentamers that lack the γ_2-subunit do not show modulation by benzodiazepines[22,24]. Furthermore, depending on the α-subunit that forms the α–γ interface, selective modulator effects of the benzodiazepines have been noted[25]. By contrast, the binding of GABA relies on a domain at the interface of α- and β-subunits[22]. These differences are illustrated schematically in FIG. 2a, which shows the relationship between amino acids that are thought to contribute to the allosteric benzodiazepine binding site and the two orthosteric sites for GABA on the $\alpha_1\beta_2\gamma_2$ pentamer[26]. In general, receptors that are oligomeric are natural drug targets for allosteric modulators, as the likelihood of multiple interacting binding domains is high. Indeed, the recognition of functional diversity of $GABA_A$ receptors as a consequence of LGIC subunit composition has already led to the discovery of a range of selective allosteric modulators that span a spectrum of positive to negative effects[27,28].

Although recent evidence indicates that GPCRs can also form oligomeric arrangements[18,29,30], most of the biological properties of these receptors have traditionally been accommodated in models that treat them as monomeric proteins. Nevertheless, mutagenesis experiments that have been conducted on some GPCRs[31–38] have identified ligand binding domains within the receptor monomer that are unequivocally distinct from those that comprise the orthosteric binding site, as shown in FIG. 2b for the M_2 muscarinic acetylcholine receptor. It can be seen that the allosteric site on the M_2 receptor is close to the orthosteric site, but at a more extracellular level[33,39]. So, receptors that show high sequence homology within the orthosteric domain but not in the allosteric binding domains are another class of candidate targets for allosteric modulators, as the likelihood of subtype selectivity can be enhanced if drug discovery programmes target receptor allosteric sites.

The second mechanism for receptor subtype selectivity of allosteric modulators is related to cooperativity rather than affinity. It is known that the affinity of a modulator for its binding site is not correlated with the degree of cooperativity that exists between orthosteric and allosteric sites[40]. Hence, a modulator might have the same affinity for different subtypes of a receptor, but still exert a selective effect by having different degrees of cooperativity at each subtype. The ideal expression of this phenomenon has been termed 'absolute subtype selectivity'[41], and is observed when a modulator remains neutrally cooperative at all receptor subtypes except the one that is being targeted for therapeutic purposes. For example, although the allosteric modulator *N*-chloromethylbrucine has almost identical affinities for the allosteric sites on the M_3 and M_4 muscarinic acetylcholine receptors (pK_A~4.3), it is positively cooperative ($\alpha = 3.3$) at the M_3 receptor, but effectively neutrally cooperative ($\alpha = 1.03$) at the M_4 receptor[41].

Given the current bias of most drug discovery programmes towards orthosteric ligands, the potential clinical advantages of allosteric modulators that are outlined above remain to be validated for many drug targets. However, the success of the benzodiazepines as positive allosteric modulators of the $GABA_A$ LGIC already attests to the therapeutic utility of allosteric ligands, as well as the degree to which the theoretical advantages of these ligands can actually be met in practice. Even more encouraging are some recent studies that have identified novel ligands from GPCR drug discovery programmes that do not seem to use orthosteric mechanisms[38,42–44]. One important example is the phenylalkylamine derivatives NPRS467 and NPRS568, which are positive allosteric modulators of the calcium-sensing GPCR. Compounds that are related to these modulators are now in clinical trials for the treatment of primary hyperparathyroidism[45],

