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Abstract	Selectivity of a drug for a desired response as compared to undesirable responses (side effect) is a key goal of drug development. Early concepts to achieve this were based on molecular selectivity for a molecular target as compared to others, pharmacokinetic factors to achieve high concentrations in the target tissue as compared to low concentrations in others, differential efficacy in the target vs. others tissues, and leveraging the concept of cell type and tissue differences in expression levels of receptors and their related signaling molecules, which can be further complicated by alterations of such ratios in disease. Biased agonism occurs when one response is activated preferentially over another after accounting for the others. Thus, assessment of ligand bias is not always easy. β-Adrenoceptors have played a relevant role in our understanding of the phenomenon of biased agonism. Several clinically used β-adrenoceptor ligands were proposed to exhibit biased agonism, but the findings often are inconclusive, at least partly based on the overall complexity of assessment of biased signaling. These complexities also make it challenging to determine the desired biased profile of a ligand at the start of a drug research and development project, particularly for innovative applications. Thus, biased agonism has potential to contribute to functional target selectivity, but its prospective use remains challenging.	
Keywords (separated by '-')	Biased agonism - Drug development - β_1-adrenoceptor - β_2-adrenoceptor - β_3-adrenoceptor	

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Biased Agonism at β-Adrenoceptor Subtypes: A Drug Development Perspective

Martin C. Michel and Ongun Onaran

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Contents

1 Introduction
2 What Constitutes Biased Agonism?
3 Methodological Challenges in the Assessment of Bias at β-Adrenoceptor Subtypes
4 Biased Signaling via β_1-Adrenoceptors
5 Biased Signaling via β_2-Adrenoceptors
6 Biased Signaling via β_3-Adrenoceptors
7 Conclusions and Perspectives
References

Abstract

Selectivity of a drug for a desired response as compared to undesirable responses (side effect) is a key goal of drug development. Early concepts to achieve this were based on molecular selectivity for a molecular target as compared to others, pharmacokinetic factors to achieve high concentrations in the target tissue as compared to low concentrations in others, differential efficacy in the target vs. others tissues, and leveraging the concept of cell type and tissue differences in expression levels of receptors and their related signaling molecules, which can be further complicated by alterations of such ratios in disease. Biased agonism occurs when one response is activated preferentially over another after accounting for the others. Thus, assessment of ligand bias is not always easy. β-Adrenoceptors have played a relevant role in our understanding of the

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phenomenon of biased agonism. Several clinically used β-adrenoceptor ligands were proposed to exhibit biased agonism, but the findings often are inconclusive, at least partly based on the overall complexity of assessment of biased signaling. These complexities also make it challenging to determine the desired biased profile of a ligand at the start of a drug research and development project, particularly for innovative applications. Thus, biased agonism has potential to contribute to functional target selectivity, but its prospective use remains challenging.

Keywords

Biased agonism · Drug development · β_1-adrenoceptor · β_2-adrenoceptor · β_3-adrenoceptor

Abbreviations

AR	adrenoceptor
CHO	Chinese hamster ovary
ECAR	extracellular acidification rate
EPAC	exchange proteins directly activated by cAMP
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
MAPK	mitogen-activated protein kinase
PTX	pertussis toxin

1 Introduction

Adrenoceptors (ARs) mediate the effects of the endogenous catecholamines adrenaline and noradrenaline, including positive inotropic, chronotropic, dromotropic, and lusitropic effects on the heart; modulation of smooth muscle tone, e.g., of blood vessels, airways, prostate and urinary bladder; and modulation of glucose homeostasis by acting on glycogenolysis, thermogenesis, and pancreatic insulin release and of brain function including ocular function. This wide range of physiological functions led to the development of a plethora of drugs. Many of these AR-ligands have become standard of care in therapeutic areas including the cardiovascular system, respiratory medicine, urogenital conditions, migraine, or ocular disease. As research on this receptor class started more than a century ago, ARs, particularly the β_2-AR, have become prototypes for our understanding of the action of G protein-coupled receptors (GPCRs) (Michel 2024).

