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Fulfilling the Promise of 'Biased' GPCR Agonism

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1

Running Title: Climate change in GPCR drug discovery?

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List of Nonstandard Abbreviations: AngII, angiotensin II; $AT_{1A}R$, angiotensin II type 1A receptor; GPCR, G protein-coupled receptor; $5HT_{2A}R$, 5-hydroxytryptamine type 2A receptor; □□□, κ opioid receptor; LSI, latent semantic indexing; MOR, μ opioid receptor; PTH, parathyroid hormone; PTH_1R, type 1 parathyroid hormone receptor

Abstract

The fact that over 30% of current pharmaceuticals target heptahelical G protein-coupled receptors (GPCRs) attests to their tractability as drug targets. While GPCR drug development has traditionally focused on conventional agonists and antagonists, the growing appreciation that GPCRs mediate physiologically relevant effects via both G protein and non-G protein effectors has prompted the search for ligands that can 'bias' downstream signaling in favor of one or the other process. Biased ligands are novel entities with distinct signaling profiles dictated by ligand structure, and the potential prospect of biased ligands as better drugs has been pleonastically proclaimed. Indeed, preclinical proof-of-concept studies have demonstrated that both G protein and arrestin pathway-selective ligands can promote beneficial effects *in vivo* while simultaneously antagonizing deleterious ones. But along with opportunity comes added complexity and new challenges for drug discovery. If ligands can be biased, then ligand classification becomes assay dependent, and more nuanced screening approaches are needed to capture ligand efficacy across several dimensions of signaling. Moreover, because the signaling repertoire of biased ligands differs from that of the native agonist, unpredicted responses may arise *in vivo* as these unbalanced signals propagate. For any given GPCR target, establishing a framework relating *in vitro* efficacy to *in vivo* biological response is crucial to biased drug discovery. This review discusses approaches to describing ligand efficacy *in vitro*, translating ligand bias into biological response, and developing a systems-level understanding of biased agonism *in vivo*, with the overall goal of overcoming current barriers to developing biased GPCR therapeutics.

Introduction

Early models of GPCR signaling envisioned the receptor as existing in equilibrium

between discrete 'off' and 'on' states distinguished by their ability to trigger downstream

responses. Ligands were thought to act by perturbing this equilibrium, and were

classified as agonists if they elicited a maximal response, partial agonists if they

generated a sub-maximal response at saturating ligand concentration, antagonists if they

lacked intrinsic efficacy but competitively inhibited agonist responses, and inverse

agonists if they preferentially stabilized the 'off' state leading to a suppression of basal

receptor activity (Black and Leff 1983; Black et al. 1985; Samama et al. 1993; Weiss et

al. 1996). This conceptualization began to evolve in the mid-1990s, driven by the

recognition that many GPCRs couple to several different G protein families, enabling a

single receptor to engage multiple signaling pathways simultaneously (Offermans et al.

1994; Laugwitz et al. 1996) or to activate them differentially in different tissues (Jin et al.

2001; Mahon et al. 2002). As appreciation of the complexity of GPCR signaling grew, so

did evidence that changes in ligand structure can affect the efficiency of receptor

coupling to different downstream effectors (Sagan et al. 1996; Berg et al. 1998; Maudsley

et al. 1998; Takasu et al. 1999; Holloway et al. 2002). The finding that structurally

distinct ligands can activate the same GPCR in different ways indicated that most, if not

all, GPCRs possess more than one 'active' receptor state, and that ligand structure can

'bias' downstream signaling relative to that of the native agonist (Kenakin 1995;

Christopoulos and Kenakin 2002).

Adding to the complexity was the recognition that GPCRs signal through both G

protein and non-G protein effectors. The first non-G protein effectors to be described

were the arrestins, a small family of cytosolic proteins originally characterized for their

role in GPCR desensitization (Ferguson 2001). Beginning in 1999 with the report that

arrestin2 bound the nonreceptor tyrosine kinase, c-Src, and recruited it to activated β2

adrenergic receptors (Luttrell et al. 1999), studies linking arrestins to GPCR activation of

novel enzymatic effectors began appearing with regularity. Putative arrestin-regulated

effectors include Src family kinases (Barlic et al. 2000; DeFea etal. 2000a), components

of the extracellular signal-regulated kinase 1/2 and c-Jun N-terminal Kinase 3 mitogen-

activated protein kinase cascades (DeFea et al. 2000b; McDonald et al. 2000; Luttrell et

al. 2001), the E3 ubiquitin ligase, Mdm2 (Shenoy et al. 2001), the cAMP

phosphodiesterases, PDE4D3/5 (Perry et al. 2002), diacylglycerol kinase (Nelson et al.

2007), the inhibitor of nuclear factor-κB, IκBα (Witherow et al. 2004), the Ral-GDP

dissociation stimulator, Ral-GDS (Bhattacharya et al. 2002), the actin filament-severing

protein, cofilin (Zoudilova et al. 2007), and the Ser/Thr protein phosphatase 2A (Beaulieu

et al. 2004; Beaulieu et al. 2005). With time, it became clear that GPCRs signal via a

variety of non-G protein effectors besides arrestins, including PDZ domain- and non-PDZ

domain-containing scaffolds (Walther and Ferguson 2015), and that interaction with

accessory proteins is a critical determinant of both the ligand binding and effector

coupling specificity of many GPCRs (Kenakin and Miller 2010). The current model of

GPCR pharmacodynamics recognizes that efficacy arises from the bidirectional allosteric

interplay of ligands that stabilize unique ensembles of receptor states, and the

complement of intracellular effectors, accessory proteins, and environmental influences

that translate those conformations into cellular responses while at the same time

modulating the conformational ensemble through allosteric interaction (Kenakin and

