Bias Translation: The Final Frontier?

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September 4, 2023

Abstract

Biased signaling is a natural result of GPCR allosteric function and should be expected from any and all synthetic agonists. Therefore, it may be encountered in all agonist discovery projects and must be considered as a beneficial (or possible detrimental) feature of new candidate molecules. While bias is easily detected , the synoptic nature of GPCR signaling makes translation of simple in vitro bias to complex in vivo systems problematic. The practical outcome of this is a difficulty in predicting the therapeutic value of biased signaling due to the failure of translation of identified biased signaling to in vivo agonism. This is discussed in this review as well as some new ways forward to improve this translation process and better exploit this powerful pharmacologic activity.

Review ms: British Journal of Pharmacology

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keywords: Drug discovery, biased signaling, receptor agonism

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Running Title: Translation of Agonist Bias

 # ms pages

 # figures: 5

 # Tables: 0

#references: 124

 # words abstract: 125

 # words: Introduction 591

 # words Discussion: This is a review so essentially the rest of the paper is Discussion- 7147

Authorship contribution: Terry Kenakin wrote the manuscript:

Funding footnote: This work received no external funding.

Financial Disclosure Statement: The author has no actual or perceived conflict of interest with the contents of this article.

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Biased signaling is a natural result of GPCR allosteric function and should be expected from any and all synthetic agonists. Therefore, it may be encountered in all agonist discovery projects and must be considered as a beneficial (or possible detrimental) feature of new candidate molecules. While bias is easily detected , the synoptic nature of GPCR signaling makes translation of simple in vitro bias to complex in vivo systems problematic. The practical outcome of this is a difficulty in predicting the therapeutic value of biased signaling due to the failure of translation of identified biased signaling to in vivo agonism. This is discussed in this review as well as some new ways forward to improve this translation process and better exploit this powerful pharmacologic activity.

Introduction

It has been 28 years since the first papers describing signaling bias were published but we are just beginning to fully capitalize on this unique property of GPCRs; at present this represents a prominent example of a scientific mechanism failing to live up to it's promise. GPCR signaling bias is the result of natural protein allostery and is an established property of receptors and agonists. Simple *in vitro*assays can identify biased agonists which hopefully then can be used to produce unique (and in many cases beneficial) *in vivo* phenotypic agonism. A practical barrier to the effective exploitation of this property, however, is the confounding effect of the cellular host. It is not clear if this is the reason for biased molecule failures *in vivo* but it could be a consideration. Thus while signaling bias remains a viable valuable property of new synthetic agonists, unless ways to predict accurate translation can be solved, it may remain an untapped resource in drug therapy.

Agonists produce cell response because they have affinity for the receptor and also the fact that, when bound, they change the conformation of the receptor through the property of efficacy. Molecular dynamics predicts that binding is not a passive property but rather that the binding of a ligand to a protein necessarily will alter the conformation of that protein (Kenakin and Onaran, 2002). Therefore, ligand binding is expected to change receptor conformation which, in turn, will be discerned by the cell. Historically, whole cell or tissue response has revealed graded strengths of efficacy but since whole cell response or single signaling pathways were chosen for assays, no texture in the quality of efficacy could be discerned. When pharmacologists

acquired the ability to selectively measure different signaling pathways emanating from the same receptor with agonist activation, a rich allosteric world of texture in agonist response was revealed.

In the time honored Pharmacologic tradition of 'Occam's Razor' (keep it simple), receptors were thought of as switches that were activated by agonists to form a state which then interacted with cellular signaling components to produce response. Though receptors are known to be pleiotropic with respect to the number of signaling pathways they influence, it was assumed that the full cadre of available pathways coupled to the receptor were activated by agonists in a generally uniform manner as a function of strength of signal. The main reason why this could not be challenged at the time was the paucity of readouts of agonist efficacy, i.e. these were complex outcomes of agonist activation such as whole cell response or monotonic signals chosen by the assay such as cyclic AMP or calcium.

The first indications that this simple model was not tenable were reports that, when more than one signaling pathway coupled to a single receptor could be measured, there were deviations from homogeneous activation. Although the mechanism for this was not specified, reports began to indicate the possibility that different agonists might select different signaling pathways (i.e Roth and Chuang, 1987). A subsequent wave of publications indicated that signaling was more heterogenous than previously thought; various groups around the world published these ideas and each had their own name for the phenomenon ('Stimulus Trafficking'; Kenakin, 1995; 'Biased Agonism' ; Jarpe et al, 1998 ; 'Functional Selectivity', Lawler et al, 1999; 'Functional Dissociation' Whistler et al, 1999; 'Biased Inhibition', Kudlacek et al, 2002; 'Differential Engagement',Manning, 2002; 'Collateral Efficacy', Kenakin, 2005). The first proposed mechanism for this was the selective stabilization of different active receptor conformations by different agonists (Kenakin, 1995; Kenakin and Morgan, 1989).

Molecular Mechanism of Biased Signaling

GPCRs are Nature's prototypic allosteric proteins designed to bind a ligand that subsequently modifies the interaction of the receptor with another body (i.e. G protein, β-arrestin, etc.); bias is the result of standard probe dependent protein allostery where 'probes' (signaling proteins) are affected differentially by agonist receptor activation. The link between biased signaling and standard receptor allostery are evident in the mathematical models used to describe both phenomena. Thus, it can be shown that the standard equations describing receptor allostery and agonism also can be used to describe biased signaling (Kenakin, 2021).

Allosterism is a modification of an immensely complex tertiary protein structure and if more than one entity interacts with the receptor, there are no rules to dictate that the effect of a change in conformation (such as that produced by an allosteric ligand like an agonist) will be unform for the two interactions at different sites (in fact, experimental evidence shows that this rarely if ever is the case). Thus the multiple interrogators of receptor information in the cell cytosol are excellent reporters of different receptor conformational states and this is the source of allosteric heterogeneity.

