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ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iedc20

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To cite this article: Karim Nagi (2023): How can we improve the measurement of receptor signaling bias?, Expert Opinion on Drug Discovery, DOI: 10.1080/17460441.2023.2204225

To link to this article: https://doi.org/10.1080/17460441.2023.2204225

Published online: 19 Apr 2023.

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EXPERT OPINION ON DRUG DISCOVERY
https://doi.org/10.1080/17460441.2023.2204225

EDITORIAL

How can we improve the measurement of receptor signaling bias?

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ARTICLE HISTORY Received 28 February 2023; Accepted 14 April 2023

Keywords Biased agonism; Biased ligand; Drug discovery; Functional selectivity; GPCR; Screening; Receptor signaling; Signaling bias; Signaling pathways

1. Introduction

G protein-coupled receptors (GPCRs) are flexible and dynamic signaling entities that can adopt multiple active conformations upon activation [1,2]. However, each agonist stabilizes a distinct receptor conformation at a definite point of time allowing transmission of a specific conformational information to downstream transducers and effectors. This signaling modality known as biased agonism or functional selectivity has rapidly attracted interest as a means to improve drug discovery by screening for drug candidates that can direct their stimuli toward pathways that are therapeutically beneficial while avoiding those associated with adverse effects. Although appealing as a work plan, screening for biased ligands is a challenging process that needs to be correctly assessed and interpreted. In fact, numerous studies reported identifying compounds with biased signaling properties, but very few of those compounds have progressed to clinical testing, leaving open the question of whether it would be possible to translate a biased drug stimulus into a therapeutically desired response.

2. Strategies to bypass challenges in detecting and measuring biased agonism

Signaling bias has a great potential in identifying more effective and better tolerated drug candidates. However, it needs to be accessed with caution to prevent any confusion about what has really been measured in assays and what can be concluded. For that to be done correctly, a drug action mediating the receptor signaling needs to be purified from any confounder that may affect its signaling activity and assessed in a biological environment that reflects the disease condition to be treated (Figure 1). Detecting signaling bias could be challenging at the fundamental (pathways to target, assays to use) and experimental (system, kinetic, and location bias) levels as detailed below. However, several possible solutions could be incorporated at the experimental design and data processing to optimize bias assessment and eliminate any apparent signaling artifact.

2.1. Which signaling pathways should we target in drug screening?

Advances in the field of biased agonism allowed identification of new drug candidates based on hypotheses that allocated desired and undesired effects to specific signaling pathways. For example, associating G protein signaling to desired (analgesia) and β-arrestin to side effects (constipation and respiratory depression) of opioid analgesics [3,4]. However, a number of recent studies have challenged some of these hypotheses [5]. And still for most GPCR targets, the signaling cascades employed to drive particular therapeutic outcomes are not well defined. In addition, receptors can homo- or heterodimerize, which stretches the complexity even further as different ligands may preferentially bind to homo or heterodimers and generate different physiological responses.

To tackle this issue, defining the physiologically relevant signaling pathways for a particular disease is an essential first step in drug discovery. In this regard, designing biased ligands that could avoid activation of a specific signaling pathway and testing them *in vivo* could help revealing the physiological outcome of that pathway. Moreover, it became relevant from several studies that introducing point mutations into a target receptor could result in pathway-selective receptor variants (biased receptors) [6]. Characterizing biased receptors signaling *in vitro* and defining the phenotype of biased receptor-mutant mice models (knock-in mice generated by replacing the wild-type receptor with a biased receptor) could also reveal the importance of the affected downstream signaling pathways.

2.2. How to identify a suitable screening assay?

Choosing the right functional screening assay that can characterize the pharmacological activity of drug candidates with a large response window for detecting the target protein signals is critical in early drug discovery. The assay needs to have a certain degree of screening flexibility that will allow researchers to use it for monitoring different signaling pathways while using the same experimental system. Ideally, the assay should also be non-radioactive, easily adaptable to standard microplate formats and automation platforms, and robust to perform under high-throughput conditions.

Moreover, studies suggest that a ligand can stabilize different receptor conformations over time, which can change its bias toward different signaling pathways. Therefore, assays used for screening must require a short

