

A pluridimensional view of biased agonism

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List of non-standard abbreviations: GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding protein; GRK, G protein-coupled receptor kinase; 5HT, 5-hydroxytryptamine; PI, phosphoinositide; PACAP Pituitary adenylate cyclase-activating polypeptide; cAMP, cyclic adenosine monophosphate; IP3, inositol trisphosphate; PKA, protein kinase A; PKC, protein kinase C; NMR, nuclear magnetic resonance; ERK1/2, extracellular signal-regulated kinases; V2R, V2 vasopressin receptor; PTHR, parathyroid hormone receptor; CCR7, C-C chemokine receptor type 7; CCL19, chemokine (C-C motif) ligand 19; CCL21, chemokine (C-C motif) ligand 21; AT1R, angiotensin II type 1 receptor; K_{on} , association rate; K_{off} , dissociation rate; RGS, regulators of G protein signaling; PLC, phospholipase C; iPS, induced pluripotent stem cells; BRET, bioluminescence resonance energy transfer.

Abstract:

When studying GPCR signaling and ligand-biased agonism, at least three “dimensional spaces” must be considered: 1) the distinct conformations that can be stabilized by different ligands promoting the engagement of different signaling effectors and accessory regulators; 2) the distinct subcellular trafficking that can be conferred by different ligands, which results in spatially distinct signals; and, 3) the differential binding kinetics that maintain the receptor in specific conformation and or subcellular localization for different periods of time, allowing for the engagement of distinct signaling effector subsets. These three pluridimensional aspects of signaling contribute to different faces of functional selectivity and provide a complex, interconnected way to define the signaling profile of each individual ligand acting at GPCRs. In this review we discuss how each of these aspects may contribute to the diversity of signaling, but also how they shed light on the complexity of data analyses and interpretation. The impact of phenotype variability as a source of signaling diversity, and the influence of novel and more sensitive assays in the detection and analysis of signaling pluridimensionality, is also discussed. Finally, we discuss perspectives for the use of the concept of pluridimensional signaling in drug-discovery, where we highlight future predictive tools that may facilitate the identification of compounds with optimal therapeutic and safety properties based on the “signaling signatures” of drug candidates.

Introduction:

For many decades, GPCRs have been described as plasma membrane proteins transducing signals from a variety of extracellular molecules to the intracellular milieu by coupling to and activating G proteins (e.g. Wess, 1997; Dohlman et al., 1991). The original model proposed that binding of agonists would promote an active receptor state that could engage and activate its cognate G protein, whereas binding of competitive antagonists would block activation by impeding agonist interaction. Therefore, GPCR functionality was measured and interpreted in a linear way as a switch turning a given signaling pathway “ON” or “OFF”. Nowadays it is known that a plethora of GPCRs signals can occur and are modulated by multiple mechanisms, including interaction with different G proteins, β -arrestins, GRKs and other effectors.

The first evidence that select GPCRs could interact with different G proteins isoforms was published in the 1980's (Burch et al., 1986; Brown and Goldstein, 1986; Murayama and Ui, 1985; Kelly et al., 1985; for a review see Garcia-Sainz, 1987). Further insight regarding diversity of signaling and biased agonism, its possible therapeutic applications, and potential underlying mechanisms were introduced by Roth and Chuang in 1987. “The 5HT₂ receptors may be coupled, directly or indirectly, to: (1) PI metabolism; (2) voltage-gated calcium channels and (3) prostaglandin metabolism. If these responses represent distinct coupling mechanism, it might be possible to design selective 5HT agonists and antagonists that alter only one or another of these systems” (Roth & Chuang, 1987). To explain such a phenomenon, it was then hypothesized that receptors could be stabilized in different active conformations, and that ligands with different structures would be able to

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stabilize such different conformations of the receptor (Kenakin & Morgan, 1989). Federman and colleagues in 1992 showed that the α 2-adrenoceptor could couple to both Gi and Gs isoforms, a profile that could be detected depending on the used cell type due to the distinct repertoire of co-expressed receptors and intracellular effectors (Federman et al., 1992). In 1994, using the same receptor, Eason and coworkers reported that Gs or Gi selection could be defined by the agonist in use (Eason et al., 1994). In this article, Stephen Liggett's group showed that six different agonists presented very similar efficacies towards Gi coupling, whereas significant differences were observed in Gs signaling. One year before, in 1993, Spengler and coworkers showed that splicing variants of the PACAP receptor triggered different kinds of response after stimulation with the same ligands. These authors provided clear evidence of the reversal of potency for the ligands PACAP-27 and PACAP-38 when triggering cAMP and IP3 responses (Spengler et al., 1993). The term agonist-receptor trafficking was introduced in 1995 by Terry Kenakin (Kenakin, 1995), and few years later, the term "biased agonism" was used for the first time (Jarpe et al., 1998).

