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Perspective

Is the Quest for Signaling Bias Worth the Effort?

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RUNNING (SHORT) TITLE : Is Bias Worth the Effort?

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Abbreviations: PTH- Parathyroid Hormone; SII -[Sar1, Ile4, Ile8]-AngII;TRV120027- D-Alanine,N-methylglycyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl

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Abstract : The question of whether signaling bias is a viable discovery strategy for drug therapy is discussed as a value proposition. On the positive side, bias if easily identified and quantified in simple *in vitro* functional assays with little resource expenditure. However, there are valid pharmacological reasons why these *in vitro* bias numbers may not accurately translate to *in vivo* therapeutic systems making the expectation of direct correspondence of *in vitro* bias to *in vivo* systems a problematic process. Presently, *in vitro* bias is used simply as a means to identify unique molecules to be advanced to more complex therapeutic assays but from this standpoint alone, the value proposition lies far to the positive. However, pharmacological attention needs to be given to the translational gap to reduce inevitable and costly attrition in biased molecule progression.

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Is the Quest for Signaling Bias Worth the Effort?

The ability of different agonists to stabilize different active receptor states can lead to signaling bias whereby some cytosolic signaling pathways are preferentially activated at the expense of others (Kenakin and Morgan, 1989). This effect can (1) emphasize beneficial signaling pathways (i.e. PTH-mediated bone building for osteoporosis; Gesty-Palmer and Luttrell, 2011;Gesty-Palmer et al, 2013), (2) de-emphasize harmful signaling pathways (i.e. respiratory depression for opioid analgesics, Raehal et al, 2005; Kelly, 2013; Koblish et al, 2017), (3) de-emphasize harmful pathways and prevent the natural agonist from activing these pathways (i.e. biased angiotensin blockers for heart failure, Violin et al, 2006; 2010), and (4) allow pursuit of previously forbidden drug targets due to side effects (i.e. κ-opioid receptor analgesics; White et al, 2014; Brust et al, 2016). The consideration of biased signaling has revitalized seven transmembrane receptors as therapeutic drug targets (Kenakin, 2015a); from this point of view, bias is a strategy to improve drug therapy. However, there are clear challenges to the pursuit of signaling bias elegantly elucidated in the paper by Michel and Charlton (this vol) leaving the question, is bias a practical way forward for drug discovery at this time? It is worth considering biased signaling in terms of a value proposition.

Ideally, enough information about cell signaling should be available to design a target bias profile. The first viable biased ligand, SII (and later the analog TRV120027) was designed with a defined profile of β-arrestin activation and absence of Gq protein activation for congestive heart failure (Wei et al, 2003) and there have been many other similar proposals for other molecules (i.e. opioid analgesics and other agonists). However, as discussed by Michel and

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Charlton ( ), our knowledge of signaling is still insufficient to adequately predict how the bias observed in signaling assays will translate to *in vivo* therapy for most receptors, leaving a large translational gap between *in vitro* definition and *in vivo* activity. The reasons for this gap relate to the differences between the simple isolated pathway assays used to detect bias and the eminently more complicated systems *in vivo* in which this bias must make a difference.

Agonist bias is most commonly revealed as differences in agonist relative potency (either potency ratios for full agonists or $Log(\tau/K_A)$ or $Log(max/EC_{50})$ values for full and partial agonists (Kenakin, 2017)). In fact, in the 1980's diversity in relative potencies was the first sign that Stephenson's model of a single receptor active state need not be the singular mechanism for agonism; these published reports were harbingers of biased signaling (i.e. see Roth and Chuang, 1987). The simple model of selective stabilization of different receptor active states accommodates the various effects seen in functional *in vitro* assays. However, differences in potency ratios with cell type (Christmanson et al, 1994), stoichiometry in receptors and G proteins (Watson et al, 2000), and measurement of agonist signals at different points along the stimulus-response cascade (Peters et al, 2007; Peters and Scott, 2009) also indicate that this bias can change with disposition of the receptor stimulus beyond the allosteric vector of agonist/receptor/signaling protein, ie. functional systems modify bias. The dissociation comes from the fact that *in vitro* bias measured in single functional pathway assays such as second messenger or BRET association assays quantifies the differences in allosteric vectors characterizing the ternary complex. These numbers are made up of α and β values for probe dependent allostery (Kenakin, 2015b) and thus adhere to fairly stable molecular rules (with comparison to reference agonists, system and assay bias cease to be an issue). However, once