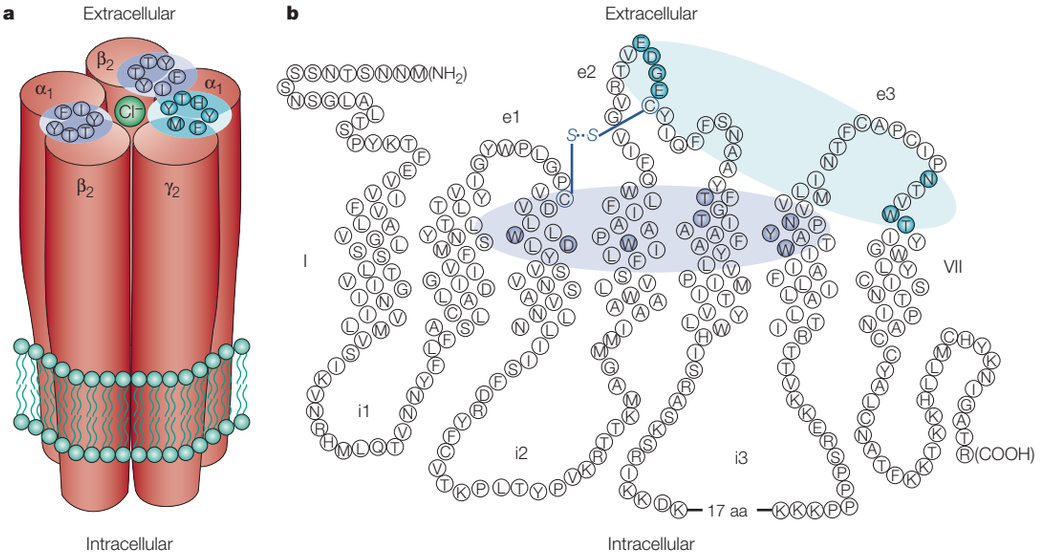


Figure 2 | **Allosteric sites as receptor-selective drug targets. a** | Hypothetical model of the $\alpha_1\beta_2\gamma_2$ γ-aminobutyric acid A ($GABA_A$) ligand-gated ion channel. The amino acids that are believed to constitute the orthosteric site are indicated in purple, whereas those that contribute to the allosteric benzodiazepine site are shown in turquoise. **b** | Structure of the M_2 muscarinic acetylcholine G-protein-coupled receptor. Transmembrane domains are arranged from I to VII (left to right), extracellular loops are indicated by the letter 'e', whereas intracellular loops are indicated by the letter 'i'. Amino acids (aa) that were identified from mutagenesis experiments to contribute to orthosteric ligand binding are shown in purple, whereas those that contribute to the binding of allosteric modulators are shown in turquoise. It can be seen that even on a monomeric protein, different domains (shaded) are available for targeting by drugs.

and could represent the first GPCR allosteric modulators to reach the clinic specifically because of their allosteric properties.

Detecting allosteric effects

Until recent years, the primary assay for high-throughput screening in the drug discovery process has been radioligand binding. This assay uses a radiolabelled 'probe' to directly monitor occupancy of the binding site on the receptor with which the probe interacts, and has been very successful in screening for orthosteric drugs because it is relatively efficient, reproducible and highly scalable towards sampling large compound libraries. However, the features that make binding assays useful for detecting orthosteric ligands do not necessarily apply to the detection of allosteric ligands; although an allosteric modulator might have a striking effect on orthosteric ligand binding in some instances, it might have minimal or no effects in others, because allosteric phenomena are probe dependent[46]. This finding might explain the current paucity of clinically available allosteric drugs — radioligand binding assays are biased towards the detection of orthosteric effects.

Even when radioligand binding assays are able to measure allosteric interactions, the assays might need to be modified to ensure maximum likelihood of detection. Although the TCM describes allosteric phenomena in terms of changes in orthosteric ligand binding affinity, some allosteric mechanisms can also involve changes in ligand efficacy rather than direct effects on affinity (BOX 1). For LGICs, this involves an effect on channel open/shut states; for a GPCR, the corollary would be a direct effect on receptor–G-protein coupling and/or receptor isomerization into an active state. Whereas 'pure' effects on orthosteric ligand affinity will be reflected as changes in the occupancy of the probes, allosteric effects on ligand–receptor efficacy can lead to a dissimilarity in the detection and classification of the allosteric ligand. For example, agonist probes of GPCRs predispose the screen towards detecting ligands that bind to the high-affinity state of the receptor that is coupled to the G protein. By contrast, inverse agonists prefer receptors in the G-protein-uncoupled, or inactive, state, whereas neutral antagonists show no discernible bias and so indiscriminately sample the entire receptor population[5]. The presence of an allosteric modulator that selectively perturbs receptor–G-protein coupling without an effect on orthosteric site occupancy can therefore have opposite effects on agonist and inverse-agonist probes, while remaining undetected by neutral-antagonist probes. Because of the probe specificity of allosteric effects, drug discovery programmes that are specifically aimed at allosteric modulators must consider the following two points: first, if possible, the probe that is used should be the endogenous orthosteric ligand for the receptor of interest; and second, if this is not possible, then the probe might still detect an allosteric interaction, but the magnitude and direction of that interaction can be quite different from the situation with the endogenous-ligand probe.