The first evidence was also obtained during the 1980s showing that agonist stimulation leads to GPCR phosphorylation catalyzed either by second messenger-dependent kinases (e.g. PKA and PKC) or by G protein coupled receptor kinases (GRKs), leading to desensitization of the response (e.g. Nambi et al., 1985; Bouvier et al., 1987; Bouvier et al., 1988; for a review see Lefkowitz et al., 1990). GRK-promoted phosphorylation was then shown to promote the recruitment of the regulatory protein β -arrestin to the receptor, leading to internalization and termination of G protein signaling (e.g. Benovic et al., 1987;

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Lohse et al., 1990; Ferguson et al., 1996; for reviews see Lefkowitz, 1998; Zhang et al., 1997). Although such findings revealed the roles of new important partners, the overall view of GPCR functionality remained linked to G protein signaling. However, these findings introduced the notion that modifications in the receptor structure could regulate its ability to interact with different intracellular partners, and thus modulate its signaling properties.

An understanding of the dynamic nature of the equilibrium between active and inactive conformations of the receptor was significantly enriched by the first reports of ligands that were able to decrease the basal level of receptor activity (e.g. Costa & Herz, 1989; Costa et al., 1990; Chidiac et al., 1994; Barker et al., 1994; Samama et al., 1994; Leeb-Lundberg et al., 1994). Such reports underpinned the concept that receptors could spontaneously isomerize between active and inactive conformations, and that the equilibrium state in the absence of ligand determined the level of constitutive activity (Bond et al., 2001). These observations led to the discovery of inverse agonists, which shift the equilibrium from the active to the inactive conformation of the receptor and silence constitutive activity. This is in contrast to agonists, which promote the equilibrium shift towards the active state. Although the concept of inverse agonism added the idea of dynamic transition between different receptor conformations with important mechanistic and possibly therapeutic implications, the concept still fits into the perspective of receptor activation by engagement of G proteins.

The paradigm of G protein coupling as the unique effector of GPCRs' activation was challenged by the first reports describing that some compounds could either promote cellular responses even when unable to trigger G protein

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activation or even act as inverse agonists on G protein-dependent signaling (Azzi et al., 2003; Baker et al., 2003; Wei et al., 2003, Gbahou et al., 2003). These studies indicated that the G protein-independent component of GPCR activation could occur via β -arrestins, consistent with previous reports demonstrating that after interacting with receptors, β -arrestin serves as a scaffolding protein coordinating the actions of other effectors, including kinases, involved in downstream signaling (Milano et al., 2002; McDonald et al., 2000; Luttrell et al., 1999; Luttrell et al., 2001). The discovery of molecules bearing the capacity of activating distinct pathways after interacting with the same receptor resulted in the concept of functional selectivity and biased agonism. Therefore, agonists able to preferentially trigger β -arrestin-dependent signaling pathways became known as β -arrestin-biased agonists, and those able to preferentially trigger G protein-dependent pathways as G protein-biased agonists. More recently, compounds biased toward distinct G protein subtypes were described (Busnelli et al., 2012; Saulière et al., 2012).

The concept of biased agonism therefore shifted our view of GPCR from unidimensional regulators of linear signaling cascades to multidimensional transducers that can engage diverse signaling pathways and differentially regulate them. This has clear implications for pharmacological nomenclature, and indeed it has become insufficient to qualify a ligand as “agonist” or “inverse agonist” and it is now necessary to specify toward which pathway a given ligand is an agonist or an inverse agonist. This new vision of GPCR signaling also raises the issue of how to quantify the relative efficacy toward the different pathways. There are currently rich and active discussions about the best way of calculating bias. This topic is out of the scope of this mini-review, but articles

addressing it can be found elsewhere (e.g. Stallaert et al., 2011; Kenakin 2014; Van der Westhuizen et al., 2014; Kenakin et al., 2012; Kenakin and Christopoulos, 2013).

The pluridimensionality of signaling and of biased agonism:

Different structures/conformations, different active states:

The main hypothesis proposed to explain biased signaling tenders that differences from the ligand structure are reflected in differences of the final structures/conformations of the receptor, which in turn underlie the selective engagement of different subsets of effectors and/or the stabilization of different conformational states of the effectors. As will be discussed further below, in addition to this conformational hypothesis, different kinetics of ligand binding, differential post-translational modification, as well as distinct subcellular localization of the signaling complexes most-likely also contribute to the pluridimensionality of GPCR signaling and biased signaling.

