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Commentary

Strategies for the identification of allosteric modulators of G-protein-coupled receptors

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ABSTRACT

Once considered a pharmacological curiosity, allosteric modulation of seven transmembrane domain G-protein-coupled receptors (GPCRs) has emerged as a potentially powerful means to affect receptor function for therapeutic purposes. Allosteric modulators, which interact with binding sites topologically distinct from the orthosteric ligand binding sites, can potentially provide improved selectivity and safety, along with maintenance of spatial and temporal aspects of GPCR signaling. Accordingly, drug discovery efforts for GPCRs have increasingly focused on the identification of allosteric modulators. This review is devoted to an examination of the strategies, challenges, and opportunities for high-throughput screening for allosteric modulators of GPCRs, with particular focus on the identification of positive allosteric modulators.

1. Introduction

G-protein-coupled receptors (GPCRs), also known as seven transmembrane domain (7TM) receptors, are the largest family of integral transmembrane proteins and account for roughly 2% (700–800 functional genes) of the human proteome [1]. GPCRs have also proven to be an extremely valuable target class for therapeutic drug discovery and development with at least one-third of all currently marketed small molecule drugs targeting this receptor superfamily [1,2].

GPCRs bind and are activated by a wide variety of structurally diverse environmental stimuli, such as peptides, neurotransmitters, and ions, and mediate a wide array of physiological processes, including neurotransmission, immune and cardiac function, olfaction, taste and vision. Binding of activating (agonist) ligands stabilizes an active receptor conformation which is in turn communicated to intracellular signal transducers including heterotrimeric G proteins, β-arrestins, G-protein-coupled receptor kinases and other receptor interacting proteins [3]. Receptor-activated signal transduction is in turn coupled to the regulation of cellular second messengers such as Ca^{2+} and cAMP, ion channels, and mitogen-activated protein kinase (MAPK) cascades. Endogenous agonists bind to GPCRs at the orthosteric binding site. Classically, GPCR functioning is understood to involve binding of activating (agonist) or inhibitory (antagonist) ligands to this orthosteric binding site. Indeed, the vast majority of marketed medicines that target GPCRs act by interacting with the receptor's orthosteric binding site, and orthosteric site interactions form the foundation of receptor pharmacological theory [4]. However, for the past 40 years it has been appreciated that proteins, whether receptors, channels or enzymes, can be regulated by allosteric binding sites topologically distinct from the orthosteric binding site (for a recent review [5]). Indeed, there is now ample evidence that GPCR signaling in response to endogenous agonists can be modulated by synthetic small molecules that bind to such allosteric binding sites [6–9]. These molecules are termed "allosteric modulators" and can exert positive or negative effects on endogenous ligand signaling. Such modulation of receptor activity can be explained in terms of the ability of the allosteric modulators to stabilize particular receptor conformations that in turn promote or inhibit receptor signaling. The term allosteric (meaning "other site") refers to any chemical that binds to a site other than the orthosteric binding site (defined as the site where the endogenous agonist binds). Allosteric GPCR ligands can either positively or negatively modulate the activity of orthosteric agonists, can exert agonist activity on their own, or can simply bind to the receptor at an allosteric site without influencing receptor activity whatsoever.

The GPCR superfamily has been grouped into different subfamilies on the basis of phylogenetic analyses of primary sequences. One useful classification scheme defines five families,

Abbreviations: GPCR, G-protein-coupled receptor; PAM, positive allosteric modulator; NAM, negative allosteric modulator; SAM, silent allosteric modulator; NAL, neutral allosteric ligand; HTS, high-throughput screening; cAMP, 3′-5′-cyclic adenosine monophosphate; mGluR, metabotropic glutamate receptor; CaSR, calcium-sensing receptor; MAPK, mitogen-activated protein kinase.

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which accommodate most of the known and presumed GPCR proteins. These five families are known by the acronym, GRAFS, which stands for Glutamate, Rhodopsin, Adhesion, Frizzled, and Secretin [10]. The Glutamate Receptor Family, which includes receptors for calcium (Ca^{2+}), glutamate, GABA, and the T1R taste receptors, is characterized by an extremely large N-terminal domain that contains the orthosteric ligand binding pockets. The Rhodopsin Family is typified by the visual photoreceptor, rhodopsin, and includes receptors for diverse ligands such as biogenic amines, fatty acids, and chemokine peptides, as well as olfactory molecules. The Adhesion Receptor Family consists of receptors with seven transmembrane domains and adhesion-like motifs in the N-terminus, such as EGF-like repeats and mucin-like regions. The Frizzled Family includes the 10 frizzled receptors as well as the T2R taste receptors. The Secretin Family receptors bind large peptide ligands, such calcitonin, glucagon and glucagon-like peptides, parathyroid hormone and vasoactive intestinal peptide. It should be noted that G protein coupling and signal transduction for receptors in the Adhesion and Frizzled families remains controversial. Importantly for the subject of this review, allosteric modulators have been described for receptors in the Rhodopsin, Secretin and Glutamate receptor families (Table 1).

1.1. Types of allosteric ligands

There are four "flavors" of allosteric ligands: allosteric antagonists, also known as negative allosteric modulators (NAMs); potentiators, also known as positive allosteric modulators (PAMs); allosteric agonists (ago-allosterics or allo-agonists); and silent allosteric modulators (SAMs).

1.1.1. NAMs

Ligands that bind to an allosteric site of the receptor resulting in inhibition of receptor function are considered NAMs. NAMs produce rightward and/or downward shifts in agonist concentration–response curves. This can result from the NAM decreasing agonist affinity (measured at equilibrium) by stabilizing a lower-affinity receptor conformation, or from the NAM increasing the energy barrier for transition to the active state, or both. The degree of right- or downward shift observed with increasing concentrations of NAM is finite, reaching a maximum as the allosteric site becomes fully occupied with the NAM. This is in contrast to competitive (orthosteric) antagonists, which produce ever greater rightward shifts at increasing concentrations with no theoretical limit, because the orthosteric antagonist directly competes for the agonist binding site. Because NAMs can reduce the affinity (increase in K_D value) of orthosteric ligands by changing receptor conformation, they can reduce ligand binding and therefore resemble competitive antagonists in radioligand binding experiments. However, the two mechanisms can be differentiated by determining the off-rate (k_{off}) of the labeled ligand in the presence and absence of the competitive antagonist or NAM. An antagonist that reduces binding of the labeled ligand through a competitive mechanism should have no effect on k_{off}, whereas a NAM that reduces binding by lowering affinity (increase K_D, defined as k_{off}/k_{on}) will usually increase the k_{off} value.

1.1.2. PAMs

PAMs have three major mechanisms of action: (i) Most GPCR PAMs produce leftward shifts in agonist concentration–response curves. This can result from the PAM increasing agonist affinity (measured at equilibrium), or from the PAM lowering the energy barrier for transition to the active state by stabilizing an intermediate conformation, or both. The degree of leftward shift observed with increasing concentrations of PAM is finite, reaching a maximum as the allosteric site becomes fully occupied with the PAM. (ii) Some PAMs increase the magnitude of signaling that occurs when the receptor is active (i.e., produce upward shifts in the agonist concentration–response curve). This mechanism is less frequently observed for GPCR PAMs than for PAMs of other target classes such as ligand-gated ion channels. Increased maximal efficacy in the presence of a PAM, compared to the maximal efficacy of the orthosteric agonist alone, may occur in a cellular assay system that lacks spare receptors (low receptor reserve).

Table 1
Examples of allosteric modulators of GPCRs.