The second feature that must be considered when using binding assays to screen for allosteric modulators is the strength of the cooperativity. Although it represents a therapeutic advantage in many instances,

limited degrees of cooperativity also pose a practical problem for drug discovery because they lead to small windows of detection of the allosteric effect. An example is shown in FIG. 3, in which the effects of a weak positive modulator ($\alpha = 4$) and a weak negative modulator ($\alpha = 0.25$) on the occupancy of an orthosteric ligand are viewed from the point of view of orthosteric saturation binding assays (centre panel), or the more commonly used method of inhibition (or potentiation) binding assays; that is, monitoring the effects of the different concentrations of modulator on a fixed concentration of orthosteric probe (outer panels). It can be seen that low levels of radioligand occupancy allow for the maximum detection window for allosteric effects — as the concentration of probe is increased, the saturability of the allosteric phenomenon reduces the ordinate window and therefore makes it much more difficult to discern the allosteric effect. In practice, a trade-off will need to be made between the lowest possible concentration of radiolabelled probe that is attainable and the resulting signal-to-noise ratio (that is, specific to non-specific binding) of the assay. When screening for antagonist compounds, it should also be noted that a failure to cause complete inhibition of specific orthosteric binding might be a telltale sign of a negative allosteric modulator. However, complete inhibition of radioligand binding by an antagonist need not imply strict competitive binding; it could be that the test compound is an allosteric modulator with high negative cooperativity. Sometimes, this latter property can be confirmed by carrying out the binding assay using very high concentrations of radioligand[47], although it might often be impractical. An alternative is to use a different type of binding assay to validate the allosteric effect.

One useful radioligand-based alternative to equilibrium binding assays for detecting and quantifying allosteric phenomena exploits the fact that allosteric changes in receptor conformation lead to changes in the rates of orthosteric ligand association and dissociation. Indeed, allosteric effects on the binding kinetics of orthosteric ligands are often more sensitive indicators of

