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Biased agonism: An emerging paradigm in GPCR drug discovery

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Abstract

G protein coupled receptors have historically been one of the most druggable classes of cellular proteins. The members of this large receptor gene family couple to primary effectors, G proteins, that have built in mechanisms for regeneration and amplification of signaling with each engagement of receptor and ligand, a kinetic event in itself. In recent years GPCRs, have been found to interact with arrestin proteins to initiate signal propagation in the absence of G protein interactions. This pinnacle observation has changed a previously held notion of the linear spectrum of GPCR efficacy and uncovered a new paradigm in GPCR research and drug discovery that relies on multidimensionality of GPCR signaling. Ligands were found that selectively confer activity in one pathway over another, and this phenomenon has been referred to as ‘biased agonism’ or ‘functional selectivity’. While great strides in the understanding of this phenomenon have been made in recent years, two critical questions still dominate the field: How can we rationally design biased GPCR ligands, and ultimately, which physiological responses are due to G protein versus arrestin interactions? This review will discuss the current understanding of some of the key aspects of biased signaling that are related to these questions, including mechanistic insights in the nature of biased signaling and methods for measuring ligand bias, as well as relevant examples of drug discovery applications and medicinal chemistry strategies that highlight the challenges and opportunities in this rapidly evolving field.

Keywords

Biased ligand; Functional selectivity; GPCR; Drug discovery

G protein-coupled receptors (GPCRs) are seven-transmembrane domain proteins that function to transmit signals into the intracellular space. It is estimated that nearly 36% of all FDA-approved drugs target at least one member of the GPCR gene family.^{1,2} Classically, the categorization of GPCR-ligands has been done based on their efficacies for activation of G proteins, as full agonists, partial agonists, antagonists, or inverse agonists, depending on their abilities to elicit a receptor-mediated response. However, it has become evident that

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GPCRs can mediate multiple signaling outcomes. For instance, β arrestins, which have been long associated with receptor desensitization, can also lead to signaling events.³

Arrestins are cytosolic proteins originally discovered in the visual system, which include arrestin-1 (also referred to as visual arrestin), arrestin-2 (β arrestin1), arrestin-3 (β arrestin2) and arrestin-4 (termed a cone arrestin).^{4,5} While arrestins-1 and -4 are localized in retinal rods and cones, molecular cloning revealed two ubiquitously expressed isoforms termed β arrestin1 and β arrestin2 due to their high homology with the visual arrestin and their potent functional regulation of the β_2 adrenergic receptor (β_2 AR).^{6–8} More recently, two additional families of arrestin-related proteins have been found to be broadly expressed in eukaryotes, namely, a group of vacuolar protein sort 26 (Vps26)-related proteins and α arrestins. Their tertiary structure is similar to that of the visual and β arrestins, although their physiological role and potential involvement in regulating GPCR signaling are still not fully understood.^{9,10}

While arrestins were named based on their initially discovered ability to arrest (turn off) GPCR signaling, it is now evident that β arrestins regulate GPCR trafficking as well as G protein-independent signaling.^{11–13} A general scheme for the β arrestin-mediated regulation of GPCR function is depicted in Figure 1. Agonist binding to GPCR stabilizes an active conformation(s) of the receptor that promotes its interaction with heterotrimeric G proteins, $G\alpha\beta\gamma$ (Fig. 1a). This is followed by the GDP to GTP exchange at the $G\alpha$ subunit, and the subsequent dissociation of G proteins (Fig. 1b). The dissociated G proteins interact with and modulate the activity of downstream effectors (e.g., adenylyl cyclase and phospholipases) that produce second messengers such as cAMP and inositol phosphate (G protein-dependent signaling).¹⁴

Since one activated receptor can sequentially couple to multiple G proteins with signal amplification occurring through to the enzymatic activity of receptor second messengers (e.g., cyclases and phospholipases), desensitization mechanisms have evolved to turn off the potentially deleterious effects of sustained signaling.¹⁵ Desensitization is considered a two-step process, which starts with phosphorylation of the agonist-occupied GPCR by the second messenger-dependent protein kinases, such as protein kinase A or C (PKA or PKC), or by a corresponding GPCR Kinase (GRK, Fig. 1c).⁴ This triggers the second desensitization step, which involves the recruitment of β arrestin to active phosphorylated receptors and the consequent steric hindrance of further G protein activation (Fig. 1d).¹⁶ The initial discovery of the critical involvement of β arrestins in GPCR desensitization was subsequently followed up by studies that indicated their important role in receptor internalization. It has been found that the receptor-bound β arrestins interact with several key components of the receptor endocytotic process (Fig. 1e), including the adaptor-protein2 (AP2) complex,¹⁷ the clathrin heavy chain,¹⁸ and the E3 ubiquitin ligase Mdm2.¹⁹ The complex formed upon the binding of clathrin/AP2 to the receptor-bound arrestin translocates to the clathrin-coated pit, which is then sequestered off the plasma membrane and hindered from subsequent stimuli.²⁰