The conformational hypothesis of ligand-biased signaling is supported by an increasing number of studies directly demonstrating that chemically diverse ligands can stabilize different conformations of receptors as assessed by NMR studies (Nygaard et al., 2013). Energy landscape can be used as an approach to visualize the various conformational states of proteins (Henzler-Wildman & Kern, 2007) and also of GPCR through their activation steps (Deupi & Kobilka, 2010). In Figure 1 we represent part of an activation coordinate path for a hypothetical GPCR and the corresponding energy landscapes until reaching the G protein active state(s). In panel A, we represent one possible active conformation, which is correlated to the G protein signaling pathway. Our

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representation on panel B highlights the existence of different intermediate active conformations of the ligand-receptor complex, which besides being able to interact with G proteins, are also able to interact with additional effectors. Distinct ligands can lead to stabilization of different conformations, hence generating signaling diversity.

Cellular compartments and microdomains unveils new dimensions of signaling:

The role of distinct membrane microdomains, receptor trafficking, and endosomal signaling are among the most actively debated topics in the field of GPCR signaling (e.g. Eichel et al., 2016; Irannejad et al., 2013; for a review see Calebiro et al., 2010). The concept of endosomal signaling has first been proposed for receptors of the tyrosine kinase family (for review see Wiley & Burke, 2001). Evidence has also accumulated indicating that endocytosed GPCRs can activate the mitogen-activated protein kinases ERK1/2 in a β -arrestin-dependent manner (Luttrell et al., 2001; Luttrell & Lefkowitz, 2002). More recently, sustained cAMP signaling has been proposed to result from a second wave of activation of Gs in the endosomes (for a review see Vilardaga et al., 2014). For instance, such non-canonical activation of Gs has been shown for the vasopressin type 2 receptor (V2R) and the parathyroid hormone receptor (PTHr), and that long lasting receptor-arrestin complexes correlate with different endocytosis patterns and sustained Gs signaling from endosomes (Feinstein et al., 2011; Vilardaga et al., 2012; Wehbi et al., 2013; Feinstein et al., 2013).

Such distinct signaling location, that was neglected until recently for GPCR drug discovery, may have a significant impact as it may be a key factor

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controlling response to drugs. Supporting this notion, it was recently reported that the cAMP produced in endosomes promotes a different gene regulation profile than that resulting from cAMP generated at the plasma membrane (Tsvetanova & von Zastrow, 2014).

The role of distinct subcellular compartment signaling takes particular importance when considering that different ligands can promote distinct endocytosis and differential subcellular trafficking and recycling paths. For instance, it has been described that activation of the CCR7 receptor by CCL19 or CCL21 resulted in different patterns of receptor internalization despite the ability of both ligands to similarly induce recruitment of β -arrestin 2. It was also shown that activation by CCL19 resulted in receptor phosphorylation by GRK3 and GRK6, while CCL21 only involved GRK6. Such differences probably yielded distinct phosphorylation patterns involved in differential engagement of the internalization and trafficking machinery (Zidar et al., 2009). The finding that different β 2-adrenergic ligands can promote distinct receptor phosphorylation patterns leading to different conformations and function of the engaged β -arrestin (desensitization vs MAPK signaling) lead to the concept of “barcode”, related to different phosphorylation patterns in the receptor by GRKs (Nobles et al., 2011). Stabilization of distinct β -arrestin conformations by different ligands has been adding a new dimension to the concept of biased signaling (Zimmerman et al., 2012; Santos et al., 2015), and recently a β -arrestin “conformational signature” has been elegantly proposed (Lee et al., 2016). It has also been recently shown that different ligands for the AT1R were able to promote distinct intracellular trafficking (Szakadati et al., 2015; Namkung et al., 2016)

The kinetic component of biased signaling:

When considering biased ligand signaling, it is also critical to bear in mind the influence of time on determined signaling profiles. Time and kinetics can indeed influence the interpretation of signaling data on different fronts. Among these, times of measurements and ligand binding kinetics represent the most obvious parameters that need to be considered.

Signal and assay time courses:

The different signals triggered by receptor activation as well as the assays used to monitor them can vary significantly. Therefore, two ligands with similar affinities for the same receptor and in the same given system may yield similar or completely contrasting signaling profiles depending on the time of data acquisition. Kinetics analyses of different pathways and the use of different readouts should reveal the best time for data acquisition for each pathway and also suggest which pathways can be compared at a given time-point. For instance, the same time point cannot be used for comparing transient signals, such as calcium or ERK1/2 activation with the more sustained cAMP accumulation. As illustrated in Figure 2, pathways **A** to **H** are activated by a given ligand to a similar extent at 5 and 10 minutes after stimulus, while only pathways **A**, **B**, and **F** maintain a similar profile of activation when comparing data acquired at 5 and 30 minutes. In this hypothetical example, if a kinetics analyses had not been carried out, one or the other time of data acquisition could have been chosen (e.g. 5 or 30 minutes), therefore leading to completely

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different interpretation. It follows that time-course profiles of responses should be carried out for each pathway considered and the consequence of different kinetics taken into consideration when analyzing apparent bias. In a recent study, Thompson and coworkers (2016) revisited their own data on biased signalling of opioid ligands, taking into consideration various cellular background and assay format parameters. Although the ligands considered as biased in previous studies retained their uniqueness, different profiles were observed providing broader knowledge on the characteristics of each ligand (Thompson et al., 2016). Also, in an elegant work by Rob Lane and colleagues, the influence of experimental kinetics and the kinetics of ligand-receptor interaction in signaling results, along with the possible misinterpretations about biased agonism that may arise were reported. Among other interesting data, the authors state that the distinct choice of time acquisition can even lead to a reversal of bias direction for some agonists for the D2 receptor (Klein Herenbrink et al., 2016).