There are biochemical and biophysical assays that can directly identify agonist-specific receptor states. Biosensors have been employed to detect separate receptor active states (Ghanouni et al, 2001) as in the recent study with the angiotensin II type (Devost et al, 2017). BRET experiments also have been used to identify δ-opioid receptor agonist-selective receptor conformations (Audet et al, 2008).^{19}F-NMR has been used to identify agonist-selective β_2-adrenergic receptor agonist-receptor complexes (Liu et al, 2012). Distinctly different receptor active states also have been identified through receptor structure (El Daibani et al, 2023; Wingler et al, 2019; 2020).

Data suggests that Nature has hard wired 'bias' into natural neurotransmitters and hormones according to the needs of physiology. While natural agonists are commonly referred to as 'non-biased' or 'balanced' these actually have 'Nature's' bias with little relation to*in vitro* assays of varying sensitivity. Terms such as non-biased or balanced have no intrinsic meaning as arbitrarily they are controlled by the sensitivity of *in vitro* pharmacologic assays. For practical application of bias to therapy, so called 'non-biased' or balances' (natural) agonists only serve as a point of reference for synthetic agonists to demonstrate different signaling profiles in the therapeutic environment. It can be seen that Nature's bias is exploited in natural systems for

fine tuning multi agonist signals for receptors; for example the chemokine receptor CXCR7 has two natural agonists (CCL19, CCL21); one recruits β-arrestin and the other does not (Kohout et al, 2004; Sarma et al, 2023).

Experimentally, once pharmacologists had the means to separately measure agonist mediated receptor activation of different signaling pathways, probe dependence was revealed in the form different signaling patterns for different agonists, i.e. biased signaling. Importantly, natural probe dependent allostery then suggests that the quality of efficacy (mixture of signaling pathways to the cell) would be *expected to differ* for a synthetic agonist, i.e. *we should not expect synthetic agonists to have the same quality of efficacy as natural agonists.* Operating on the premise that biased signaling should be an expected property of a new synthetic agonist, it is useful to consider the methods to detect this molecular property

Identifying and Measuring Bias

There are simple *in vitro* techniques that can be used to detect and quantify bias but the most reliable is to simply express two signaling responses for an agonist for the same receptor as functions of each other; this is a 'bias' plot and clearly indicates bias without models or equations. For example, Fig 1 shows the obviously different bias of four K-opioid agonists for G-protein and β-arrestin response; the data indicate that these agonists stabilize two distinctly different active receptor conformations, one preferring G proteins and one preferring β-arrestin (White et al, 2014). For exploitation of biased signaling in discovery programs, models have been derived to put numbers on the magnitude of bias to allow medicinal chemists to systematically modify chemical scaffolds (Onaran et al, 2017; Kenakin, 2019; Kolb et al, 2022). Basically these procedures identify exemplar agonists as being special, the assumption being that they stabilize a unique active receptor state conformation. When these effects are seen, there are important criteria that must be met before biased signaling is considered seriously.

1. Lack of response does not necessarily signify bias but rather only that the agonist efficacy is insufficient to demonstrate where along the concentration axis of a dose-response (DR) curve the response occurs in a particular assay. Therefore, a **measurable CR curve must be observed** to accurately determine bias.
2. If no response in a particular pathway is observed for a molecule (appearance of 'perfect' bias) it should be tested as an antagonist of other molecules that do activate the pathway to assess the interaction with the receptor and determine affinity.
3. Bias has no translatable meaning in itself but only as a ratio to another agonist, i.e. bias always refers to a comparison to another agonist, usually the natural agonist.
4. Potency ratios must be used as this cancels measurement bias caused by differences in the sensitivities of different functional assays, i.e. second messenger assays are more sensitive than BRET assays. In this regard, the separation between two pathway DR curves does not designate the relative concentrations producing effect *in vivo* ; this occurs over the same concentration range but just with different magnitudes.

Finally, it should be recognized that 'bias' does not designate whether or not selective agonism will be seen; this is determined by the intrinsic efficacy of the agonist. However, when the system is sensitive enough and/or when the agonist has sufficient efficacy, bias determines the relative strength of signal for a given pathway when agonism is observed.

It should be recognized that assays identifying biased signaling, whether it be therapeutically applicable or not, identify an important agonist species, i.e. an agonist that produces a unique active state receptor of the receptor. This is the important deliverable of an *in vitro* bias assay but the *selected pathways used to detect the receptor conformations may not be the relevant pathways* for therapeutic bias. In essence, the *in vitro* detection bias measurements can be considered a method of detecting a harbinger of complex signaling heterogeneity to come that eventually may culminate in therapeutically beneficial bias. The main utility of bias scores is that they enable classification of new agonists for further testing *in vivo* and once bias is identified at the level of the receptor, it can be considered a rogue property and a unique uncontrolled signal

initiator that then imparts a unique activation pattern on cells.

In terms of thermodynamics, the complete description of a ligand activity on a receptor must include both affinity and efficacy and the operational model index of $\mathrm{Log}(\tau/\mathrm{K_A})$ fulfils that criterion (Kenakin et al, 2012). However, while bias can arise through differences in either affinity or efficacy (or both), the most robust differences come from differences in efficacy (Rajagopal et al, 2011). This is in keeping with the fact that agonism is much more resistant to changes in tissue sensitivity when it is based on high efficacy as opposed to high affinity (Kenakin, 1984).

From it's inception, bias has been considered a binary readout, i.e. if cyclic AMP and β-arrestin assays are used to discern signaling, compounds would fall into one of two groups (or perhaps a third that did not discern pathways). However, these are crude indicators of a more sophisticated phenomenon, namely the stabilization of a unique receptor active state and this active state could go on to selectively interact with a number of other proteins in the cell to show further differentiations. Therefore, from the first binary test of agonism in two pathways, **a further series of experiments could be done** to delineate finer texture in the signaling produced by the molecule.

