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Practical guide for calculating and representing biased signaling by GPCR ligands: A stepwise approach ☆

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ABSTRACT

Signaling bias makes reference to the capacity of G-protein coupled receptor (GPCR) ligands to direct pharmacological stimuli to a subset of effectors among all of those controlled by the receptor. This new signaling modality has added texture to the classical notion of efficacy. In doing so, it has opened new avenues for the development of therapeutic GPCR ligands that specifically modulate signals underlying desired effects while sparing those that support undesired drug actions. Essential to taking advantage of this texture is the ability to identify, quantify and represent bias in a reliable and intuitive manner that ensures comparison among ligands. Here, we present a practical guide on how the operational model may be used to evaluate ligand efficiency to induce different responses, how differences in response may be used to estimate bias and how quantitative information derived from this analysis may be graphically represented to recreate a drug's unique signaling footprint. The approach used is discussed in terms of data interpretation and limitations that may influence the conclusions drawn from the analysis.

1. Introduction

Representing ~4% of the protein-coding genome, G protein-coupled receptors (GPCRs) are the largest family of membrane proteins involved in signal transduction and constitute the target for more than 30% of therapeutic agents that are clinically available. At the same time, it is also widely known that successfully-targeted receptors represent only a small percentage of all family members, leaving countless GPCRs yet to be explored. Despite this wide availability of druggable targets, traditional pharmacological approaches have consistently declined in their ability to yield novel therapeutic ligands [1], pressing for a change in strategy in drug development. Of considerable value for the development of more efficacious and safer drugs has been the realization that GPCR ligands may distinctively activate a specific set of signals among all those controlled by a receptor. The discovery of this signaling modality has been transforming because it raises the possibility of specifically directing the pharmacological stimuli that is imparted by agonist binding to the receptor towards therapeutically relevant pathways. The capacity to direct pharmacological stimulus towards a distinct signaling outcome is supported by receptor ability to adopt multiple and distinct signaling conformations.

The notion that GPCRs may exist in more than one active state was initially formulated in a theoretical report in which the effects of two agonists were simulated at a receptor that was allowed to promiscuously couple to two different transducer G proteins [2]. The simulation showed that when the availability of the two G proteins was inversed (as could possibly happen in two different tissues), the rank order of potency of the two agonists was also reversed. The prospect that this reversal might also happen in cell signaling was exciting and compelling, contrasting with the prevailing model at the time which favoured the idea that relative drug potencies/efficacies were maintained across different tissues and/or responses. The conservation of rank orders was based on the model's assumption that ligands of different efficacies stabilized a single active state of the receptor whose accumulation was determined by efficacy. The reversal in the rank order of potencies that was predicted by the simulation presented by Kenakin and Morgan [2] was not explained by simple accumulation and implied the existence of multiple active receptor states. Early

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experimental corroboration for the reversal of agonist potencies was provided for the pituitary adenylate cyclase activating polypeptide (PACAP) receptor. It was found that two agonists displayed reversed potency to produce cAMP stimulation as compared to inositol phosphate production [3]. Factual functional evidence of the existence of multiple signaling conformations immediately suggested the possibility of these states being specifically stabilized by different agonists. This new ligand-specific signaling modality was proposed under the name of agonist-trafficking of stimulus [4]. Since then, examples of agonist-specific signaling to different effectors have multiplied and alternative names such as biased signaling [5] or functional selectivity [6] have been proposed and are now in use. Independent of nomenclature, the essential pharmacological property responsible for the signaling imbalance is the stabilization of ligand-specific conformations. This property is inherent to the drug, it can be carried over to biological *in vivo* systems and is the one sought in drug development.

2. Identifying and quantifying ligand-specific signals

The most common approach to search for biased agonists is the use of cell-based assays which allow to measure and compare ligand signaling efficacies in different pathways. However, to confidently identify ligands of interest, it is important to keep in mind that the signaling imbalance being sought is the one that results from the stabilization of ligand-specific conformations. We are thus searching for a molecular property by looking at cellular responses. In this context, it is essential to keep in mind that responses available to us through cell-based assays are not solely determined by drug signaling properties, but also by the way the cell and the different assays "perceive" the generation of this response. These different "perceptions" of the pharmacological stimulus may themselves determine an imbalance among signals from different pathways which are known as "system" and "assay bias" [7]. What is common to these two types of influences and distinguishes them from proper ligand-bias is that the signaling imbalance they create influences all ligands in the same way. Methods that quantify the ligand-dependent component of a biased response 'dissect away' system and assay confounding influences [8–11]. One way of doing so is by normalizing, to a common standard, the response produced by each ligand in different pathways of interest. Since standard and ligand are similarly affected by the same confounders, the use of reference-weighed responses allows to cancel these out, which explains why comparison to a standard is implicit in the estimation of bias.