The contribution of ligand's interaction kinetics to conformation stabilization:

Another kinetic aspect to be considered in the context of biased signaling concerns ligand binding on-rates and off-rates. Although some ligands have similar affinities determined by equilibrium radio-ligand binding or competition assays, they may have distinct K_{on} and K_{off} rates. Such differences in the "time of residence" of ligands bound to a GPCR can ultimately play a pivotal role in stabilizing specific conformations of the receptor for longer periods of time. This can lead to significant differences in signaling profiles, notably those that

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depend on lower affinity interaction with effectors (Zhang et al., 2015; Guo et al., 2014). Although we are aware that such approaches cannot be easily implemented in drug discovery high-throughput screenings, recent data highlights the relevance of the ligand's association and dissociation rates in addition to characterizing the properties of drug candidates and assessing the contribution of their K_{on} and K_{off} to the biased signaling observed (Guo et al., 2016; Klein Herenbrink et al., 2016). The observation that binding kinetics may influence the signaling profile of ligands adds the dimension of time to those of conformation and localization, all of which control GPCR functional selectivity.

The influence of phenotypic diversity to signal texture:

Subtle changes in ligands' structures can ultimately result in different receptor conformations, but it is also predicted that other factors such as membrane composition, presence of different effectors, and interacting proteins or other receptors will also impact the conformation of the receptor and influence its stabilization in distinct active states that may lead to distinct signaling profiles. Also, the relative abundance of the receptor's signaling partners influences the propensity of the receptor to engage some of the pathways simply by the law of mass action. For example, the low expression level of a given G protein in a specific cell type may allow a receptor that is normally highly coupled to this G protein to now engage other G protein subtypes for which it has moderate affinity. In a recent article, Broad and coworkers analyzed the functional profiles of κ -opioid receptors present in heterologous systems and in primary cell cultures. ICI204448 and Asimadoline are usually referred to as full agonists for the G_i coupled pathway, and G_i

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activation assays performed in this study using HEK293 cells expressing the human κ -opioid receptor indeed showed that both drugs acted with similar potencies and seemed to be full agonists for this pathway. On the other hand, in human colon cells, ICI204448 acted at very low concentrations while Asimadoline was only effective in much higher concentrations (Broad et al., 2016), an effect that we believe probably includes differences in receptor expression levels when comparing heterologous and primary systems.

Even when comparing cells having similar G protein repertoires, differences in the expression levels of other signaling partners such as regulators of G protein signaling (RGS), G protein receptor kinases (GRKs), second messenger-dependent kinases, phosphatases, other proteins involved in downstream signaling cascades, as well as other GPCRs that may indirectly interact by functional crosstalk or directly by receptor dimerization undoubtedly impact the signaling profiles observed. For instance, μ - and δ - opioid receptor heterodimerization has been proposed to affect subcellular trafficking, which results in lower cell surface expression (Décaillot et al., 2008), and unveiled a pertussis toxin-resistant signaling pathway (George et al., 2000).

The role of GRK expression levels in defining the signaling profile of the receptor has also been exemplified for the μ -opioid receptor, which in many cell types has been shown not to recruit and activate β -arrestin upon morphine treatment. However, over-expression of GRK2 has been shown to allow the recruitment of β -arrestin to the morphine-activated receptor and to restore its endocytosis (Zhang et al., 1998). These data indicate that the functional selectivity of morphine may be affected by the relative expression levels of GRKs in different tissues.

The composition of biological membranes might also play a significant role in defining the activation profile of a given GPCR. This can be easily understood when considering Gq/11 coupled receptors, which are functionally linked to PLC action on membrane phospholipids. Cholesterol has also been proposed to influence GPCR signal transduction (e.g. Pontier et al., 2008; Levitt et al., 2009; for reviews see Head et al., 2014; Oates & Watts, 2011). Obviously, differential membrane composition of molecules involved in internalization and/or endosome formation processes, such as lipids, clathrin and caveolae, could also have critical impact on signal transduction (for review see Head et al., 2014). Because membrane compartmentalization, endosome formation and trafficking are also dependent on the repertoire of membrane and intracellular proteins, it becomes clear that different cell systems may result in distinct profiles, and therefore must be taken into account when designing, analyzing and interpreting signal transduction data and biased signaling.