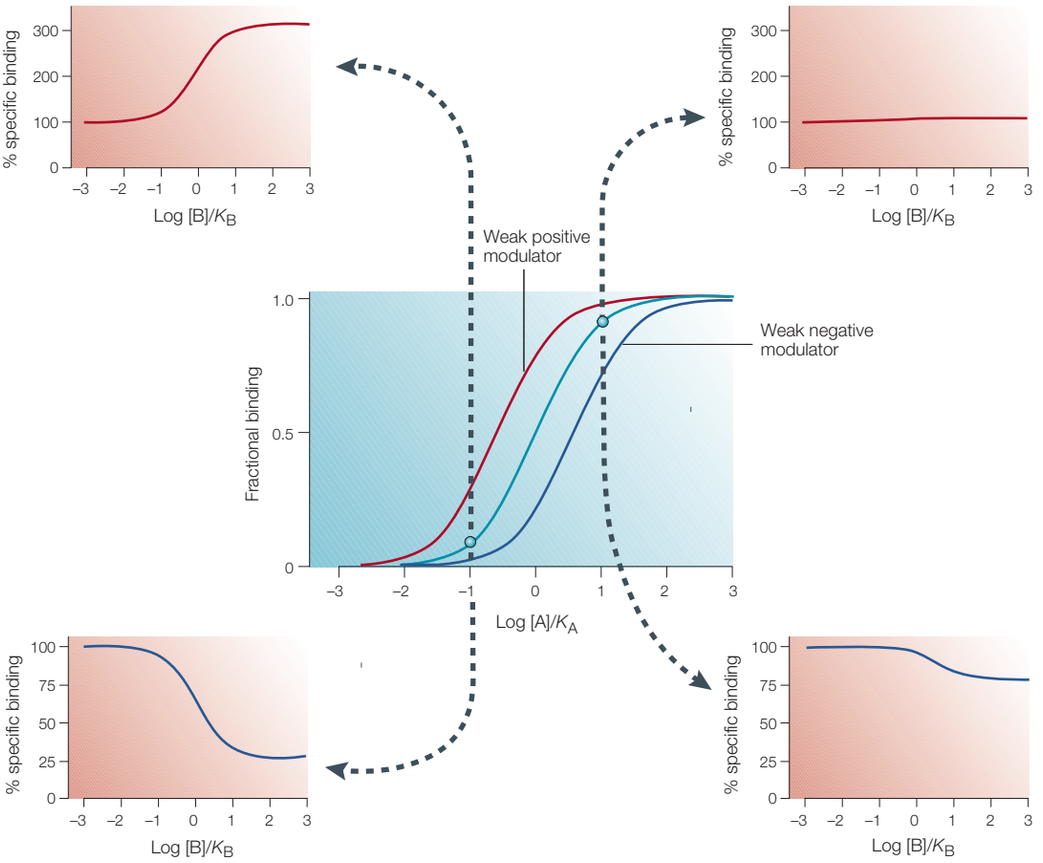


Figure 3 | **Detection of allosteric effects on orthosteric ligand binding.** The centre panel illustrates the effects of a weak positive (red curve; $\alpha = 4$) and weak negative (blue curve; $\alpha = 0.25$) modulator on the saturation binding of the orthosteric-ligand probe A. The outer panels show the effects of the modulator on the binding of a fixed concentration of orthosteric ligand that initially yields low orthosteric occupancy (left panels) or high orthosteric occupancy (right panels). It can be seen that low levels of orthosteric occupancy by a radioligand provide the maximal window for detecting allosteric effects in terms of the percentage change in specific orthosteric binding. However, high levels of orthosteric occupancy can be used diagnostically to differentiate allosteric antagonists — which will cause variable degrees of maximal inhibition depending on the concentration of orthosteric probe — from competitive antagonists, which will always be able to completely inhibit orthosteric radioligand binding. [A], concentration of A; [B], concentration of B; K_A, dissociation constant of A; K_B, dissociation constant of B.