To be able to detect bias, it is first necessary to evaluate ligand signaling efficacy in the pathways of interest. The ability of a drug to elicit a response in any system can be classically estimated from fitting the data of a concentration–response curve to a logistic equation of the type:

$$\text{Response} = \min + (\max - \min)/1 + 10^{(\text{Log}(EC_{50}) - x)^* \text{Hillslope}},$$

where response is the dependent variable, and the independent variable x corresponds to the logarithm of the concentration that elicits that response. The terms "min" and "max" are equation parameters to which response tends at infinitely low or high concentrations of the ligand and EC_{50} is an equation parameter which, in this case, corresponds to the concentration of agonist which produces a response half-way between "min" and "max". The Hill slope is the steepness of the curve and gives an idea of the efficiency with which the signaling machinery transforms a dose increment into a larger response. The EC_{50} parameter and the "max" asymptote of this equation are related to drug properties as envisaged by classical receptor theory [12–15] and are highly influenced by how the cell processes the pharmacological stimulus to produce the response we measure. Concerning drug properties that contribute to the value of these parameters, "max" is influenced by efficacy while EC_{50} depends both on binding affinity and efficacy. Thus, it can be seen that the ratio $Emax/EC_{50}$ encapsulates information about the overall efficiency with which a drug activates a given response. In particular conditions in which the Hill coefficient is not different from the unity, comparison of $Log(Emax/EC_{50})$ ratios observed across different responses may be used to quantify bias, provided that appropriate standards are used in order to control for system and/or assay confounders (see below).

An alternative way of estimating ligand signaling efficiency is the operational model developed by Black and Leff [16]. In their formulation of the model, the authors considered experimental response as the product of successive steps involving occupation of the receptor by the agonist, communication of the pharmacological stimulus to the system and processing of the stimulus by the system that produces the response. The first step is determined by the affinity with which the ligand interacts with the receptor and was represented by the equilibrium dissociation constant of the agonist–receptor complex (K_A). The intensity of the pharmacological stimulus and it's conversion into the measured experimental response were considered non-dissociable. They are represented by a ratio given by the total amount of receptors in the system ($[R_{tot}]$) and the positional parameter of a function (K_E) that represents the system's ability to translate the pharmacological stimulus into a response. The $[R_{tot}]/K_E$ ratio was equalled to tau (τ) and the complete conceptualization of the model was formalized in the following equation:

$$E/Emax = \tau^n \times [A]^n/(K_A + [A])^n + \tau^n \times [A]^n$$

where, in addition to the parameters mentioned just above, E/Emax corresponds to the fractional response observed at a specific agonist concentration [A], Emax is the maximal response allowed by the system and "n" describes the presence of cooperative steps that lead from receptor occupation to response production. In the absence of such cooperativity, the value of "n" is one. Like $[R_{tot}]$, Emax and "n" are shared by all agonists and describe the unique properties of the system in which response is evaluated. For example, a change in cellular background could be represented by a change in the total amount of receptors ($[R_{tot}]$), in the cooperative steps leading to response (n) or in both. The system is also described by K_E but, unlike the latter, this parameter also contains information about the drug. It is in fact contained within the definition of τ and its contribution to response can be considered as an indicator of how efficiently immediate downstream signaling partners respond to the activated receptor (note the then that two different cascades in the same cell are represented as different systems). Thus, because τ is embedded within system parameters ($\tau = [R_{tot}]/K_E$), direct comparison of τ values provides meaningful information of drug relative efficacy only when it is done within the same system. To allow for cross-system comparisons, differences in system-related parameters should be offset by normalizing the responses to a common standard.

In turn, K_A informs us about "*functional*" (or "*operational*") affinity, which represents the affinity the ligand is assumed to have for the receptor given the functional response it produces. Conceptually, K_A represents the weighed affinity of the drug for the receptor state(s) present in the signaling ensemble. Its value may or may not correspond to the equilibrium dissociation constant as measured in binding experiments [17].

Both drug-descriptive parameters (τ and K_A) may be directly obtained by fitting experimental data to the operational equation and can be expressed as transduction coefficients $Log(\tau/K_A)$. As proposed by Kenakin et al. [10], comparison of a ligand's standard-