Receptor	Coupling	Example modulator(s)	Reference(s)
RHODOPSIN FAMILY			
Adenosine A_1–A_3	Gαs, Gαi	PD 81723, LUF 5484, amilorides, VU5455, VU8504, DU124183	[77]
Adrenoceptor α_1, α_2	Gαq/11, Gαi	Amilorides, benzodiazepines, conopeptide ρ-TIA	[78–80]
Adrenoceptor β_2	Gαs	Zinc	[81]
Chemokine CXCR1, CXCR2	Gαi	Repertaxin	[82]
Chemokine CXCR4	Gαi	RSVM, ASLW, Trichosanthin	[83]
Chemokine CCR1, CCR3	Gαi	UCB35625	[84]
Chemokine CCR5	Gαi	AK602, AK530, TAK779, SCH 351125, 873140	[85,86]
Dopamine D_1–D_4	Gαs, Gαq/11	Zinc, L-prolyl-L-leucylglycinamide, amilorides	[87–91]
Glycoprotein hormone Receptors	Gαs, Gαq/11	Thienopyrimidines, pyrazoles, thiazolidinones	[92]
Muscarinic M_1–M_5	Gαs, Gαq/11	Gallamine, alcuronium, brucine, W84, C7/3-phth, WIN 62577, AC-42, thiochrome, MT7, MT3, strychnine, staurosporine, tacrine, McN-A-343	[93–95]
Serotonin $5HT_{2C}$	Gαq/11	PNU-69176E	[96]
SECRETIN FAMILY			
CRF1	Gαs	Antalarmin, NBI 35965, DMP696, NBI 27914	[97]
CGRP		BIBN4096BS	[98]
Glucagon	Gαs	Bay27-9955, L-168049	[97]
GLP1	Gαs	T-0632, quinoxalines	[23]
GLUTAMATE FAMILY			
CaSR	Gαs	Cinacalcet, NPS 467, NPS 568, l-amino acids	[99]
$GABA_B$	Gαi	CGP7930, CGP13501, GS39783	[100,101]
Metabotropic glutamate			
mGluR1	Gαq/11	(-)-CPCCOEt, BAY36-7620, Ro 67-7476, Ro 01-6128	[22,102]
mGluR2	Gαi	LY 487379	[102]
mGluR4	Gαi	(−)–PHCCC	[103]
mGluR5	Gαq/11	MPEP, CPPHA, CDPPB	[22,102–105]
mGluR7	Gαi	AMN082	[106]
mGluR8	Gαi	AZ12216052	[107]

Under these circumstances the endogenous agonist may behave as a partial agonist, and a PAM might then be expected to increase the overall efficacy of the response. (iii) A compound may function as a PAM by blocking desensitization of the receptor. To date, no GPCR PAMs have been reported which act through this type of mechanism. However, there are examples of PAMs of ligand-gated ion channels that exert their effects by blocking desensitization [11–13], and by analogy we may expect that such a mechanism would be possible for GPCR PAMs as well. The above-listed mechanisms are not mutually exclusive, and a given PAM may function through a combination of these actions.

1.1.3. Allosteric agonists

Many allosteric compounds can exhibit agonist activity on their own, in the absence of another agonist [14]. Such compounds are referred to as allosteric agonists. Evidence for allosteric agonism was first documented for the adenosine 2a receptor twenty years ago [15], and additional evidence has accumulated over the years indicating that allosteric modulators can possess agonist activity in their own right [6,16–18]. Some compounds which display PAM activity also display allosteric agonist activity in the absence of an orthosteric agonist. Typically, agonist activity is seen at concentrations higher than those needed for PAM activity to be observed [6,14,16].

1.1.4. SAMs

Finally, a fourth type of allosteric ligand exists: compounds that bind to an allosteric site on the receptor but do not affect receptor function. These compounds are referred to as "silent allosteric modulators" (SAMs; somewhat of a misnomer since they do not modulate receptor activity) or as "neutral allosteric ligands" (NALs). Because SAMs can bind to the same allosteric site as NAMs, PAMs, and allosteric agonists, they have the ability to compete for the allosteric site and thus produce rightward shifts in the concentration–response curves for other allosteric ligands, in a way that is analogous to the effect that neutral orthosteric antagonists have on the concentration–response curves for orthosteric agonists and inverse agonists. While SAMs likely have no therapeutic utility, they can be very useful as research tools. It is becoming increasingly evident that for allosteric drug discovery programs it will be important to adopt testing approaches that have the ability to detect SAMs, both because of their utility as tools and to better inform our understanding of the chemical structure-activity relationship during lead optimization.

It should be noted that the flavor of allosteric ligands is dependent on the specific orthosteric agonist being modulated (probe dependence) [19,20] as well as the functional assay used (functional selectivity) [21]. Therefore, ligands can only be labeled as PAMs, NAMs, SAMs, or allosteric agonists within the context of the assay and orthosteric probe used to assess their activity. For additional discussion of probe dependence and functional selectivity, and their impact on screening for allosteric modulators, please see Section 3.4 and Section 5 below, respectively.

1.2. Potential advantages of allosteric modulators

Allosteric modulators have a number of potential advantages over traditional (orthosteric) agonists or antagonists as therapeutic agents. First, allosteric ligands have the potential to exhibit greater selectivity for receptor subtypes than orthosteric ligands. For example, several of the metabotropic glutamate receptors (mGluRs) 1–8 have long been seen as attractive drug targets from a biology perspective. Because all eight mGluR subtypes have evolved to bind the same ligand, the orthosteric binding site is highly conserved between all mGluRs, which has dramatically hampered attempts to identify selective ligands. However, there is presumably much less evolutionary pressure for conservation of receptor structure outside of the glutamate binding site, resulting in binding pockets that discriminate between receptor subtypes. In fact, allosteric approaches to mGluRs have proven to be very successful in identifying selective compounds [7,22]. Even when selectivity is not an issue, an allosteric site, if present, can offer an opportunity to identify synthetic ligands for a receptor whose orthosteric binding site has proven to be chemically intractable. One example is the GLP-1 receptor, for which small molecule orthosteric agonists have not been identified despite years of research. Examples of small molecule allosteric agonists of GLP-1 have now been reported [23].

In addition to providing practical advantages from the standpoint of drug discovery, allosteric modulators may provide a number of functional advantages over traditional orthosteric agonists and antagonists. NAMs offer a potential advantage over orthosteric antagonists in that it is possible for a NAM to have only partial antagonist activity without exhibiting any degree of agonist activity [24], as opposed to orthosteric antagonists, which can only be partial antagonists to the extent that they are also partial agonists. By limiting the maximal amount of antagonism produced, it is easy to imagine that a partial NAM could exhibit a greater safety index than a full antagonist. PAMs have a potential advantage over direct agonists because they do not exhibit any biological activity in the absence of the endogenous agonist. While PAM activity is typically tested *in vitro* in the presence of an exogenously added orthosteric agonist, the intended clinical use would be for the PAM to be administered alone. Therefore, the drug would be expected to have an effect only when and where the endogenous agonist is present. Although the magnitude of receptor signaling is increased, normal physiological regulation of signaling, including proper temporal regulation, remains unchanged [7]. This is potentially advantageous, for example, for many neurotransmitter systems where the timing and pulsatile nature of signaling is important for proper function. In addition to maintaining the temporal fidelity of physiological signaling patterns, the spatial fidelity would be maintained as well–a PAM would be expected to have an effect only in tissues (and even individual cells) where the native agonist is acting. This is in sharp contrast with a direct agonist, which essentially activates all of its receptors in all tissues in the body. It is easy to imagine that maintaining the tissue specificity of native receptor signaling could in many cases lead to dramatically improved side effect profiles for PAMs over direct-acting agonists. Finally, because activation of the receptor is not constant, as with a direct agonist, it is likely that PAMs may also be free from rapid desensitization and other compensatory mechanisms which can produce tolerance and limit the therapeutic effectiveness of direct agonists.