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these diverse signals escape the ternary complex and make their way into the cytosol, the cellular milieu can modify them in cell and system dependent ways and thus the stable allosteric-based quantification of vector bias dissociates from cellular signaling (Kenakin and Christopoulos, 2013; Kenakin, 2016). In fact, a shortlist of how system modification of vector bias can be modified includes differences in:

- cell phenotype
- relative stoichiometry of receptors to signaling proteins
- cell density of receptors
- temporal profiles of different cell signals
- whether agonist potency is affinity or efficacy based

So there are good pharmacological reasons why simple *in vitro* bias will fail to translate accurately to *in vivo* systems. In view of this potential hazard, how can *in vitro* bias add value to a drug discovery program?

An answer to this question may lie in the practical way that bias is used in industrial and academic drug discovery, namely as a means to identify different molecules. A general theme in discovery and development is that the more complex the assay, the more textured the output. Thus, a series of agonists elevating cytosolic cyclic AMP levels may simply differ in the strength of the signal they produce but otherwise appear to be doing the same thing. In contrast, these same agonists, when put into more complex assays (i.e. *in vivo*) where many other systems come into play, may diversify; i.e. efficacy can vary in ***quality*** as well as ***quantity***. Therefore, identifying fundamentally different agonists (i.e. those that stabilize different receptor active

states) in simple *in vitro* bias assays becomes a cost effective way of choosing the optimal molecules to advance from a screen to a more complex assay. Using this approach the probability of revealing different phenotype responses in complex therapeutic assays will be much higher than if only the most potent hits from a single assay screen are blindly progressed.

It is well established that efficacy is 'pluridimensional' (Galandrin and Bouvier, 2006), (i.e. agonists most often have many efficacies in terms of activation of different signaling pathways) and these elements can be identified in bias assays. These combinations of signal pathway activation profiles can be depicted as radar plots (i.e. 'webs of efficacy', Evans et al, 2010; Zhou et al, 2013) or clustered groups (Huang et al, 2009; Kenakin,2015a) and when this is done, it is seen that every agonist tested has a unique efficacy fingerprint. The elements of these fingerprints are what cells use to produce a mixed phenotypic response, rather like a mixture of primary colors to yield a subtle hue (see Fig 1). Thus, simple bias numbers may function as a code for complex cellular phenotype responses. Once a particular hue (phenotype cell response) is identified as beneficial, then medicinal chemistry can be employed to optimize it. However, such complex outputs may be difficult to systematically modify raising another possible benefit of *in vitro* bias profiles. Specifically, if the cellular phenotype could be deconstructed to the mixture of elements (i.e. signaling pathways and the relative activation of these through bias) then these simple systems may be used to more easily manipulate efficacy quality through medicinal chemistry.

In vitro bias can readily be identified and quantified to characterize agonists. The quantification processes differ slightly depending on whether affinity and efficacy are considered in the measurement or whether only efficacy is used (Kenakin et al, 2012, Kenakin and

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Christopoulos, 2013; Onaran et al, 2017) but the differences are usually minor and do not affect identification of exemplar molecules. In light of the other pitfalls in translation of *in vitro* bias to *in vivo* systems, the expectation that *in vitro* bias numbers represent immutable codes becomes relative unimportant; experience suggests it is probably best to progress biased molecules into the therapeutically relevant systems as soon as possible and not waste time and resources on model systems. In this regard, while an ideal scenario would be to move into the final therapeutic in vivo system, this is often impractical due to resource constraints. A useful interim step would be to take recombinant bias data and next text compounds in a natural cell system more closely related to the therapeutic situation.