Based on the above discussion, it is predictable that the same ligands could have distinct signaling profiles in different cellular system as a result of the specific components present in each cell (Devost et al., 2016). However, if as theoretically proposed, functional selectivity is an intrinsic property of a ligand-receptor complex, it would be reasonable to propose that compounds sharing similar signaling profiles in a given cell type will still share a similar signaling signature in a different cellular background, albeit different from the first cell type. If this prediction is borne out, it would allow the clustering of compounds in different signaling groups in generic cell lines and the testing of representatives of these groups for their biological and clinical outcomes (i.e., therapeutic efficacy, side effects, and safety). Although such classification of

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compounds may not allow for the identification of the specific pathways involved in the desirable or undesirable effects, it may still be very useful to stratify different drug lead series identified in drug discovery programs. To validate this hypothesis, the signaling profile of the same ligands would need to be tested in different cell types, including primary cells and/or iPS cells differentiated in physiologically relevant cells to determine if the compounds cluster in the same subgroups independently of the cell background (Figure 3). If it is the case, it will then be important to determine how many signaling pathways need to be tested in order to establish “signaling signatures” of clusters that bear predictive biological and clinical values.

Discovering new signaling dimensions:

The discovery of ligand-biased signaling stimulated the development of assays with the required sensitivity to monitor GPCR functional selectivity, which allowed for the detection of multiple signaling pathways. Among these, bioluminescence resonance energy transfer (BRET)-based assays monitoring G protein activation (Galés et al., 2006), β -arrestin engagement (Angers et al., 2000) and activation (Charest et al., 2005), as well as second messenger generation (Jiang et al., 2007; Leduc et al., 2009) have attracted considerable attention and have been used to describe the signaling profile of an increasing number of ligands and receptors (Saulière et al., 2012; Quoyer et al., 2013; for reviews see Schann et al., 2013; Lohse et al., 2012). The granularity and sensitivity of the assays used is of primary importance to allow the full characterization of the signaling texture of ligands. As illustrated in Figure 4A, activation of pathways by different compounds may appear similar or even

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equal when tested by poorly sensitive assays, whereas a more sensitive assay and/or the broadening of the evaluation to downstream targets could reveal differences (Figure 4B and 4C) that would be neglected otherwise. Examples of the importance of the assay format on the detection of biased signaling involve β -arrestin; using an assay with high sensitivity detected the conformational rearrangement of β -arrestin following its activation, and this allowed researchers to distinguish the β -arrestin activation profile of the angiotensin AT1 receptor (AT1R) ligands that otherwise recruited β -arrestin to a similar extent to the receptor (Zimmerman et al., 2012). More recently, we analyzed downstream signaling profiles of two β -arrestin biased ligands in the AT1R, and observed significant differences, unveiling that broader analyses of signaling pathways and downstream targets can generate a more complete activation profile for a given ligand (Santos et al., 2015).

Discussion:

How the pluridimensional aspects discussed above can help or confuse biased agonism measurement and interpretation.

Analyses of diverse and pluridimensional aspects of GPCR signaling certainly will bring significant knowledge to the field and broaden our concept of biased agonism. The frequently used dichotomy of biased agonism towards β -arrestin or G protein can substantially change with inclusion of other pathways, as well as inclusion of spatiotemporal perspectives. On the other hand, it is important to remember that the concept of biased agonism is intrinsically dependent on a comparison with a second ligand acting on the same receptor that should ideally be under identical conditions. Therefore, differences in

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parameters such as the assay system or assay conditions (temperature, time of acquisition, etc) can lead to data misinterpretations or even contradictory data.

Perspectives for pluridimensional signaling in drug discovery:

A key point when considering analyses of large amounts of complex data is to extract those pathways that are indeed crucial for characterizing a given molecule in a predictive way in a drug discovery program. In other words, how much is it necessary to know? Is it mandatory to evaluate all possible pathways that are conceivably activated by a given ligand-receptor complex (including those of very low activation levels)? Or would it be possible to identify some pathways that when presenting a certain pattern of activation, would be enough to suggest the next directions? Perhaps those questions cannot be fully answered yet, but what we believe is that in the coming years, the increasing level of data granularity about GPCR pluridimensional signaling obtained in different systems, combined with pre-clinical and clinical data from successful molecules, will allow the creation of a predictive signaling/outcome framework. More importantly, meta-analyses extraction and predictive software should allow identification of the key pathways and corresponding patterns of activation, generating a “signaling signature” that is relevant for a given drug discovery pipeline or indication (see Figure 3). Therefore, whereas the methodological advances and sensitivities of the assays will allow us to discover the diversity of signaling pathways, the use of artificial intelligence tools should facilitate the early identification of promising molecules based on “learned data” from previous successful molecules. Such machine learning tools should allow us to predict physiological outcomes based on the signaling

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patterns previously associated with specific physiological responses. Similarly to a cell phone predictive text typing application that “learns” from our regular insertion of the most used/required words, the predictive signaling pathway identification tool would “learn” from the continuous insertion of pluridimensional signaling data combined with physiological outcomes from pre-clinical and clinical studies.