Currently, there are two marketed drugs that act by targeting GPCR allosteric binding sites. Maraviroc (UK-427857) is a noncompetitive antagonist of the CCR5 chemokine receptor and prevents HIV entry into host cells expressing the CCR5 co-receptor [25]. Maraviroc inhibits the binding of the HIV envelope protein, gp120, to CCR5. Maraviroc also allosterically inhibits the binding of two endogenous chemokine ligands of CCR5, MIP1a and RANTES. The safety profile of maraviroc is quite good, lending credence to the hypothesis that allosteric modulators may exhibit improved safety over their orthosteric counterparts [26]. However, since there are no anti-HIV medicines that target CCR5 orthosterically, a definitive conclusion with respect to the safety of maraviroc is not possible.

Cinacalcet is a positive allosteric modulator of the calcium-sensing receptor (CaSR) and has been approved for reducing parathyroid hormone secretion in primary hyperparathyroidism and in the treatment of secondary hyperparathyroidism resulting from chronic renal failure. Cinacalcet does not bind to the calcium orthosteric binding pocket located in the large extracellular

domain of the CaSR but instead interacts with an allosteric binding site located between the 6th and 7th membrane-spanning segments [27]. Binding of cinacalcet or related phenylalkylamine compounds to the CaSR induces a conformational change in the receptor that results in an increased sensitivity to extracellular calcium [28]. As with maraviroc, cinacalcet appears to be well-tolerated therapeutically, again consistent with the idea of enhanced safety profiles of allosteric agents [29].

2. The operational model of allosterism

Development of appropriate HTS campaigns to identify GPCR PAMs is greatly aided by an understanding of the pharmacological principles that describe receptor activation. The effects of allosteric ligands on receptor systems can best be described by an operational model where experimental data can be applied to obtain useful values which medicinal chemistry can use to track the structure-activity relationship for allosteric ligands in any particular cell system. This model has been reviewed extensively [7,8,19,30] and only its final derivation is written here:

$$E = \frac{Em(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}.$$

In this model, E is the pharmacological effect, and K_A and K_B denote the equilibrium binding constants for the orthosteric ligand, A, and the allosteric ligand, B, at the receptor. The binding cooperativity factor, α, denotes the effect of the allosteric ligand on orthosteric ligand binding affinity, and vice versa. An activation cooperativity factor, β, denotes the effect that the allosteric ligand has on orthosteric agonist efficacy. Agonism constants τ_A and τ_B, represent the intrinsic activity of the orthosteric agonist and any intrinsic activity of the allosteric ligand, respectively, which is dependent on the cell context and receptor expression level of the cell system, and intrinsic efficacy of the ligands used. The remaining parameters, Em and n, denote the maximal responsiveness of the system, and the slope, respectively.

For purposes of allosteric ligand drug development, medicinal chemistry can track a combination of $\alpha \times \beta$ which gives a value that relates to the degree of curve shift that an allosteric ligand will have on the orthosteric agonist concentration–response curve.

3. Screening/Hit identification strategies

Allosteric modulators may or may not have effects on orthosteric ligand equilibrium binding. Therefore, to identify allosteric modulators, functional screening assays are commonly used. For primary high-throughput screening (HTS), the screening method for identifying allosteric ligands can be different depending on the functional assay used, and whether the screen is looking to identify agonists and PAMs or antagonists and NAMs.

3.1. Ca^{2+} flux (dynamic assays)

A popular screening assay measures Ca^{2+} mobilization following phospholipase C activation by $G_{\alpha q/11\alpha}$ or $G_{\beta\gamma}$ subunits [31]. Using either Ca^{2+}-sensitive fluorescent dyes detected by a fluorescence imaging plate reader (FLIPR^{TM}) or flash luminescence readouts (e.g. apo-aequorin), IP_3-induced Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane, can be measured in real-time [32]. The responses are generally very rapid (within a few seconds) and require instrumentation which can detect signal as the compound and cells are mixed in the well. Ca^{2+} mobilization assays can use endogenous receptors or recombinant expression of receptors in cell lines. GPCRs that may not induce ligand-dependent Ca^{2+} mobilization (i.e., G_s and G_i-coupled receptors) can be targeted to the Ca^{2+} mobilization pathway by expression of either promiscuous G proteins ($G_{\alpha 15}$, $G_{\alpha 16}$) or chimeric G proteins (G_{qi5}, G_{qs5}) [33] in the cells.

To identify agonists and positive allosteric modulators (PAMs), Ca^{2+} mobilization assays should be run consecutively, in the same assay plate, first in agonist mode and then in potentiator mode. In agonist mode, cells are exposed to test compounds and the cellular response is measured to characterize any agonist activity present. After an incubation period which allows for any agonist-induced Ca^{2+} increase to return to basal levels, potentiator activity can then be measured, in the same plate, by addition of a low concentration (typically $\sim EC_{20}$ but see Section 4, "HTS challenges", below) of orthosteric agonist. PAMs can be detected as an elevated signal above the EC_{20} background response in the potentiator assay. Antagonists (both NAMs and orthosteric antagonists) can be identified by screening in a similar manner using a higher dose ($\sim EC_{80}$) of orthosteric agonist, and are detected by an inhibition of the agonist response. In both potentiator and antagonist modes, compounds are often pre-incubated with cells for a short time prior to the addition of orthosteric agonist, to allow allosteric ligands with slow on-rates to bind to receptor prior to the addition of orthosteric agonist. Co-addition of orthosteric agonist with test compounds to the cells may lead to false negatives, due to the relatively slow on-rates of allosteric and orthosteric compounds, compared with the orthosteric agonist used and the relatively fast read time of Ca^{2+} flux assays (1–60 s). Some groups have also used a triple addition assay to detect agonists, PAMs and antagonists, all from the same plate, sequentially adding compound, EC_{20} agonist and then EC_{80} agonist [1].

3.2. Challenges with Ca^{2+} flux assays

Dual mode screening for agonists and PAMs in Ca^{2+} flux assays is necessary, because some allosteric ligands can have agonist activity on their own, as well as being allosteric potentiators of the orthosteric agonist. These compounds can be readily detected in the agonist mode portion of the screen. However, the agonist response can result in receptor internalization/desensitization, depletion of the detection reagent, and/or emptying of the Ca^{2+} stores, which in the continued presence of the compound do not re-fill [34]. This can result in a desensitization of the Ca^{2+} response to further challenges with agonists or PAMs. In such cases, when the PAM mode portion of the assay is performed, the response elicited by the addition of a low concentration (EC_{10}–EC_{20}) of orthosteric agonist, in the presence of the PAM, may be attenuated, resulting in false negatives. Allosteric ligands with dual agonist and PAM activities are often more potent as potentiators of the orthosteric agonist than as agonists. This can result in bell-shaped concentration–response curves when the compound is dosed against a single concentration of agonist (EC_{10} - EC_{20}) in potentiator mode. An example of this is shown in Fig. 1. Screening of compounds at a single concentration (usually around 10 μM) in potentiator mode alone may miss compounds which have dual agonist and PAM behaviors. It has been our experience that compounds displaying such dual activity are quite common [1,6,14,16]. Hits identified in such dual-mode Ca^{2+} flux assays can be deconvoluted by performing separate agonist mode and PAM mode assays during hit confirmation.