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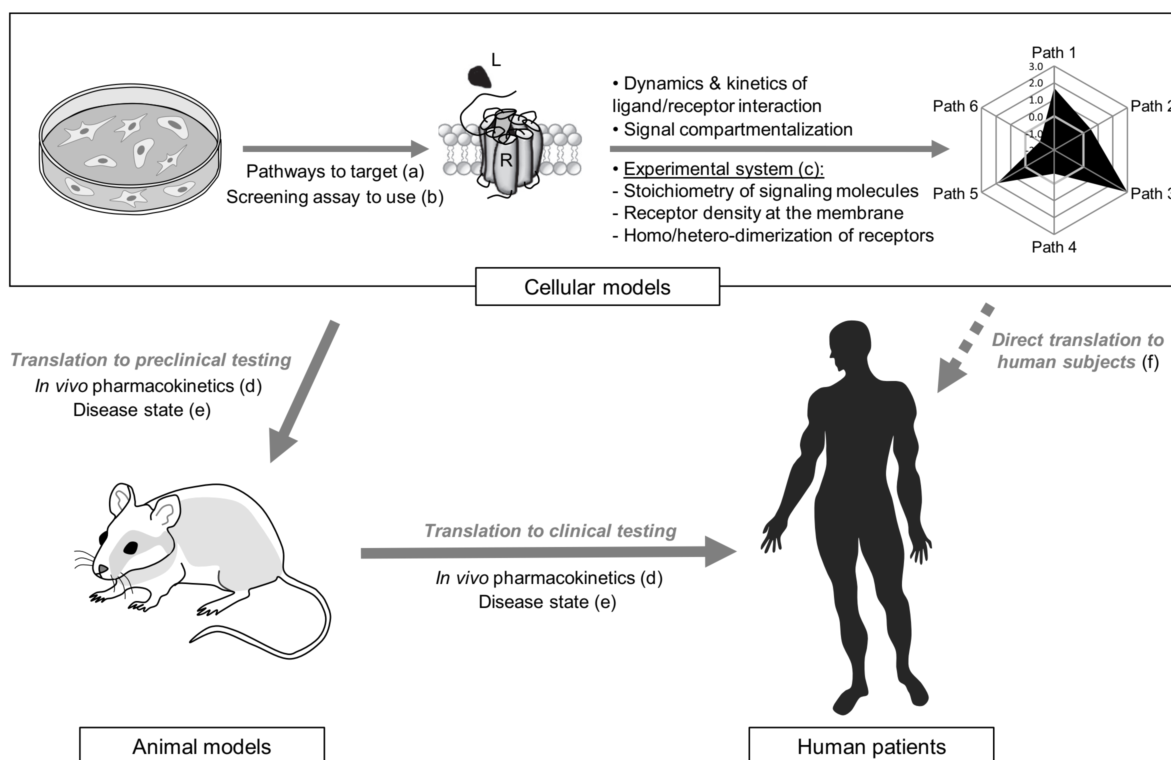

Figure 1. Factors that can influence measurement of receptor signaling bias.

(a) The choice of optimum time points for data collection is essential as each signaling pathway has a distinct activation and desensitization kinetics.

(b) A detailed experimental protocol should be reported as inconsistency between assays in incubation times required for signal detection might affect the directionality of bias.

(c) Using the same cellular system, the effect of this factor on bias measurement could be normalized by quantifying relative to a reference ligand.

(d) Difference in ligand exposure at the site of action or formation of active metabolites that could differ in their signaling from the parent molecule could affect the directionality of bias.

(e) This factor could modulate the stoichiometric ratios of various signaling components as well as the membrane expression, homo/hetero-dimerization and compartmentalization of receptors.

(f) Direct translation from cellular models to human subjects is feasible by associating cellular responses to human side effects (for details, see reference 14).

incubation time for signal detection and should be sensitive enough to assess early and late signaling events following receptor stimulation.

Also, it is preferable to choose assays with similar levels of amplification [7] as high protein overexpression might influence the signaling bias in a ligand-independent manner. However, some assays require the use of a recombinant system, where target proteins such as the receptor, transducer, or effector proteins can be overexpressed and/or genetically modified (fused with a tag sequence). Although overexpression and modifications could have an effect on the system used, the signaling bias generated can still serve as an identifier for molecular mechanisms to be further elucidated in animal models. Furthermore, it must be noted that assays carried out using different experimental conditions may lead to different biased responses, which indicates the importance of reporting the detailed experimental protocol used when considering biased agonism.

2.3. Can the experimental system or the assay type used in screening affect the signaling bias of drugs?

Once activated by a ligand, a receptor can adopt distinct active conformations as it interacts with different downstream signaling partners such as transducers or effectors [2]. Therefore, the stoichiometry of signaling molecules in each experimental system could play a significant role in directing ligand signaling toward certain specific pathways (effect referred to as system bias) [8].

While quantifying bias using the same cellular system, system bias will equally affect all the tested ligands and could therefore be eliminated by quantifying bias relative to a reference ligand. Ideally, the endogenous agonist is used as a reference ligand to check how drug candidates differ from the natural signaling. Otherwise, a ligand with very similar signaling through different pathways (balanced), is used as reference. Definitely, a ligand referred to as ‘balanced’ might become ‘biased’ if tested in another system or assay. Likewise, a tested ligand may be unbiased, relative to the reference ligand, in one particular pathway but biased in another. Hence, designations such as ‘biased’ or ‘balanced’ cannot be used as an absolute descriptor for a ligand, but should be reported and interpreted in the context of a specific receptor, reference ligand, and/or pathways.