Currently, sophisticated readouts of receptor activation of signaling components are being used to detect and quantify bias in ways that go beyond a binary readout. For example, measuring biased signaling through monitoring G protein subunits allows estimation of imbalances in neurotransmission (Park et al, 2023). New assays have been developed to determine micro-interactions of receptors with the complete complement of G proteins in a cell (Olsen et al, 2020). The application of pathway-selective bioluminescence resonance energy transfer (BRET) biosensors that monitor the engagement and activation of signaling effectors downstream of G proteins (including phospholipase C (PLC), p63RhoGEF, protein kinase C (PKC), and Rho), allow clustering of compounds into different subfamilies of biased ligands. These effects, in turn, show bias among G protein subtypes, in terms of not only functional selectivity between Gαq and β-arrestin but, interestingly, bias through the engagement of different G proteins to activate a common effector. Through a suite of BRET biosensors, a fingerprint of angiotensin receptor agonism has the ability to provide a view of signaling at various levels of GPCR activation – see Fig 2 (Namkung et al, 2018). Nanoluciferase-based complementation assays also have been used to provide textured readouts of biased signaling (Laschet et al, 2019) and genetically-encoded fluorescent biosensors have been employed to illuminate spatiotemporal biased signaling (Kayser et al, 2023).

Therapeutic Applications of Bias

Bias By Design: Biased agonism emerged as a pharmacologic force through pre-conceived notions of how agonist activity could be improved. These ideas emerged from consideration of the impact of various signals on physiology and the resulting outcomes. The first foray into this area was with the angiotensin receptor biased agonist/antagonist TRV027. This molecule was designed to improve the then current therapy of losartan (an angiotensin receptor blocker designed to block angiotensin-mediated vasoconstriction) (Violin and Lefkowitz, 2007); TRV027 provided angiotensin blockade of vasoconstriction with β-arrestin positive cardiac effects by being a biased agonist toward β-arrestin away from Gαq stimulation (Violin et al, 2006; 2010; 2014). From a theoretical point of view it is difficult to imagine a better exemplar biased molecule for the testing of this hypothesis in therapy. There have been many more prospective ideas presented for molecules with modified signaling profiles to improve drug therapy- see Table 2 of Kenakin, 2019. In general these ideas center on the emphasis of beneficial signaling pathways (eg. β-arrestin for PTH bone building in osteoporosis, [Ferrari et al, 2005]; β-arrestin-mediated glucagon-like peptide-1 insulin secretion in diabetes [Sonoda et al, 2008], or the elimination of negative signaling [opioid-mediated respiratory depression and or negative behavioral effects mediated by β-arrestin (Raehal and Bohn, 2011; Raehal et al, 2005; Bohn et al, 2003; Urs and Caron, 2014]). There are many more recent proposals for improved drug candidates based on the biased concept including agonists for muscarinic receptors (Randakova and Jakubic, 2021; McDonald et al, 2022), opioid receptors (Che et al, 2021; Conibear et al, 2020), Ghrelin (Mende et al, 2018), Neurotensin (Krumm et al, 2023), Glucocorticoids (Mao et al, 2023), and 5-HT2A (Pottie et al, 2023; Allen et al, 2011). Interestingly, efforts to achieve selectivity through allosteric modulation in studies for Alzheimer's disease

and schizophrenia fall short of yielding the selectivity needed without added biased signaling (in this case, induced bias through allosteric modulation) (van der Westhuizen et al, 2021).

Currently new approaches to the active quest for biased molecules have been developed (i.e. DNA-encoded libraries (Cai et al, 2023). At the receptor level techniques employing molecular dynamic simulations (Suomivuori et al, 2020), receptor structure (Cao et al, 2023; Sengmany et al, 2020; Vuckovic et al, 2023), receptor/β-arrestin/GRK structures (Chen and Tesmer; 2022), and combinations of techniques (eg atomic-level molecular dynamic simulations and functional assays) have been used to design biased molecules (El Daibani et al, 2023). Interestingly, a case where bias has been associated with structure is the identification of an allosteric binding site on PTH1R (and possible other members of class B GPCRs) which mediates only receptor interaction with G proteins and not β-arrestin (Zhao et al, 2023).

Interest in previously forbidden targets (where activation of the target initiates negative signaling responses) has been resurrected through considering biased signaling. An example of this is the K-opioid receptor which could be involved in cognition, reward, mood and perception. Agonists for these receptors have possible utility as antidepressants and anxiolytics in affective disorders, drug addiction, and psychotic disorders. However, this receptor also produces β-arrestin-mediated disturbing hallucinations thereby precluding therapeutic application. Analyses of biased signaling of K-opioid agonists suggests that the harmful effects of K-opioid agonism may be modulated through biased signaling and convert this receptor to be a viable therapeutic candidate protein (White et al, 2014; Che et al, 2021).

A variation on seeking bias in a new molecule is to intentionally control the intrinsic efficacy of the molecule to diminish an unwanted signaling effects. An added bonus of this approach is that the biased molecule will function as an antagonist of the negative signals that would have been produced by the natural agonist. This actually is an important part of the biased profile for the angiotensin molecule for heart failure TRV027; specifically, while it produces β-arrestin agonism with an EC_{50} of 10 nM, it also is a competitive antagonist at the same concentration blocking natural angiotensin mediated agonism of vasoconstriction at the same concentration (pKi=8.0: Violin et al, 2010). In fact the low intrinsic efficacy of the new biased opioid agonist TRV130 is postulated to be an important part of this molecule's therapeutic profile (Che et al, 2021). Specifically, estimation of the relative efficacy of TRV130 (vs morphine) with the operational model of agonism (Black and Leff, 1983) shows that TRV130 has 33% of the efficacy of morphine for G protein and only 15% of the efficacy of morphine on β-arrestin (Singleton et al, 2021). Partial agonism of opioid receptors is being pursued as a means of bias for other molecules as well (Lutz et al, 2023). In addition, low efficacy for β-arrestin has been identified as an important feature of new GLP-1 biased agonists such as tirzepatide (Willard et al, 2020; Yuliantie et al, 2020; Jones; 2021).