Miller 2010; van der Westhuizen et al. 2015). With respect to the dichotomous G protein- and arrestin-mediated pathways, GPCR signaling may be thought of as the minute-to-minute balance between highly amplified G protein-mediated signals that are rapidly desensitized by arrestin binding, and more durable, but less amplified, non-canonical arrestin-mediated signals that arise from stoichiometric GPCR-arrestin 'signalsome' complexes (Ferguson 2001; Luttrell and Lefkowitz 2002). Whereas second messengers generated via G protein-dependent activation of enzymatic effectors account for most of the classical short term consequences of GPCR activation, arrestin-mediated signals appear to perform numerous functions, among them enhancing second messenger degradation, regulating cytoskeletal dynamics controlling vesicle trafficking, exocytosis and cell migration, and promoting cell survival, growth and hyperplasia (Luttrell and Gesty-Palmer 2010; Luttrell 2013).

Importantly for our story, G protein- and arrestin-coupled components of the receptor ensemble are pharmacologically dissociable, such that highly efficacious activators of G protein signaling may not promote engagement of arrestins (Walters et al. 2009; Zhou et al. 2013), rendering them effectively non-desensitizing, while ligands display no efficacy for G protein coupling may yet serve as agonists for arrestin-mediated signaling events (Wei et al. 2003; Zimmerman et al. 2012; Azzi et al. 2003; Gesty-Palmer et al. 2006). This conjunction of 'pluridimensional efficacy' (Galandrin and Bouvier 2006) and ligand 'bias' has set the stage for development of novel pharmaceutical agents that act *in vivo* as pathway-selective agonists or as mixed agonist-antagonists that promote the activation of some pathways while inhibiting the capacity of the endogenous ligand to stimulate others.

What 'bias' is – And what it isn't

Translating orthosteric ligand bias into novel therapeutics requires both a formal understanding of what functional selectivity is, and a means to quantify it so that the efficacy profiles of different ligands can be meaningfully compared. The current evolution of the Black-Leff operational model as applied to receptor allostery envisions GPCRs not as binary switches, but as 'ensembles' of tertiary conformations (Black and Leff 1983; Kenakin and Christopoulos 2002; Kenakin 2007; Griffin et al. 2007; Kenakin 2009; Ehlert 2008; Kenakin and Miller 2010). Since there is no *a priori* reason that a receptor conformation that links it to one downstream effector would necessarily couple it with equal efficiency to all possible effectors, one can posit that a finite number of discrete active and inactive states exist within the sterically permissible conformational ensemble. Thus, the biological activity of the system at any instant is a reflection of the distribution of the receptor population among the component microstates that make up the ensemble. Within this framework, there is likewise no *a priori* reason that the distribution of receptor conformations stabilized by one ligand should be identical to that generated by a structurally distinct ligand. In this case 'functional selectivity' may arise from differences in the efficiency with which ligands stabilize different active states.

Evidence generated using intramolecular fluorescence probes to monitor the effect of ligand binding on receptor conformation supports the concept that ligands with different structure stabilize unique receptor ensembles (Yao et al. 2006; Yao et al. 2009). 1D ^{19}F-NMR spectroscopic analysis of conventional and biased ligand binding to the β2 adrenergic receptor indicates that G protein activation correlates with movement of transmembrane helix VI, whereas arrestin-biased ligands predominantly affect the

conformation of helix VII (Liu et al. 2012). In this study, helix VI and helix VII were found to move independently, providing a physical basis for biased agonism and a conformational 'signature' predictive of arrestin selectivity. While to date there is little direct x-ray crystallographic structure of GPCRs bound to conventional and biased agonist ligands, computational modeling of agonist docking within the ligand binding pocket of class A GPCRs suggests that efficacy correlates with engagement of certain residues and exclusion of interaction with others (Katrich et al. 2012; Jacobson and Costanzi 2012; Costanzi 2014).

Historically, the experimental hallmark of orthosteric ligand bias has been 'reversal of potency', where two ligands exhibit opposite rank order of potency for two downstream responses measured in the same system (Sagan et al. 1996; Berg et al. 1998; Takasu et al. 1999), or 'reversal of efficacy', where a single ligand exhibits opposing efficacy towards two different downstream responses (Gray and Roth 2001; Holloway et al 2002; Sneddon et al. 2004). Such phenomena can only be explained by the existence of more than one active receptor conformation, and thus offered the initial proof that different ligands can activate the same receptor in qualitatively different ways. Importantly for drug discovery, the existence of bias dictates that ligand classification is both assay and context dependent; a biased ligand may be classified as an inverse agonist, antagonist, or agonist depending on the assay or cell/tissue system used to interrogate it. Biased ligands are novel pharmacological entities and they cannot be adequately described using low-dimensionality screening techniques.

Central to this definition is that 'bias' must be demonstrable when two measures of receptor activity are examined in a common cellular background. Since GPCRs that

couple to multiple downstream effectors may do so with different efficiencies, increasing

ligand occupancy will cause the most efficiently coupled response(s) to be activated first,

followed by less efficiently activated processes. In this case, variations in the level of

receptor or effector expression between systems can cause the appearance of 'new'

signaling processes (Zhu et al. 1994; Cordeaux et al. 2000; Nasman et al. 2001) and even

create the illusion of unique functional states (Kenakin 1995). In the case of partial

agonists, responses that are tightly coupled or highly amplified will persist because

fractional receptor activation is sufficient to generate a maximal response, while

responses that are weakly coupled or unamplified, where full receptor activation is

necessary for a maximal response, will be very sensitive to changes in receptor or

effector expression and will appear or disappear depending on cellular background. This

form of variability, arising from differences in 'signal strength', does not require the

existence of multiple active states, and the rank order of potency of a series of

conventional full and partial agonists will not vary when compared in different systems

(Kenakin 1995; Kenakin 2007).