Achieving a good *efficacy/tolerability profile* requires selecting a drug candidate that causes the desired effect with as little as possible undesired effects. This can be achieved in multiple, not mutually exclusive ways (Box 1). Firstly, it is possible to

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design compounds that interact with the desired molecular target selectively as compared to other molecules, such as subtype-selective receptor ligands. This is the oldest approach obtaining a favorable efficacy/tolerability profile in AR research. Following the initial proposal to subdivide AR into the subtypes α and β (Ahlquist 1948), further subdivision into α_1 and α_2 (Langer 1974; Starke 1987) and β_1 and β_2 emerged (Lands et al. 1967). However, shortly thereafter it became clear that even that was insufficient (Harms et al. 1977). Following the availability of more selective tool compounds and the cloning of AR subtypes, it ultimately became accepted that the AR family consists of three subfamilies (α_1, α_2 and β) including three subtypes each (α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3) (Bylund et al. 1994; Hieble et al. 1995). The selective targeting of AR subfamilies or subtypes proved to be a very successful strategy in the design and development of novel drugs that are efficacious and have limited side effects. Examples are β_1-AR antagonists in cardiovascular indications (Bond et al. 2022), β_2-AR agonists in obstructive lung disease (Kume et al. 2015), or β_3-AR agonists in the treatment of the overactive bladder syndrome (He et al. 2023). The pioneering work in the development of selective β-AR antagonists was honored with a Nobel prize to Sir James Black (1989).

Box 1: Means to Achieve Selectivity for a Targeted Drug Response

- Selectivity for molecular target
- Enrichment of exclusion of tissue on pharmacokinetic grounds
- Degree of agonism/inverse agonism
- Cell type/tissue specific stochiometry of molecular target and associated signaling molecules
- Biased agonism at the receptor level

A second approach to improve the efficacy/tolerability ratio of AR ligands was the identification of compounds with particular pharmacokinetic properties that enrich the target as compared to other tissues (Michel 2010) or the opposite, i.e., the sparing of the brain by drug candidates that exhibit limited penetration through the blood-brain barrier (Callegari et al. 2011). Other than exploiting inherent target tissue selectivity of a drug, this can also be achieved by local administration, for instance, in the administration of β_2-AR agonists by inhalation in the treatment of obstructive lung disease (Kume et al. 2015).

A third approach relies on the observation that ligands can exhibit different degrees of *efficacy* at the molecular target. While original concept of drug action on ARs dichotomized ligands as activators (agonists) or inhibitors (antagonists) of the receptor, it turned out that efficacy covers a continuous spectrum from full agonists (e.g., isoprenaline for β-AR) to antagonists, with partial agonists in between (Jasper and Insel 1992; Clarke and Bond 1998). Finally, this spectrum has been extended beyond the competitive antagonism to include inverse agonists with negative efficacy, i.e., ligands that can reduce the constitutive activity of GPCRs, including ARs in the absence of agonist (Schütz and Freissmuth 1992; Michel et al.

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2020). Of note, although efficacy represents an invariant quantity encoded in the chemical identity of ligand, receptor, and transducer, its manifestation in the observed response may differ among cell types, most likely due to the differential expression of signaling molecules mediating a response (Michel et al. 2020). Thus, *inverse agonism* is easier to detect in systems with high receptor expression, such as those often achieved after transfection of the receptor. Depending on the degree of expression of a receptor and/or associated signaling molecules, occupation of as little as 5% of the receptors may be sufficient to reach a close to maximal response, as in the case of positive inotropic effects of β-AR agonists in the heart (Brown et al. 1992). This phenomenon is sometimes referred to as receptor reserve, which arise from the nonlinearity of receptor-effector coupling. Thus, a weak partial agonist may be desirable if the target cell type or tissue exhibits a large receptor reserve whereas tissues mediating side effects exhibit little or no receptor reserve.

A fourth approach can also be based on the fact that the efficiency of stimulus-response coupling depends on the relative expression levels of signaling molecules involved in the relevant pathways. This may provide additional means of selectivity when we consider the fact that a signal starting from a single receptor type can lead to multiple responses via branching signaling pathways. In case of β-AR, for example, the receptor can activate multiple G proteins, adenylyl cyclases, arrestins, protein kinase A, mitogen-activated protein kinases (MAPK), or exchange proteins directly activated by cAMP (*EPAC*s). The magnitudes of such responses depend on the relative concentrations of these players in a cell type (Ostrom et al. 2000). This may lead to a single receptor subtype preferentially activating response X in one cell type, where signaling molecules involved in this response are highly expressed. On the other hand, preferential activation of response Y may occur in cases where signaling molecules involved in that response are highly expressed. This phenomenon is called cellular or tissue bias and does not necessarily involve biased agonism inherent to a ligand. Of note, subtypes of β-AR exhibit intrinsically divergent affinity and efficacy in coupling to G_s and to arrestins, which may make ligands acting on both partner molecules look as being biased, whereas this may reflect system bias (see below) and not biased signaling by the ligand (Casella et al. 2011).