While the receptor–G protein interaction that takes place upon receptor activation is unstable and brief, the receptor–arrestin complex can be relatively stable and exist on a time scale of minutes to hours.^{21,22} Analysis of the agonist-mediated arrestin translocation to multiple

GPCRs identified two major classes of receptor–arrestin complexes, based on their strength and longevity. Class A complexes are transient and receptor– β arrestin interactions rapidly dissociate upon receptor internalization, as exemplified by the β_2 adrenergic, μ -opioid, endothelin type A, dopamine D₁, and α_1 adrenergic receptors. Receptors in this class are rapidly recycled back to the plasma membrane. In contrast, Class B complexes have more stable receptor– β arrestin interactions that persist even as the receptor undergoes endosomal sorting. Receptors in this class are sequestered in endosomes and recycled slowly or undergo degradation, for example, the angiotensin II type 1A, neurotensin 1, vasopressin V₂, thyrotropin-releasing hormone, and substance P receptors.²³

Recent β arrestin crystallographic,²⁴ mutation,²⁵ and biophysical studies²⁶ suggest that β arrestins undergo extensive conformational changes upon binding to the phosphorylated GPCR. In their basal cytosolic receptor-unbound state, arrestins are elongated molecules, which consist of two (N- and C-) domains (Fig. 2a) and the C-terminus anchored in a polar core between them, unavailable for interaction with partner proteins (Fig. 2b).²⁷

The recently reported crystal structure of β arrestin1 in complex with a fully phosphorylated 29-amino-acid carboxy-terminal (derived from the vasopressin receptor-2 (V₂Rpp) and shown to functionally and conformationally activate β arrestin1) revealed extensive conformational changes in the C-terminus, which is released and becomes available for interactions with clathrin and AP2 (Fig. 2c).²⁴ The V₂Rpp– β arrestin1 crystal structure also revealed a 20° twist between the N- and C-domains. A similar 20° rotation has been observed in the crystal structure of the mouse visual arrestin bound to a constitutively active form of human rhodopsin.²⁸ It has been suggested that the twisting movement of the two domains is part of the general mechanism by which arrestins, upon activation, may expose an additional interface for interacting with their numerous binding partners.

Over the past decades, a growing list of binding partners has implicated β arrestins in a number of important cellular functions in addition to GPCR desensitization and trafficking. In particular, arrestins have been found to link activated GPCRs to signaling effectors such as the Src family tyrosine kinases,²⁹ components of the extracellular signal-regulated kinase 1/2 (ERK 1/2) and the c-Jun N-terminal kinase 3 (JNK3),³⁰ mitogen-activated protein kinase (MAPK) cascades,³¹ Akt,^{32,33} and effectively convey the G protein-independent signaling (Fig. 1f). Because arrestin binding can uncouple G protein from the activated receptor, the G protein-dependent and arrestin-dependent signaling can be segregated. Furthermore, the two signaling pathways, proved to be spatially and temporally distinct events, are also pharmacologically separable.³⁴ In recent years, there has been a movement to develop ligands that will preferentially segregate GPCR to one pathway or another. These functionally selective ligands, also referred to as the ‘functionally selective’ or ‘biased’ agonists, have demonstrated the potential to harness GPCR signaling and have collectively opened new possibilities in GPCR drug discovery.³⁵

Although the exact molecular mechanism of biased signaling is not well understood, the studies reported to date suggest that the GPCR conformation, stabilized by a G protein-biased agonist, is distinct from the conformation stabilized by a β arrestin-biased agonist. For example, the fluorescence-based study on the arginine vasopressin type 2 receptor (V₂R)

activation using biased and unbiased agonists provided experimental evidence suggesting that the transmembrane helix 6 (TM6) and third intracellular loop (icl3) movements are associated with selective G protein signaling, whereas the movements between its TM7 and helix 8 (H8) region are required for selective β arrestin recruitment.³⁶ Similar conclusions resulted from the ¹⁹F NMR study of β_2 AR in complex with a range of ligands with different functional profiles.³⁷ The NMR signal changes of the site-specific ¹⁹F-labels located in the cytoplasmic region revealed that binding of an unbiased agonist (e.g., formoterol) shifts the receptor conformational equilibrium toward the specific 'G protein active' state of TM6, while the β arrestin-biased ligands (e.g., carvedilol) impact primarily the conformational state of the TM7 helix. These findings are consistent with the crystallographic³⁸ and electron microscopy³⁹ studies of the β_2 AR–G $\alpha\beta\gamma$ heterotrimer complex that showed that the G α subunit contacts primarily the intracellular regions of TM5 and TM6.