Bias by design also may be approached through the mechanism of allosteric agonists. Specifically, there are data to show that allosteric agonists tend to have a different signaling bias from standard orthosteric agonists; for example, in a series of muscarinic m2 receptor agonists a bias plot for orthosteric and allosteric agonists producing [^{35}S] GTP-S and ERK1/2 responses in CHO cells shows that the conventional orthosteric agonists are generally biased toward GTP-γ-S while the allosteric agonists are biased toward ERK1/2 (Gregory et al, 2010).

Bias By Cross-Screening: Another approach is through the evaluation of biased signaling in agonist therapy in retrospect, i.e. cross screening molecular libraries to identify exemplar selective agonists that show preference to one pathway over another. This identifies molecules that specifically activate certain signaling pathways over others which then can be explored separately to identify possible therapeutic utility. For example, a comparative screen of adenosine A1 receptor agonists in calcium and cyclic AMP assays demonstrates a wide dispersion of biased compounds with little correlations (Aurelio et al, 2018). This approach plays on the natural expectation of allosteric probe dependence yielding bias thus reversing the question. . . 'should an agonist program seek bias?' into '. . . Bias will eventually seek an agonist program'.

The Therapeutic Impact of Biased Signaling:

In general, prior art suggests that identifying agonists that stabilize unique receptor states is not a difficult

task. However, the linking of these exemplar molecules to attainable biased signaling states of benefit in therapy is a more formidable challenge. As a preface to considering this, it is useful to review the current impact of bias on drug therapy.

Retrospective Analyses of Known Drugs: Over the past years, literature reports have linked favorable therapeutic profiles of drugs retrospectively with identified bias. An example of this is the drug carvedilol, an inverse agonist for Gαs-mediated cAMP production in heart failure where beneficial effects have been attributed to β-arrestin–mediated partial agonist activity for activation of ERK1/2 (Wisler et al, 2007; Kim et al, 2008). This shared profile also has been cited for the useful responses to propranolol (Azzi et al, 2003; Baker et al, 2003), nebivolol (Erickson et al, 2013), and alprenolol (Baker et al, 2003). Further, the reduced respiratory depression potential of the opioid analgesic levorphanol (over morphine) has been attributed to its biased signaling profile (lack of β-arrestin2 recruitment) (Le Rouzic et al, 2019). Interestingly, the dependence liability of oxycodone, hydrocodone/paracetamol, and hydromorphone has been attributed to biased signaling to G protein over β-arrestin (Johnson et al, 2017). Other reportedly unique biased profiles for established drugs have been reported for the adenosine 2b receptor agonist capadenoson (cardioprotective and cardiac fibrosis–modulating properties through biased cAMP activity; Baltos et al, 2017) and the β_2-adreoceptor agonist fenoterol (biased activation of Gαs protein over nonspecific dual Gαs and Gαi activation (Jozwiak et al, 2010). While it is not clear whether the noted biased signaling for these molecules is the discerning property in their beneficial therapeutic effects, it is notable that beneficial profiles are associated with biased profiles in these drugs. These drugs were developed before bias was considered a viable drug property; since then, molecules consciously have been developed with known biased signaling properties in attempts to improve therapeutic profiles.

Current Biased Molecules: As mentioned previously, the first biased molecule designed to enter the therapeutic arena was TRV027 for congestive heart failure. This molecule has a unique lack of efficacy for angiotensin-mediated vasoconstriction (via Gαq protein) and a residual and measurable efficacy for angiotensin receptor-mediated β-arrestin activation. Notably, an acute randomized double-blind placebo-controlled Phase IIB dose-ranging trial (BLAST-AHF) did not show superior efficacy for TRV027 illustrating the difficulties in bias translation from the *in vitro* to *in vivo* arena; in this case, the trial design could have been the reason for failure. Specifically, a molecule such as TRV027 would be expected to enhance beneficial cardiac remodeling over many months. In contrast, the trial design was a short term 24-28 hr infusion of TRV 027 followed by a 30 day endpoint of symptoms and survival which really would not detect the possible beneficial effects of this molecule. In addition , a possible lack of activation of the renin-angiotensin system in the heart failure patients could have caused a negative background for this molecule coupled with shortcomings in study design and duration of treatment (Pang et al, 2017; Sugihara et al, 2017).

Data from early experiments with morphine and other opioids had suggested that bias away from β-arrestin could be beneficial (Raehal and Bohn, 2011; Raehal et al, 2005; Bohn et al, 2003; Urs and Caron, 2014) and this led to continued research into biased opioid agonists for pain management (Le Rouzic et al, 2019; Conibear et al, 2019). This is a very active area of research and presently there is a viable biased agonist opioid agonist (TRV130) approved for moderate to severe pain (Lambert et al, 2020; Viscusi et al, 2019).

A recent and very active research into biased signaling is in the field of incretin agonists for metabolic diseases such as obesity and diabetes. Several peptides are under development for GLP-1R and either the glucagon receptor (GCGR) and/or glucose-dependent insulinotropic peptide receptor (GIPR) as dual- or tri peptide agonists. It has been shown that many novel peptides have distinct biased agonism profiles relative to either natural agonists or each other (Darbalaei et al, 2020). Examples of this are biased signaling for GIP mono-agonists (Pro3GIP, Lys3GIP) towards ERK1/2 phosphorylation (pERK1/2) relative to cAMP accumulation at GIPR, the dual GIPR/GLP-1R agonist, LY3298176, biased towards pERK1/2 relative to cAMP accumulation at both GIPR and GLP-1R (vs endogenous ligands), and the triple agonist GLP-1R/GCGR/GIPR tirzepatide biased towards pERK1/2 relative to β-arrestin2 recruitment. In addition, studies have shown reduced β-arrestin recruitment for tirzepatide leading to less GLP-1 receptor desensitisation and downregulation (Yuliantie et al, 2020). While there is an abundance of data on the biased signaling for these agonists,

it is not yet clear to what extent the bias contributes to their beneficial action vs dual and tripartide agonism (Jones, 2021).