Another level of complexity occurs when some signaling molecules undergo regulation in disease or upon drug treatment. For instance, heart failure may lead to redistribution of β_1- and β_2-ARs (Nikolaev et al. 2010), upregulation of β_3-ARs (Dal Monte et al. 2020), downregulation of $G_{\alpha s}$, and upregulation of $G_{\alpha i}$ proteins (Lohse et al. 2003). Expression level of β_2-AR affects interaction with the GRK/arrestin pathway in transgenic mice (Dorn II et al. 1999). Prolonged exposure to a β-AR agonist may lead not only to changes in receptor and G protein expression but also to a diminished activity of adenylyl cyclase (Okeke et al. 2019), thereby altering the relative concentrations of the signaling molecules involved in a response. This phenomenon has been called "*dynamic bias*" (Michel et al. 2014).

A fifth possibility to achieve the selective activation of a desired response came from the observation that the rank order of agonists to elicit one response via a single AR subtype may differ from that eliciting another response, if this phenomenon cannot be explained by any of the above factors (Nagi and Onaran 2021). This fifth

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dimension of selectivity has been described by various names including functional selectivity, agonist-directed trafficking, protean agonism, or ligand-directed signaling (Michel and Alewijnse 2007) but is now generally referred to as biased agonism (Kenakin and Miller 2010). While early conceptual work on biased agonism involved several GPCRs including opioid receptors and angiotensin II type 1 receptors, β-AR were important prototypes for our understanding of biased agonism (Ippolito and Benovic 2021). For instance, *propranolol* and ICI 118,551 are antagonists for G_s-mediated β-AR signaling but can promote the phosphorylation/activation of *extracellular signal-regulated kinase* (ERK) via arrestin, independent of G_s or $G_{i/o}$ proteins (Azzi et al. 2003; Baker et al. 2003; Shenoy et al. 2006). Interestingly, biased agonism can not only be elicited by small molecules but also by nanobodies (Staus et al. 2014). Pioneering work on biased agonism at β-AR particularly came from the groups of Terry P. Kenakin at Glaxo Research Laboratories (Kenakin and Morgan 1989) and Roger Summers at Monash University (Evans et al. 2013).

In the following, we will first discuss conceptual issues with the assessment of biased agonism at β-ARs, then review the evidence for biased agonism at each of the three β-AR subtypes, followed by a discussion of the implications of biased agonism for translational research, drug research and development, and clinical medicine. We explicitly acknowledge informative previous reviews in this area (Evans et al. 2010; Patel et al. 2010; Baker et al. 2011; Reiter et al. 2012; Ippolito and Benovic 2021).

2 What Constitutes Biased Agonism?

A rank order of potency and/or efficacy of two ligands, that is, A > B for response X but B > A for response Y, can point to the presence of biased agonism inherent to a ligand but may potentially also be explained by the four other determinants of selectivity mentioned above, particularly if responses X and Y are measured in different cell types or even different tissues (cell/tissue bias). These alternative explanations include molecular target/receptor subtype selectivity, tissue selectivity, degree of agonism, cell/tissue bias, or dynamic bias (Fig. 1). It follows from this complexity that biased signaling of ligands cannot easily be evaluated when the ligands being compared are tested in different cell types/tissues. Moreover, biased agonism inherently is a drug property relative to a reference compound. Even minor chemical changes within a molecule can markedly affect its biased signaling; for instance, 12 stereoisomers of *fenoterol* exhibited distinct biased profiles across six molecular and cellular assays (Reinartz et al. 2015). Thus, conclusive statements on ligand bias should be based on a direct comparison of two or more ligands within a cell type (Onaran et al. 2014; Kenakin 2015; Kolb et al. 2022). Computational methods to quantify ligand bias have been reported (Rajagopal et al. 2011; Reinartz et al. 2015).