The efficiency with which any given orthosteric agonist will promote receptor

coupling to downstream signaling pathways may vary between effectors, e.g.

heterotrimeric G protein species or arrestins. While the observed signaling output may

vary in different cell backgrounds, intrinsic efficacy is an innate property of the ligand,

i.e. a ligand can only be 'biased' in relation to the intrinsic efficacy of some other ligand

acting on the same receptor. For classification purposes, this 'reference ligand' is usually

the native hormone or neurotransmitter. Yet ligand 'bias' is not merely a product of

synthetic pharmacology. Nature has been exploiting bias far longer than pharmaceutical

scientists have been working to discover clinically useful biased drugs. This is apparent in the case of chemokine signaling where there are more than twice the number of endogenous chemokines than chemokine receptors. For example, two endogenous ligands for the CC chemokine receptor 7, CCL19 and CCL21, while retaining similar G protein coupling efficacy, differ in their capacity to promote receptor desensitization, arrestin recruitment, and ERK1/2 activation (Kohout et al. 2004; Zidar et al. 2009). Differential activation of serotonin receptors by metabolic derivatives of serotonin and 'trace amines' is another physiologically relevant example of endogenous bias. In response to serotonin, the serotonin $5HT_{2A}$ receptor ($5HT_{2A}R$) activates Akt in mouse cortex and cortical neurons via an arrestin3-Src dependent mechanism. However, this signaling complex does not form when the $5HT_{2A}R$ is activated by N-methyltryptamines (Schmid et al. 2010). These differences in neuronal signaling translate into agonist-induced behaviors, where the $5HT_{2A}R$-mediated activation of the head twitch response in mice requires the production of N-methyltryptamine metabolites to activate the non-arrestin-dependent pathway utilized by serotonin (Schmid et al. 2008; Schmid et al. 2010). Thus, it appears that ligand bias a common solution to the need for fine regulation in complex signaling systems and that GPCR systems may already be poised to response selectively to variations in ligand structure.

Quantifying bias

When considering a single dimension of efficacy, the actions of a ligand can be described by two terms: the equilibrium dissociation constant of the ligand-receptor complex (K_d) and the maximal observed change in receptor activity (V_{max}), that together specify the relationship between receptor occupancy and system response. Any single parameter

used to describe ligand efficacy in a given pathway needs to incorporate both of these

factors.   One approach, based on the Black-Leff operational model (Black and Leff

1983), is to determine a 'transduction coefficient', $\log(\tau/K_A)$, by fitting the dose-response

curve to the operational model below, where τ_A is the efficacy of the ligand (A) for the

pathway, K_A is the equilibrium dissociation constant for the agonist-receptor complex,

and E_M is the maximum response capability of the system:

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

The term τ encompasses both the intrinsic efficacy of the ligand and system-dependent

factors such as receptor density and coupling efficiency. Since the latter factors are

constant for any dose-response curve determined in the same cell for any given signaling

pathway, the ratio of τ values for any two agonists in the same system will yield an

intrinsic efficacy ratio for activation of the pathway that is independent of receptor

number or coupling efficiency. Once determined for each ligand of interest, the

efficiency of ligands producing activation of a pathway relative to a reference agonist,

e.g. the endogenous ligand, can be quantified by the normalized transduction coefficient

$\Delta\log(\tau/K_A)$ (Kenakin 2009; Kenakin et al. 2012; Kenakin 2014; Stahl et al. 2015).

  An alternative approach that is valid for dose-response data described by curves

with Hill coefficients near unity is to determine 'intrinsic relative activity' (RA_i) from

EC_{50} and E_{MAX} data according to the method of Ehlert, where A and B are the reference

and test ligands, respectively (Ehlert 2005):

$$RA_i = \frac{E_{max-B}\, EC_{50-A}}{E_{max-A}\, EC_{50-B}}$$

Since EC_{50} and K_d are equal when the Hill slope of the dose response curve is 1.0, the two approaches yield similar results as long as this condition is fulfilled. Similar to the operational model, when applied correctly $\Delta\log(RA_i)$ provides a measure of ligand efficiency relative to a reference agonist.

To quantify ligand bias, it is then necessary to compare the $\Delta\log(RA_i)$ value obtained for each agonist/pathway of interest. For any two pathways j1 and j2, a 'bias factor' can be derived as the $\Delta\Delta\log(\tau/K_A)$ or $\Delta\Delta\log(RA_i)$ (Kenakin et al. 2012):

$$\text{bias} = 10^{\Delta\Delta\log(\tau/K_A)\,j1-j2}$$

where

$$\Delta\Delta\log(\tau/K_A)_{j1-j2} = \Delta\log(\tau/K_A)_{j1} - \Delta\log(\tau/K_A)_{j1}$$

Since GPCR efficacy can be profiled in as many 'dimensions' as there are assays, a convenient way to visualize efficacy is by preparing multi-axial graphic displays of bias factors determined using a series of ligands in a panel of assays. As shown in **Fig 1**, such plots provide a depiction of ligand bias that is easily comprehended and permits identification of ligands with similar efficacy profiles.

