GPCR Allosterism and Accessory Proteins: New Insights into Drug Discovery


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Abstract
Allosteric modulators of G-protein-coupled receptors interact with binding sites that are topographically distinct from the orthosteric site recognized by the receptor’s endogenous agonist. Allosteric ligands offer a number of advantages over orthosteric drugs, including the potential for greater receptor subtype selectivity and a more ‘physiological’ regulation of receptor activity. However, the manifestations of allosterism at G-protein-coupled receptors are quite varied, and significant challenges remain for the optimization of screening methods to ensure the routine detection and validation of allosteric ligands.

Introduction
The GPCRs (G-protein-coupled receptors) are the largest receptor superfamily and are extremely tractable drug targets. Traditional approaches to drug discovery at GPCRs focus on mimicking or inhibiting the actions of the endogenous agonist for a target receptor, usually by exploiting the structure–activity relationships associated with the binding site of that agonist [1]. Although this approach has yielded a number of blockbuster drugs, there remain situations where the development of therapeutically useful GPCR ligands is hampered due to undesirable side-effects or lack of acceptable selectivity.

Allosteric modulation of protein function has long been recognized as an important mechanism by which the activity of a protein can be regulated by ligands binding to domains that are topographically distinct from the active site of the protein [2]. Although GPCRs are well known to possess an allosteric binding site for G-proteins, the realization that they possess additional allosteric sites for small molecule ligands has only recently gained some prominence. This shift in thinking reflects an ongoing need for attaining greater GPCR–ligand selectivity and/or efficacy, and the move towards functional HTS (high-throughput screening), which has resulted in the detection of a wider array of biologically active molecules. Allosteric modulators of GPCRs represent a potentially rich region of untapped therapeutic chemical space, but a full appreciation of the effects such ligands mediate and the best means of screening for modulators are still areas of significant challenge.

Concepts and terminology
Some of the difficulty in reconciling experimental observations with allosteric mechanisms of drug action relates to the word ‘allosteric’, which has been used to describe interactions between multiple binding ‘sites’ on a given protein as well as multiple conformational ‘states’ that the protein can adopt [2–5]. For this review, the most recent NC-IUPHAR nomenclature recommendations [6] will be used. Thus the site to which the endogenous agonist binds will be referred to as the ‘orthosteric’ site; an ‘allosteric’ site is defined as any domain on the GPCR that is topographically distinct from the orthosteric site, such that the GPCR can

Key words: allosteric interaction, drug discovery, G-protein-coupled receptor, kinetics, ternary complex model.

Abbreviations used: ATCM, the allosteric ternary complex model; GPCR, G-protein-coupled receptor; HTS, high-throughput screening.

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concomitantly bind both orthosteric and allosteric ligands. The two binding sites are conformationally linked, such that binding to one site can change the nature and extent of binding to (or signaling via) the other site; this is referred to as an ‘allosteric interaction’.

**Allosteric models of GPCR drug action**

The binding of an allosteric ligand to a GPCR will change the conformation of the receptor. In theory, this can lead to a variety of functional consequences. For instance, the resulting conformation can exhibit altered binding properties towards orthosteric ligand, altered signalling properties in the absence and/or presence of orthosteric ligand or a combination of both. Herein lies the greatest challenge to drug discovery based on allosteric modulators, namely the ability to ascribe and to validate an allosteric mechanism of action. In this regard, the use of mechanistic models of ligand–receptor interaction is indispensable. The simplest manifestation of an allosteric interaction occurs when the binding of the allosteric modulator either enhances or inhibits the affinity of the orthosteric ligand for the receptor [5,7–9]; in the absence of orthosteric ligand, the modulator is assumed not to mediate any effect in its own right. This simple mechanism is referred to as the ATCM (allosteric ternary complex model; Figure 1). In this model, the binding of orthosteric and allosteric ligands to the receptor is governed by the concentration of each ligand, their equilibrium dissociation constants and an additional parameter, the ‘co-operativity factor’ α, which describes the magnitude of the allosteric change in ligand affinity that occurs between the two sites when they are concomitantly bound. Since the affinity of a given ligand for its receptor is determined by the ratio of its association to dissociation rates, a molecular mechanism for the effects of allosteric ligands that behave according to the ATCM is the modulation of orthosteric ligand association and/or dissociation [5,10]. In terms of drug discovery, structure–activity studies of allosteric modulators need to focus on both modulator affinity and co-operativity, as these parameters are not correlated [11]. There are a number of advantages in using allosteric modulators as preferred therapeutic agents over classic orthosteric ligands. For example, GPCR allosteric binding sites have not faced the same evolutionary pressure as orthosteric sites to accommodate an endogenous ligand [1]. Therefore greater GPCR selectivity may be obtained by targeting allosteric sites, as exemplified in numerous studies of the family A muscarinic acetylcholine and adenosine GPCRs [12,13], as well as more recent examples using Family C GPCRs [14–18]. Subtype selectivity with allosteric modulators can also be obtained by manipulating the co-operativity between orthosteric and allosteric sites rather than by ligand affinity [19]. An additional advantage of allosteric modulators that behave according to the ATCM is that the normal spatial and temporal pattern of neurohumoral signal generation and termination is preserved [5,20]. A third advantage of these modulators is a decreased potential for toxic effects, since modulators with limited co-operativity will have a ceiling level to their effect, irrespective of the administered dose.