A study of the patterns of β_2 AR phosphorylation by individual GRKs, a prerequisite for β -arrestin binding, showed that GRKs target primarily the short H8 helix adjacent to the TM7 region.⁴⁰ In addition, a detailed mapping of the β_2 AR phosphorylation in this study demonstrated that the β arrestin-biased ligand (carvedilol) induced a phosphorylation pattern that was distinct from that of isoproterenol, an unbiased full agonist.⁴⁰ This suggests that by inducing distinct receptor conformations, the biased ligands are able to recruit distinct GRKs and, consequently, produce a distinct phosphorylation pattern. In fact, the experimental evidence accumulated over the recent years indicates that the biased ligands can stabilize both the receptor and β arrestin in conformations that are distinct from those associated with unbiased ligands. For example, by employing an intramolecular bioluminescence resonance energy transfer (BRET)-based biosensor of β arrestin2 and a combination of biased ligands and/or biased mutants of three different GPCRs, Shukla and colleagues at Duke University provided evidence suggesting that β arrestins can adopt multiple 'active' conformations.⁴¹ One can speculate that these multiple β arrestin conformers with distinct surface topologies complex with different binding partners, and thereby engage in different downstream signaling pathways.

Another important advance toward unraveling the molecular basis of biased signaling was the recently reported crystal structure of two 5-hydroxytryptamine serotonin receptor subtypes, namely 5-HT_{1B} and 5-HT_{2B}, both bound to ergotamine (ERG, Fig. 3a).^{42,43} ERG is an alkaloid produced by the ergot fungus, which has been shown to exhibit a strong β arrestin-biased agonism at 5-HT_{2B}, and a relatively weak bias at the 5-HT_{1B} receptor.

A close inspection of the two data sets revealed a small shrinkage of the ligand binding pocket in the ERG-5-HT_{2B} structure, with ERG in slightly different binding conformation and hydrophobic contacts with several additional residues when compared with the 5-HT_{1B} structure (Fig. 3b). Intriguingly, a similar network of additional interactions has been found in the structure of carvedilol, a β arrestin-biased agonist bound to the β_1 AR.⁴⁴ The analysis of the two crystal structures also revealed that the ERG-bound 5HT_{1B} complex exists in a classical full agonist-induced active-like state, whereas the ERG-bound 5HT_{2B} structure displays conformational characteristics that are attributed to both the active and inactive states. For example, both structures showed the conserved NPxxY motif (TM7) in an active-like conformation with the intracellular TM7 helix moved toward the receptor core.

However, only the ERG-5HT_{1B} structure displayed another highly conserved GPCR feature, the DRY motif in TM3, in an active state with the salt bridge between the Asp¹⁴⁶–Arg¹⁴⁷ residues being broken. In the ERG-5HT_{2B} structure, the DRY motif salt bridge between Asp¹⁵² and Arg¹⁵³ was intact, which is consistent with all inactive-state GPCR structures known to date (Fig. 3c). Consequently, it is tempting to attribute this ‘intermediate’ state of the 5HT_{2B} receptor structure to the β arrestin-biased signaling conformation characterized by a strong β arrestin and an inefficient G protein coupling. However, it is important to note that the observed difference between the two structures may also reflect distinct signal transduction mechanisms between the two receptor subtypes used in this study. Additionally, it is critical to take into account the fact that crystal structures represent only a static snapshot of a potentially complex ensemble of conformations. It is now widely recognized that GPCRs are highly dynamic systems that assume many conformations associated with different signaling states, which are stabilized by different ligands.^{45,46} Further research efforts, most likely involving crystallography in conjunction with other biophysical and computational⁴⁷ methods to study GPCRs as dynamic systems, will be required to reliably delineate the biased signaling mechanism at the molecular level.

Functional selectivity (biased signaling): definition, assays, quantification

The definition and identification of biased ligands has been challenging. It appears that initially, differences in relative efficacy or potency were used as the determinants of ligand bias.^{48–51} The realization that more efficient approaches for determining bias were needed, led to the development of different methods for identifying biased ligands.⁵² However, not always a consensus was reached about the most appropriate way of measuring functional selectivity.^{53,54} Both qualitative and quantitative methods have been described.^{52,55,56} These methods compare test ligands with reference agonists in order to determine if a compound is biased for one signaling pathway relative to another.⁵² In the case of the quantitative methods, a ‘bias factor’ defining the degree of bias of the test ligand is generated.⁵²

One of the most simplistic methods, developed by Ehlert and colleagues,^{57,58} utilizes data from standard four-parameter Hill equations that display slopes close to unity. This method uses ratios of relative efficacies ($E_{\max}$) by potencies (EC_{50}) (relative activities) to quantify ligand bias, as shown in the equation below:

$$\begin{aligned} \text{Bias factor} &= \log \left(\frac{\text{relative activity}_{12,\text{lig}}}{\text{relative activity}_{12,\text{ref}}} \right) \\ &= \log \left(\left(\frac{E_{\max_{\text{path1}} \times EC_{50-\text{path2}}}{EC_{50-\text{path1}} \times E_{\max_{\text{path2}}}} \right)_{\text{lig}} \div \left(\frac{E_{\max_{\text{path1}} \times EC_{50-\text{path2}}}{EC_{50-\text{path1}} \times E_{\max_{\text{path2}}}} \right)_{\text{ref}} \right) \end{aligned}$$