Bias Translation: Possible Reasons for Failure

In terms of translation of biased agonism found in human cells *in vitro* , there are two considerations. The first relates to drug development in that a given pharmacologic profile must be linked to a potential therapeutic indication and often that is done through testing in animal models. Therefore, this involves the transfer of bias from a human receptor to an animal orthologue receptor. For orthosteric ligands this usually is not a serious impediment as residues required for natural ligand binding and structural integrity of receptors are highly conserved whereas residues critical for allosteric signaling are poorly conserved (Leandera et al, 2020). In general, orthosteric natural agonist recognition sites for families of receptors with a common agonist (such as the 5 member family of acetylcholine receptors) is difficult due to the similarity of the conserved acetylcholine binding sites (Gentry et al, 2013; Myslivecek, 2022). However, since bias involves protein allostery, there are data to suggest that bias transduction could be more sensitive to receptor structure and a.a. homology than standard orthosteric agonism even through nuances of agonists binding differently at the same binding site (metabotropic glutamate receptor 5 (Hellyer et al, 2020). In fact allosteric effects have been shown to be particularly sensitive to minor differences in a.a. homology possibly because the residues involved in allosteric function go beyond those involved in agonist recognition. Therefore, differences in residues distant from the natural agonist binding site can be critical to the effect of a ligand produced at the receptor binding site and single amino acid mutations have been shown to produce serious effects on allosteric molecular function. For example, the muscarinic receptor positive allosteric modulator BQCA produces >10 fold potentiation of acetylcholine effects in native receptors but a single amino acid mutation ($Y381^{6.51}A$) in the receptor completely negates the effects of BQCA (Abdul-Ridha et al, 2014). Similar differences in biased signaling are seen with the dopamine D1 receptor agonist bias for agonists at dopamine D2 receptors. While in the Wild Type Dopamine D1 Receptor DPAT D1 is biased toward ERK, a single a.a. mutation reverses DPAT bias toward cAMP (Tschammer et al, 2011). Differences in a.a. sequence, as seen with human and animal receptor orthologues, can show large differences in allosteric modulators as in the case of allosteric potentiating modulators of the glutamate receptor Type 1; these show large differences in activity between human and rat receptors (Cho et al, 2014). Since bias is allosteric modulation, this may pose special problems testing translation of *in vitro* human signaling bias to animal therapeutic models to assess significance.

The second issue with bias translation does not inolve species differences but rather, translation across different human systems. Progress in new technologies has brought vast improvements in screening hit rates and the development of new molecules but in spite of these advances, the success rate of actual drug candidates that are useful therapeutically is still surprisingly low. One estimate suggests that 50% of all new drug candidates fail because of lack of efficacy (Arrowsmith, 2011) where here efficacy is defined as the candidate performed as required in human therapeutic settings. Aside from commercial and safety issues, this rate of failure indicates a serious shortcoming in the drug discovery process in that it represents the fact that, after rigorous state of the art application of pharmacology and discovery science, seemingly optimal candidate molecules still do not do what they were supposed to do in humans. Literature analyses suggest that the difficulties may be related to failure to verify compound exposure and to demonstrate physical target engagement in the relevant therapeutic tissue (Morgan et al, 2012; 2018; Bunnage et al, 2013; Cook et al, 2014) but with biased ligands, there may be other issues. Obvious reasons for miscalculation are failure to recognize what efficacy is needed to treat the disease, and/or a wrong choice of biological or chemical target. However, another factor that may be under-estimated is a failure to adequately characterize the true efficacies of the candidate molecule; this may especially be true in the case of biased molecules. From this standpoint the question could be asked, is it enough to characterize receptor-mediated bias for a candidate molecule without further classifying possible texture in biased signaling in the cell?

There are a number of possible dissimulations between initial bias estimates at the receptor level and complex *in vivo* signaling profiles; it is worth considering these. An *in vitro*identification of biased signaling

furnishes a premise that the ligand in question will produce differential signaling compared to the natural agonist. Historically, initial ideas on how bias could improve therapy (see Kenakin, 2019) were gained from simplistic comparison of agonism in two quantifiable assay systems, cyclic AMP and β-arrestin. Presently there are several more sophisticated analyses to predict favorable prospective biased signaling than previously considered when only G protein and β-arrestin signaling were the options (*vide infra*).

Unknown Downstream Outcomes from Receptor-based Bias

While bias most often is detected at the level of the receptor, the stabilized receptor active state conformation may code for unknown events further on down into the cytosol. In light of the complexity of signaling in cells, it is possible that the benefcial effects of a biased agonist may be lost in the milieu of signals and biochemical cascades. Dependence on a single readout of bias at the receptor level may not identify molecules that produce unique effects further down the signaling cascade. For example, BRET association of receptors with β-arrestin indicates increased association but does not necessarily augur receptor internalization and/or signaling (*vide infra*). Recent data with β-arrestin suggest that heterogeneity in β-arrestin conformations may lead to trafficking of stimulus in various ways after receptor-β-arrestin interaction (Chen et al, 2023). Specifically, while BRET analysis of receptor association with beta arrestin may be found, it may be critical which beta arrestin confromation is involved (i.e. 'tail' or 'core', (Cahill et al, 2017) to further delineate internalization vs internalization for the formation of a 'supercomplex' of the receptor and β-arrestin in endosomes providing sustained signaling (Chen et al, 2023). Therefore, a program seeking an internally signaling agonist for prolonged cellular activity might test compounds for preferential β-arrestin bias (assuming β-arrestin is the vehicle for transport to the endoplasmic reticulum) but further experiments might discern agonist activity toward that desired endpoint. For example, fig 3 shows that agonist 11 is identified as the most biased for β-arrestin. However, further testing could be done at this stage to differentiate which β-arrestin conformation is preferred; in this case, it may be that agonist 11 would not be the preferred compound as it is not biased toward the predicted β-arrestin conformation that could lead to ER signaling. In general, testing sub-groups of biased agonists may further characterize useful activity, especially in cases where an initial testing of a biased agonist does not provide a more informative outcome. Follow up studies with other biased molecules could provide a clearer answer; as shown in the figure, identfication of agonist 11 as an exemplar biased agonist in this case would not yield the required signaling as the stimulus bifurcates throughout the cell whereas agonists 13 to 16 might have provided a better choice.