It should be noted, however, that the increased focus on discovering more selective modulators of GPCRs has led to the identification of ligands that do not appear to behave according to the ATCM. In some instances, such observations are simply due to modulator-specific experimental artifacts (see the next section). In other cases, these observations indicate a more complex mechanism of action. For example, allosteric ligands have been identified that modify the signalling properties of the receptor in addition to, or instead of, affecting orthosteric ligand affinity [14,15,21–26]. Perhaps most disconcerting is the apparent system dependence of this phenomenon for some modulators. As shown in Figure 2, the prototypical allosteric modulator of muscarinic M2 acetylcholine receptors, gallamine, behaves as a simple antagonist of acetylcholine when tested in most systems; yet, under certain experimental conditions, it can reveal allosteric agonist-like properties.

Findings such as those described above have necessitated extensions to the ATCM to accommodate modulator effects on efficacy as well as affinity [5,27,28]. Unfortunately, there may be additional allosteric ligands that are not accommodated by any of the current ternary complex model variants [29], highlighting the need for further studies on the molecular mechanisms underlying the allosteric modulation of GPCRs.

**Screening and validating allosteric ligands at GPCRs**

One of the most significant developments in GPCR-based HTS within the last decade has been the adoption of cell-based functional assays over radioligand binding as the primary screening method [30,31]. This shift has had both positive and negative effects on the detection and validation of GPCR allosteric modulators.

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**Figure 1** | An allosteric ternary complex model

Orthosteric ligand, A, and allosteric modulator, B, interact with the unoccupied receptor, R, according to their equilibrium dissociation constants, $K_A$ and $K_B$ respectively. The symbol $\alpha$ denotes the magnitude by which the affinity of each ligand is modified by the concomitant binding of the other.
Allosteric modulators can affect affinity or efficacy, such approaches are only as successful as the conformity of either orthosteric or allosteric mechanisms of action [5], but use of pharmacological null methods can certainly support directly determining where a ligand binds on the receptor. The site. To our knowledge, no functional assay is capable of modulator binding to a domain other than the orthosteric is the ability to demonstrate that it is mediated through modulator’s effect. The key to validating an allosteric effect in validating the receptor-based nature of an allosteric of choice in screening for allosteric ligands is the difficulty major disadvantage in using functional assays as the method that modify receptor activity in their own right [31]. The in independent of any effect on affinity, as well as the modulators that alter orthosteric ligand efficacy almost the overall time required for attaining equilibrium [37]. For ligand dissociation by an allosteric modulator will increase ‘equilibrium’ conditions. A slowing of orthosteric radio-ligand binding assays that are ostensibly conducted under ‘equilibrium’ conditions. A slowing of orthosteric radio-ligand binding assays has the advantage of significantly. The ability of many allosteric modulators to directly validating whether a test compound is acting allosterically. The most straightforward approach, of course, is to label an established, high-affinity allosteric ligand and directly monitor its interaction with test compounds at the receptor of interest. Although recent examples of the use of radiolabelled allosteric probes have been published [34,35], they remain few; this practice is, therefore, not amenable to the majority of GPCRs known to possess allosteric binding sites. Hence, binding assays based on orthosteric ligands as probes will probably remain a major approach for screening or validating allosteric ligands for some time.