In conclusion, although the phenotypic diversity, the influence of binding kinetics and/or time of data acquisition, and other factors can complicate the analysis of biased signaling, we believe that such information can be taken into consideration and factored in formal analyses in order to provide robust prediction of the biological outcomes of different ligands.

Authorship contribution:

Designed the review: Costa-Neto, and Bouvier.

Wrote the review: Costa-Neto, Parreiras-e-Silva, and Bouvier.

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Legends to the Figures:

Figure 1. Representation of energy landscapes for GPCR activation. The energy landscape of GPCR is represented as a function of the activation path of a receptor from the resting state to the “G protein active state” following stimulation with ligands. The represented minima in each landscape graph correspond to stable conformations. Panel **A** illustrates the classical view that the unbound receptor (R), upon ligand binding (RL), would reach a unique ternary complex ligand-receptor-G protein (RLG*) low energy state, corresponding to the active conformation that triggers G protein dependent signaling. Panel **B** illustrates our current view that upon ligand binding, the receptor can be stabilized in several intermediate active conformations. All such intermediate active conformations allow coupling to G proteins, but also bear structural differences that could be reflected in coupling/activation of other pathways. In panel **B**, the distinct intermediate active states are represented by LRG^{*a} , LRG^{*b} , LRG^{*c} , and LRG^{*d} . Panel **B** also illustrates that Ligands X and Y, despite being able to both trigger G protein activation, may yield distinct signaling profiles since they differentially stabilize different subsets of intermediate active conformations.

Figure 2. Impact of different data acquisition times on signaling profiles.

The signaling profiles for each ligand is illustrated using a web representation of activity where each radius of the web represents the extent of activation of a specific pathway arbitrarily named **A** to **H** in the present hypothetical example. The webs represent the hypothetical activation of a given receptor by the same

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ligand, where activation of signaling pathways **A** to **H** are measured at 5, 10, and 30 minutes after stimulus. As can be observed in the red and blue overlapping web-chart, pathways **A** to **H** reach similar extents of activation at 5 and 10 minutes after stimulus. On the other hand, the blue and green overlapping web-chart shows that only pathways **A**, **B** and **F** display a similar activation profile when comparing data acquired at 5 and 30 minutes. This figure highlights that conclusions about the distinct signaling profiles of different ligands can be greatly influenced by the choice of data acquisition times.

Figure 3. Schematic representation of a hypothetical platform for “functional prediction” based on clustering of compounds with similar “signaling signatures” in different cell lines. Although the signaling pathways triggered by a given compound are expected to be different in different cell lines, compounds sharing similar signaling profiles in a given cell line would share a common “signaling signature” in another cell line allowing a clustering of compounds in distinct groups. In the example, although compound **A**, **D** and **F** have different signaling profiles in the 3 cell types tested, they remained clustered within each of the cell types representing a “signaling signature”. Such clustering would then be used to link signaling signatures to specific biological/clinical outcomes and make predictions about the efficacy and safety of drug candidates.

Figure 4. Hypothetical heat-map representing signaling activation patterns triggered by different compounds. Panel **A** shows the activation patterns of compounds **A** to **F** in pathways 1 to 5 using a low sensitivity assay. As

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highlighted, compounds A and E seem to yield a similar activation profile in pathway 4. Panels **B** and **C** illustrate that when a more sensitive and/or amplified assay is used, such as evaluation of downstream targets or gene expression, important differences can be unveiled.