Signaling bias has been verified in many systems and studied by many experimental groups over the years where it has been given a variety of names (*stimulus trafficking*, Kenakin, 1995; *functional dissociation*, Whistler et al., 1999, *biased agonism*, Jarpe et al., 1998, *biased inhibition,* Kudlacek et al., 2002; *differential engagement*, Manning, 2002; *discrete activation of transduction* Gurwitz et al., 1994; *functional selectivity* ,Lawler et al., 1999; Kilts et al., 2002; Shapiro et al., 2003; *ligand directed signaling*, Michel and Alewijnse, 2007) but everyone basically was and is talking about the same thing. The effect was discovered through the observation that the single active state mechanism for response, that spawned the pharmacological tool of agonist potency ratio, is not the only mechanism available to physiology for production of agonist response. To date, biased agonism has been shown to be a ubiquitous mechanism in pharmacology even used by natural systems to fine tune signaling (Kohout et al, 2004) and at present, it is impossible to prove that a given receptor will not demonstrate signaling bias. In general, it is a readily discoverable and ubiquitous property of any molecule

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that could add value in the compound progression process. From this standpoint, the value proposition for determining bias falls heavily towards the positive. However, the problems of translation still remain and predict failures of biased molecules in therapeutic systems. There are certain approaches that can be taken to mitigate the translational hazards and perhaps reduce attrition at this stage.

One factor determining the robustness of a measured bias across different tissue systems is common to all agonist programs, namely the comparison of the efficacy of the synthetic (biased) agonist to that of the natural endogenous agonist. High potency can be achieved either through high affinity or high efficacy but the ability of agonists to activate a range of tissues with varying sensitivity is dependent on whether efficacy or affinity is the main potency factor. For example, a high affinity / low efficacy agonist such as the α-adrenoceptor agonist oxymetazoline is a more potent agonist than norepinephrine in sensitive tissues (it has a positive potency bias) but reverts to being less active in less sensitive tissues (bias reverts to norepinephrine being more sensitive) in less sensitive tissues (Kenakin, 1984); the same effect will be true for biased signaling. Thus a biased agonist depending on high affinity will essentially 'run out of gas' in less sensitive tissues. From this standpoint it should be noted that bias numbers only describe differences when response is ***observed*** but do not predict whether or not agonism will be observed; this remains in the realm of efficacy (Kenakin,2015c). Ostensibly this may suggest that efficacy-based scales of bias are better suited for discovery (Onaran et al, 2017). However, efficacy-based scales fail when full agonism is observed for both pathways as no differentiation between agonists then can be achieved without an independent estimate of agonist affinity and this is problematic (Kenakin et al, 2012; Kenakin and Christopoulos, 2013). Once bias has been

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established, separate experiments should be done to quantify the relative efficacies of the signals. In this regard, the limits of these bias numbers need to be realized. For instance, the lack of observed signaling in a pathway is not indicative of 'perfect bias' but rather simply of an insufficient assay sensitivity; methods are available to estimate a minimal estimated bias under these conditions (Stahl et al, 2015; Kenakin, 2015c).

Another fruitful approach to reduce translation attrition is discussed by Michel and Charlton, namely gaining more detailed knowledge of cell signaling. It has been shown that receptor stimulus can be modified through the stimulus-response amplification cascade in a cell to modify receptor-based bias. Thus potency ratios measured at the level of cyclic AMP production change in the very same cell when the response is measure further down the cascade with label free methods (Peters et al, 2007). These stimulus processing effects have been explored in more detail in other systems. For example, the effects of biased PTH agonists have been studied for down-stream stimulus-response cascade effects. Specifically, the β-arrestin signaling activity of hPTH(1-34) vs that of the β-arrestin biased analog [D-Trp^{12},Tyr^{34}]-bPTH(7-34) can further be differentiated in terms of transcriptomic signatures in different tissues (the β-arrestin biased signal differentiates further down the cellular stimulus-response cascade); this type of fine tuning in terms of bias-characterization may reduce the failure rate of biased molecules in the translational process (Maudsley et al, 2016). The application of label-free signaling technology (i.e. integrative pharmacology-on-target, iPOT methods) can further link *in vitro* bias estimates with *in vivo* cell type diversity to further predict *in vivo* biased effects (Ferrie et al, 2011; Deng et al, 2013; Morse et al, 2011).