Additional methods use the Black and Leff operational model to quantify functional selectivity. As shown in the equation below, the Black and Leff model defines the terms τ (coupling efficiency) and K_A (conditional affinity) as the ligands’ (A) intrinsic efficacy and dissociation constant of agonist–receptor–signal transducer complex, respectively (n = transducer slope).⁵⁹

$$\text{Response} = \frac{E_{\max} \times \tau^n \times [A]^n}{\tau^n \times [A]^n + ([A] + K_A)^n}$$

The Black and Leff operational model is as a mathematical rearrangement of two distinct Hill equations (concentration of agonist by receptor occupancy and receptor occupancy by effect) with the introduction of τ (τ = receptor density/receptor occupancy needed for producing 50% of the system's maximal effect).⁵⁹ By plotting ligand's functional data into the Black and Leff equation, ratios of τ/K_A quantify pharmacological responses regardless of signal amplification and receptor reserve.^{52,60} This feature is very desirable for ligand bias analyses and has been developed by Kenakin and colleagues as 'transduction coefficients' according to the following equation:⁶⁰

$$\begin{aligned} \text{Bias factor} &= \Delta \log \left(\frac{\tau}{K_A} \right) \\ &= \left(\log \left(\frac{\tau}{K_A} \right)_{\text{lig}} - \log \left(\frac{\tau}{K_A} \right)_{\text{ref}} \right)_{\text{path 1}} - \left(\log \left(\frac{\tau}{K_A} \right)_{\text{lig}} - \log \left(\frac{\tau}{K_A} \right)_{\text{ref}} \right)_{\text{path 2}} \end{aligned}$$

The transduction coefficients and the relative activity ratios become proportional (and, thus, result in the same bias factors) when slopes of 1 are obtained from the dose-response curves.^{52,55} The transduction coefficients have been recently modified in order to more accurately accommodate data from weak partial agonists.⁶¹ This new method entitled 'competitive model' utilizes ligand curves in agonist and antagonist mode to more precisely define τ and K_A values for weak partial agonists.⁶¹

The utility of the methods of measuring ligand bias has been studied. For instance, the transduction coefficients have been successfully incorporated into SAR studies to aid in the development of biased ligands for the dopamine D₂ and the κ -opioid receptors.^{62–64} It also appears that these analyses can provide mechanistic insight on the activation of more complex signaling pathways that are downstream of immediate GPCR signal transducers.⁵⁵ Moreover, bias analyses have been successfully implemented in studies to investigate ligand-specific functional consequences of different point mutations in the muscarinic M₁ receptor.⁶⁵ It is important to note that bias is a result of how a ligand interacts with the receptor (molecular bias, which translates across assays and expression systems) plus how the receptor response is observed (an experimental artifact that is system- and assay-dependent and may not translate to other expression systems or assays).⁵² As shown in the methods above, the solution found to correct for this observational issue has been to use a reference ligand for comparisons of relative activities within specific assay systems.⁵² Therefore, bias is always a measure relative to the reference ligand and assay systems. A reference agonist is typically chosen based on its ability to robustly activate the receptor in the assays that are to be compared. In some cases, the endogenous ligand of a receptor is used as the reference compound. However, nature may also exploit biased signaling and, thus, endogenous ligands may also present functional selectivity.^{66–71} This allows for a test ligand being biased compared to an endogenous agonist, but that does not preclude the

endogenous ligand from displaying bias compared to another reference ligand.⁵² Therefore, it is important to emphasize that bias is not an absolute property, but is highly dependent on context of the assay and performance of the reference compound.

Another type of bias that needs to be considered in functional selectivity studies is the case of 'perfect bias'. Ligands that display perfect bias are agonists for one signaling pathway and do not display any detectable activity for another pathway.⁵² Caution should be taken in the classification of those ligands, since assay systems that display low amplification or have low receptor reserve might not result in any detectable responses for partial agonists. Therefore, it has been suggested that those ligands should be defined as 'extremely biased'.⁷² Different assay systems with higher signal amplification and receptor reserve should be employed for the analyses of ligands that display this type of bias.

The cases of perfect or extreme bias also expose a deficiency in the current quantitative methods for measuring ligand bias. That is, these methods cannot accommodate data from compounds that display antagonism or inverse agonism for one of the signaling pathways analyzed.⁵⁵ Extremely biased compounds have been described.^{49,73,74} However, a framework for quantitatively ranking the degree of bias of these compounds has only just begun to emerge.^{61,75} As these quantitative, mechanistic methods become more accepted and elaborative there is certain to be a broader interest in both the discovery and interpretation of the molecular and physiological implications of extremely biased ligands. Again, it is important to emphasize that bias is context dependent and bias observed in one condition (i.e., cell-based signaling assay) may not translate to the receptor expressed in an endogenous setting (i.e., synapse).