Classic equilibrium binding assays can be useful for detecting and quantifying modulator actions that behave according to the ATCM [9]. However, a disadvantage of these types of experiments is the potential insensitivity in detecting compounds that exhibit low degrees of co-operativity with the radioligand [1]. Since the co-operativity between a given pair of orthosteric and allosteric ligands is dependent on their respective chemical structures [1], changing an orthosteric radioligand to a different structure may lead to a greater (detectable) degree of co-operativity, but this practice must be weighed against time and cost considerations. Similar cost–benefit considerations apply to another approach that has been used successfully in the past to differentiate allosteric from orthosteric ligands, namely the assaying of test compounds in the presence of different concentrations of radioligand [5]. At a minimum, it is suggested that any assay using orthosteric probes to detect allosteric modulators should endeavour to use two kinds of probes, the first being the endogenous orthosteric agonist for the receptor of interest and the second being a high-affinity orthosteric ligand of a different chemical class to the agonist.

An alternative to the equilibrium binding assay is the dissociation kinetic assay [9,10]. This method exploits the fact that a change in the dissociation rate of a pre-equilibrated orthosteric radioligand–receptor complex can only occur if the conformation of that complex is allosterically perturbed, e.g. by a ligand binding to a topographically distinct site on the complex [36]. In addition to being more sensitive than equilibrium assays in detecting receptor conformational changes, dissociation kinetic assays have the advantage of directly validating whether a test compound is acting allosterically. The ability of many allosteric modulators to change the rate of orthosteric ligand kinetics also has significant implications for the design and interpretation of radioligand binding assays that are ostensibly conducted under ‘equilibrium’ conditions. A slowing of orthosteric radioligand dissociation by an allosteric modulator will increase the overall time required for attaining equilibrium [37]. For modulators that slow orthosteric ligand dissociation almost...
Figure 3 | Effects of the allosteric enhancer alcuronium on the binding (A) or dissociation rate $K_{\text{off}}$ (B) of $[^3\text{H}]$N-methylscopolamine at wild-type (○) or Y177A/T423A mutant (○-○) $M_2$ muscarinic acetylcholine receptors, stably expressed in CHO cell membranes; $n = 3$

completely, equilibrium may never be attained within the time course of a typical experiment, leading to complex binding curves [38] (e.g. Figure 3A). Analytical methods have been developed that allow for the determination of ATCM parameters from such 'non-equilibrium' assays [9,38], but it should be noted that such curves can readily be misinterpreted in terms of more complex reaction mechanisms if prior knowledge of the kinetic effects of the modulators is lacking.

Dissociation kinetic assays have also been used to probe for the effects of GPCR mutations on the potency of allosteric modulators [39–41]. Figure 3 illustrates the effects of the allosteric enhancer alcuronium on the binding of the orthosteric antagonist, $[^3\text{H}]$N-methylscopolamine, at wild-type human $M_2$ muscarinic acetylcholine receptors and at a receptor that has the double-point mutation Y177A (Tyr177 $\rightarrow$ Ala) and T423A. The bell-shaped curve in Figure 3(A) reflects the profound slowing effects of high concentrations of modulator on the radioligand equilibrium; as shown in Figure 3(B), the modulator can essentially retard the dissociation of radioligand almost to zero. Both data sets must be globally fitted to a kinetic ATCM [38] to derive individual estimates of modulator affinity and cooperative with $[^3\text{H}]$N-methylscopolamine; this is essential since mutation of the receptor can affect one or both of these properties. Mutation of Y177A, in the second extracellular loop, and T423A, at the top of transmembrane domain VII, has a profound effect on the potency of the modulator, consistent with the hypothesis that prototypical allosteric modulators of the muscarinic acetylcholine receptors utilize attachment points that are more extracellular to the classic orthosteric site [12].

The above discussion has highlighted some of the difficulties inherent in screening and validating allosteric modulator actions at GPCRs. It is unlikely that functional HTS will be discarded in favour of radioligand binding as the primary screening assay of choice, but binding assays will probably remain important secondary assays for validating modulator actions. The dissociation kinetic assay can be particularly informative, both in validating an allosteric mode of action and for probing the structural basis of allosteric modulator effects. However, this approach is currently not widespread enough to gauge how generally applicable it is to all GPCRs.

If the allosteric nature of the modulator cannot be confirmed using the kinetic binding assay, then the only alternative is to demonstrate that the effect of the candidate modulator is absent from cells that lack the target GPCR.

Conclusion

Allosteric modulation of GPCRs promises to yield drugs with potentially greater receptor subtype selectivity, a more 'physiological' signalling profile and a greater efficacy/safety profile. However, this promise is currently offset by significant challenges to modulator-based drug discovery, including the probe dependence of allosteric phenomena and difficulty in validating allosteric effects.

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References


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