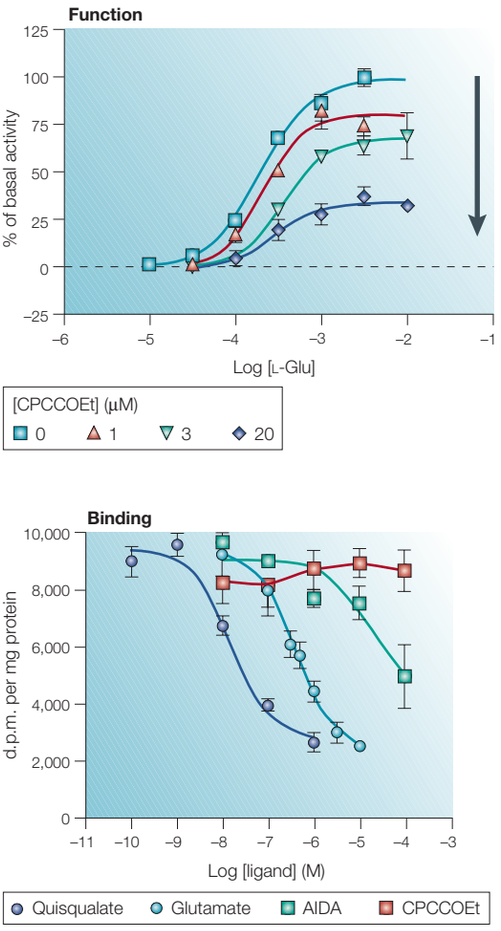


Figure 4 | **Detection of allosteric effects on orthosteric ligand function.** The effects of the metabotropic glutamate receptor 1 (mGlu1) allosteric antagonist, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), on glutamate-mediated phosphoinositide hydrolysis in Chinese hamster ovary (CHO) cells (top panel) or [^{3}H]glutamate binding in baby hamster kidney (BHK) cells (bottom panel). In contrast to other orthosteric ligands, CPCCOEt had no discernible effect on [^{3}H]glutamate binding, but was able to significantly diminish glutamate signalling over similar concentrations as those used in the binding assay. [CPCCOEt], concentration of CPCCOEt (μM); d.p.m., disintegrations per minute; [L-Glu], concentration of L-glutamate (M); [ligand], concentration of ligand (M). Redrawn with permission from REF. 50 © (1999) American Society for Pharmacology and Experimental Therapeutics (ASPET).

allosteric mechanisms than effects on equilibrium binding properties. For example, neutral cooperativity ($\alpha = 1$) might result in unaltered orthosteric binding at equilibrium, but measurements of orthosteric dissociation kinetics can still reveal that an allosteric mechanism is operative[48]. So, drug discovery programmes that specifically target allosteric modulators should consider the extra rigour that is provided by secondary validation assays based on radioligand dissociation rate measurements[14,49].

In comparison to radioligand binding, functional receptor assays have not been used as often in the past as the primary high-throughput assay of choice in drug discovery. However, this has now changed owing to the advent of new and improved technologies, including reporter systems, yeast and melanophore systems, and high-throughput, fluorescence-based, intracellular calcium-detection systems. The obvious advantage of using assays of receptor function as the primary screen is that the desired physiological end point — that is, an alteration of cellular responsiveness — is directly determined. It is too early at the moment to gauge the full impact of these technologies on the screening of allosteric modulators, but it is worth noting that small-molecule modulators of receptor function are now being detected in functional assays that might otherwise have been missed in binding assays. For example, FIG. 4 shows a clear lack of concordance between effects on receptor function, on the one hand, and receptor binding, on the other, of the allosteric antagonist 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) at the metabotropic glutamate receptor 1 ($mGlu_1$). Whereas CPCCOEt causes a marked attenuation of glutamate-mediated signalling with no significant effect on agonist potency, it fails to perturb the binding of [^{3}H]glutamate to the orthosteric site[50]. The allosteric properties of CPCCOEt would therefore not have been detected in standard assays of radioligand binding.

Another advantage of using functional assays to detect allosteric ligands is the ability of the assay to provide a sensitive readout for drugs that modify efficacy in their own right; for example, by directly altering receptor–G-protein coupling in the absence of orthosteric ligand. However, the probe dependence of allosteric effects can also lead to a problem in functional assays when it comes to validating the receptor specificity of allosteric agonists. For example, the endogenous fatty acid oleamide can activate 5-HT_7 receptors (5-hydroxytryptamine receptor 7) that are transfected into HeLa cells in the absence of the endogenous agonist 5-HT by interacting at an allosteric site[51,52]. However, the receptor specificity of this effect could not be confirmed by the classic approach of using a selective receptor antagonist to block the agonist-mediated response, as the high-affinity 5-HT_7 antagonist clozapine failed to attenuate the oleamide effect[51]. A marked attenuation in signalling through oleamide was noted, however, in cells that were not transfected with the 5-HT_7 receptor. So, the lack of a response in non-transfected cells might be a prerequisite for identifying allosteric agonists.