An important advantage of the operational model is its capability to quantify the full range of agonism from submaximal effects to amplified effects in very sensitive systems with effective receptor reserve (Black and Leff 1983; Black et al. 1985). Further, $\log(\tau/K_A)$ ratios determined in one system are applicable to all systems without the need to independently quantify functional selectivity in each (Kenakin 2009; Kenakin et al. 2012). Variation in receptor density and coupling efficiency between systems might change the ability of all agonists targeting a given receptor to activate a particular pathway, but it will not change the pathway selective 'bias' of different ligands relative to

one another or to a reference agonist run in parallel in all assays. On the other hand,

using the operational model to describe bias requires generating extensive dose response

data and K_A measurements for the test agonists and reference agonist in in multiple

assays. While clearly useful, it is not readily adaptable to high-throughput screening

programs, which historically depend on 'single shot' screens of vast numbers of

compounds in single assays of cellular response. Moreover, quantifying bias by

assessing cell-based responses alone provides no information about the degree of bias

needed to produce a physiologically relevant change in signaling activity, nor does it help

in defining the type of bias needed to dissociate therapeutic from deleterious ligand

effects *in vivo*.

Translating bias

The therapeutic promise of biased agonism resides in its capacity to elicit biological

responses *in vivo* that cannot be obtained via conventional agonist/antagonist ligands. In

effect, a GPCR bound to a biased ligand is a different functional entity, with different

signaling characteristics, than the same receptor bound to a conventional agonist or

partial agonist. This presents both an opportunity and a major challenge to developing

biased therapeutics. If the appropriate *in vitro* efficacy profile is known, then judicious

multiplex screening should identify compounds that produce the desired physiological

effects. On the other hand, since biased ligands activate GPCRs in 'unnatural' ways

compared to their conventional agonist counterparts, the relationship between the *in vitro*

efficacy profile and the desired *in vivo* biological response is not necessarily obvious, and

cannot simply be inferred based on prior knowledge of the native ligand. In most cases,

the effect of ligand bias *in vivo* has to be determined empirically (Luttrell 2013; Appleton

and Luttrell 2013; Schmid et al. 2013).

Several examples illustrate both the promise and pitfalls encountered in translating ligand bias to complex *in vivo* systems. One very promising area for biased drug development involves G protein pathway-selective opioid receptor agonists (Dewire et al. 2013; Schmid et al. 2013; Zhou et al. 2013). From the initial discovery that the impaired µ opioid receptor (□□□) desensitization observed in arrestin3 null mice led to enhanced morphine analgesia with less tolerance (Bohn et al. 1999; Bohn et al. 2000), it was apparent that non-desensitizing opioid analogues might be superior analgesics. The additional benefit became evident when it was realized that the side effects of constipation, respiratory suppression and physical dependence were lessened in the absence of arrestin3, suggesting that MOR-arrestin3 signaling may underlie some of these physiological responses (Raehal et al. 2005; Bohn and Raehal 2006; Raehal et al. 2011a, Raehal et al. 2011b). While efforts are still ongoing to try to capture the endogenous signalsomes utilizing arrestins downstream of MOR, the generation of G protein biased ligands has allowed for testing the role of G protein versus arrestin-mediated effects *in vivo*.

The first generation of G protein biased MOR agonists has shown promise in mouse models. This compound, TRV-130, is weakly biased (bias factor of 3 fold for G protein over arrestin signaling), yet the authors reported a favorable separation between analgesic efficacy and gastrointestinal and respiratory side effects when tested in rodent models (Dewire et al. 2013). Currently in clinical trials, TRV-130 has proven to be a very potent analgesic in humans (Soergel et al. 2014). However, the results of the phase II clinical trial have not revealed a significant reduction in side effects, raising the

question of whether biased agonists at MOR can dissociate analgesia from respiratory

depression/constipation, or if a greater degree of bias is necessary to translate into human

therapeutic responses. To fully test this hypothesis, compounds of different chemical

structures and that display more bias for G protein signaling over arrestin signaling

should be developed and tested.

Selective κ opioid receptor (KOR) agonists also produce anti-nociceptive effects

in animal models through activation of $G_{i/o}$ family heterotrimeric G proteins (Gullapalli

and Ramarao 2002), but unlike MOR agonists do not cause physical dependence or

respiratory depression (Charbogne et al., 2014). However, their clinical utility is

hampered by an array of undesirable side effects, including dysphoria, sedation, diuresis,

hallucination, and depression (Pfeiffer et al. 1986; Togashi et al. 2002; Land et al. 2008;

Van't Veer and Carlezon 2013). Interestingly, it has been proposed that the dysphoric

component of KOR agonism is mediated via a process involving arrestins (Bruchas et al.

2006; Redila and Chavkin 2010), suggesting that G protein biased KOR agonists may

deliver the desired analgesic effect while circumventing deleterious mood disturbances

(White et al. 2014; Schmid et al. 2013; Zhou et al. 2013).

In animal models, certain properties, such as antinociception, are retained with G

protein signaling-biased KOR agonists; however the first reports from *in vivo* testing

show that biased agonists derived from Salvinorin A analogs still promote aversive

behaviors in mouse conditioned place preference tests (White et al. 2015). Since

aversion can be produced by any substance that an animal associates with an unpleasant

sensation, whether due to direct neurological stimulation or to visceral discomfort, it is

difficult to determine whether such findings disprove the hypothesis that arrestin-

dependent signaling mediates the KOR aversive response. While other behavioral

models, such as altering reward thresholds using intracranial self-stimulation, offer better

models of dysphoria, it is clear that the development of additional ligands across diverse

chemical scaffolds with conserved bias properties will be necessary to assign causality.

Expanding the spectrum of bias to include ligands with different degrees of bias among

downstream effectors, such as ERK activation, will provide valuable tools for navigating

the complex effects of signalsome engagement. Recent examples of this approach have

been described for KOR agonists, where a triazole series was modified to maintain G

protein over arrestin bias while altering selectivity for ERK1/2 activation (Lovell et al.

2015).