For antagonist screens, any agonists or Ca^{2+}-mobilizing compounds pre-incubated with cells may appear as hits in antagonist mode (desensitizing the receptor and/or Ca^{2+} response when an EC_{80} agonist is added), resulting in hits which are false positives. It should be noted that antagonist screens can be run in a single antagonist mode (without the agonist mode portion), because agonists will be identified as false positives rather than

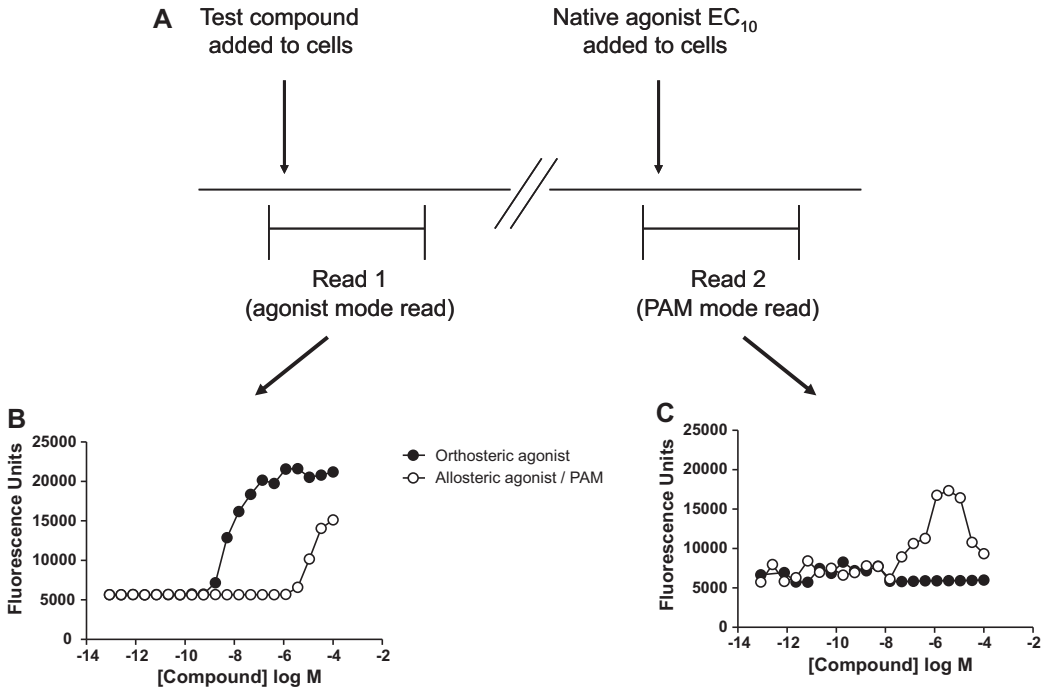

Fig. 1. Dual mode screening for allosteric agonists and PAMs in Ca^{2+} flux assays. The top panel (A) shows a schematic representation of the time-course of a "dual mode" Ca^{2+} flux assay designed to detect both agonists and PAMs. Panels (B) and (C) show concentration–response curves for an orthosteric agonist (filled symbols) and for an allosteric modulator that has both PAM activity and allosteric agonist activity (open symbols), tested using the assay format depicted in panel (A). Peak Ca^{2+} responses are measured in cells expressing the GPCR target of interest; first upon addition of the test compound (agonist mode, panel B), and again upon subsequent addition of the native agonist at its EC_{10}, to the same wells, several minutes later (PAM mode, panel C). Agonist responses can be measured immediately upon compound addition (agonist mode read, panel B), but are transient and are no longer detected during the PAM mode read (panel C). The allosteric compound (open symbols) has intrinsic agonist activity at concentrations >1 μM (panel B); while at lower (30 nM–1 μM) concentrations it has no agonist activity on its own, but potentiates the EC_{10} response to the native agonist (panel C). The agonist response that occurs immediately upon the addition of this compound at concentrations >1 μM desensitizes the subsequent Ca^{2+} response seen when the EC_{10} of the native agonist is added. Therefore, a bell-shaped curve is observed in PAM mode–the measured PAM response (panel C, open symbols) decreases at those concentrations where the compound exhibits agonist activity (panel B). This phenomenon has critical implications for single-point compound screening. If compounds were tested at 30 μM or higher in PAM mode only, the allosteric compound shown would be missed. Therefore, it is important to include an agonist mode read in addition to a PAM mode read when screening for PAMs.

false negatives. In this scenario, hits can be assessed for agonist activity at either the retest or concentration–response curve stage of the HTS.

3.3. Accumulation assays

Other popular functional assays for GPCRs are homogeneous second messenger accumulation assays detecting cAMP or IP_1, which measure the product of adenylyl cyclase and phospholipase C activity, respectively [31]. Cells are stimulated with test compounds for a defined period (usually 15 min–2 h), followed by cell lysis and detection of the second messengers.

A potential advantage of accumulation assays over Ca^{2+} flux assays is that they can identify both orthosteric and allosteric ligands in a single addition screening mode, i.e. either in the presence of an EC_{20} of orthosteric agonist (for agonists/PAMs) or an EC_{80} of orthosteric agonist (for antagonists), as long as the activity of compounds exceeds the noise of the assay. As with the Ca^{2+} flux assay described in the preceding sections, the test compounds should be allowed to bind to the receptor prior to the addition of orthosteric agonist. If compounds which are allosteric modulators have slow on-rates then it is conceivable that their effects will not be present during the early phase of functional activity generated by the co-addition of the orthosteric agonist. Also, the agonist response may quickly desensitize the receptor so that slow on-rate modulators have no apparent effect. Agonist and PAM activity can be deconvoluted at a later stage by running the assays in the absence and presence of an EC_{20} of orthosteric agonist, or by "curve shift" assays which measure the leftward movement of the orthosteric agonist concentration–response curve in the presence of increasing concentrations of the test compound.

A hypothesis that has yet to be validated is the concept that allosteric modulators may be identified more readily in dynamic screening assays as opposed to accumulation assays. For example, a PAM of the GLP1 receptor was identified through an assay coupling this G_s-coupled GPCR to Ca^{2+} via promiscuous G proteins in a dynamic FLIPR assay. However, the PAM activity was not observed in a cAMP accumulation assay [35]. Interestingly, a dynamic cAMP assay using a biosensor was able to identify the PAM activity. A proposed explanation for this finding is that if the PAM response has an activity that is transient, then, in an accumulation assay the fraction of the PAM response compared to the total accumulated response over time is small, and may not be significantly above the background of the assay. However, by following a dynamic assay the peak activity of the PAM would be detected. Again, to date there is no truly compelling evidence to support this hypothesis, and there are a number of other possible explanations for the above observations. Certainly, many screens utilizing accumulation assays have successfully yielded PAMs. It is likely that the optimal format for any screen is target-dependent.

3.4. Functional selectivity and probe dependence

It is now apparent that various agonists acting through the same receptor can induce functionally distinct conformations that differ not only in the magnitude of the functional response produced (efficacy), but also the relative degree to which different functional pathways are activated. This phenomenon has been

termed functional selectivity, biased agonism, ligand-directed trafficking of receptor stimulus, or protean agonism [36–38]. HTS campaigns are often executed early in drug discovery programs, when the therapeutically relevant signaling mechanism may not be fully elucidated. This poses significant challenges when screening for orthosteric and allosteric ligands, as more often than not, a single functional pathway is assayed. This concept has also created confusion in labeling of ligands as agonist, antagonists, etc. For example, propranolol is a β_2-adrenergic receptor antagonist of G protein-mediated cAMP accumulation, but an agonist of β-arrestin recruitment and activation of ERK1/2 MAP kinases via the same receptor [39].