Bias translation involves the synoptic nature of pharmacologic response (i.e. the necessary partnership of the activated receptor with complex signaling patterns) and brings into consideration the cellular milieu of the active state receptor-cell mixture. In addition to the production of a variety of ligand-bound receptor active states, comes the subsequent interaction of these states with another constellation of (for example) possible β-arrestin conformations. Specifically, the conformational flexibility of β-arrestin allows GPCR-induced conformational rearrangement to expose distinct binding surfaces that allow recruitment of different effectors for specialized signaling complexes (Haider et al, 2023; Luttrell et al, 2018; Shukla et al, 2014). This can lead to considerable heterogeneity in cytosolic signaling and factorial combinations of outcomes are possible. Possible other dissimulations with respect to expected therapeutic outcomes at this stage include:

1. **Different signaling partners in different cell lines** Agonist activity on receptors transfected into different cell lines have shown differences in relative agonist potency. For example, transfections of calcitonin receptors into two different host cell lines (CHO cells, COS cells) show large differences in the relative potency for porcine Cal, human Cal and h CGRP. Specifically, in CHO cells the relative potency is hCGRP/hCal/pCal of 1/ 10/500 whereas in CHO cells the potency ratios are 1/2/8 (Christmanson et al, 1994). Total synoptic agonist response can be revealed through label free assay formats such as cell impedance; these have been to used to measure the relative potency of dopamine agonists in two types of cells (U-2 cell, SK-N-MC cells) where the cell type made a 4-fold difference in the relative potency of agonists dopamine and A77636 (Peters and Scott, 2009). This translates into differences in cell bias as function of cell type
2. **Variant stoichiometry between receptors and signaling components** . The most obvious

variable operative here is the relative stoichiometry of receptors and signaling components in various cells. The relative stoichiometry of receptors and signaling proteins is a well established variable in functional pharmacology and this brings into play the role of the host cell in bias measurement and detection. Thus, a paucity of an important signaling partner (i.e. G protein) could negate a bias seen in a system where this is not the case i.e. Eason et al, 1992. This is particularly relevant to low efficacy agonists where response could disappear with low signaling coupler in a given cell; this may be a factor in the bias seen with the biased opioid receptor TRV130 (Singleton et al, 2021). An even more surprising effect can be seen for truly biased agonists in receptor systems without limitations in coupling proteins. For example, the relative potency of the full calcitonin agonists eel and porcine calcitonin for human calcitonin receptors transfected into in wild type HEK 293 cells is EC_{50}(eel Cal)/ EC_{50} (pCal) = 0.4; Co-transfection of Gαs protein to enrich the natural Gαs content produces a complete reversal of the relative potency of eel and porcine calcitonin. In this enriched cell line, the relative potency is reversed to EC_{50} (eel Cal)/ EC_{50} (pCal) = 8 (a 32-fold difference) (Watson et al, 2000). Reduction of key signaling components in a cell clearly can limit low efficacy agonists from utilizing pathways but a recently interesting variation on the theme of receptor-signaling protein relative stoichiometry suggests that actual increases in cellular receptor can affect the observed bias (Li et al, 2023). Differences in receptor expression levels in cells also can introduce a temporal dissociation for response as in the case of GPR84 where a delayed and suppressed activation of Akt was found in low expressing cell lines (Luscombe et al, 2023).

3. **ἄριαντ Στοιχηιομετρψ οφ ΓΡΚς ανδ β-Αρρεστιν:** There are striking cases of ligand-directed signaling to the β-arrestin system through targeting GRKs. For example, the chemokine receptor CCR7 has two natural agonists (CCL19, CCL21) and while CCL19 leads to receptor phosphorylation and β-arrestin recruitment through GRK2 and GRK6, CCL21 activates only GRK6. This differential activity on GRKs leads to different cytosolic consequential responses for these two chemokines (Zidar et al, 2009). Other studies indicate with receptor structural studies that the interaction of the neurotensin 1 receptor with GRK2 guides the receptor-β-arrestin interaction for signaling (Duan et al, 2023). GRK may not be the only player as β-arrestin/GRK complexes also may require the presence of ubiquitin to direct receptor selectivity (Liu et al, 2023). In general, data with GRK knockout cells reveal the importance of GRKs as major players in the cytosolic signaling of GPCRs (Drube et al, 2022) therefore variation in cellular GRK levels can modify GPCR signaling from initial *in vitro* bias determinations in other cell lines.
4. **Varying temporal differentiation of agonist signals in cells**I*n vitro* assays are snapshots in time with no real regard to the timescale of real life physiology but rather are optimized for accuracy of measurement. The two main *in vitro* assays used to detect bias (second messenger G protein vs β-arrestin) have very different timescales for steady-state and maximal effect making comparisons possibly dependent on when the measurements are made. For example, a study of dopamine D1 receptor agonist bias on cyclic AMP and β-arrestin shows a discernible temporal difference thus introducing a possible dissimulation in the assessment of signaling bias (Klein Herenbrink et al, 2016). These temporal differences extend to the cell where G protein activation of ERK is rapid and transient and β-arrestin activation of ERK are more sustained involving translocation to the nucleus (Liu et al, 2023). Temporal dissociations extend beyond short acute timespan responses to the production of transcription effects leading to protein expression. In general, the time bias measurements are taken yield somewhat arbitrary indices of differential signaling that may have unpredictable effects in real time physiology. These dissimulations in time emanate from the cellular translation of receptor activation and not necessarily from the timescale of ligand-receptor interaction. In fact it has been shown that the bias of opioid agonists are independent of the rate of interaction of the molecules with the receptor (Pedersen et al, 2020). New techniques are being used to explore this variant in bias, i.e. genetically-encoded fluorescent biosensors have been employed to illuminate spatiotemporal biased signaling (Kayser et al, 2023).
5. **Location bias:** Agonists that target receptors to β-arrestin leading to internalization can show bias with respect to the location (and function) of the internalized species (Eiger et al, 2022;Wang et al,

2023). For example, the chemokines RANTES and AOP-RANTES both internalize CCR5 receptor (for prevention of HIV-1 infection) but whereas RANTES internalizes the receptor which then rapidly re-emerges through recycling, AOP-RANTES internalizes the receptor to shunt it to lysosomal destruction (Mack et al, 1998). Thus the actual receptor conformation stabilized by the agonist may determine the fate of β-arrestin bound receptors. Location bias also has been noted for GLP-1 agonists where in studies all GLP-1 agonists activate nuclear ERK1/2 activity but the agonists liraglutide and oxyntomodulin (biased towards pERK1/2 relative to cAMP when compared to GLP-1 and exendin-4), show spatiotemporal control by also stimulating pERK1/2 activity in the cytosol (Fletcher et al, 2018).