It is therefore obvious that both binding and functional assays have specific advantages and disadvantages when it comes to screening for allosteric modulators of cell-surface receptors. Overall, however, assays of receptor function are now generally sensitive enough and have the capacity to match, if not exceed, assays of radioligand binding in terms of compound sampling volumes. The choice of a high-throughput functional assay as the primary screen can be an efficient compromise for drug

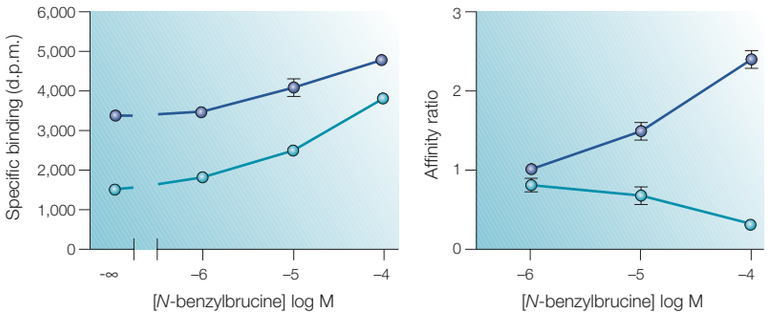


Figure 5 | **An example of the semi-quantitative 'affinity-ratio' assay for the detection of allosteric modulator effects.** The effects of the modulator *N*-benzylbrucine on the binding of the orthosteric-antagonist probe [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) in the absence (blue) or presence (green) of the agonist probe acetylcholine at the cloned human M_2 muscarinic acetylcholine receptor. The left panel shows the raw data for the binding of [^{3}H]NMS, whereas the right panel shows the conversion of the data to affinity-ratio plots; that is, plots of the ratio of apparent affinity of orthosteric probe in the absence, to that in the presence, of each tested concentration of allosteric ligand. Note that conversion of the raw data to affinity ratios allows for a simple visual detection of the direction and magnitude of the cooperativity between the modulator and each orthosteric probe. d.p.m., disintegrations per minute; [*N*-benzylbrucine], concentration of *N*-benzylbrucine (M). Redrawn with permission from REF. 41 © (1998) American Society for Pharmacology and Experimental Therapeutics (ASPET).

discovery programmes that target one type of compound (for example, agonist, antagonist or modulator), while still allowing for the maximal detection of allosteric effects. The obvious advantages of functional assays are a measurable physiological end point, the ability to detect inverse agonists and, importantly, the ability to detect allosteric agonists as well as allosteric enhancers or antagonists that do not affect orthosteric binding. Potential disadvantages of the functional assays include a higher endogenous hit rate — that is, the activation of non-target receptors or non-receptor signalling mechanisms — and a slightly less sensitive detection window for antagonist ligands[53]. However, these disadvantages can be offset in many instances by the use of radioligand binding assays as secondary screens, provided that the probe dependence of allosteric interactions is considered in the experimental design.

Quantifying allosteric effects

Once an allosteric effect is detected, there are several methods available for quantifying the interaction. A detailed discussion of these methods is beyond the scope of this review, but they are generally based on the TCM and its variants as applied to allosteric phenomena. For example, equilibrium binding data can often be fitted directly to EQN 1 to derive the relevant affinity and cooperativity values that define the interaction[13,14,49,54]. Functional data can be analysed in terms of the TCM by using a variation of the classic null method of Arunlakshana and Schild[55], which is adapted for allosteric effects[13], as well as models that are based on the analysis of combinations of orthosteric and allosteric ligands[56–58]. In general, most of these methods are not always amenable to data that are generated from routine screening assays, as they require larger data sets to accurately define model parameters.