True ligand bias is based on the interaction between a biological and a chemical phenomenon. Biologically, a given β-AR subtype may not only couple to α-subunits of G_s, which is its prototypical signaling pathway (Alexander et al. 2023), but also to

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those of other G proteins including forms of G_i and G_q, to βγ-subunits of G proteins, and even to non-G protein targets such as arrestins (Wenzel-Seifert and Seifert 2000; Lefkowitz and Shenoy 2005; Stallaert et al. 2012; Wess et al. 2023). The degree of coupling to multiple pathways may partly differ between β-AR subtypes; for instance, β_2-ARs may be more prone to couple to G_i proteins than β_1-ARs (Abramson et al. 1988; Xiao et al. 1995), and β_3-ARs may exhibit an even greater affinity for G_i proteins (Cao et al. 2000). Based on their molecular interaction with the receptor, some ligands inherently favor/induce conformational states that couple to one as compared to another signaling pathway present in substantial quantity in a cell (Bokoch et al. 2010; Kahsai et al. 2011). To further complicate the matter, the degree of intrinsic efficacy for the activation of individual signaling pathways may differ between ligands.

3 Methodological Challenges in the Assessment of Bias at β-Adrenoceptor Subtypes

Several approaches are used to explore biased signaling at β-ARs. This includes the *extracellular acidification rate* (ECAR) (Hutchinson et al. 2005), GPCR kinase phosphorylation and receptor trafficking (De Pascali et al. 2022), and impedance responses (and integrated indicator of multiple signaling pathways) (Stallaert et al. 2012). However, the most frequently used comparator to cAMP formation is the phosphorylation (as proxy for activation) of ERK and other MAPKs; however, ERK/MAPK is a complex response. Thus, other than arrestin, cAMP itself can lead to ERK phosphorylation (Vossler et al. 1997; Stork and Schmitt 2002), G_i may be involved in the ERK phosphorylation response even for primarily G_s-coupled receptors (Daaka et al. 1997; Galandrin et al. 2008), and Src can contribute with or without the involvement of arrestin (Luttrell et al. 1999; Ma et al. 2000; Sun et al. 2007; Galandrin et al. 2008). Moreover, such contributions are not only cell-dependent (Qiu et al. 2000; Lefkowitz et al. 2002) but may also depend on the state of the same cell clone (Kaya et al. 2012). Therefore, the fact that ERK phosphorylation is a complex response possibly involving multiple mechanisms should be kept in mind when interpreting the phosphorylation data. Similarly, glucose uptake into mouse soleus muscle can be stimulated by multiple ligands including BRL 37344 and clenbuterol, but this may involve distinct signaling G_s- and G_i-mediated pathways (Evans et al. 2013; Ngala et al. 2013). AU4

Off-target effects can obscure the detection of true biased agonism (van der Westhuizen et al. 2014). For example, the clinically used β-AR antagonist carvedilol has been proposed to be a biased agonist based on its ability to stimulate arrestin signaling (Wisler et al. 2007); this appears to be mediated by a G_i protein when acting on β_1-ARs, but not when acting on β_2-ARs (Wang et al. 2017). Moreover, carvedilol has additional actions not shared by most other β-AR antagonists. It has been reported to be an antagonist at α_1-AR, exhibit antioxidative effects and clash with NO production (Yoshioka et al. 2000; Afonso et al. 2006), inhibit endothelin production (Saijonmaa et al. 1997; Ohlstein et al. 1998), and at least in high

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concentrations block voltage-operated Ca^{2+} channels (Ruffolo et al. 1990). Another example of this problem is the β_1-selective antagonist *nebivolol* that can also stimulate arrestin signaling (Erickson et al. 2013). It has also been proposed to exhibit α_1-AR antagonism, antioxidative effects, and stimulating effects on *NO production* through activation of NO synthase; the latter was proposed to occur through β_3-AR agonism (Rozec et al. 2006), but others found no evidence for nebivolol being a β_3-AR agonist in transfected cell lines or rat bladder tissue (Frazier et al. 2011). Linking observed effects, particularly in vivo, to biased agonism is close to impossible with such "dirty" ligands as the observed effect may be due to any of these effects or any combination thereof.