Like orthosteric agonists, allosteric ligands can show differential activity depending on the functional readout that is assayed [40,41]. Therefore, labeling ligands in terms of their pharmacological properties (e.g., allo-agonists, PAMs, NAMs or SAMs) should only be done in the context of the functional assay described [40]. Furthermore, the functional behavior of allosteric modulators can depend not only on the functional endpoint which is measured, but also vary significantly based on what orthosteric agonist is used. A given allosteric ligand may potentiate the activity of one orthosteric agonist while having no effect or even reducing the activity of a different agonist (e.g., [19,20]). This phenomenon is referred to as probe dependence. Because of the potential for the effects of allosteric compounds to be probe-dependent, when screening in antagonist or PAM mode it is important to use the endogenous (and therefore physiologically relevant) agonist whenever possible. This can add an additional layer of challenge and complexity for GPCR targets that may have more than one endogenous activator, or for orphan GPCR targets whose native agonist is unknown.

3.5. Selection of a ligand library

Selection of the compound set to be screened is obviously an important part of any HTS strategy. Assaying one's entire compound collection ensures coverage of the entire range of chemical diversity thus far assembled, and is usually preferred as it provides the richest information for a discovery program. Yet there are situations where such a "full deck" screen is not practical, such as when resources are limited or for elucidation of signaling pathways. To this end, compound libraries of known GPCR ligands are commercially available, including natural product libraries as well as synthetic molecules. However, these libraries currently are primarily built around orthosteric GPCR ligands, and there is no *a priori* reason to assume that these libraries will be at all enriched with regard to allosteric GPCR ligands. In fact, it has been our experience that, while such focused libraries are indeed useful for the identification of orthosteric GPCR ligands, they are no better than random libraries when looking for allosteric compounds.

Natural product extracts have historically been a rich source for the discovery of therapeutically useful drugs [42]. Interestingly, of the allosteric modulators listed in Table 1, at least seven are natural products (conopeptide ρ-TIA, zinc, trichosanthin, brucine, strychnine, staurosporine, and l-amino acids). In addition, salicylate, oleamide, and cannabidiol have been reported to be allosteric modulators of endothelin, serotonin and opioid receptors, respectively [43–45]. This suggests that structurally diverse natural products libraries may in fact be good starting points for allosteric modulator discovery.

There is some question as to whether an "allosteric GPCR" focused screening deck approach has much potential. One of the advantages of targeting allosteric sites on GPCRs is that greater receptor selectivity can be achieved based on the fact these sites are not likely to be as evolutionarily conserved as orthosteric ligand binding sites. Therefore, one might expect compounds that bind to allosteric sites on GPCRs to be much more chemically diverse than orthosteric GPCR ligands. However, it is also possible that there are certain chemical features that make a given compound more likely to function as an allosteric modulator. As more and more allosteric GPCR ligands are discovered and reported, we can be certain that pharmaceutical companies will empirically test the utility of this strategy by using a computational chemistry approach to design screening libraries intended to be enriched in allosteric GPCR modulators. Within the next few years we will likely gain a clearer understanding of whether an allosteric GPCR focused screening approach is useful or not.

While the utility of generic GPCR allosteric focused screening libraries remains questionable, there is no question that computational chemistry approaches can be very successful for identifying allosteric modulators of a specific known receptor [46], and both homology-based and structure-based *in silico* screening approaches have already proven successful for the identification of allosteric GPCR ligands (see also Section 6.4, below).

4. HTS challenges

If known allosteric modulators are available for a target, these can be used to validate and optimize the HTS assay. For instance, a known PAM can be used to fine-tune the concentration of orthosteric agonist used so that the window between the orthosteric agonist response in the presence and absence of PAM can be maximized empirically. Also, using a maximally effective concentration of a control PAM to represent 100% activity in the assay can help to provide a realistic assessment of signal window and Z', a parameter used to quantify the quality of an assay designed for a high-throughput screen [47], for detecting PAMs.

One of the main challenges for detecting allosteric ligands in HTS is that the functional responses that allosteric ligands exert in the presence of orthosteric agonist can be relatively small when compared with compounds that are full agonists and antagonists. Allosteric ligands typically exert functional effects by either shifting the orthosteric agonist concentration–response curve to the left or right, depending on the modulator's effects on orthosteric agonist affinity and efficacy, or by having intrinsic efficacy in the absence of orthosteric agonist. Depending on the magnitude of the curve shift that it produces, the maximal signal generated by an allosteric ligand can be smaller than the signal that can be observed with traditional orthosteric agonist and antagonist assay modes of screening. As can be seen in Fig. 2, a PAM that generates a 10-fold curve-shift of the orthosteric agonist is capable of generating only about 50% of the available response that is seen with a maximally effective concentration of an orthosteric full agonist when the slope of the curve is unity (Fig. 2A–C).

Fig. 2 further illustrates the relationship between the response generated by the orthosteric agonist concentration used and responses generated for PAMs that result in a 2, 3, 5 and 10-fold curve shift of the agonist response curve. Maximal PAM effects are observed using between an EC_{20}–EC_{40} of orthosteric agonist. However, changes in the slopes of agonist curves can have dramatic effects on the response window for identifying PAMs (Fig. 2D–I). Slopes of greater than unity have sometimes been observed for agonist curves in Ca^{2+} mobilization assays, depending on the receptor, cell line and agonist used.

Consistently adding a concentration of agonist that is equivalent to an EC_{20} is not always straightforward. Day-to-day or run-to-run variability in the response observed at a given concentration of orthosteric agonist can have large implications in an assays' ability to identifying PAMs above the activity cut-off of the screen. This concept is expanded upon in Fig. 3.