The *in vivo* effects of arrestin pathway-selective biased agonists are even less

predictable. Since loss of function in arrestin null animals could arise either as a

consequence of enhanced G protein pathway activation or due to loss of arrestin-

mediated signaling, determining the role of arrestin signaling *in vivo* is best accomplished

by empirically examining the effects arrestin-biased ligands in a wild type background.

Arrestin-dependent signaling by the type 1 parathyroid hormone receptor (PTH$_1$R) is a

case in point. The native hormone, PTH(1-84), and its conventional agonist N-terminal

fragment human PTH(1-34), promote intestinal calcium absorption, renal calcium

retention, and osteoblastic bone formation. PTH-dependent osteoblast activation

produces coupled activation of bone-resorbing osteoclasts through G protein-dependent

elaboration of osteoclast activating cytokines, like RANKL, the receptor activator of

NFκB ligand. Although hPTH(1-34) is FDA approved as an anabolic treatment to build

new bone in patients with severe osteoporosis, its beneficial effects depend on the

16

kinetics of administration, as continuous exposure to PTH leads to hypercalcemia and net

bone resorption, whereas intermittent exposure stimulates bone formation in excess of

resorption (Qin et al. 2011). Thus, wild type C57BL/6 mice given daily injections of

PTH(1-34) experience a net increase in bone mass, characterized by increases in

trabecular bone volume and cortical thickness, osteoblast number, bone matrix deposition

and mineral apposition rate. Reflective of osteoblast-osteoclast coupling, osteoclast

numbers also increase, along with markers of bone resorption, and treated mice develop

hypercalciuria from PTH_1R regulated G_s signaling in the proximal renal tubule (Mohan et

al. 2000; Sebastian et al. 2008).

Surprisingly, wild type mice treated with (D-Trp^{12},Tyr^{34})-bPTH(7-34), an arrestin

biased PTH_1R agonist (Gesty-Palmer et al. 2006), also exhibit increased bone formation

with greater trabecular bone volume, increased osteoblast number, matrix deposition and

mineral apposition (Gesty-Palmer et al. 2009; Gesty-Palmer et al. 2013). Unlike the

conventional agonist, (D-Trp^{12},Tyr^{34})-bPTH(7-34) fails to activate the G protein-

mediated processes regulating osteoclast number, bone turnover and calciuresis, thereby

accomplishing the potentially beneficial feat of 'uncoupling' PTH_1R-mediated bone

formation from bone resorption.   All responses to (D-Trp^{12},Tyr^{34})-bPTH(7-34) are either

absent or reversed in arrestin3 null mice, suggesting its actions *in vivo* result from arrestin

biased signaling. Given that prior work had attributed the bone-forming capacity of PTH

solely to its ability to activate G_s-cAMP-protein kinase A signaling (Mohan et al. 2000;

Lian et al. 2006; Sebastian et al. 2008; Deng et al. 2008), the finding that arrestin-

dependent signaling is sufficient to promote osteoblastic bone formation is both

paradoxical and not predicted from the study of conventional PTH_1R agonism.

Understanding bias at a systems level

Orthosteric GPCR agonists, whether conventional or biased, mediate their cellular effects by interacting with the ligand-binding pocket and changing the distribution of conformations within the receptor ensemble. Everything that follows, whether it is short-term modulation of intermediary metabolism, cellular contractility, or membrane potential, or long term changes in cell proliferation, differentiation and survival, is entrained and functionally encrypted at that point in time. The concept of conformational selection, upon which allosteric models of GPCR function are based, posits that biased ligands may stabilize active receptor conformations in different proportions than the native ligand, but do not force receptors into sterically unfavorable conformations or couple them to novel effectors. If true, then the actions of a biased ligand, at least in the short term, must comprise a subset of those produced by a conventional pluripotent agonist. In fact, experimental data examining the actions of biased agonists *in vitro* using a wide range of readouts tend to bear this out (Swaminath et al. 2005; Rajagopal et al. 2006; Aplin et al. 2007; Kendall et al. 2011; Liu et al. 2012; Sauliere et al. 2012; Gesty-Palmer et al. 2013). Even 'unbiased' comparisons of conventional and arrestin biased GPCR signaling *in vitro* using quantitative global phosphoproteomics suggest that biased agonist responses are subsumed within the larger whole of conventional agonism. For example, a phosphoproteomic comparison of the conventional angiotensin AT_{1A} receptor agonist, angiotensin II (AngII), and the arrestin biased agonist, [Sar^1,Ile^4,Ile^8]-AngII, that identified 1183 regulated protein phosphorylation sites following 5 min of ligand exposure, found that 756 (64%) were unique to AngII, 369 (34%) were regulated by both AngII and [Sar^1,Ile^4,Ile^8]-AngII, and only 58 (5%) were unique to [Sar^1,Ile^4,Ile^8]-AngII

(Christensen et al. 2010).

In vivo, however, where drugs must exert their therapeutic effects, the limited systems level data available suggest that conventional and biased agonists may be dramatically different (Appleton and Luttrell 2013). **Fig 2A** illustrates this point using the 3-dimensional data visualization application *Omnimorph* to depict the transcriptomic fingerprint of the conventional PTH_1R agonist, hPTH(1-34), and the arrestin biased agonist, (D-Trp^{12},Tyr^{34})-bPTH(7-34), in the kidneys of mice exposed to vehicle or ligand for 4 weeks (Maudsley et al. 2015a). As shown, at a gestalt level the global transcriptomic effects of hPTH(1-34) and (D-Trp^{12},Tyr^{34})-bPTH(7-34) appear to be markedly different in wild type mice, and while the effects of the G protein-competent conventional ligand are largely conserved between wild type and arrestin3 null backgrounds, the loss of arrestin3 profoundly disrupts the effects of the arrestin-biased ligand. Similar divergence is evident when the transcriptomic effects of the two ligands are compared at the pathway level by geneset enrichment analysis (Gesty-Palmer et al. 2013; Maudsley et al. 2015a). **Fig 2B** illustrates the substantial degree of non-overlap observed in calvarial bone following long term hPTH(1-34) or (D-Trp^{12},Tyr^{34})-bPTH(7-34) treatment. Unlike data from short-term phosphoproteomic surveys, transcriptomic datasets from six different tissues show only 1.4% to 38.9% commonality at the pathway level between conventional and arrestin biased PTH_1R agonists (Maudsley et al., 2015a). Whereas in bone hPTH(1-34) primarily affects pathways classically associated with enhanced bone turnover, including collagen synthesis, matrix mineralization, and osteoclast activation, (D-Trp^{12},Tyr^{34})-bPTH(7-34) primarily affects pathways regulating cell cycle progression, cell survival, and migration (Gesty-Palmer et al. 2013).