6. **Variation of the magnitude of bias significant with respect to the overall cell response** While 'bias' can be detected in *in vitro* systems, there is no guide as to the significance of that bias to whole body physiology. Bias indices can range from fairly modest (2-3 fold) to values >10-20 raising the question, what level of bias is physiologically significant? While this probably will be system dependent, a measure of how powerful apparently small bias values can be is demonstrated by diazepam, an anxiolytic with known prominent therapeutic activity. Specifically, diazepam produces a mild two-fold sensitization of GABA response but this translates to an 80% increase in GABA response and a well-known significant physiological effect (Skerrit and MacDonald, 1984). This suggests that bias values of 2 or greater might have a significantly affect on agonist phenotypic activity. Calculation of the bias of the opioid analgesic TRV130 for cyclic AMP over β-arrestin using $\Delta\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values (Kenakin, 2017) yields a value of 3.39 (Singleton et al, 2021), ostensibly a relatively low value but of possible significance in light of data with diazepam. It should also be noted that the low efficacy of TRV130 for β-arrestin signaling (TRV130 has 33% of the efficacy of morphine for G protein and only 15% of the efficacy of morphine on β-arrestin) may significantly contribute to the beneficial profile of this molecule. Considering a relatively 'balanced' agonist that generally does not distinguish signaling proteins versus a biased agonist that does, the question arises does the degree of bias influence variability in terms of translation (i.e. variation in potency with differences in cell type and/or tissue sensitivity)? A theoretical model of a single receptor interacting with two coupling proteins (Kenakin, 2003) (Fig 4A) indicates that the potency ratio of two balanced agonists (or two agonists of identical bias) will not deviate in two cells lines of varying G protein make-up (CellA = [G1]/[G2] = 1 ; CellB = [G1]/[G2] = 10; Fig 4B). In contrast, for two agonists of different bias , the difference in G protein composition can produce radical differences in relative potency (Fig 4C). It can be seen that the relative potencies of non biased agonists remains the same whereas the relative potencies of the agonists of different bias actually reverses with the change in G protein composition. These simulations suggest that the degree of bias may contribute to the variability of translation of agonist effect in different cell types.
7. **Different levels of assessment of bias within the stimulus-response cascade in the cytosol:** When considering bias it is also relevant to think about where in the cytosolic signaling cascade the bias may make a difference. Standard *in vitro* bias assays generally assess differences at the receptor level but as signals bifurcate throughout the cell, the emphasis on discrete pathways may vary. For example, Fig 5 shows the effect of seven dopamine agonists measured from the point of view of six response pathways. When bias is assessed for each pathway through $\Delta\Delta\text{Log}(\text{max}/\text{EC}_{50})$ values, it is interesting to note that the magnitudes of the bias indices vary with pathway indicating that as the signal propagates from the cell, it is differentially modified in an agonist dependent manner (Klein Herenbrink et al, 2016). These types of effects reveal the texture of bias as a function of the number of vantage points used to make the measurements. For example, G protein selective PTH analogs build bone through cAMP and β-arrestin selective analogs would not be predicted to be as efficacious since the β-arrestin activity terminates G protein signals. However, paradoxically, β-arrestin selective analogs also build bone mass *in vivo* largely through regulation of cell-cycle, survival and migration/cytoskeletal dynamics (Luttrell et al, 2018). Arrestin-focused response signatures can further be explored through arrestin-dependent transcriptome signatures to predicted outcomes of biased agonism (Maudsley et al, 2016).

Improving Bias Translation:

There are numerous theoretical and practical hurdles to the accurate translation of *in vitro* bias to complex *in vivo* systems raising the question, how can these be minimized to optimally design biased agonist programs for success? The first step is to identify bias in a molecule and this can be done through cross-screening in two assays and comparing the results with a bias plot. Considering the complexity of allosteric differences with different receptor conformations, it probably is not too important which two pathways are chosen; G protein signaling and β-arrestin historically have been the standards. The main function of this first step is the identification of a candidate biased molecules which may produce a useful agonist phenotypes *in vivo*by stabilizing unique receptor conformations. However, out of an array of biased candidates, their 'robustness' in terms of resilience of bias to varying cellular conditions could be tested:

1. **Identify 'Efficacy-based' over 'Affinity-based' bias** : Bias based on differences in efficacy (Rajagopal et al, 2011) are more resilient to changes in cell sensitivity than those based on affinity.*Experiment* : Test bias for immutability in cells of varying sensitivity (i.e. varying levels of receptor expression).
2. **Measure the intrinsic efficacy of the candidates in single pathways** : Low efficacy in a negative pathway can be useful to strengthen bias under a range of *in vivo* conditions and a measure of agonist efficacy can be obtained through manipulation of levels of receptor expression (Jiang et al, 2022). *Experiment* : Measure the relative efficacy of the candidate to the natural agonist with the operational model.
3. **Measure variation of bias in different cellular backgrounds:**Bias could be measured in a range of host cell lines to gauge variation in biased signaling. For example, GRKs have been shown to affect signaling bias and cells have variable GRK levels and variable levels of GRKs can be used as a variable to assess the impact of GRK levels on agonist bias (Matthees et al, 2021). *Experiment:*Assess bias in cells with varying levels of GRKs and/or varying cell types.
4. **Measure the temporal dependence of bias estimates:** Some agonists yield time dependent estimates of bias which could make them unstable predictors of *in vivo* bias; for instance, while dopamine cAMP/β-arrestin bias is stable when measurements are made over 90 min, aripiprazole changes by a factor of 10 (Klein Herenbrink et al, 2016). *Experiment:* Measure bias at two separated timepoints.
5. **Apply more textured estimates of signaling heterogeneity:**While simple assays such as cyclic AMP and β-arrestin **BRET**have been used to good measure in this field, the availability of first line assays to further differentiate active state signaling can offer advantages. Thus while cyclic AMP may augur effects of the agonist-activated receptor on Gαs signaling, assays that differentiate all G protein signaling (such as TRUPATH, (Olsen et al, 2020) may offer rapid first-line separation of agonist profiles.*Experiment:* Utilize more textured G protein assays (Soave et al, 2020) as the first differentiator of bias.
6. **Measure agonist receptor off-rates:** Slow dissociation of molecules from receptors can cause pharmacodynamic-pharmacokinetic dissociation and favorable *in vivo* target coverage. This is a property separate from potency and/or bias but is essential in the characterization of a future *in vivo* candidate.*Experiment:* Measure agonist off-rates.

Conclusions

In general, initial biased assays are measures designed to identify molecules that appear to stabilize a unique receptor active state; however, subsequent experiments further measuring nuances and textures in bias may identify molecules less sensitive to pharmacologic environment and physiological conditions and thus identify 'robust' biased signaling. Beyond that these assays should not be seen as guarantees against lack of bias translation *in vivo* . Considering the myriad of possible variation in the bias translation process to*in vivo* therapy, the best approach may be to identify exemplar biased molecules and then compare them head-to-head in the therapeutically relevant system as soon as possible. However, considering the case of TRV027, the *in vivo* testing conditions also should be considered carefully as the initial clinical test will color the future testing of a biased molecule thereafter, i.e. would TRV027 have shown valuable activity in another set of patients (higher renin-angiotensin elevated activity) or under another set of conditions? In this case, TRV027 was a molecule designed to enhance cardiac remodeling over many months yet the trial was short

term (48-96 hr infusion) with endpoints that would not detect the possible beneficial effects of this molecule. The risk with inadequate tertiary testing of biased molecules is the possibility of 'throwing out the baby with the bathwater' and losing a potentially valuable entity through poorly devised tests.

Further dissection of bias in experiments may discover texture in bias that may be useful should the candidate molecule fail *in vivo*,i.e. to eliminate further study with similarly textured biased molecules. In any case, the increasing tide of biased molecules coming into development will be a positive force in assessing the value of this pharmacologic effect for therapy.

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Figure Legends:

Fig 1 Bias plot showing the recruitment of β-arrestin-2 (Tango assay) as the abscissae and activation of a genetically engineered firefly luciferase cAMP biosensor (GloSensor; Promega, Madison, WI) as the ordinates. Four agonists for the K-opioid receptor were compared:*GR89696* , 4-([3,4-dichlorophenyl]acetyl)-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumarate salt, *ICI 199,441* , 2-(3,4-dichlorophenyl)-N-methyl-N-([1S]-1-phenyl-2-[1-pyrrolidinyl]ethyl)acetamide hydrochloride; *RB 48,* 22-thiocyanatosalvinorin A and *RB 64* , 22-bromosalvinorin A. Plot shows that the salvinorin analogues RB64 and RB48 are biased toward G protein while ICI199441 and GR89696 are biased toward β-arrestin. Data from White et al, 2014.

Fig 2 A. Angiotensin receptor II type 1 receptor downstream signaling pathways and BRET sensors for G protein and β-arrestin activation, specifically heterotrimeric G proteins and activation of βarr2. cAMP, adenosine 3',5'-cyclic monophosphate. B. Heatmap of relative activity ($\Delta\text{Log}(\tau/K_A)$ (Kenakin et al, 2012) values for angiotensin analogs showing activation of various G proteins and β-arrestin. Data from Namkung et al (2018)

Fig 3. Theoretical scheme for bias testing showing how secondary bias assays may further differentiate and better characterize an initial bias estimate.

Fig 4: Concentration-response (CR) curves for two agonists (denoted by black and red curves) A. Theoretical model showing receptor R interacting with an agonist A and two G proteins G1 and G2 (Kenakin, 2003). B. Non biased agonist parameters: Agonist_1 (black curve): $\alpha = 10$, $\gamma_1 = \gamma_2 = 2$, $K_{G1} = K_{G2} = 3$: Agonist_2(red curve): $\alpha = 10$, $\gamma_1 = \gamma_2 = 10$, $K_{G1} = K_{G2} = 1$. Composition of Cell Types: CellA = $[G_1] = 1$, $[G_2] = 10$: CellB = $[G_1] = 10$, $[G_2] = 1$. C. Biased agonist parameters: Agonist_1 (black curve): $\alpha = 10$, $\gamma_1 = 1$, $\gamma_2 = 10$, $K_{G1} = 3$, $K_{G2} = 1$: Agonist_2 (red curve): $\alpha = 10$, $\gamma_1 = 10$, $\gamma_2 = 1$, $K_{G1} = 1$, $K_{G2} = 3$. Cell type composition as in Panel B.

Fig 5. Bias at different levels within the cell. A. Schematic diagram showing three general zones where assays may access bias. Usually it is detected at the level of the receptor. As the receptor-mediated signals spread through the cell various other components become involved which can modify bias in different areas. Finally, whole cell response integrates these signals to yield a quantity and quality of efficacy. B. Data for seven dopamine agonists producing six responses in the cell. Radar plot shows the relative bias of the agonists for each pathway (color coded) through $\Delta\Delta\text{Log}(\text{max}/EC_{50})$ values. Note how the bias for the agonists differs depending on which part of the signaling pathway is assessed. Data from Klein Herrenbrink et al, 2016.