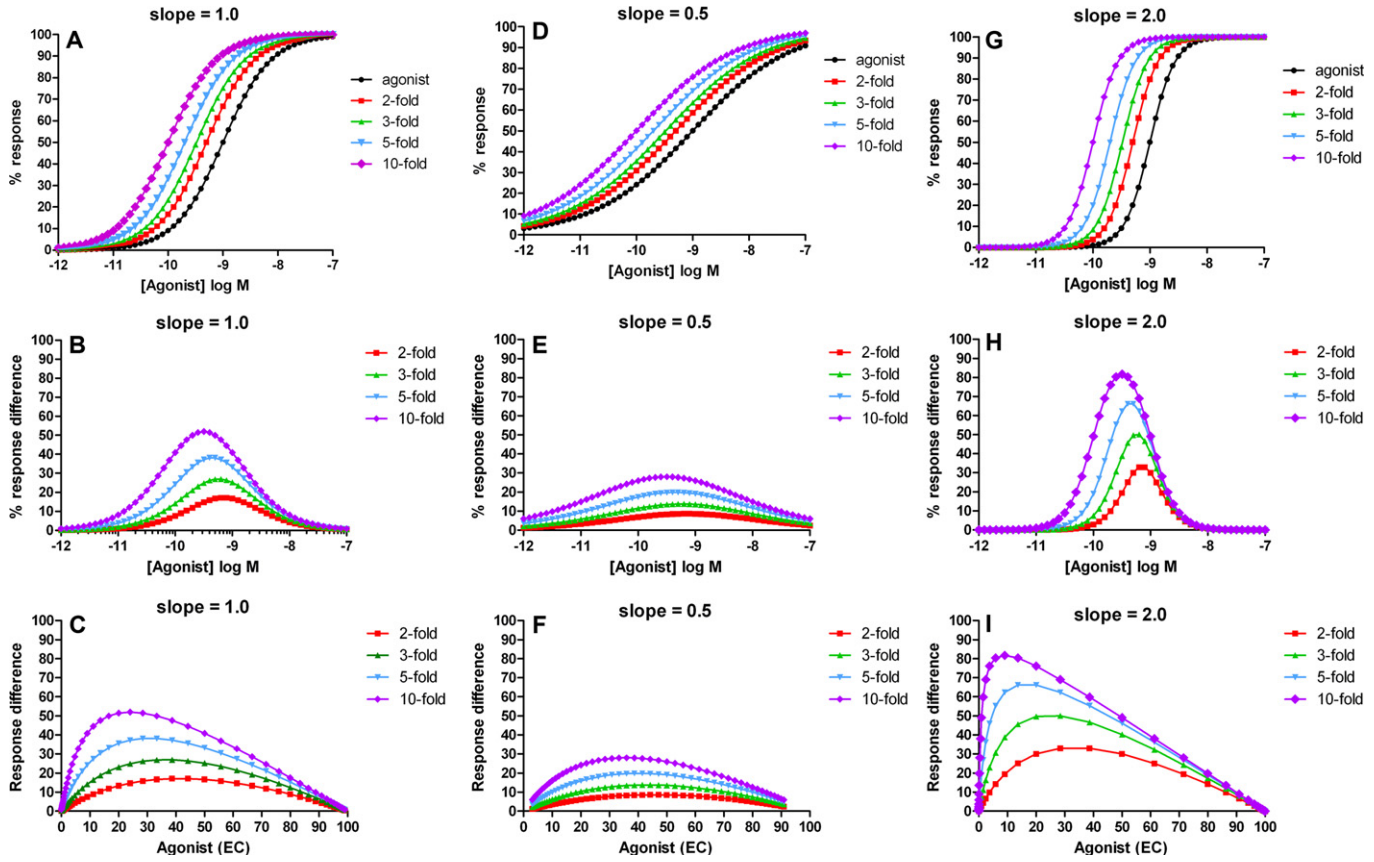

Fig. 2. Relationship between orthosteric agonist concentration and the degree of curve shift induced by PAMs. Positive allosteric modulators (PAMs) can increase the sensitivity of orthosteric agonist-mediated responses, resulting in a leftward shifting of the orthosteric agonist CRC (for reviews see [6–8]). They do this by increasing affinity and/or efficacy of the orthosteric agonist at the receptor. The degree of PAM activity or "fold shift" that is required therapeutically is not currently well understood, and will likely be target-specific. In screening assays, the effects of compounds on orthosteric agonist curves can be used to assess PAM activity. However, measuring orthosteric agonist "curve shifts" requires multiple wells per compound screened and is expensive for HTS. Instead, HTS screening assays typically incubate a single concentration of compound with a single concentration of orthosteric agonist in a single well. Panel (A) shows the curve shifts produced by each of four hypothetical PAMs which cause 2, 3, 5 and 10-fold leftward shifts of the orthosteric agonist CRC, respectively. In this simulation, the orthosteric agonist has a maximal response of 100%, an EC_{50} of 1 nM and a slope of unity. Panel (B) shows the difference in response between the screening concentration of orthosteric agonist used (*x*-axis) and the response in the presence of the PAM. Panel (C) shows the same response change data as a function of the orthosteric agonist effective concentration (EC) that was used. From these data, it can be seen that a range of orthosteric agonist concentrations from an EC_{20} to an EC_{40} provides the best window for screening for PAM activity. For an HTS to effectively capture as many PAM hits as possible (including PAMs that produce a two-fold curve shift) an agonist concentration closer to an EC_{40} would theoretically be optimal. In reality, the concentration of orthosteric agonist used in the potentiator mode screen will depend on the response window and Z' that can be produced and the cut-off response used to select hits. Because of these considerations, functional screening using an orthosteric agonist concentration greater than an EC_{20} is not always practical. In Panels (D–F) and (G–I), the Hill slope of the orthosteric response has been changed to 0.5 and 2, respectively. With steeper curves, a lower concentration of orthosteric agonist may be optimal (i.e., EC_{10}–EC_{30}). In the case of more shallow curves, a higher concentration of orthosteric agonist should be used (i.e., EC_{30}–EC_{50}). As can be seen in the Figure, functional assays with slopes >1 may provide better signal windows for detection of PAMs.

5. Follow up/Hit assessment

Following primary screening, hits are retested in duplicate or triplicate at the screening concentration used for the primary screen, or at multiple concentrations (depending on the number of hits identified). Confirmed hits are then assayed in concentration–response assays to determine potency and intrinsic activity of the compounds with the cell system used. It is also common at this point to assess the target specificity of the compound responses. This is done by evaluating compound activity in the parental cell line lacking recombinant expression of the target of interest. For an agonist screen, compounds are incubated with cells and cellular responses determined. If the parental cell line and cell line expressing recombinant target receptor produce a similar activity to the compound, then the hits are segregated out as being potentially non-target-mediated. Defining the target specificity of allosteric modulators or antagonists requires additional steps. For orthosteric antagonists and NAMs, a response must be elicited in the parental cell line to evaluate the inhibition of that signal by the hits. This can be done by using an agonist to a different GPCR that is either endogenously expressed in the parental cell-line, or to a different receptor recombinantly expressed in the same cell background, which couples through the same second messenger pathway. A similar process can be used for assessing the receptor specificity of PAMs. Following confirmation of target specificity, target-specific hits can then be assessed in concentration–response assays. In the case of PAMs, "potentiator efficacy" can also be assessed by measuring the magnitude of the curve shift produced for the orthosteric agonist, in the presence of multiple concentrations of the hit compound.

Further hit assessment can be carried out by using additional pharmacological tools such as orthosteric or allosteric radioligand binding (if such tools exist) to verify that binding occurs at the target receptor. However, radioligand binding assays are typically not available for allosteric sites, and determining that a compound is acting directly at the receptor target is not always straightforward. This is particularly true for endogenously expressed receptors, where no parental cell-line counterscreen can be used to determine whether activity is observed in the absence of receptor.