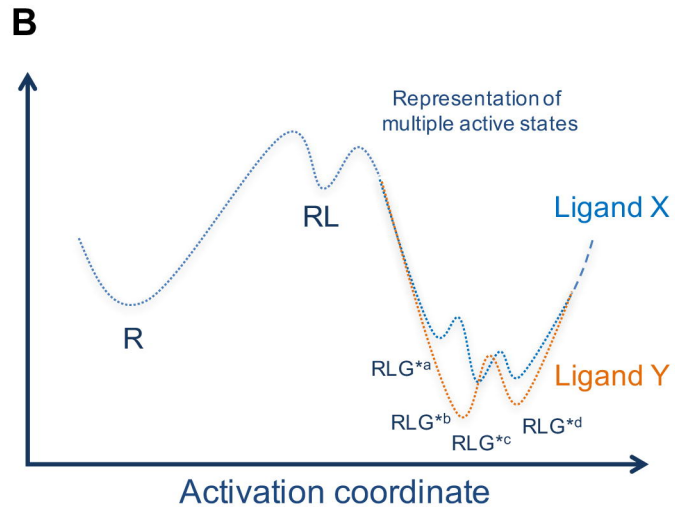
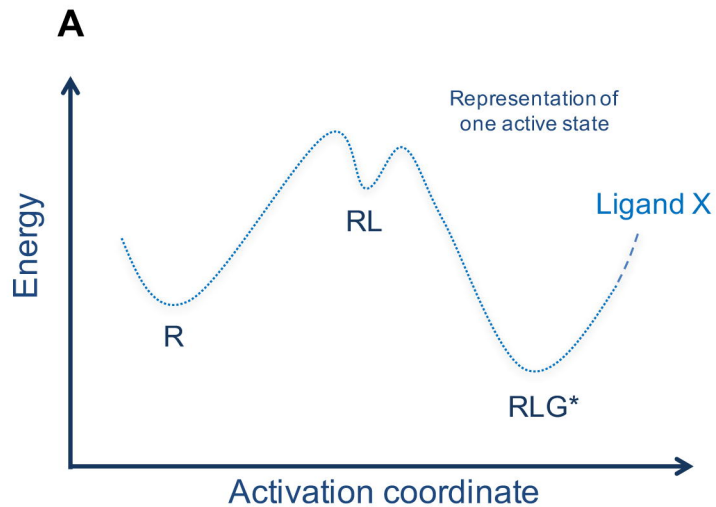
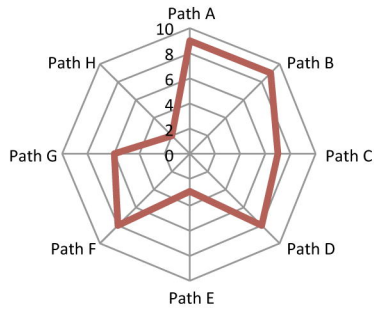
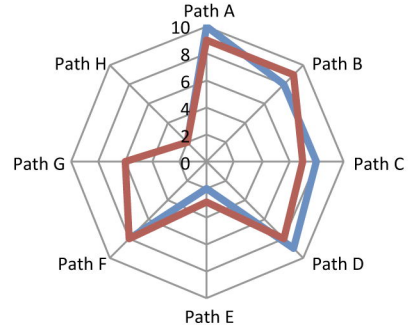


Figure 1

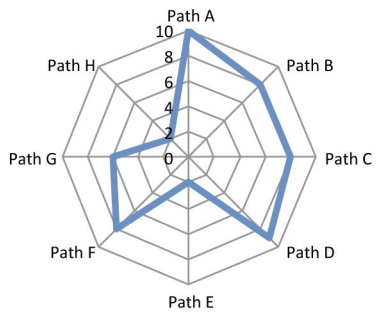
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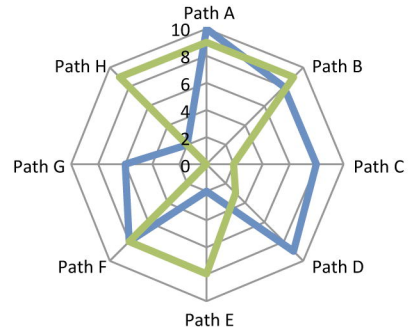
5 min vs. 10 min