Drug discovery and medicinal chemistry highlights

The realization that β arrestins mediate cellular signaling, which is independent and pharmacologically separable from G protein signaling, has changed a previously held notion of the linear spectrum of GPCR efficacy and uncovered a new paradigm in drug discovery that relies on the multidimensionality of GPCR signaling. Over the past couple of decades a number of GPCR ligands displaying biased profiles were reported in the literature, most discovered retrospectively. In this section we discuss the hypothesis-driven drug discovery and medicinal chemistry efforts that were intentionally focused from the outset on the design and development of biased GPCR ligands toward safer and/or more efficacious medicines. For a more general coverage outside of the scope of this section readers are referred to recently published reviews of this topic.^{76,77}

One of the earliest identified opportunities for the biased GPCR agonist approach in drug discovery steamed from the studies reported in 1999, 2000 and 2005 by Bohn and colleagues. These studies showed that morphine administered to β arrestin2 KO mice produce enhanced and prolonged analgesia with reduced tolerance and fewer adverse events, when compared to wild-type mice.⁷⁸⁻⁸⁰ More recently, a similar profile of improved morphine potency with reduced side effects has been observed in mice and rats with β arrestin ablated by siRNA.^{81,82} Opioids produce powerful analgesia, by activating μ -opioid receptors (MOR), in addition to many efficacy-limiting adverse effects, which include

tolerance, nausea, vomiting, sedation, constipation, and respiratory depression.^{83–85} Numerous MOR agonists have been developed over the years as part of a global effort to improve the opioid safety and tolerability profile. However, all of these structurally diverse opioids display morphine-like adverse effects, suggesting that the analgesic efficacy, as well as the side effects, is directly related to MOR activation. Intriguingly, experiments in the β arrestin2 KO mice imply not only that β arrestins reduce morphine's analgesic potency, but it may also be associated with producing morphine-induced side effects.

This hypothesis suggests that it would be beneficial to develop agonists that could selectively activate the G protein signaling pathway without engaging β arrestins as a means to preserve analgesic efficacy while avoiding side effects. The first ligand identified that displayed this separation between signaling pathways was a neoclerodane diterpene derived from the kappa opioid receptor natural product agonist, salvinorin A.⁸⁶ This compound, herkinorin, is structurally intriguing as it has no basic nitrogen, a highly conserved property of GPCR ligands.^{87,88} In antinociception studies assessing formalin-induced pain responses, herkinorin proved efficacious without tolerance; supporting the general hypothesis that the antinociceptive properties could be dissected from tolerance by driving G protein signaling over β arrestin2 recruitment.⁸⁹ However, herkinorin was not shown to be acting at the same sites as morphine and therefore, it is difficult to conclude whether its bias properties produced the differences in physiological responses.

Further motivated by this hypothesis, Chen and colleagues at Trevena Inc. set out to identify G protein-biased MOR agonists as potential novel analgesics with improved efficacy and side effect profile.⁹⁰ They initiated a hit finding effort by screening the internal compound collection in the cAMP accumulation assay (G protein signaling) and the β arrestin2-based β -galactosidase fragment compartmentalization assay (β arrestin signaling). Both assays were developed in the HEK-293 cell line expressing human MOR.

This effort led to the discovery of tetrahydropyran **1** (Table 1) with a submicromolar G protein MOR potency and a low MOR β arrestin recruitment activity (32% vs 100% in comparison to morphine), which was deemed a suitable starting point for an optimization program. The optimization efforts around compound **1**, which primarily focused on improving the MOR G protein potency while diminishing β arrestin recruitment, resulted in the discovery of thiophenyl analogue **2**. It is worth noting that some closely related analogues displayed an unbiased full agonist profile, such as compound **3**, which showed high efficacy in both the cAMP and β arrestin assays (104% and 197%). Displaying the desired in vitro profile, compound **2** was progressed to the in vivo evaluation and benchmarking against morphine's efficacy and potential for adverse effects. These studies showed that at equianalgesic doses, established in the mouse hot-plate assay, morphine (6 mg/kg) exhibited a more severe constipation effect than compound **2** (3 mg/kg) in a mouse glass bead retention model of colonic motility (158 min vs 65 min retention times, respectively). However, despite the favorable in vivo pharmacology profile, the potent hERG channel inhibition ($IC_{50} = 2.3 \mu M$) and the cardiac abnormalities observed in the rabbit ventricular wedge assay prevented further progression of compound **2**. Encouraged by the initial in vivo data that reaffirmed the original hypothesis, the authors continued their optimization efforts around **2**, which led to the discovery of 3-methoxy thiophenyl derivative

4 with not only an improved biased MOR profile (Table 1), but also reduced cardiovascular liabilities with hERG IC_{50} >200-fold over MOR cAMP EC_{50} value of 8 nM. The authors calculated a bias factor of 3 based on the equiactive comparison method based on the intrinsic relative activity model outlined by Griffin et al.⁵⁸ and Rajagopal et al.^{56,91}