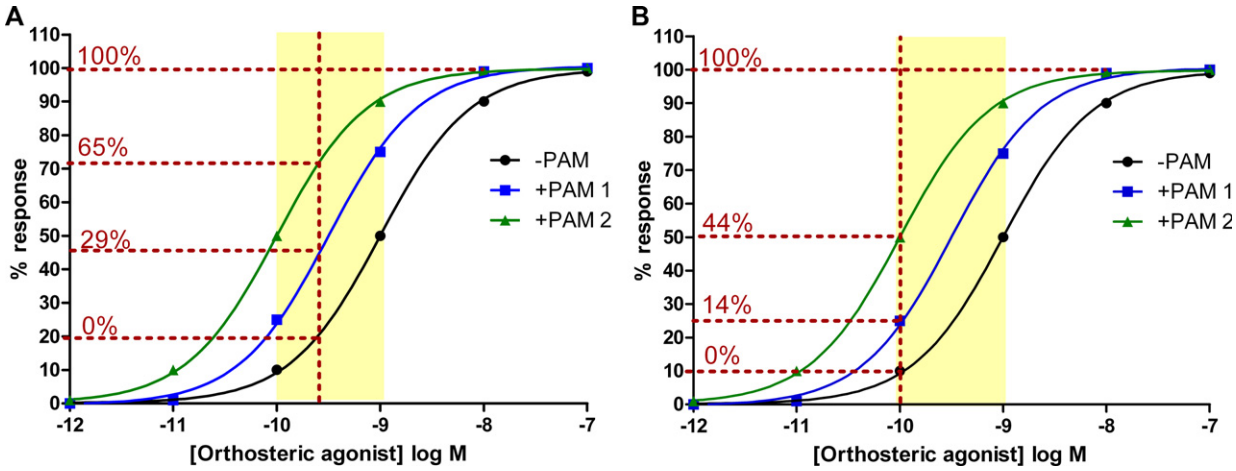

Fig. 3. Predicted activity for PAMs in a screening campaign using an EC_{20} or EC_{10} of orthosteric agonist. Modeled orthosteric agonist response curves produced by PAM compounds that generate 3- and 10-fold (PAM1 and PAM2, respectively) leftward shifts in the orthosteric agonist response curve (−PAM). Red numbering along the *Y*-axis represents normalized agonist activity in which 0% and 100% are defined as the responses produced by the low concentration of orthosteric agonist used in the potentiator mode assay alone, and the maximum response produced by the orthosteric agonist, respectively. By comparing the activity values for the PAM responses with the % activity cut-off for the primary screen, one can predict how much of a curve shift is required to detect a PAM hit above the background noise of the screen when using an EC_{20} (A) vs. an EC_{10} (B) of agonist. In this example, when using an EC_{20} of agonist (A), PAMs that generate a 3-fold and 10-fold curve shift would be expected to produce a response of 29% and 65% normalized activity (in red), respectively. If the primary screening cut-off were 30% normalized activity, then PAMs producing a curve shift of three-fold or less would likely be missed in the screen. Variability of the response to the orthosteric agonist concentration used (either over the time period of the run, or day-to-day variability) will also affect the % activity values generated for the PAMs. If the low agonist concentration used resulted in an EC_{10} response (B), then the normalized activity cut-off to detect a compound that produces a three-fold shift in the agonist curve would need to be <14%. This additional challenge suggests that screening cut-offs should be calculated on a run-by-run (or even plate-by-plate) basis, dependent on the actual response elicited by the concentration of orthosteric agonist.

SAMs can be particularly useful tools for evaluating the receptor specificity of compound effects, because they can block the activity of NAMs, PAMs, or allosteric agonists, while exerting no effects on their own. However, like allosteric radioligands, known SAMs are not available for the vast majority of GPCR targets, and most drug discovery programs will not have the luxury of using SAM tool compounds unless they are discovered internally. Discovery of SAM compounds presents its own set of challenges, because on their own they exert no effects in the functional assays typically employed in allosteric GPCR drug discovery programs.

Compound effects in mutated receptors (mutated either at the orthosteric or known allosteric binding pockets) can be assessed, giving additional information about where the ligands bind to the receptor. Mutational data must be interpreted with caution, however, as many mutations have been observed to affect receptor signaling in unpredicted ways, including affecting the binding and/or activity of compounds that bind to sites distal to the mutation. Even more confusing, the effects observed are often ligand-dependent: two different ligands binding to the same allosteric site can be affected in dramatically different ways by the same mutation (R.B., unpublished observations). Combined, these phenomena can make interpretation of mutational data very difficult. Nonetheless, mutational data can be a useful method for triaging HTS hits by providing a method for prioritizing compounds that appear to bind to a site of interest.

One of the perceived advantages of allosteric modulators is their potential for improved selectivity for one receptor subtype over another. This is based on reduced evolutionary constraint imposed on the allosteric binding sites compared with the orthosteric binding pocket, which binds the same endogenous agonist ligand. To demonstrate receptor subtype selectivity of the compound responses, cell lines expressing each of the receptor subtypes must be developed (preferably in the same cell background as the target receptor) and the hits assessed functionally in these cell lines.

An inconvenient corollary to the potential for allosteric ligands to display improved receptor subtype selectivity is that, again because the allosteric binding pocket need not be evolutionarily constrained, differences may be observed at the target receptor from different species. Therefore, it is especially important to evaluate the functional effects of hits at the human receptor and also at the orthologous receptor from the species of animal used for *in vivo* experiments (typically mouse and rat).

A potential artifact of allosteric modulator screening (even in cell lines expressing recombinant receptor) can be that compounds interfere with the receptor agonist rather than the receptor itself. In the case of PAM screening, the compound may increase the affinity of the agonist for the receptor by binding to the agonist, or make more agonist available to the receptor by displacing some non-specific binding of agonist from the plastic of the assay plate. The latter can be particularly problematic when screening at receptors where the endogenous agonists are peptides (J.W., unpublished observations).

Evidence in the literature also suggests that allosteric ligand behavior is not only probe-dependent and pathway-dependent, but may also depend on the cell line that the receptor is expressed in. Such "context-dependent" signaling may occur when the receptor conformation induced by the allosteric ligands may change its binding with different interacting proteins that are present in different amounts in different cell lines [48].

Ultimately, allosteric modulators identified from HTS need to be tested in primary tissues or cell lines natively expressing the receptor of interest, as a prerequisite to *in vivo* studies. This should be performed as early as possible in the hit assessment process, in order to gain a better understanding of how these molecules are likely to behave *in vivo*. Functional responses in cell systems where the target receptor is heavily over-expressed may differ substantially from cellular responses in native expression systems. This can be caused by differences in receptor density (receptor reserve) and by the milieu of downstream interacting proteins that differ from one cell line to another, resulting in apparent changes in efficacy and potency of the allosteric modulator. Specifically, it is common for many PAMs to show intrinsic agonist activity in recombinant systems, while displaying only potentiator activity at endogenously expressed receptors in native tissue. As mentioned above, this can conceivably be attributed to the very high level of

receptor expression that is typical in recombinant cell lines used for screening, and it could be hypothesized that compounds which exhibit an amount of potentiator activity (α and/or β factor; see Section 2 above) that is beyond a certain threshold will display agonist activity in such over-expressing receptor systems. However, we have observed that compounds that appear to have similar potentiator activity (produce similar fold-shifts of orthosteric agonist concentration–response curves) can nonetheless show dramatically different levels of direct agonist activity in a recombinant system, implying that the reasons for the differences in allosteric agonist activity seen between native and recombinant receptor systems may likely reflect something more complicated than simply differences in receptor number.

6. Future directions

The past decade has seen profound advances in our understanding of other areas of GPCR structure and function. It is likely that these advances will influence the ways in which allosteric modulators are identified and analyzed. In this section we will examine some of these areas and their possible impacts on allosteric modulator discovery.

6.1. Physiological relevance

As discussed above in Section 5, it is important to test allosteric modulator screening hits in stem cells, primary tissues or other physiologically relevant systems using natively expressed receptors as early as possible in the "hit assessment" process, because of the potential for allosteric ligands to produce different effects in native tissue than they do in the very artificial recombinant systems typically used in screening. Advantages include appropriate receptor expression levels in an appropriate cellular milieu. In fact, it may be possible to produce stem cells and primary cell cultures that express disease-relevant targets in the context of a disease-relevant phenotype. It is hoped that performing pharmacological assays in such cell systems will result in better understanding of compound behavior and improved drug candidate selection [49]. Indeed, our increasing understanding of phenomena such as biased agonism, probe-dependence of allosteric effects, and especially context-dependent signaling has led to a growing interest in performing the HTS itself in physiologically relevant systems/tissues, with physiologically relevant end points.