However, this normalization procedure cannot be applied to compare ligand signaling preferences in different screening environments. Using experimental systems that express different levels of signaling molecules or altering the stoichiometry

of target proteins in the same cellular system will likely affect both the magnitude and directionality of bias. Therefore, it is important to point out that when characterizing the same signaling pathway, bias values obtained from different experimental systems are not quantitatively comparable. Instead, a ligand rank order of bias factors could be used to detect any apparent ligand bias [7].

Furthermore, it is of interest to mention that the stoichiometric ratios of various signaling components within cells can be modulated by disease states. Thus, the signaling preference of ligands for a specific pathway in a healthy organism might change in disease conditions. Consequently, the use of primary and/or disease-specific cells, in the case available, to evaluate ligand bias would be preferred as it will provide a better understanding of the therapeutic manipulations that could lead to a disease-modifying effect.

2.4. How to deal with kinetic and location bias?

The receptor conformations stabilized by different biased ligands might change over time and be influenced by the dynamics and kinetics of the ligand–receptor interaction [5,9]. Consequently, a ligand's effect is time-dependent. Thus, assays that require different incubation times for signal detection have the potential of producing time-dependent differences in signaling bias. In addition, every signaling pathway has distinct activation and desensitization kinetics that could be distinctively initiated by different ligands. Therefore, choosing the optimum time points for data collection is essential. Sampling time points are generally chosen as being the most physiologically relevant or those that can capture the maximum ligand effect. Kinetic measurements of dynamic events, whenever applicable, could also be of great value.

Moreover, some findings consider a long ligand residence time at the receptor as an opportunity to induce more signaling as several internalized receptors were found to signal from subcellular organelles [10]. The effect of signaling from different cellular locations through the same transducer and producing distinct signaling responses has been referred to as 'spatial/location' bias [7]. In that context, several biosensors targeted to the plasma membrane or subcellular compartments were developed to investigate, in real-time, the importance of signal compartmentalization upon GPCRs activation [10,11]. Despite that the potential therapeutic consequences of modulating receptor signaling from different cellular locations remain largely unexplored, the use of such biosensors will advance our understanding of the spatiotemporal regulation of signal transduction and will provide insights into how factors such as time and location can influence the generation of a biased response.

3. Expert opinion

The directionality of bias could differ when ligands are tested in different cellular environments [5,12] (Figure 1). Therefore, identifying the intracellular signaling network within a diseased tissue, by monitoring changes in proteins expression and interactions using single-cell phenotyping and high-throughput sequencing strategies, could be used to identify a suitable preclinical disease model for testing ligand candidates. Using a preclinical model that mimics the diseased tissue by its stoichiometry of proteins expressed could allow a more accurate prediction of the therapeutic value of ligands.

Next, it would be necessary to determine how the conformational information generated by each ligand tested is transmitted from the receptor to downstream signaling proteins to mediate distinct regulatory responses. For instance, a recent study used bitopic fentanyl derivatives to identify the importance of a conserved sodium pocket among class A GPCRs in the modulation of signaling at the mu-opioid receptor [13]. While combining crystallography in conjunction with computational methods could solve the structural features of receptors dynamic interactions, other biophysical technologies such as the use of BRET/FRET biosensors could allow monitoring the efficacy of ligands toward distinct pathways and the spatiotemporal characteristics of their signaling. It is essential to represent the multifaceted mechanisms of receptor signaling by examining the ability of ligands to trigger multiple signaling cascades since classifying ligands as 'biased' after measuring a few signaling outputs such as G protein activation and arrestin recruitment to the receptor might be too reductive.

The large generated datasets need then to be analyzed and by using machine learning clustering techniques, it would be possible to classify ligands according to similarities in their structural and signaling properties [14,15]. Such classification methods could provide a broad visualization of the data for better identification of stimulus bias profiles [16]. It could also help to infer the clinical activity of new biased ligands by clustering them with drugs of known clinical effects and predicting that ligands showing the same stimulus bias profile might have similar therapeutic outcomes [14].

Furthermore, it is therapeutically essential to distinguish 'partial' from 'biased' receptor agonists. A number of recent studies showed that the improved side effect profile of some ligands could be explained by their low efficacy at stimulating the receptor (partial agonism), and not necessarily because of their greater efficacy for activating a specific signaling pathway over another (signaling bias) [14,17]. However, despite all efforts in refining our approaches for predicting the therapeutic effect of biased agonists using simple cellular responses, failures in the clinic should be expected as additional levels of translational testing that associate *in vivo* responses with specific *in vitro* signaling effects might be required. This, however, should not diminish our interest in bias signaling as a viable therapeutic approach in drug discovery.

Acknowledgments

Open Access funding provided by the Qatar National Library.

Funding

This paper was not funded.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with

the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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