This divergence may not be surprising. *In vitro*, biased agonists activate a subset of the signaling pathways regulated by a conventional agonist, while *in vivo* they might be expected to exert mixed agonist-antagonist effects, generating some signals while antagonizing activation of others by the endogenous ligand. The individual components that comprise downstream signaling may not be qualitatively different, but they will be quantitatively and stoichiometrically 'unbalanced' compared to the native ligand. As these unbalanced signals propagate, differences in the tissue response arising from even small differences in signal strength or the kinetics of pathway activation/inhibition have the potential to introduce unpredicted, and perhaps unpredictable, biological outcomes.

Given the myriad signaling events attributed to arrestins (Luttrell and Gesty-Palmer 2010; Whalen et al. 2011) and the relative paucity of information about which might be physiologically relevant, developing a systems level appreciation of arrestin biased agonism *in vivo* might facilitate the rational development of biased GPCR ligands. One attempt to develop a comprehensive description of the core activities of (D-Trp^{12},Tyr^{34})-bPTH(7-34) through bioinformatic analysis of *in vivo* transcriptomic data is illustrated in **Fig 2C-D** (Maudsley et al. 2015b). For this analysis, the most conserved transcripts populating the most conserved signaling pathways from a comparison of the actions of (D-Trp^{12},Tyr^{34})-bPTH(7-34) in six different murine tissues was analyzed using the reverse latent semantic indexing (LSI) application *Textrous!* (Chen et al. 2013a). LSI-based analysis correlates the strength of association between specific genes in a dataset with user-defined interrogation terms. Individual processing investigates the links between scientifically relevant words and individual transcripts, while collective processing generates a hierarchical word cloud indicating the word groups most strongly

associated with the entire input dataset (Chen et al. 2013b). As illustrated, at the individual processing level arrestin biased signaling correlates most prominently with regulation of protein kinase activity and phosphorylation (**Fig 2C**), while at the collective level a bias towards cellular growth, apoptosis/survival, remodeling, histone regulation and cell cycle control is evident (**Fig 2D**). Adding credence to the analysis, the major functions identified are consistent with the *in vitro* characterization of (D-Trp^{12},Tyr^{34})-bPTH(7-34) actions in primary calvarial osteoblasts (Gesty-Palmer et al. 2013) and data emerging from the cancer field suggesting that arrestins function as regulators of tumor cell proliferation, survival and metastasis (Buchanan et al. 2006; Dasgupta et al. 2006; Moussa et al. 2008; Chun et al. 2009; Lakshmikanthan et al. 2009; Li et al. 2009; Rosanò et al. 2009; Liu et al. 2011; Bonnans et al. 2012; Fereshteh et al., 2012).

Systems level transcriptomic studies of PTH$_1$R agonism *in vivo* suggest that an arrestin biased ligand, with its limited signaling repertoire, has narrower and more cross-tissue conserved effects than its pluripotent conventional agonist counterpart. Thus, arrestin biased ligands may possess greater stability and predictability of activity across multiple cell types and perhaps across diverse pathological contexts (Maudsley et al. 2012). It remains to be determined, however, if the transcriptomic 'signature' of arrestin biased PTH$_1$R agonism is transferrable across different GPCRs, or whether each GPCR uses arrestins to its own purpose. Nonetheless, current evidence suggests that systems level studies may contribute to a conceptual framework within which the *in vivo* outcomes of arrestin-biased signaling may be generalized.

Conclusions

Table 1 summarizes some of the opportunities and challenges currently facing efforts to

21

translate biased GPCR agonism into the clinic. Clearly, the field possesses a sound theoretical basis for coping with ligand bias, numerous assays capable of detecting both G protein and arrestin signaling that are amenable to high-throughput screening, and mathematical tools to quantitatively compare ligands across multiple dimensions of efficacy. The major challenge from the screener's perspective is that we do not have a good idea what to screen for, i.e. the link between *in vitro* efficacy and biological activity is at best tenuous for many potential biased agonist targets. Even in settings where a sound rationale exists for favoring G protein or arrestin signaling, the extent to which a 'biased' ligand must favor one pathway over another to produce a therapeutic benefit is unknown. True 'selective agonism', i.e. opposing efficacy toward different effectors, is rare. Most ligand 'bias' is more subtle, i.e. quantitative variance from the native ligand in the efficiency of coupling to different effectors. In most cases, neither the direction of bias, e.g. G protein versus arrestin selectivity, nor the magnitude of bias needed for optimal clinical outcomes, is necessarily obvious. In such a near vacuum, the cost and complexity of screening every possible dimension of GPCR efficacy is prohibitive.