One example of an analytical procedure that is specifically applicable to the routine screening of allosteric modulators is the determination of 'affinity ratios'[14]. This procedure was developed to detect and provide semi-quantitative estimates of allosteric cooperativity from equilibrium binding assays, especially in instances in which an allosteric agent is tested against an unlabelled orthosteric probe, such as an endogenous agonist, in the presence of a second, radiolabelled orthosteric probe, such as a high-affinity antagonist. These types of combination experiment are often necessary because of the lack of availability of a radiolabelled version of a particular orthosteric probe (for example, an endogenous agonist); however, they can yield complex binding curves that are not easy to interpret. Affinity-ratio methodology offers the advantages of allowing these types of screening assay with minimal data points, as well as being graphically versatile and amenable to a semi-quantitative analysis by simple visual inspection of the data[14,49]. In essence, the procedure involves determining the ratio of the apparent affinities of an orthosteric probe (radiolabelled or unlabelled) in the absence and presence of a fixed concentration of modulator. FIG. 5 illustrates the application of this approach to the interaction between the allosteric modulator *N*-benzylbrucine (NBB) and the orthosteric radioligand [^{3}H]*N*-methylscopolamine ([^{3}H]NMS), in the absence or presence of the unlabelled orthosteric probe acetylcholine. Although NBB exerts positive cooperativity with [^{3}H]NMS and negative cooperativity with acetylcholine, this is not readily evident in the raw binding data, which only show the changes in [^{3}H]NMS binding. However, conversion of the data to affinity ratios clearly shows the effects of NBB on either ligand; the direction of the change indicates the type of cooperativity and the midpoint of the curves yields an estimate of the affinity of the allosteric modulator for the probe-occupied receptor.

The usefulness of radioligand dissociation rate assays to detect allosteric modulators has already been mentioned in the preceding section. Radioligand dissociation is an exponential process, and often the rate of dissociation as a function of time can be described by the simple mono-exponential relationship below:

$$B_t = B_0 \exp(-k_{off} t) \quad (3)$$

where B_t denotes specific radioligand binding at time t, B_0 denotes specific radioligand binding at time $t = 0$, and k_{off} denotes the radioligand dissociation rate constant. In the presence of an allosteric modulator, the value for k_{off} can change, with dissociation either being enhanced[16,59] or retarded[57,60,61]. If the kinetics of binding of the allosteric modulator to the allosteric site are more rapid than those of the orthosteric probe to its site, then the radioligand dissociation kinetic curves will remain mono-exponential for all concentrations of modulator[14]. Theoretically, this means that the determination of radioligand dissociation at two time points (that is, B_0 and one other time) is sufficient to estimate the apparent k_{off} value, both in the absence

and presence of modulator, according to EQN 3 above[62]. So, the use of such 'two-point' kinetic assays to determine radioligand k_{off} values in the absence and presence of a test compound is often sufficient to detect allosteric mechanisms, provided that other binding artefacts that can yield similar effects are excluded[49]. Another advantage of dissociation kinetic assays is that they can also be used to quantify the interaction and to independently check results obtained from equilibrium binding assays. For example, by carrying out these experiments for a range of modulator concentrations, the resulting relationship between modulator concentration and effect on the radioligand k_{off} can be used to derive a measure of the affinity of the allosteric modulator for the radioligand-occupied receptor, which can then be compared with values derived from TCM analysis of data obtained using equilibrium binding assays[14,49].

Concluding remarks

Allosteric interactions represent a mechanism by which small molecules can exert profound effects on large proteins. The structure–activity relationships that govern classic orthosteric effects do not apply to allosteric sites, and so a greater degree of flexibility becomes available with respect to the development of receptor-subtype-selective ligands that target allosteric sites. Given that most ideal therapeutic agents are small molecules, drug discovery programmes should not discount the search for allosteric ligands as lead compounds. Furthermore, the potential advantages of allosteric modulators can lead to novel therapeutic regimens that provide maximal benefit with minimal side effects. The current paucity of clinically available allosteric modulators, especially for GPCRs, most probably reflects the historical bias of drug discovery towards orthosteric ligands, but it is now evident that newer methodologies that are based on high-throughput functional screening can detect small molecules that modulate receptor function through allosteric effects. Allosteric enhancers of cell-surface receptors should be suitable for most systems in which orthosteric agonism is desired, but especially in situations in which enhancement of the endogenous signal is the preferred therapeutic outcome, such as in the treatment of neurodegenerative disorders. The use of allosteric inhibitors could be an attractive approach if antagonism is required but needs to be titrated to avoid excessive inhibition, as is the case for dopamine antagonists in schizophrenia and adrenoceptor antagonists in hypertension. Provided that drug discovery programmes recognise and accommodate the nuances that are involved in detecting allosteric effects, the search for allosteric modulators of cell-surface receptors could become a routine feature of drug discovery in the new millennium.

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