On the basis of its in vitro profile, compound **4** (TRV-130) was progressed for in vivo evaluation, where it displayed a 5–10 times higher potency than morphine in the mouse (ED_{50} values 0.9 mg/kg vs 4.9 mg/kg) and rat (ED_{50} 0.32 mg/kg vs 3.2 mg/kg) hot-plate assays, as well as an improved safety profile in the mouse glass bead colonic motility assay (E_{max} = 53% for TRV-130 compared to 100% for morphine at equianalgesic doses). Despite showing at higher doses an effect on blood gases, TRV-130 therapeutic index proved superior to morphine in respect of the analgesia versus respiratory suppression in the rat, with no effect on blood CO_2 and O_2 levels at doses of up to eight-fold over the equianalgesic dose.⁹¹ Interestingly, the in vitro MOR biased profile of compound TRV-130 was found to be species dependent. It showed higher β arrestin potency and efficacy at the mouse- (EC_{50} = 12.6 nM; efficacy = 74%) and dog- (EC_{50} = 9.4 nM; efficacy = 24%) receptors, compared to the rat MOR (inactive) and human MOR (EC_{50} = 40 nM; efficacy = 14%). This phenomenon, potentially resulting from differences in phosphorylation sites and/or receptor expression, is important to remember when designing or interpreting translational in vivo data for biased ligands.

Gratifyingly, the observed pre-clinical profile of TRV-130 proved translatable in the clinic, where in healthy volunteers it produced analgesia with less reduction in a respiratory drive and a less severe nausea than morphine.⁹² However, in a recently reported phase 2, randomized, placebo- and active-controlled study in acute pain following bunionectomy, intravenously administered TRV130 produced greater categorical pain relief compared with morphine ($P < 0.005$), with tolerability similar to morphine and no serious adverse effects; the respiratory effects in this study did not provide a statistically significant benefit over morphine but it is hoped that the improved analgesic benefit will offset this difference.⁹³ These results provide an early clinical translation of ligand bias as an important new paradigm in GPCR-targeted pharmacotherapy. However, considering a profound effect seen in β arrestin2 KO mice, one may be disappointed with the relatively limited improvements brought about by TRV-130 in both the animal models and clinical trials. For example, in comparison to morphine, TRV-130 showed only approximately a two-fold improved therapeutic index (analgesia vs respiratory depression).⁹² This raises the question of whether this result is the maximum one could expect from this approach, or if a more biased G protein MOR agonist would show a safer profile with an even greater therapeutic index than TRV-130.

Similarly to the MOR for pain, the dopamine D_2 receptor has been for a long time a focus of research and drug discovery for psychiatric disorders. With the exception of aripiprazole **5**, reportedly a D_2 partial agonist, all clinically used antipsychotics are unbiased antagonists of the D_2 receptor.⁹⁴ The D_2 antagonism is thought to underlie the therapeutic benefit in the treatment of psychotic symptoms, as well as serious extrapyramidal side effects including catalepsy and other dyskinesias.⁹⁵ Investigating a possibility of separating the D_2 antipsychotic efficacy from the target-related side effects, Chen and colleagues were

interested in developing a β arrestin signaling biased D_2 receptor agonist.⁹⁶ They selected the FDA approved antipsychotic aripiprazole as a starting point because of its unique D_2 profile and hoping that its closely related analogues may share its favorable pharmacokinetic properties, including CNS exposure. A range of aripiprazole analogues were synthesized using diversity-oriented high throughput synthetic methods. The compounds were profiled in the cAMP accumulation (G protein signaling) and β arrestin recruitment assays, in which aripiprazole itself displayed an unbiased partial agonist profile (Table 2).

This effort resulted in the discovery of potent and β arrestin-biased D_2 agonists **6** and **7** (Table 2). Compound **6** displayed a potent partial agonist profile in the D_2 β arrestin assay, similar to the parent compound **5**, while in the same assay **7** was found to be a slightly less potent but full agonist. Importantly, both compounds displayed a potent antipsychotic-like efficacy in the amphetamine-induced hyperlocomotion studies in mouse, without inducing motor side effects that are characteristic for unbiased D_2 antagonists such as haloperidol. At the same doses both compounds produced a haloperidol-like catalepsy in β arrestin2 KO mice.⁹⁷ Taken together, these results suggest that the D_2 receptor β arrestin2 signaling blocking is not required for antipsychotic efficacy, while the β arrestin2 agonist activity may protect from the motor side effects triggered by D_2 -mediated G protein antagonism. It remains to be seen if these encouraging preclinical data for the β arrestin-biased D_2 agonists can be translated to the clinic.

A similar literature-inspired approach in the design of β arrestin-biased D_2 receptor ligands has been taken by Shonberg and colleagues at Monash University.⁶² The focus of their structure–functional selectivity exploration was on cariprazine **8**, another D_2 partial agonist currently in clinical development for schizophrenia.⁹⁸ Again, to assess G protein-related signaling, the compounds were profiled in the D_2 cAMP accumulation assay, whereas β arrestin signaling was evaluated by measuring phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2). A particularly interesting aspect of this work is the application of the transduction coefficients, as shown in Table 3.