Another level of complexity comes from the general observation that 50% or more of reported findings in biology are not robust (Vollert et al. 2022). For instance, controversial data on biased agonism of *fenoterol* and *salmeterol* at β_2-AR have been reported (see below). Thus, the presence of ligand bias of many if not most known adrenergic agonists remains undecidable for the moment (Onaran et al. 2017). Thus, it seems impossible to associate confidently the therapeutic advantage of some bronchodilators (like salmeterol) with bias. Very few if any β-AR ligands exhibit a robustly proven bias for a signaling pathway, e.g., compound C1-S apparently favoring G_s over arrestin when acting on β_2-AR (Kim et al. 2021). Finally, allosteric modulators may affect signaling. Thus, compound-6 was reported to be a positive allosteric modulator enhancing agonism at β_2-AR; among a panel of structurally diverse β-AR antagonists, compound-6 potentiated arrestin but not Gs-mediated effects of *carvedilol*, including activation of ERK, receptor endocytosis, or receptor trafficking to lysosomes (Pani et al. 2021), implying that biased signaling may in some cases depend on allosteric modulation.

4 Biased Signaling via β_1-Adrenoceptors

Given the widespread use of β_1-AR, specifically antagonists, in more than 30 indications (Bond et al. 2022), studies concerning biased agonism at this subtype are surprisingly sparse. A study using transfected human embryonic kidney (HEK) cells compared isoprenaline, bucindolol, and propranolol for their effects on ERK activation, which are full, partial, and inverse agonists, respectively, for cAMP formation (Galandrin et al. 2008). Despite their differential effectiveness for cAMP formation, all activated ERK to a similar extent. This was dependent on Src for the three ligands; for bucindolol and propranolol, in contrast to isoprenaline, this was independent of G protein activation.

The β_1-selective antagonist nebivolol was reported to stimulate arrestin signaling (Erickson et al. 2013). However, based on multiple other effects of nebivolol, perhaps including those on β_1-AR (Rozec et al. 2006; Frazier et al. 2011), it remained difficult to determine whether this represents biased agonism. Metoprolol induced an interaction between the β_1-AR and arrestin-2 but not arrestin-1; accordingly, metoprolol-induced cardiac fibrosis was abolished in arrestin-2 knockout mice (Nakaya et al. 2012). Carvedilol was reported to active the cGMP pathway in the

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murine heart in concentrations having little effect on the cAMP pathway (Wang et al. 2021). This is unlikely to represent a system bias as it was shared by nebivolol but not by other β-blockers such as metoprolol, propranolol, or sotalol.

5 Biased Signaling via β_2-Adrenoceptors

Some groups have examined large panels of ligands for potential biased agonism at β_2-AR. The group of Michel Bouvier from Montrèal tested 14 ligands in HEK cells and various transfected variants thereof in assays for cAMP accumulation, ERK activation, cell impedance assay, or Ca^{2+} elevation (Stallaert et al. 2012). Based on their findings, they categorized ligands into five groups. Group I ligands (isoprenaline, adrenaline) similarly stimulated cAMP accumulation and ERK phosphorylation. Group II ligands (*salbutamol*, *salmeterol*) strongly activated cAMP accumulation but were weak partial agonists for ERK phosphorylation. Group III ligands (alprenolol, bucindolol, labetolol, pindolol) were weak partial agonists for cAMP and ERK responses. Group IV ligands (propranolol, carvedilol) were very weak partial agonists in both assays. Finally, group V ligands (atenolol, ICI 118,551, metoprolol, timolol) were inverse agonists for the cAMP and the ERK responses. A follow-up study from the same group tested 19 (mostly clinically used) β_2-AR ligands for cAMP production, ERK activation, Ca^{2+} elevation, and receptor endocytosis (van der Westhuizen et al. 2014). This data supported the clustering into five groups. While apparent biased signaling of nine compounds could be explained by off-target effects, the clustering into five groups was confirmed with the remaining 10 compounds. Calculation of relative effectiveness within a pathway and of bias factors between pathways for each compound using an operational model of agonism showed a global signaling signature of all compounds relative to isoprenaline with most being biased toward ERK activation relative to the other pathways. Finally, a collaboration between German, Japanese, and US investigators tested 65 β_2-AR ligands (40 classically considered as agonists and 25 as antagonists) in HEK cells for their ability to recruit β-arrestin 1 and 2 to the β_2-AR (Littmann et al. 2015). Many agonists showed only weak partial agonism for arrestin recruitment with potency and/or efficacy increasing depending on the number of chirality centers in (R) configuration; no (S)-configured enantiomer was more effective in arrestin recruitment than the eutomer. Generally, β-arrestin 2 was recruited more effectively than β-arrestin 1. Several agonists showed strong bias for activation of Gαs relative to arrestin recruitment. Compounds classically considered as antagonists did not promote arrestin recruitment.