Yet the potential of biased therapeutics seems clear. Solid evidence indicates that G proteins and arrestins mediate distinct physiological processes and that the two GPCR signaling modes are pharmacologically dissociable, permitting the development of biased ligands with unique efficacy profiles *in vivo*. Moreover, in a handful of cases there is clear proof-of-principle that biased agonists can produce biological effects that cannot be attained using conventional agonists or antagonists; at least in pre-clinical animal models. For most potential targets, the missing link is an understanding of how G protein- and arrestin-mediated signals both contribute to the biological actions of specific GPCRs.

Biased agonists are unique pharmacological entities and the phenotypic consequences of treatment cannot necessarily be predicted from existing knowledge of receptor function. Early forays into probing arrestin biology at a systems level suggest there may be a conserved core of transposable arrestin-mediated functions, but it is still early days. Finally there is the specter of unanticipated 'on-target' effects resulting from activating GPCRs in non-physiological ways.

Future progress will depend on knowing, for each potential GPCR target, what efficacy profile will deliver the most desirable physiologic response; whether that of a conventional agonist or antagonist, or a G-protein or arrestin-selective biased agonist. Assuming that the number of proximal effectors, e.g. G protein and arrestin isoforms, engaged by any given GPCR is finite and that agonists simply select from this preset menu, then the number of 'flavors' of biased agonism should likewise be finite (Maudsley et al. 2005). Initially, it may be helpful to generate multi-dimensional efficacy profiles encompassing receptor coupling to all G protein families, arrestin isoforms, second messengers, small G proteins, and functions like cell proliferation, survival, permeability and migration (Sauliere et al. 2012; Schann et al. 2013), in order to cluster ligands into a smaller number of functional classes.

Even so, translating *in vitro* efficacy to *in vivo* biological function is an endeavor that must be very carefully approached. Just as cellular context can determine whether a ligand appears as an agonist or antagonist and which intracellular signaling partners are engaged (Kenakin 2007), the body as a whole presents multiple variables that can interfere with the valid comparison of a proposed "biased" agonist with the endogenous hormone or clinically validated agonist used for reference. Pharmacokinetic variables

23

that affect the compound's ability to reach its target, e.g. absorption, bioavailability, and

metabolism, must be overcome to assure that the ligand has every opportunity to occupy

the receptor before its biological activity can be attributed to differential signaling arising

from pharmacodynamic bias. Ultimately, primary cell cultures and tissue-based assays

will provide the intermediary platforms for reducing the impact of such variables. Thus,

for any given target, a thoughtful combination of high throughput screening to classify

chemically diverse sets of compounds, followed by characterization in primary cells to

determine the impact of different forms of bias in a native context, and finally testing in

preclinical animal models to understand the physiological impact of ligand bias, will be

needed to empirically determine the most effective form of bias and the degree of bias

factor necessary to translate *in vitro* efficacy to the *in vivo* state.

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Figure Legends

Fig. 1. Example of ligand bias described using the operational model. Transduction efficiencies $[\Delta\log(\tau/K_A)]$ for five different assays of κ opioid receptor signaling were used to calculate bias factors $(\Delta\Delta\log(\tau/K_A)_{j1-j2})$ that are presented in a multi-axial graphic format. The κ opioid receptor-selective agonist, U69,593, was used as the reference ligand, hence its bias factor conforms to unity in all assays. The pathways represented are: membrane $[^{35}S]GTP\gamma S$ binding (*mG protein*); βarr2 enzyme fragment complementation (*βarr2 EFC*), βarr2 fluorescence imaging (*βarr2 imaging*), cellular impedance (*impedance*), and whole cell $[^{35}S]GTP\gamma S$ binding (*wcG protein*). As depicted, the two test compounds, 1.1 and 1.2, display bias for G protein signaling over arrestin recruitment. This research was originally published in *The Journal of Biological Chemistry*: Zhou L, Lovell KM, Frankowski KJ, Slauson SR, Phillips AM, Streicher JM, Stahl E, Schmid CL, Hodder P, Madoux F, Cameron MD, Prisinzano TE, Aubé J, Bohn LM. Development of functionally selective, small molecule agonists at kappa opioid receptors. *J Biol Chem* 2013; **288**: 36703-36716. © American Society for Biochemistry and Molecular Biology.

Fig. 2. Systems level characterization of conventional and arrestin biased PTH$_1$R agonism *in vivo*. **(A)** *Omnimorph* structures depicting a high-dimensionality z score representation of the transcriptional responses to hPTH(1-34) (*panels 1-2 WT; panels 5-6 Arrb2 KO*) or (D-Trp^{12},Tyr^{34})-bPTH(7-34) (*panels 3-4 WT; panels 7-8 Arrb2 KO*) in wild type (WT) and arrestin3 null (Arrb2 KO) murine kidney. **(B)** Significantly-populated canonical signaling pathways induced by bPTH(7-34) (*red bars*) or by (D-Trp^{12},Tyr^{34})-bPTH(7-34) (*black bars*) in murine calvarial bone identified using Ingenuity Systems$^®$

Pathways Analysis. Each histogram bar represents the signaling pathway score. Signaling pathways within the yellow block represent coherently regulated common pathways between the two ligands. The associated Venn diagram indicates the functional signaling separation between bPTH(7-34) and (D-Trp^{12},Tyr^{34})-bPTH(7-34). **(C)** Word cloud interpretation of the individual *Textrous!* output (*dismantled noun–phrases*) performed using the most cross-tissue conserved (D-Trp^{12},Tyr^{34})-bPTH(7-34) regulated transcripts. **(D)** Word cloud interpretation of the collective *Textrous!* output performed using the most cross-tissue conserved (D-Trp^{12},Tyr^{34})-bPTH(7-34) regulated transcripts. *Textrous!* is an LSI-based analytical tool that correlates the strength of association between specific genes in a dataset with user-defined interrogation terms, in this case biomedical words and noun-word phrases extracted from PubMed, Online Mendelian Inheritance in Man, and the Mammalian Phenotypes Database at the Jackson Laboratories Mouse Genomic Informatics portal (Chen et al. 2013a). Research originally published in: Maudsley S, Martin B, Gesty-Palmer D, Cheung H, Johnson C, Patel S, Becker KG, Wood WH 3rd, Zhang Y, Lehrmann E, Luttrell LM (2015) Delineation of a conserved arrestin-biased signaling repertoire in vivo. *Mol Pharmacol* **87**: 706-717. © American Society for Pharamcology and Experimental Therapeutics.