In this testing paradigm, cariprazine **8** displayed a 230-fold bias toward the cAMP pathway compared with dopamine, whereas a closely related carbamate **9** showed a 42-fold bias decrease. Interestingly, while all cariprazine derivatives disclosed in this Letter showed a bias toward the G protein signaling pathway, subtle changes of the D_2 unbiased partial agonist **10**, a structurally related aryl piperazine reported by Tschammer et al.,⁹⁹ led to analogues that displayed a strong bias toward β arrestin signaling, for example, **11**;

$$\log(\tau/K_A) = -1.2 \text{ (Fig. 4).}$$

Cardiovascular disease is another therapeutic area where biased agonists are extensively investigated as an approach to improve the current standards of care. Angiotensin II is a powerful endogenous vasoconstrictor that exerts its physiological effects through activation of angiotensin II receptors (AT1R), a G_{α_q} -coupled GPCR.¹⁰⁰ AT1R antagonism proved to be an effective approach for the treatment of hypertension since the 1985 launch of losartan, the first of the AT1 receptor blockers (ARBs) currently used in the clinic.¹⁰¹ However, in addition to blocking adverse effects of angiotensin II, such as hypertension and cardiac remodeling, ARBs also prevent beneficial effects of angiotensin II, including the AT1R-

mediated inotropy which supports cardiac function. Consequently, reduced cardiac output was long thought to be an inseparable side effect associated with AT1R antagonism and ARBs in general. This notion has been recently challenged by studies showing that, in a stark contrast to angiotensin II (a balanced AT1R agonist) a β arrestin-biased analogue Sar¹Ile⁴Ile⁸-angiotensin (SII) robustly promotes contractility of isolated cardiac myocytes.¹⁰² This finding suggested that the AT1R G protein signaling pathway is the primary mediator of the angiotensin II vasoconstrictive effect, whereas β arrestin signaling is solely responsible for the inotropic effect of AT1R stimulation. In line with this hypothesis, another biased AT1R ligand that blocks the G proteins but activates the β arrestin signaling pathway, Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH (TRV027), has been shown to reduce blood pressure and improve cardiac performance in rats, whereas the unbiased ARB losartan decreased both blood pressure and cardiac performance.¹⁰³ It has been shown that the in vivo effect of a selective β arrestin-biased AT1R agonist on cardiac contractility is lost in β arrestin2 KO animals, which further supports the hypothesis that positive inotropic effects of AT1R stimulation are specific to β arrestin-mediated signaling.¹⁰⁴ Since administration of a β arrestin-biased AT1R agonist does not lead to increased intracellular levels of secondary messengers such as calcium, IP1 or DAG, it is hypothesized that the observed β arrestin-dependent inotropic effect is likely related to enhanced sensitivity of the myofilament to calcium. In addition to the positive effect on cardiac contractility, β arrestin-biased AT1R agonists were also found to promote cell survival during cardiac injury. Together, these findings present intriguing opportunities for cardiac therapy in congestive heart failure in general and ischemic injury in particular, which is often characterized clinically by peripheral arterial vasoconstriction and diminished cardiac contractility. Indeed, the preclinical efficacy and safety profile of TRV027 has been translated into the clinic where it lowered blood pressure in healthy volunteers on a salt restricted diet, as well as in patients with acute heart failure (AHF), without significant adverse safety signals. On the basis of these data TRV027 has recently progressed to a larger Phase 2b study in AHF patients.¹⁰⁵

Concluding remarks and future perspectives

The classical understanding of GPCR signaling^{106,107} has undergone major revisions in recent years with the introduction of the biased agonism paradigm.^{108–111} The realization that β arrestin has a role in GPCR signaling and the discovery of ‘biased’ or ‘functionally selective’ ligands that can produce specific receptor activation profiles have stimulated significant interest in the physiological, pharmacological and biostructural mechanisms that underline this paradigm.^{33,64,78–80,112–116} Based on experimental evidence suggesting that the physiological effects of GPCR activation are pathway dependent, functional selectivity has been proposed as a strategy for improving current drug therapies that target GPCRs.⁶⁶

Emerging reports of drug discovery programs aimed to evaluate potential therapeutic benefits of the functional selectivity paradigm provide an insight into the current challenges and opportunities in this rapidly evolving field. For example, understanding SAR data is one of the commonly reported challenges for medicinal chemists working on biased ligand programs, since even small structural modifications are often found to result in large shifts in the functional selectivity. Interestingly, this phenomenon has been successfully leveraged as an effective hit generation strategy, where a systematic SAR exploration around a known

ligand, regardless of its functional selectivity, led to identification of a suitable starting compound with a desired biased profile. However, these steep and often unpredictable ‘SAR cliffs’ present a significant challenge at the hit/lead optimization stage, when SAR-driven medicinal chemistry efforts are focused on preserving the desired biased profile while optimizing other properties that are required in a successful clinical candidate. Great strides have been made in unravelling the biostructural mechanisms of the biased signaling.¹¹⁷ However, to better understand the SAR and enable a rational design of biased ligands, more atomic-level biostructural information about the nature of ligand–receptor–effector interactions will be required. The most useful information in this context will likely come from research that effectively combines the crystallographic and other biophysical and computational methods to study GPCRs as dynamic systems.