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The information available to date suggests that there are valid pharmacological reasons for predicted failure in the translation of *in vitro* to *in vivo* bias. Specifically, the easily quantifiable numbers that can be obtained *in vitro* to measure bias should not automatically be assumed to carry over into therapeutic *in vivo* systems; this leaves a 'translation-gap' in the application of biased signaling to drug development. Moreover, the theoretical advantages of biased signaling have yet to be realized therapeutically. In spite of these negatives, the promise far outweighs the cost as the insertion of *in vitro* screens into the discovery process to identify exemplar molecules is relatively minor and the potential benefits large. The increasing knowledge of complex signaling networks also augurs that better targeting of biased molecules is yet to come. Thus, the value proposition at this point suggests that a quest for biased signaling is worth the effort but also compels increasing attention to the translation of simple *in vitro* bias to whole cell and whole body systems.

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Legends for Figures:

Figure 1 Cells mix receptor stimuli produced by agonists to yield a complex synoptic response which defines the true therapeutic utility of the agonist. Thus, different biases in terms of elemental signaling pathways can produce different phenotypic cell responses; however, this also allows the cell to hijack various *in vitro* bias signatures and change them in accordance with the stoichiometry of receptor to signal protein. Once a useful phenotype has been identified, reduction of that phenotype to the *in vitro* bias signature codes may facilitate medicinal chemical modification of these complex phenotypes.

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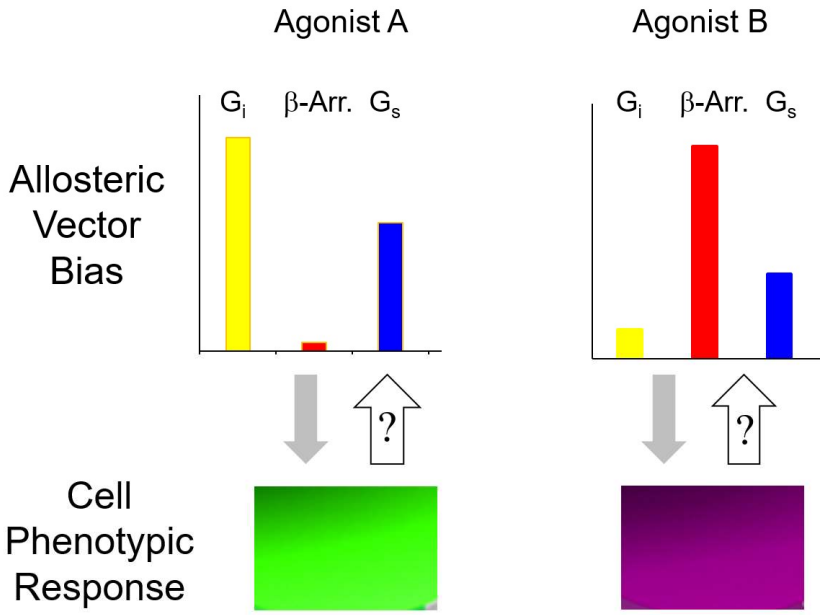

Figure 1

Fig 1 Cells mix receptor stimuli produced by agonists to yield a complex synoptic response which defines the true therapeutic utility of the agonist. Thus, different biases in terms of elemental signaling pathways can produce different phenotypic cell responses; however, this also allows the cell to hijack various *in vitro* bias signatures and change them in accordance with the stoichiometry of receptor to signal protein. Once a useful phenotype has been identified, reduction of that phenotype to the *in vitro* bias signature codes may facilitate medicinal chemical modification of these complex phenotypes.