Whether used for hit characterization or primary screening, what is often lost in cellular systems expressing physiological levels of target receptors is the magnitude and reproducibility of the signal window that can be obtained with cell lines over-expressing recombinant GPCRs. In this context, it is worth considering two assay technologies that have been used to derive biologically relevant data, often using cell preparations expressing receptors at physiological levels, high content analysis (HCA) and label-free assays. HCA is a sophisticated combination of automated microscopy, image acquisition and image analysis. Using different fluorescently labeled antibodies and biomolecules, HCA can be used to simultaneously interrogate multiple cellular pathways and features, including arrestin recruitment and receptor internalization, MAP kinase phosphorylation status and localization, mitogenic and apoptotic responses, and cyotskeletal rearrangements [50]. HCA techniques have been employed to demonstrate differential regulation of the M1 muscarinic receptor by orthosteric and allosteric agonists [51,52] The wealth of phenotypic information from HCA provides a richly contextualized picture of compound activity, but also poses significant challenges for data management, analysis and interpretation [53]. Nonetheless, HCA seems well-suited to dissect biased- and context-dependent signaling properties of orthosteric and allosteric compounds, both in lower throughput hit follow-up assays and, potentially in HTS as well.

There are two types of label-free cell-based assays, optical (resonant waveguide grating) biosensor-based and electrical (impedance) biosensor-based (reviewed in [54–56]). The optical biosensors measure changes in the distribution of certain components within the cell. These redistribution events also produce changes in cell shape, adherence, volume, and cell-cell contacts, which are measured by the impedance-based biosensors. While the details that underlie the phenotypic endpoints are obscure, it has been suggested that such global cell changes are the product of integration of numerous signal transduction cascades. Seen in this light, label-free bioassays are somewhat similar to isolated tissue assays that are the hallmark of classical pharmacology. Significantly, positive allosteric modulators of the $GABA_B$ receptor (Glutamate Family) have been characterized using an impedance biosensor label-free assay system [57]. The cost of label-free assay consumables has thus far limited the use of these platforms to lower throughput hit characterization assays, but as these assays become miniaturized they may gain a wider role in the drug discovery process.

As HTS continues to evolve, we are likely to see more screening using endogenously expressed receptors, whether in established cell lines, stem cells, primary cultures, and other physiologically relevant systems. The challenges for the development of screening assays using endogenously expressed receptors will include the identification of functional assays sensitive enough to provide an acceptable signal window with physiological levels of receptor expression, and the optimization of counter-screening methods for confirming receptor specificity under such HTS conditions.

6.2. Receptor hetero-oligomerization

Long thought of as monomeric signaling proteins, it is now widely acknowledged that GPCRs can and do form homo- and heterodimers, as well as higher order oligomers (for review see [58,59]). Receptor oligomerization has been reported for receptors from all five families of GPCRs, including adrenergic, serotonergic, opioid and vasopressin receptors (Rhopdosin Family), CRF1 (Secretin Family), γ-amino butyric acid type B ($GABA_B$) and metabotropic glutamate and multiple taste receptors (Glutamate Family). In addition, Frizzled receptor dimerization has been reported to activate wnt/β-catenin signaling [60], and the EGF module containing receptor, EMR2 (Adhesion Family) has been reported to form homo- and hetero-oligomers [61]. Importantly, hetero-oligomeric receptors can have signaling properties that are distinct from the signaling produced by monomeric or homomeric receptor configurations, as determined for the D1 and D2 dopamine receptors [62]. Allosteric modulation of hetero-oligomeric receptors can potentially arise from two ligands, binding at two sites of one of the protomers. Also, a ligand binding orthosterically at one protomer may be considered allosteric by modulating the binding and/or efficacy of the orthosteric ligand at the second protomer. Because receptor oligomerization involves interactions between sites that are distal to the ligand binding domains, allosteric ligands may have the best chance of exhibiting homomeric/heteromeric receptor selectivity. Hetero-oligomer-selective ligands would offer the potential of improved tissue specificity of responses, since both protomers must be expressed in the same cell. To date, it remains to be convincingly demonstrated that such hetero-oligomer-selective ligands can be identified. Various assay technologies that have been developed for the screening of hetero-oligomeric-selective ligands have been recently reviewed [63,64]. Continued evolution of these and other technologies will be very important in determining whether

hetero-oligomer selective ligands can be identified from high-throughput screening approaches.

6.3. Bitopic ligands

The terms "bitopic" and "dualsteric" ligand have been coined to describe a single molecule containing orthosteric and allosteric ligands connected by a linker sequence [65,66]. Bivalent ligands for GPCR homo- or heterodimers have been described, but these are almost invariably two orthosteric ligands joined by a linker moiety. It has, however, been suggested that the previously described muscarinic partial agonist/weak allosteric modulator, McN-A-543, may in fact be a bitopic ligand [67]. It has been postulated that the presence of an orthosteric binding component in McN-A-343 may explain the observation of ago-allosteric properties while the allosteric component helps explain the reported biased agonism of McN-A-543 [68]. Bitopic ligands could potentially exhibit improved subtype selectivity and/or gains in affinity relative to individual ligands, although this may depend on the pharmacology of the ligands in question (agonist or antagonist) and the receptor conformation favored/stabilized by them. Bitopic ligands could also conceivably provide an effective way to achieve the desired signal trafficking, as suggested by the McN-A-343 example. However, one of the main expected advantages of allosteric ligands as drugs is their ability to maintain the spatial and temporal aspects of cellular signaling, and this could be lost with bitopic ligands. From a drug development perspective, it remains to be seen whether the potential advantages of bitopic ligands can offset the loss of spatial and temporal signaling fidelity.

6.4. The impact of GPCR structural biology

One of the more exciting developments in GPCR biology has been the recent the elucidation of several high resolution crystal structures of GPCRs. In addition to the structure of rhodopsin, which has been available for a decade [69], available structures now include the β_2 adrenergic receptor [70], the β_1 adrenergic receptor [71], the adenosine 2a receptor [72], opsin [73], the chemokine CXCR4 receptor [74], and the dopamine D3 receptor [75].

Use of crystal structures to drive rational drug design for allosteric modulators has precedence with other types of drug targets (e.g., phosphodiesterase 4 [76]), although this approach is more difficult with GPCRs because of the challenges inherent in crystallizing integral membrane proteins. Nonetheless, this is an emerging area of research with exciting potential for GPCR drug discovery. Indeed, structure-based *in silico* compound screening has already been successfully used to discover GPCR allosteric modulators [46]. This technology, however, does not obviate the need for compound screening in biological assays (though it may reduce the number of compounds that must be tested). Additionally, there is no substitute for serendipity, and there is much to be gained by testing very large collections of "random" chemicals. Therefore, it is unlikely that advances in *in silico* drug discovery will lead to the end of HTS any time in the near future. It is, however, likely that in the next few years there will be an increasing use of both *in silico* and HTS approaches in combination for GPCR drug discovery.

7. Conclusion

Allosteric modulation of GPCRs is a rapidly developing area for drug discovery. Moreover, allosteric ligands offer a number of potential advantages over their traditional orthosteric counterparts. However, special considerations for assay design and screening must be implemented in order to maximize the chances of identifying allosteric modulators. Appropriately, screening approaches for allosteric modulators have evolved considerably within the last few years. As we continue to gain a better understanding of the behavior of all flavors of allosteric ligands, especially in consideration of GPCR functional selectivity, context-dependent signaling and hetero-oligomerization, it can also be expected that screening and hit assessment strategies will continue to evolve and be refined.

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