Availability of high quality biased ligands is a critical prerequisite to addressing the ultimate challenge of deconvoluting in vivo physiological effects that are associated with different GPCR signaling pathways. This is proving particularly difficult, as the manner in which a receptor signals is highly context dependent and the diversity of expression of GPCRs in vivo is contextually rich. When translating in vitro bias to in vivo pharmacology one should be aware of possible complications resulting from potential species differences, which may affect ligand potency or functional selectivity in vivo as indicated earlier. One should also consider potential effects of in vivo pharmacokinetics, which may result in suboptimal ligand exposure at the site of action, or formation of active metabolites with unbiased agonist profiles. For example buprenorphine, a clinically used opioid analgesic, is reportedly a partial and biased agonist of the MOR, whereas its main metabolite in humans, norbuprenorphine, is a potent, full and balanced MOR agonist.¹¹⁸ Interestingly, since norbuprenorphine is a P-gp substrate actively effluxed at the blood brain barrier, it does not affect buprenorphine’s centrally-mediated pharmacology.¹¹⁹

Moreover, many receptors were found to couple to different types of G α subunits of G proteins,^{120,121} as well as the multiple isoforms and combinations of G $\beta\gamma$ subunits, which could result in distinct signaling events and further increase the overall complexity of GPCR signaling.^{122–124} The concept of biased signaling will inevitably broaden to encompass additional complexities and continue to evolve as an important approach toward safer and more effective medications. Indeed, the sheer extent of the current scientific literature and ongoing clinical developments for a range of indications assure increasingly prominent role of the biased agonism paradigm in GPCR drug discovery.

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Figure 1. Paradigms of GPCR-mediated signaling and multiple roles of β -arrestins: binding of an agonist (a) results in activation of signaling pathways by G proteins (b), as well as β -arrestins (f), in addition to desensitization and internalization by β -arrestins (d and e).

Figure 2.

β arrestin1 crystal structures. (a) Superimposed structures of β arrestin1 in a basal state (inactive state: blue; PDB 1G4 M) and in complex with V₂Rpp (Vasopressin Receptor 2, partial protein, active state: green; PDB 4JQI) reveal marked conformational differences, including the release of the C-terminus which contains binding sites for partner proteins such as clathrin; (b) inactive state: the polar core consisting of five interacting residues keeping the C-terminus locked in the place is thought to be a critical stabilizer of the β arrestin1 inactive state (Arg³⁹³–Asp²⁹⁰ salt bridge highlighted by the broken black circle); (c) active state: upon V₂Rpp binding (omitted for clarity) the C-terminal strand residue Arg³⁹³ is displaced, and its interacting partner Asp²⁹⁷ undergoes a large movement together with the rest of the lariat loop, resulting in significant conformational rearrangement in the polar core of the activated β arrestin1.

Figure 3.

(a) Superimposed crystal structures of 5-HT_{1B} (blue; PDB 4IAR) and 5HT_{2B} (gold; PDB 4IB4) in complex with ergotamine (ERG); (b) prominent conformational difference of ERG bound to 5-HT_{1B} (gray) and 5HT_{2B} (green) is highlighted by the broken black circle. Contacts with additional residues observed in ERG-5HT_{2B} structure are also highlighted (gold); (c) conserved DRY motif exist in an 'active' state (the Asp¹⁵²-Arg¹⁵³ salt bridge broken) in 5HT_{1B} structure (blue), and 'inactive' state (the Asp¹⁴⁶-Arg¹⁴⁷ salt bridge intact) in 5HT_{2B} structure (gold).

Figure 4. Aryl piperazine analogues of cariprazine and aripiprazole with different D₂ receptor functional selectivity profiles.⁹⁹

Table 1

G protein-biased MOR agonists⁹⁰

Compound	R ¹ , R ² , R ³	MOR cAMP pEC ₅₀	MOR cAMP E _{max} (%)	MOR βarr2 pEC ₅₀	MOR βarr2 E _{max} (%)
Morphine	NA	7.4	100	6.3	100
1	NA	6.3	74	5.7	32
2	H, H, H	7.8	95	6.6	15
3	H, Me, Me	8.3	104	6.3	197
4 (TRV-130)	OMe, H, H	8.1	84	7.3	15

Table 2From aripiprazole to the β arrestin-biased D_2 agonists⁹⁶

Compound	D_2 cAMP EC_{50} (nM)	D_2 cAMP E_{max} (%)	D_2 β arr2 EC_{50} (nM)	D_2 β arr2 E_{max} (%)
5	1.0	51	4.0	62
6	Inactive	<20	1.6	47
7	Inactive	<20	50	97

Table 3

From cariprazine to the G protein-biased D₂ agonists⁶²

8, R= NMe₂, *cariprazine*
9, R= OtBu

Compound	cAMP			pERK1/2			cAMP-pERK1/2		
	pK _A	Log τ	Log(τ/K_A)	pK _A	Log τ	Log(τ/K_A)	log(τ/K_A)	log(τ/K_A)	log(τ/K_A)
8	8.71	0.85	9.59	1.04	7.61	-0.37	7.25	-1.3	2.34
9	7.11	0.76	7.92	-0.63	7.51	-0.31	7.21	-1.34	0.71