TABLE 1: Opportunities and challenges for biased drug development

I. IDENTIFICATION OF BIASED AGONISTS

Opportunities
- Sound conceptual understanding of ligand bias
- Available high-throughput screening platforms to detect G protein and arrestin efficacy
- Methods for quantifying bias allowing comparison of ligand efficacy profiles

Challenges
- Cost/complexity of employing multiplex ligand screening in early compound development
- Unclear relationship between magnitude of bias needed to produce a relevant change in physiological response
- Limited understanding of the relationship between *in vitro* efficacy profile and *in vivo* drug effect for most targets

II. LINKING *IN VITRO* EFFICACY TO THERAPEUTIC EFFECT

Opportunities
- Cellular and *in vivo* evidence that G protein and arrestin signaling pathways mediate different responses
- Proof-of-principle that biased agonists can elicit responses not attainable via conventional agonists/antagonists

Challenges
- No broad conceptual framework for arrestin-dependent efficacy *in vivo*
- Unknown phenotypic consequences of G protein- and arrestin-selective efficacy at most potential targets
- Potential for unexpected on-target effects due to 'unbalanced' receptor activation
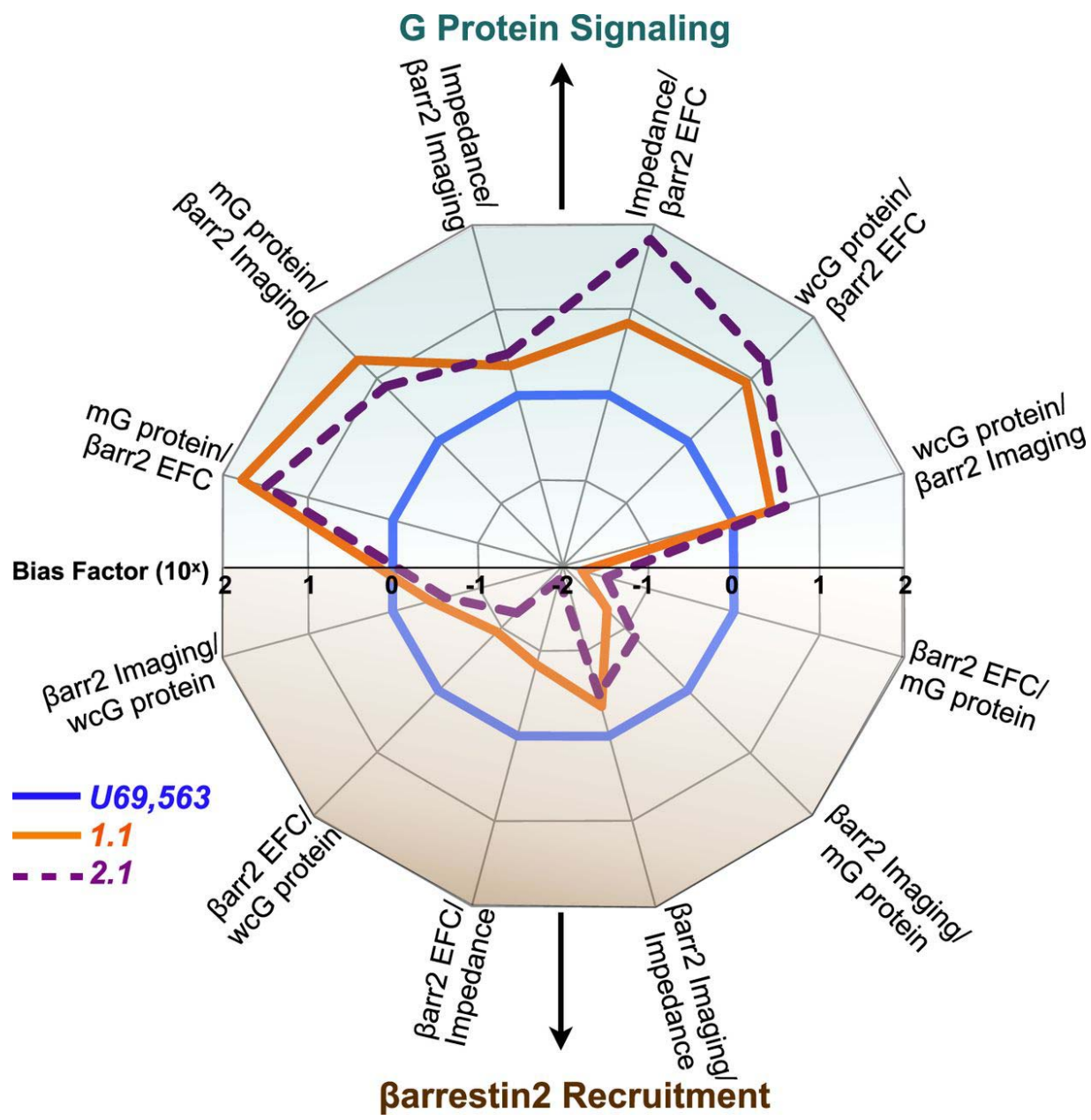
Fig 1

Fig 2