Other groups looked at smaller numbers of compounds, typically focusing on a single molecule relative to one or more reference compounds (in most cases isoprenaline). However, findings across groups were inconsistent. For instance, the β_2-AR agonist fenoterol was proposed to be G_s-biased (over G_i) by some investigators (Reinartz et al. 2015), but others did not confirm that (Onaran et al. 2017; De Pascali et al. 2022).

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Similarly, some investigators found the β_2-AR agonist salmeterol to be G_s-biased but not providing state-of-the-art bias analysis (Carter and Hill 2005; Gimenez et al. 2015), whereas others found it to be arrestin-biased (Rajagopal et al. 2011); salmeterol is not biased at all according to others (De Pascali et al. 2022) or in our hands (unpublished data). At the end, the bias status of most known adrenergic agonists remains undecidable for the moment (Onaran et al. 2017).

Finally, the β-AR antagonist carvedilol, clinically used in the treatment of heart failure, was reported to be an inverse agonist for cAMP formation but a partial agonist for β_2-AR ERK receptor phosphorylation, arresting binding, ERK activation, and receptor internalization (Wisler et al. 2007). Within a panel including acebutolol, alprenolol, atenolol, bisoprolol, betaxolol, carvedilol, ICI 118,551, labetalol, metoprolol, nadolol, oxprenolol, pindolol, propranolol, sotalol, and timolol, carvedilol was the only inverse agonist for cAMP that activated ERK.

6 Biased Signaling via β_3-Adrenoceptors

In contrast to the many indications of β_1- and β_2-AR ligands (Bond et al. 2022), the only approved use for β_3-AR agonists is the overactive bladder syndrome (Michel et al. 2023). Nonetheless, β_3-AR ligands have intensively been investigated for biased agonism. An early study reported, based on Chinese hamster ovary (CHO) cells transfected with human β_3-AR, that agonists stimulated ERK activation but not that of the related MAPK, JNK, and p38 (Gerhardt et al. 1999). In a limited panel of compounds, adrenaline and noradrenaline preferentially activated cAMP formation, whereas BRL 37,344 preferentially stimulated ERK. The latter response was sensitive to PTX, indicating an effect on G_i proteins, and the phosphatidylinositol-3 kinase inhibitors wortmannin and LY 294002.

More detailed studies on biased agonism at β_3-ARs came from a series of studies by the group of Roger Summers in Melbourne. Their initial study was performed in CHO cells transfected with cloned murine β_3-ARs, in murine 3 T3-F442 adipocytes, and in murine ileum and was based on a comparison of SR 59,230 and CL 316,243 (Hutchinson et al. 2005). The efficacy of the partial agonist SR 59,230 for stimulating cAMP formation increased with increasing levels of β_3-AR expression in the CHO cells. On the other hand, SR 59,230 was a full agonist for the ECAR response at all expression levels, an observation that could potentially be explained as by a greater receptor reserve for the ECAR as compared to the cAMP response. In 3T3-F442A cells, SR 59,230, in contrast to CL 316,243, had no agonist effects, an observation that could potentially be explained by low expression of the β_3-AR and/or by a less effective effector coupling in these cells. However, SR 59,230 had greater efficacy than CL 316,243 in the ECAR assay in 3T3-F442A cells, i.e., there was a reversal in order of efficacy that cannot be explained easily by a receptor reserve. Relaxation responses in mouse ileum were evoked by SR 59,230 in concentrations where it did not affect cAMP levels.

In a follow-up study in CHO cells transfected with the human β_3-AR, zinterol and L 755,507 caused robust, concentration-dependent increases in cAMP whereas L

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748,337 had low efficacy (Sato et al. 2008). The cAMP responses to zinterol and L 755,507 were increased after pre-treatment with pertussis toxin (PTX), indicating that the β_3-AR concomitantly coupled to G_s and $G_{i/o}$ proteins in the CHO cells. However, both L 755,507 and L 748,337 stimulated the phosphorylation of ERK and, with much lower potency, of p38 MAPK. PTX fully blocked activation of ERK and of p38 MAPK by L 748,337, whereas the p38 phosphorylation stimulated by L 755,507 was not inhibited by PTX and that of ERK phosphorylation by only about 30%. The ECAR response was elicited with similar efficacy by all three agonists. Taken together these observations were interpreted that L 755,507 couples to both G_s and $G_{i/o}$ proteins whereas L 748,337 coupled predominantly to $G_{i/o}$ proteins.