5 min



5 min vs. 30 min



30 min

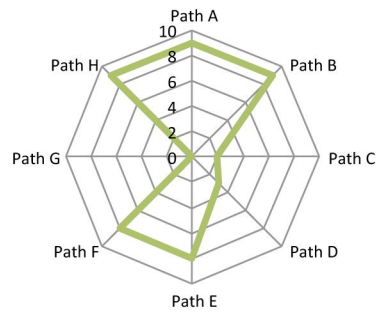


Figure 2

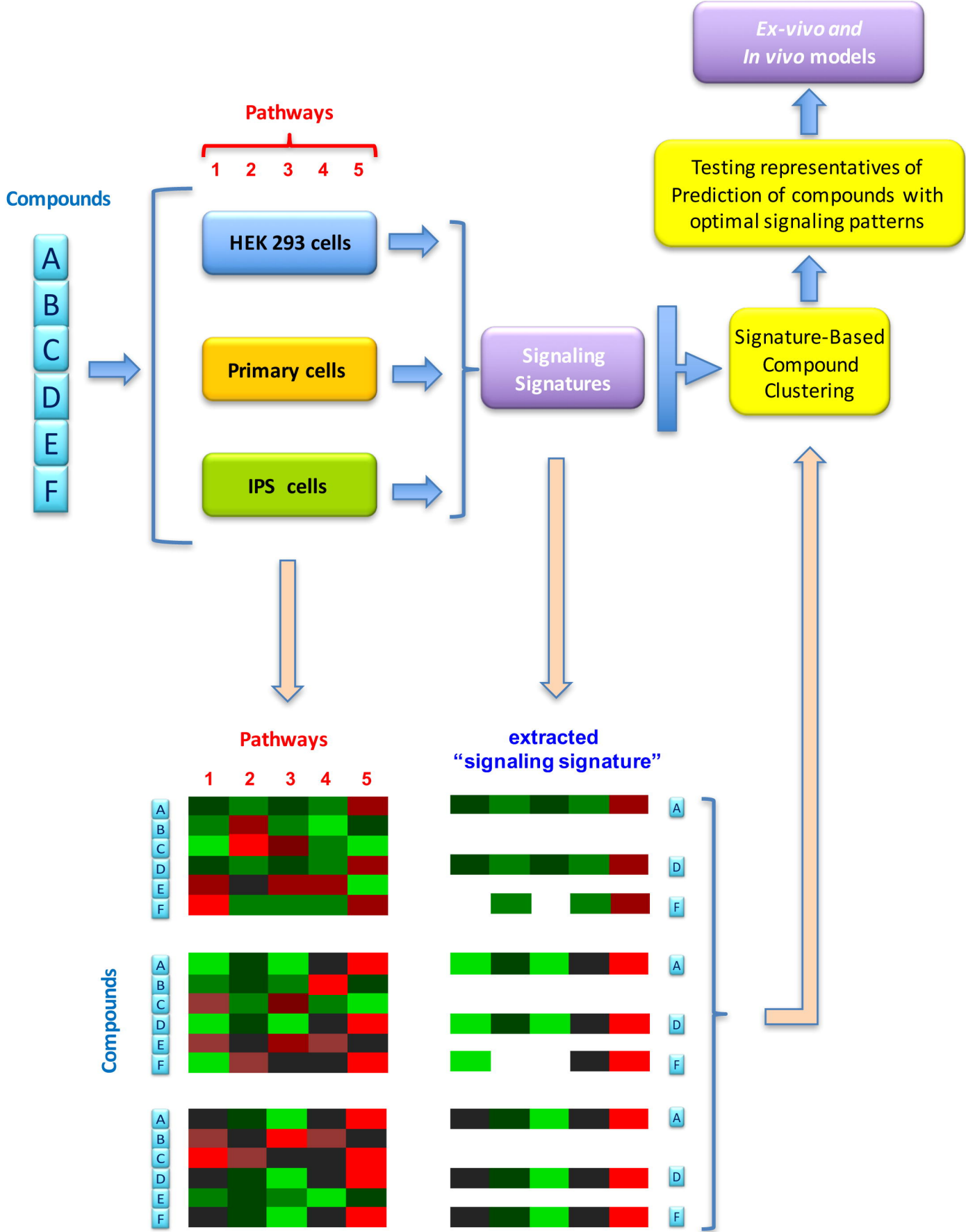


Figure 3

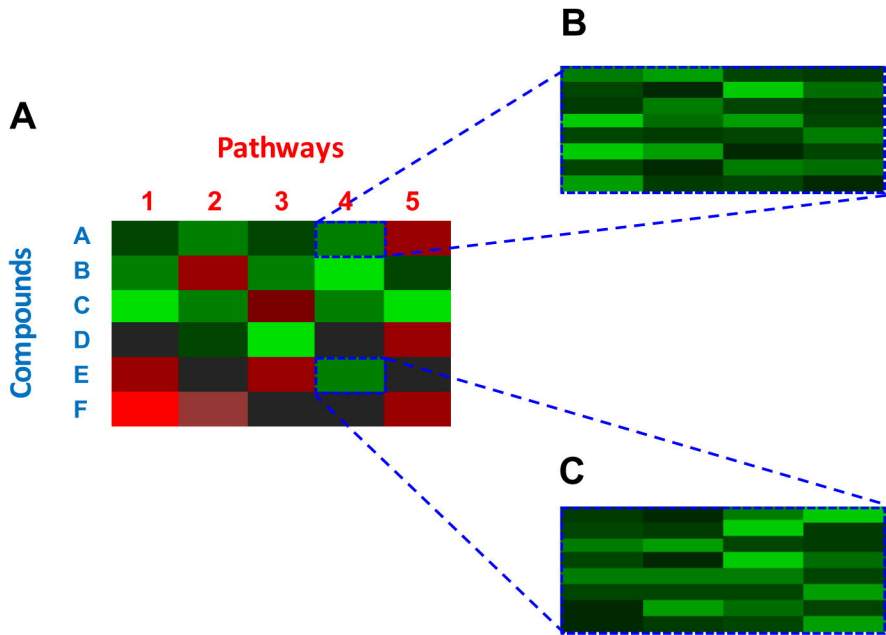


Figure 4