We have studied the desensitization of cAMP responses elicited via human β_3-ARs transfected into HEK cells at near physiological density (Okeke et al. 2019). Ligands stimulated cAMP formation with a numerical rank order of isoprenaline $\geq$ L 755,507 $\geq$ CL 316,243 > solabegron > SR 59,230 > L 748,337, an observation in line with the findings of others (Hutchinson et al. 2005; Sato et al. 2008). Except for the weakest agonist, L 748,337, pretreatment with any ligand reduced cAMP responses to freshly added isoprenaline or forskolin to a similar extent, indicating that the ability to cause desensitization of the β_3-AR did not depend on cAMP formation. On the other hand, we were unable to detect ERK phosphorylation despite testing a wide variation of conditions. In a follow-up study in transfected CHO cells, cAMP formation was stimulated with an agonist order of efficacy of isoprenaline (255% of control) $\approx$ L755,507 (247%) > CL316,243 (209%) > L748,337 (120%) $\approx$ SR59,230 (108%), whereas the order of efficacy for ERK phosphorylation was L755,507 (151%) $\approx$ CL316,243 (149%) $\approx$ isoprenaline (148%) > SR59,230 (137%) > L748,337 (122%) (Okeke et al. 2018). In these cells, desensitization of cAMP formation was caused by all ligands except for L 748,337, whereas L 748,337, but not isoprenaline, reduced ERK phosphorylation relative to a lowered baseline. Taken together, these findings demonstrate the presence of biased agonism at murine and human β_3-ARs.

7 Conclusions and Perspectives

It emerges from the above that studies of biased agonism are inherently complex because multiple factors other than biased agonism can explain different cellular, tissue, and in vivo responses when comparing two or more ligands. The possible role of allosteric modulators and frequent inconsistencies in the assessment of biased agonism across laboratories make robust conclusions on specific compounds difficult in most cases. Considering the limited robustness of reported findings, it can generally be concluded that distinguishing biased from partial agonism is challenging unless bias is extreme. That many studies in the field provided initial evidence for bias but few quantified it (Rajagopal et al. 2011; Reinartz et al. 2015) makes interpretation challenging. If we cannot/do not quantify bias relative to other drug properties such as partial agonism, rationale selection of developmental candidates based on properties such as biased agonism becomes difficult. This is exemplified by

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the discrepancy between high expectations and limited clinical success in other fields such as opioid receptor activation (Kelly 2013; Schmid et al. 2017; Gillis et al. 2020; Conibear et al. 2024).

This has sparked intensive discussions about whether the existence of biased agonism opens attractive avenues for *drug research and development*. On the one hand, it is tempting to exploit biased agonism as another path to achieve selective activation of a desired as compared to an undesirable response (Woo et al. 2015; Kenakin 2018). On the other hand, signaling heterogeneity across cells/tissues and across species within tissues as well as their changes under pathophysiological conditions can make predictions on human target tissue in a given disease close to impossible (Li et al. 2023). The roles of allosteric modulators (Pani et al. 2021) and of auxiliary signaling molecules including RGSs (De Vries et al. 2000) and AGSs (Lanier 2004) may additionally blur the picture. Similarly, the efficiency of receptor-arrestin coupling may depend on the expression levels of GRKs (Winpenny et al. 2016). Therefore, it was argued that predicting the desirable biased agonism profile at the start of a research and development project is close to impossible, unless a large body of evidence for a drug target already exists (Michel and Charlton 2018; Onaran and Costa 2021). However, drug targets with a large body of existing evidence and often many approved medicines acting on those targets have become unpopular in the pharmaceutical industry for commercial reasons. Thus, no compounds designed to be biased agonists have reached late-stage clinical development or even became marketed drugs at present.

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Uncorrected Proof

Author's Proof

Author Queries

Chapter No.: 769

Query Refs.	Details Required	Author's response
AU1	Please consider rephrasing sentence "Early concepts to achieve this…" for clarity.	
AU2	Please provide figure and caption for Fig. 1.	
AU3	Please check sentence "Biologically, a given β-AR…" for clarity.	
AU4	Please check edits to sentence "Similarly, glucose uptake…"	

Uncorrected Proof